Brain substrates of behavioural endophenotypes predictive of compulsive drug-seeking

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Declaration

The work described within this thesis was carried out between September 2017 and May 2021 at the University of Cambridge, under the supervision of Professor Jeffrey W. Dalley. The dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except that declared in the preface and specified in the text. This thesis has not been submitted in whole or in part for consideration for any other degree or qualification at the University of Cambridge or any other University of similar institution except as declared in the preface and specified in the text. The length of this thesis does not exceed the word limit of the School of Biology degree committee. The work carried out within this thesis was funded by a Medical Research Council Programme Grant (G1002231).

Abstract

Addiction, also known as severe substance use disorder, is characterised by compulsive drug-seeking and intake in the face of mounting adverse and negative consequences. Despite the prevalence of illicit drug use within society only a small subset of individuals lose control over their intake after protracted drug use. Several antecedent behavioural endophenotypes and perturbations in underlying reward-related circuitry have been linked with increased vulnerability of the development of addiction. To what extent these behavioural and neural markers pre-exist or are induced by drug exposure is still yet to be fully addressed. In this thesis, behavioural testing for risk endophenotypes was combined with a self-administration model of compulsive cocaine seeking and translationally relevant magnetic resonance imaging to investigate the behavioural and neurobiological underpinnings of vulnerability to compulsive cocaine seeking in rats. A behavioural screening procedure was employed and the underlying relationships between several risk endophenotypes was assessed. Attribution of incentive salience was unrelated to impulsivity yet was positively associated with novelty place preference and locomotor reactivity. 'Stickiness' – the tendency to repeat the same choice regardless of reward outcome – on a reversal learning procedure was positively associated with motor impulsivity. A concurrent punishment- drug-seeking procedure was next implemented to assess vulnerability to compulsive cocaine seeking. Of the behavioural risk endophenotytpes assessed, impulsivity along with stickiness significantly predicted compulsive cocaine seeking. In the final chapter, impulsivity and compulsivity were shown to predict lower grey matter volume in the infralimbic cortex and ventral striatum. Functionally, compulsivity and stickiness were predicted by decreased connectivity between the prelimbic cortex and the anterior cingulate cortex with the posterior dorsomedial striatum. In summary, this thesis supports previous work implicating impulsivity as a vulnerability marker for substance dependence in humans, and as a marker for compulsive cocaine use in rodents. These findings extend earlier reports to show that impulsivity, alongside stickiness, predicts future compulsive cocaine seeking. Furthermore, this thesis shows overlapping abnormalities in cortico-striatal networks in future compulsive animals and convergent structural deficits in the infralimbic cortex and ventral striatum of high compulsive and impulsive rats. These findings expand our understanding of vulnerability to drug-seeking by showing that pre-existing deficits in circuits contributing to impulse control and flexible, goal-directed behaviour may be precursors for the emergence of compulsive cocaine seeking.

Preface

Over the last four years I have worked with an incredible group of colleagues on a highly ambitious, multi-disciplinary project incorporating behavioural neuroscience, non-invasive magnetic resonance imaging and compulsive cocaine self-administration. As such, this project could not and would not have been completed without the hard work and dedication of multiple people for which I am extremely grateful. I would like to express my gratitude to all of the people who made this project successful, the contribution of which I shall outline below.

Dr. Bianca Jupp

As a post-doctoral scientist in Professor Jeffrey W. Dalley's laboratory between 2011-2019, Dr. Jupp was employed to work on the MRC Programme Grant (G1002231) of which the work within this thesis was funded by. As a result, both myself and Dr. Jupp contributed practically to the scanning of the animals, the behavioural training, and alongside Dr. Aude Belin-Rauscent, the cocaine self-administration experiments. In addition, Dr Jupp performed an *a priori* power analysis prior to the initiation of the work reported herein.

Dr. Stephen Sawiak

As the MRI physicist and lead imaging specialist on the programme, Dr. Sawiak was instrumental in the design and implementation of the MRI scanning sequences and post-reconstruction of the MRI images into their raw format. In addition, Dr. Sawiak provided expert theoretical guidance on a wide range of analysis techniques and imaging approaches.

Dr. Peter Zhukovsky

As a final year PhD student in Professor Jeffrey W. Dalley's laboratory at the time I started my doctoral work, Dr. Zhukovsky, alongside myself and Dr. Jupp, contributed practically to the scanning and training of the animals. Specifically, Dr. Zhukovsky ran the reversal learning behavioural training and ran the formal analysis as well as the computational modelling. Therefore, a short summary of the computational model is provided (Chapter 3) but for a more detailed description see Zhukovsky et al., 2019.

Dr. Aude Belin-Rauscent

The self-administration portion of this thesis work was carried out in collaboration with the Belin Laboratory, Cambridge. Dr. Belin-Rauscent carried out the self-administration procedure with assistance from both myself and Dr. Jupp.

Dr. Maxime Fouyssac

The self-administration portion of this thesis work was designed and validated by Dr. Fouyssac during his doctoral work (2014 - 2017). Specifically, Dr. Fouyssac carried out the hotplate sensitivity test in relation to future compulsive behaviour and produced the associated figure (see **Appendix1A-D.**).

Dr. David Belin

Dr. Belin performed all intravenous surgeries and provided important guidance on the self-administration experiments and behavioural analysis.

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Both Professor Robbins and Professor Everitt provided important theoretical insight and discussion in regard to the work presented herein.

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Named publications relating to this PhD thesis

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Jones JA., Russell B., Dalley JW. (2021) Molecular Imaging Studies in Stimulant Addiction: A Cross-Species Perspective. In: Dierckx R.A., Otte A., de Vries E.F.J., van Waarde A., Sommer I.E. (eds) PET and SPECT in Psychiatry. Springer, Cham.

Jupp B, **Jones JA**, Dalley JW. Modelling Differential Vulnerability to Substance Use Disorder in Rodents: Neurobiological Mechanisms. Handb Exp Pharmacol. 2020;258:203-230.

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Abbreviations and acronyms

2-CSRTT – 2-choice serial reaction time task
^{2nd}SOR – second order schedule of reinforcement
4-CSRTT – 4-choice serial reaction time task
5-CSRTT – 5-choice serial reaction time task
Acb – nucleus accumbens
AcbC – nucleus accumbens core
AcbS – nucleus accumbens shell
ACC – anterior cingulate cortex
ADHD – attention-deficit hyperactivity disorder
aDLS – anterior dorsomedial striatum
AI – anterior insula
ALP – active lever presses
ANOVA – analysis of variance
BA – Broadmann's area
bHR – bred high responders
BIS – Barratt Impulsiveness Scale
BLA – basolateral amygdala
bLR – bred low responders
BOLD – blood oxygen level dependent
CeA – central amygdala
CI – confidence interval
CR – conditioned response
CS – conditioned stimulus
DA – dopamine
DARTEL – diffeomorphic anatomical registration using exponentiated lie algebra
DDT – delay discounting task
DTI – diffusion tensor imaging
dlPFC – dorsolateral prefrontal cortex
DLS – dorsolateral striatum
DMN – default mode network
DMS – dorsomedial striatum
DF – degrees of freedom
DSM-5 – Diagnostic and Statistical Manual of Mental Disorders
dStr – dorsal striatum
EEG – electroencephalography
FI – fixed interval
fMRI – functional magnetic resonance imaging
FR – fixed ratio
FR-1 – fixed ratio 1
FWD – framewise displacement
GABA – gamma-aminobutryic acid
GNGT – go-no/go task
GSR – global signal regression
GT – goal-trackers
HC – high compulsive
HI – high impulsive

HK – high kappa
HPA – hypothalamo-pituitary-adrenal
HR – high responders
IC – intermediate compulsive
ICA – independent component analysis
IFG – inferior frontal gyrus
IL – infralimbic
iRISA – impaired response inhibition and salience attribution
ITI – inter-trial interval
LatOFC – lateral orbitofrontal cortex
LC – low compulsive
LFP – local field potential
LgAb – long access
LI – low impulsive
LK – low kappa
LocR – locomotor reactivity to a novel inescapable environment
LQ – lower quartile
LR – low responders
LTD – long-term depression
M1 – primary motor cortex
MDD – major depressive disorder
MedOFC – medial orbitofrontal cortex
MNI – Montreal Neurological Institute
mPFC – medial prefrontal cortex
MR – magnetic resonance
MRI – magnetic resonance imaging
MT – magnetisation transfer
NMDA – N-methyl-D-aspartate
NPP – novelty place preference
OFC – orbitofrontal cortex
OLS – ordinary least square
P&W – Paxinos and Watson
PavCA – Pavlovian conditioned approach
PCA – principal component analysis
PCA-index – Pavlovian conditioned approach index
PCC – posterior cingulate cortex
pDMS – posterior dorsomedial striatum
PET – positron emission tomography
PFC – prefrontal cortex
PND – post-natal day
pre-SMA – pre-supplementary motor area
PrL – prelimbic cortex
PVT – paraventricular thalamus
RF – radiofrequency
RI – random interval
RL – reversal learning
ROI – region-of-interest
rs-fMRI – resting state functional magnetic resonance imaging
SA – self-administration
sACC – subgenual anterior cingulate cortex

SBCA – seed-based correlation analysis
ShA – short access
SNR – signal-to-noise
SPECT – single-photon emission computed tomography
SSRT – stop signal reaction time
SSRTT – stop signal reaction time task
ST – sign-trackers
STN – subthalamic nuclei
SUD – substance use disorder
sus5-CSRTT – Sussex 5-choice serial reaction time task
SVD – singular value decomposition
T1-W – T1 weighted
T2-W – T2 weighted
TBM – tensor-based morphometry
TE – echo time
TMS – transcranial magnetic stimulation
TR – repetition time
tSNR – temporal signal-to-noise
U.K – United Kingdom
UPPS-P – Urgency, Premeditation (lack of), Perseverance (lack of), Sensation seeking, Positive Urgency, Impulsive Behaviour Scale
UQ – upper quartile
US – unconditioned stimulus
VBM – voxel-based morphometry
VI – variable interval
vlPFC – ventrolateral prefrontal cortex
vmPFC – ventromedial prefrontal cortex
VMS – ventromedial striatum
vStr – ventral striatum
VTA – ventral tegmental area
α – alpha
β – beta
κ – kappa / stickiness

Chapter 1

General introduction

1.1 Overview of illicit drug use

Drug addiction, also known as severe substance use disorder, is a debilitating, complex brain disorder affecting over 35 million people worldwide (UNODC 2019). The United Kingdom (U.K) has one of the highest prevalence of cocaine use within the European union, with cocaine consumption increasing dramatically over the last decade (UNODC 2019). There are also indications of higher-purity cocaine at lower prices with the drug being more readily and widely available (UNODC 2019). Thus, drug addiction represents a growing public health crisis with severe health and economic implications, with the illicit drug trade estimated to cost 20 billion pounds annually in the U.K (Black 2020). At the societal level, substance abuse has been linked to lower life satisfaction (Zullig et al., 2001), housing instability and unemployment (Daley 2013). However, encouragingly, the proportion of people seeking treatment for cocaine has risen since 2015 across the whole of the U.K, with a dramatic increase in Scotland, where reported treatment use has more than doubled over three years (2015 – 2018) (U.K drug situation: Focal point annual report 2019). Despite the intense research efforts over several decades there remains limited effective treatment options for substance-dependent individuals. Therefore, it is of vital importance to understand the underlying psychological and neural mechanisms that govern the transition to compulsion to facilitate the development of novel and effective therapeutic interventions.

1.2 Addiction – historical perspective and diagnosis

Since the 1950's the American Psychiatric Association's *Diagnostic and Statistical Manual of Mental Disorders* (DSM) has undergone several revisions in regards to the classification of addiction and substance use disorder (SUD). The DSM-5 is the most up-to-date classification

manual, published in 2013. It combines several abuse and dependence criterion into a single category of which there are 11 diagnostic symptoms. The classification system in the DSM-5 has been subtly changed to reflect a continuum with mild SUD characterised by individuals showing 2-3 symptoms, moderate SUD by 4-5 symptoms, and severe SUD by 6 or more symptoms – commonly referred to as addiction (Robinson and Adinoff 2016). Of note, the term addiction has been dropped from the DSM-5 due to its potential negative connotation and the stigma attached to defining one with a SUD as an addict. Whilst in this thesis the term addict has not been used, the more recognised term addiction has been used instead of severe SUD to characterise the disorder. Throughout the literature the term 'dependent' is commonly used to describe individuals with SUD. However, the precise nature of the term 'dependent' can be defined in subtly different ways across studies, and commonly the term 'met criteria for dependence/SUD' is used without explicitly highlighting which of the criteria were met. Differences may be further exacerbated by studies of past decades that use the classification system of older versions of the DSM. The widespread use of the term dependence may stem from one of the two DSM-4 subcategories used to define addiction – 'Dependence', with the latter category being 'Substance Abuse'. Previous studies using the term dependence have referred to individuals who meet the DSM-4 criteria for cocaine dependence (Moeller et al., 2001), whereas more recent studies have used this classification interchangeably with cocaine use disorder (Lim et al., 2019), whilst others have used the term dependence to describe individuals who show 4 or more symptoms on the most recent DSM-5 criteria (Ersche et al., 2020). Although different conventions have been used to define SUD, throughout this thesis the term 'dependent' has been used to reflect the naming convention used in the studies referenced and does not specifically relate to the term dependence in the context of physiological symptoms namely withdrawal and tolerance (Schuckit et al., 1999).

1.3 Behavioural endophenotypes of addiction

Most people who take drugs of abuse recreationally do so sparingly and can, when needed, exert appropriate levels of control if or when drug-taking behaviour interferes with other life events. Another subgroup of people can continue to use drugs regularly for years without any apparent side effects, escalation of use or negative effects (so-called chippers; Zinberg and Jacobson 1976; Stull et al., 2019). What allows some individuals to exert control over their drug intake, whilst others lose control is still an open question. The role of personality traits, or more specifically vulnerability endophenotypes in addiction suggests that there are several

risk traits that reflect shared genetic and/or biological aetiologies that interact with a persons' environment and social contexts to drive loss of control over intake (Badiani et al., 2011). Endophenotypes, a term adapted for psychiatry by Gottesman and Shields (1973), are measurable internal processes which reflect sub-components of a more complex aetiology. The logic behind the endophenotype phenomena is to allow for a more straightforward interrogation of the contributing genetic component underpinning the more complex, multi-factorial, polygenic disorder. The endophenotype approach has been operationalised within addiction research allowing researchers to disentangle complex disorders by evaluating the intricate contributions of multiple, simpler phenomenon to the overall clinical phenotype. In human studies, it is often difficult to interpret the causal trajectory of antecedent risk traits from the multivariate effects of chronic drug exposure, thus making it difficult to determine whether these behavioural factors pre-date onset or emerge as a consequence of drug use. One possible way to circumvent this issue is by studying the non-drug using siblings of stimulant-dependent individuals, which provides a platform to understand vulnerability through shared genetic and environmental mechanisms, likely to have predated drug dependence. Conversely, experimental approaches in rodents who naturally express recognised vulnerability traits allow for rigorous control of environmental context and level of drug exposure, thus offering a useful platform in which to probe the mechanistic trajectory towards substance dependence. Several human traits have been operationalised in animals and support the view that individual differences in these behavioural factors reflect vulnerability markers to develop certain aspects of addiction-like behaviour in rodents. These will be discussed in turn below.

1.3.1 Impulsivity

Impulsivity describes the predisposition of an individual to premature, poorly planned, and unduly risky actions and decisions. While generally considered an adaptive trait promoting sociability and appropriate risk-taking, the maladaptive expression of impulsivity is often associated with negative consequences for the individual and is co-expressed with several neuropsychiatric disorders including addiction. While impulsivity is suggested to comprise multiple domains of behaviour, this trait can broadly be defined as impaired delayed gratification, rash anticipatory behaviour and impaired action cancellation (Evenden 1999). Whilst some sub-dimensions of impulsivity are likely to be overlapping there are important differences within (Green and Myerson 2013) and between (Malanchini 2019) different sub-

dimensions of the construct. Similarly, relationships between self-report measures of impulsivity and laboratory based behavioural measures have suggested potential overlapping mechanisms across various forms of impulsivity (Jauregi, Kessler and Hassel 2018), whilst others have suggested weaker or diverging interrelationships (Green and Myerson 2013; Reynolds et al., 2006). Whilst perhaps it is too reductionist to classify impulsivity into a limited number of discrete categories, several classifications have been used and are commonly cited throughout the literature. Impulsivity can be categorised based on two overarching sub-domains, decisional or choice *versus* motoric impulsivity (Dalley and Robbins 2017; Verdejo-Garcia and Albein-Urios 2021). Decisional or choice forms of impulsivity broadly represent discounting of rewards across time such as the delayed discounting task (DDT), whereas motoric forms of impulsivity represent forms of impulsivity more closely related to withholding an already initiated motor response as in the stop signal reaction time task (SSRTT), or withholding a motor response until a time interval has elapsed, as in the serial reaction time task (Dalley and Robbins 2017). In addition, questionnaire and self-report measures of impulsivity such as the Barratt Impulsiveness Scale (BIS) (Patton et al., 1995) may represent a further sub-category, due to the observed weak correlation between self-reported and task-based measures of impulsivity (Cyders and Coskunipar 2011; Sharma et al., 2013). Impulsivity, as defined through the BIS, is separated into attentional, motor, and non-planning components, further highlighting the non-unitary nature of the impulsivity construct. Several studies have highlighted the relationship between elevated levels of impulsivity as both a consequence of drug consumption, as well as a determinant to use (de Wit 2009) suggesting that impulsivity may represent an antecedent behavioural marker for addiction, that is also increased after chronic drug use (Ersche et al., 2010). In drug-dependent individuals, high levels of impulsivity are also related to poorer treatment outcomes. Individuals with high levels of baseline impulsivity prior to treatment had higher levels of total cocaine use and also showed significantly less time in treatment (higher drop-out rates), when compared to low impulsive (LI) individuals (Moeller et al., 2001). The inability to forgo smaller short-term gains in the pursuit of longer-term beneficial rewards and delayed reward gratification is also associated with stimulant use. Thus, drug-dependent individuals show preference for small, immediate rewards and discount monetary rewards faster than healthy controls (Coffey et al., 2003), a measure closely related to the number of continuous weeks of abstinence achieved in cocaine-dependent individuals on both a 12, and 24-week treatment programme (Washio et al., 2011). Steeper discounting rates of hypothetical monetary reinforcers have repeatedly been associated with shorter durations

of cocaine abstinence, increased relapse risk and low treatment retention rates (Stevens et al., 2014; Coffey et al., 2003; Washio et al., 2011; Heil et al., 2006; Kirby and Petry 2004), highlighting the role of choice impulsivity in treatment outcomes and abuse prevention. In the 4-choice serial reaction time task (4-CSRTT), abstinent alcohol- and methamphetamine-dependent subjects also demonstrated higher levels of premature responding when compared to healthy controls (Voon et al., 2014). Several lines of preclinical evidence have also highlighted the important role of impulsivity in regulating reinforcement to cocaine and addiction-like behaviour in the rat (Belin et al., 2008; Mendez et al., 2010; Winstanley et al., 2010). For instance, high levels of impulsivity on the 5-choice serial reaction time task (5-CSRTT) are associated with enhanced self-administration (SA) of cocaine (Dalley et al., 2007), whilst high levels of discounting in the DDT is associated with enhanced cocaine SA (Perry et al., 2005) and escalation of cocaine intake during extended access conditions (Anker et al., 2009). Perhaps more relevant to the clinical picture and the American Psychiatric Association's *Diagnostic and Statistical Manual of Mental Disorders 5th edition* (DSM-5) criteria, elevated levels of impulsivity on the 5-CSRTT are associated with increased relapse rats after punishment induced abstinence of cocaine seeking (Economidou et al., 2009). In the multi-symptomatic 3-criteria model, trait-like impulsivity on the 5-CSRTT is also associated with the development of compulsive cocaine SA (Belin et al., 2008). Collectively, both clinical, and pre-clinical studies suggest that the expression of impulsivity, in its various forms, is indeed a vulnerability marker to dependence and may contribute to the pathogenesis of addiction to psychostimulants.

1.3.2 Attribution of incentive salience

The way in which we integrate and attribute significance to motivational cues in the environment is important for flexible and adaptive behaviour. Operationalised as an excessive 'wanting' for the drug in the human literature, for certain people, cues within the environment imbued with motivational and emotional significance may, as a result, promote the expression of aberrant and maladaptive behaviours to drug-associated stimuli (Robinson and Berridge 2001). In humans, 'wanting of the drug' or incentive salience may be driven by long-lasting neural system changes in response to chronic drug exposure as well as through context-dependent mechanisms when administering the drug, e.g., stress- and drug-related contexts (Robinson and Berridge 2000; Sinha 2011). However, until recently, only a handful of studies have attempted to directly address the notion of individual differences of salience

attribution in humans (Colaizzi et al., 2020; Joyner, Gearhardt and Flagel 2018). Nonetheless, a recent study back-translated the well-established Pavlovian conditioned approach (PavCA) procedure in rodents (Meyer et al., 2012) and reported to demonstrate sign- and goal-tracking behaviour in children (Joyner, Gearhardt and Flagel 2018). In the rodent paradigm, the PavCA procedure is commonly used, whereby approach behaviour to a response-independent presentation of a conditioned stimulus (CS) (often a light) that predicts the delivery of an unconditioned stimulus (US) (often a reward pellet) is measured (Meyer et al., 2012). Animals that preferentially approach the cue are phenotyped as sign-trackers (ST), whilst those that approach the location of reward delivery are phenotyped as goal-trackers (GT). ST, who have a higher propensity to attribute incentive salience to reward cues, show greater preference for cocaine over food under a cocaine/food choice procedure paradigm (Tunstall and Kearns 2015). In addition, ST also show higher levels of cocaine acquisition when compared to their goal-tracking counterparts (Beckmann et al., 2011; but see Saunders and Robinson 2010). Some evidence suggests that, when compared with GT, ST demonstrate higher levels of motivation to acquire drug of abuse, as indexed through higher final breakpoints in a progressive ratio paradigm (Saunders and Robinson 2010), whilst others have found no difference (Vanhille et al., 2014). In addition, ST also demonstrate higher sensitivity to changes in the presentation of a cocaine-associated stimulus during SA. Thus, removal of the cocaine-paired cue dramatically decreased the number of cocaine infusions per minute in ST but not GT during cocaine SA (Saunders and Robinson 2010). Moreover, ST show higher levels of reinstatement to drug-paired cues (Yager and Robinson 2013), and cocaine-primed reinstatement (Saunders and Robinson 2010). In relation to compulsive drug use, one study reported no differences between ST and GT on several different addiction relevant behaviours (Kawa, Bentzley and Robinson 2016). However, a more recent study employing the 3-criteria model found that ST displayed compulsive drug-taking behaviour and were more resistant to punishment (i.e., took more cocaine in the face of contingent foot shock punishment than GT). Although it must be noted that a dimensional analysis revealed no relationship between conditioned approach and compulsive drug-taking in this report (Pohorala et al., 2021).

1.3.3 Novelty/sensation seeking

The trait of novelty/sensation seeking is broadly associated with the pursuit of emotive and novel experiences with intense sensations (Zuckerman et al., 2010). Like impulsivity,

sensation seeking or the pursuit of novelty is generally considered to represent a continuum, the expression of which can be advantageous in certain circumstances, yet overexpression of the trait can be maladaptive and lead to negative consequences in relation to addiction. In humans, sensation seeking is often measured using questionnaire-based assessments (Zuckerman 1994) and has been shown to co-exist in individuals with SUD (Gerra et al., 2004; Hittner and Swickert 2006). In cocaine-dependent subjects', sensation seeking has been linked to increased severity of use, is negatively correlated with onset of abuse-dependence, and importantly, is negatively correlated with the age of first use (Ball et al., 1994). Along this theme, substance-dependent individuals show increased levels of sensation seeking relative to their drug-free siblings, and healthy controls, with no differences between the control and sibling groups (Ersche et al., 2010). This suggests that sensation seeking may be a consequence of drug use and chronic exposure, rather than being aberrantly expressed preceding use. These results highlight that sensation seeking may not represent a heritable risk factor for dependence (for example when compared to impulsivity; also measured in this study). Preclinical evidence suggests that sensation seeking, as assessed through locomotor reactivity to a novel inescapable environment (LocR), is a vulnerability endophenotype for initiation of use and not 'addiction' *per se* (Belin et al., 2008; 2011). Conversely, novelty-seeking assessed through a free-choice novelty place preference (NPP) paradigm is associated with the development of compulsive cocaine-taking and high resistance to punishment (Belin et al., 2011) in rodents. This aligns with previous preclinical research suggesting that these two traits are unrelated and do not correlate with each other (Hughson et al., 2019). Whereas NPP may represent a vulnerability marker for compulsive cocaine intake, sensation seeking has been shown previously to predict the propensity to acquire stimulant drug self-administration (Piazza et al., 1989), with animals displaying high levels of LocR, namely high responders (HR) rats showing increased administration of psychostimulants when compared to low responders (LR) rats (Piazza et al., 1989).

1.3.4 Cognitive flexibility

Cognitive flexibility is the ability of an organism to adapt rapidly and appropriately to changes within the environment. Reversal learning (RL) is a well-studied behavioural paradigm used to assess cognitive flexibility in humans and experimental animals. Over the last several decades, the number of published studies using RL has dramatically increased, irrespective of species (Izquierdo et al., 2017). RL is widely used to assess cognitive

flexibility across a wide range of neuropsychiatric disorders including autism spectrum disorder, schizophrenia, depression and SUD. In relation to SUD, individuals dependent on cocaine show little behavioural adaptability to contingency changes in a probabilistic RL task, showing increased levels of perseveration on the previously rewarded contingency (Ersche et al., 2008; Fernandez-Serrano 2012 but see Patzelt et al., 2014; Ide et al., 2015), a finding later revealed to be mediated by activation in the frontal, parietal and striatal regions, with strong activation specifically in the right caudate nucleus (Ersche et al., 2011). Moreover, perseverative errors have been reported to be positively correlated with lifetime duration of cocaine use (Verdejo-Garcia et al., 2015). Whether deficits in cognitive flexibility are a cause or consequence of drug use is still an open question (Spronk et al., 2013). However, one study has shown that cognitive deficits are observed in recreational users that do not meet the criterion for abuse (Colzato, Huizinga and Hommel 2009), suggesting that changes in cognitive flexibility may be a result of drug exposure. Along this line, one longitudinal study evaluated cognitive function over a 1-year period in recreational drug users, with varying levels of use/abstinence, and reported increases in cognitive function in subjects with decreasing stimulant use (Vonmoos et al., 2014). These improvements may be related to the relatively low levels of drug consumption seen in this study (when compared to dependent individuals), but nonetheless offer an insight into the potential amelioration of cognitive deficits produced by exposure to stimulants during abstinence. However, cognitive deficits in drug-dependent individuals can still be observed even after a 7-month period of abstinence (Fernandez-Serrano 2008), suggesting that the assumed neurotoxic effects of sustained drug use are long-lasting. These studies suggest that deficits in RL performance may be a consequence of drug exposure and are related to the amount of drug taken. In rodents, one study evaluated performance on an analogous spatial RL task prior to and after escalation of cocaine. At baseline, no differences in task performance were observed. However, after drug exposure differences in task performance emerged based on differential exposure to cocaine. Thus, rats displaying robust escalation of cocaine intake appeared less likely to integrate negative feedback as indexed through a decrease in lose-shift behaviour (Zhukovsky et al., 2019). This effect was only seen in high and not low drug escalators, or saline controls. This outcome echoes that observed in the human studies (above) and further suggests that the rate and overall intake of cocaine directly influences RL performance and cognitive flexibility. In a similar study, cocaine exposure decreased RL performance, decreasing the number of correct choices in the reversal phase and decreasing the likelihood of switching after an incorrect response (Groman et al., 2020). The decrease in negative

outcome updating was linearly related to the number of cocaine infusions, thus indicating that the degree of cocaine exposure and intake had a direct link with negative outcome updating, findings associated with glutamate binding in the medial prefrontal cortex (mPFC) (Groman et al., 2020). Cocaine-induced impairments in RL are long-lasting and can be induced even after relatively short exposure to the drug. Rats with only 14 days of exposure to cocaine show impairments in RL after three months of abstinence (Calu et al., 2007). Beyond the classic RL paradigm, cocaine has also been shown to impair performance on an odour discrimination paradigm (Stalnaker et al., 2006), reversal of a spatial navigation strategy on a plus-maze (Bechard et al., 2018), as well as disrupting performance on the rat gambling task, an analogue of the human Iowa Gambling Task (Cocker et al., 2020). In summary, cocaine produces powerful, long-lasting neural plasticity changes within key brain regions important in regulating cognitive flexibility and executive function. These changes can be observed several weeks and even months after exposure to relatively small drug amounts.

1.4 Neural substrates of addiction-relevant behavioural endophenotypes

1.4.1 Impulsivity

1.4.2 Questionnaire-based assessment of impulsivity

Impulsivity is often assessed in humans using subjective self-report measures; for example, the BIS (Patton et al., 1995) and the Urgency, Premeditation (lack of), Perseverance (lack of), Sensation Seeking, Positive Urgency, Impulsive Behaviour Scale (UPPS-P) model (Whiteside and Lynam 2001; Lynam et al., 2006; Cyders et al., 2007). Since its inception in 1959 (Barratt 1959), the BIS has been used extensively within the clinical and non-clinical literature to investigate the underlying relationship between impulsiveness and psychopathology and continues to be an important tool in the investigation of vulnerability mechanisms in addiction (Verdejo-Garcia, Lawrence and Clark 2008). Over the last 50 years, the BIS has been updated and revised and now consists of three second order factors which include 1) attentional impulsiveness, 2) motor impulsiveness, and 3) non-planning

impulsiveness (Stanford et al., 2009). The BIS-11 is a self-report questionnaire, where participants must rank specific statements linked to impulsive and non-impulsive personality traits such as 'I do things without thinking' and 'I am careful thinker', with the scale ranging from 'Rarely/Never' to 'Almost Always/Always'. On the other hand, the UPPS-P model separates impulsivity into five overarching domains including 1) negative urgency, 2) positive urgency 3), lack of premeditation, 4) lack of perseverance, and 5) sensation seeking (Whiteside and Lynam 2001). Scoring on the UPPS-P differs slightly to that of the BIS with items scored according to a 4-point scale (e.g., 1 = strongly agree, 4 = strongly disagree). Nonetheless, both questionnaire-based methods have been used to understand the link between impulsivity and SUD. High self-reported levels of impulsivity, as assessed through the BIS, are increased in substance-dependent individuals (Coffey et al., 2003; Verdejo-Garcia, Lawrence and Clark 2008) and both positive and negative urgency, as assessed through the UPPS-P model, are increased in cocaine-dependent individuals (Albein-Urios et al., 2012). BIS scores have also been shown to predict treatment outcome (Moeller et al., 2001) and treatment retention (Patkar et al., 2004). Although the BIS has been shown to have a high test/re-test validity and internal consistency (Stanford et al., 2009), the validity of self-report questionnaires more broadly relies on accurate introspection and can be influenced by self-reporting bias. Moreover, impulsivity may have a direct effect on reporting with the potential for high impulsive (HI) individuals to lack foresight when answering questionnaire statements. This may be one reason for the apparent lack of correlation between the BIS-11 and other laboratory-based measures of impulsivity (Reynolds et al., 2007; Cyders and Coskunpinar 2011; Reynolds, Penfold and Patak 2008). Nonetheless, other psychometric laboratory-based measures, which capture distinct aspects of the impulsivity phenotype, have been utilised to assess impulsivity in both humans and experimental animals. The assessment of impulsivity in rodents relies on analogous tasks developed for testing in humans and includes the 5-CSRTT, DDT, SSRTT and the go/no-go task (GNGT), as discussed below.

1.5 Impulsive action

1.5.1 5-choice serial reaction time task

The 5-CSRTT is an analogue of Leonard's choice reaction time task, initially developed to study attention in humans (Wilkinson 1963). The task was successfully adapted for use in rodents and has been employed to understand sustained attention, and more importantly in the context of addiction-like behaviour, impulse control deficits (Robbins 2002). Briefly, in the

5-CSRTT rodents are trained to respond (nosepoke) in one of five apertures that has previously been illuminated during the trial to earn a food reward (Bari et al., 2008). Deficits in response inhibition are observed when an animal responds prematurely in any of the five apertures prior to the illumination of the aperture, after the trial has been initiated. This period within the task is known as the inter-trial interval (ITI). For overview see **Fig1A.** Stimuli within the 5-CSRTT can be made temporally unpredictable by the introduction of changes in the frequency and predictability of the ITI duration. The ITI can be varied during the test or challenge session (commonly from 5 seconds to 7 seconds), with challenge sessions (7 second ITI) commonly interceded between two baseline sessions (5 second ITI). This short/long variable ITI task design results in reduced stimulus predictability and leads to the exacerbation of underlying deficits in response inhibitory control.

Since the development of the 5-CSRTT, a wide range of studies have interrogated the circuitry and underlying neural substrates that control premature responding on the task. In early work, the post-genual anterior cingulate cortex was found to contribute importantly to the regulation of impulse control, with lesions of the region increasing premature responding (Muir, Everitt & Robbins, 1996), a finding recently replicated in a touch screen version of the task in mice (Hvoslef-Eide et al., 2018). The early notion that this region was implicated in impulsivity was supported by neuroimaging studies where reduced [^{14}C]-deoxy-glucose uptake, an index of active metabolism, in the anterior cingulate cortex (ACC) was related to increased premature responding on the 5-CSRTT (Barbelivien 2001). However, in another study, lesions of the pre-genual ACC did not increase premature responding, whereas lesions of the infralimbic (IL) cortex did (Chudasama et al., 2003). The implied notion of the IL cortex as a critical cortical region involved in impulse control was further supported by additional evidence showing an increase in premature responding after direct infusion of the NMDA receptor antagonists R-CPP (Murphy et al., 2005) and MK801 (Benn and Robinson 2014) in the region. The IL cortex has dense top-down connections with the ventral striatum (vStr), more specifically the nucleus accumbens shell (AcbS) (Vertes 2004). Convergent evidence points to abnormalities within the vStr of HI rats; with the vStr, including the AcbS and nucleus accumbens core (AcbC) having both been shown to play an important role in regulating action impulse control (Basar et al., 2010). Thus, lesions of the AcbC exacerbate impulsive responding, increasing premature responses, whereas lesions of the AcbS decrease amphetamine-induced premature responding (Murphy et al., 2008). This is further supported by evidence showing that deactivation of the AcbS through administration of the gamma-

aminobutryic acid $(GABA)_A$ receptor agonist muscimol reduced impulse control on the 5-CSRTT (Feja, Hayn and Koch 2014). The divergent roles of the nucleus accumbens (Acb) subregions in controlling impulsive responding is further supported by work showing that activation of the AcbS through deep brain stimulation increases impulsive responding (Sesia et al., 2008; 2010), whereas stimulation of the core subregion decreases impulsivity (Sesia et al., 2008).

One hypothesis based on these findings is that impulsivity on the 5-CSRTT may be mediated by a failure of the AcbC to appropriately gate behavioural responses driven by aberrant signalling in the AcbS. Consistent with this view, Caprioli et al., showed using non-invasive MRI and voxel-based morphometry a reduction in grey matter in the AcbC of HI animals. In the same study, HI animals also showed a reduction in dendritic spines and reduced levels of GABA decarboxylase – the main enzyme controlling the synthesis of the primary inhibitory neurotransmitter GABA in the same region (Caprioli et al., 2014). Using complementary, non-invasive spectroscopy imaging, Sawiak et al., reported that HI animals exhibit a reduced concentration of GABA in the Acb (Sawiak et al., 2016). These findings support the notion that impulse control deficits are mediated by aberrant dopaminergic, and possibly GABA-ergic signalling in the vStr. One such mechanism may be related to a decreased gating of AcbS output by signalling in the AcbC, leading to a net overall increase in signalling in the AcbS. A theory supported by reports showing that HI animals have reduced dopamine (DA) release in the AcbC (Diergaarde et al., 2008).

Beyond the vStr, impulsive responding on the 5-CSRTT has also been found to relate inversely to the thickness of the anterior insula (AI) cortex (Belin-Rauscent et al., 2016). Moreover, lesions of the AI selectively reduced impulsivity in HI rats but had no effect in mid- or LI rats (Belin-Rauscent et al., 2016), suggesting that the insula may act as an ancillary node in the network contributing to impulsive responding while not necessarily playing a critical role in independently controlling impulsivity. In addition, rats with lesions of the ventral, but not dorsal hippocampus showed increased premature responding on the 5-CSRTT (Abela et al., 2013). Moreover, disconnection of the prefrontal cortex (PFC) to ventral hippocampus pathway has been shown to increase premature responding on the 5-CSRTT (Chudasama, Doobay and Liu 2012). These studies suggest that PFC interactions with the hippocampus and vStr are central to the regulation of impulsivity. In line with this,

functional disconnection of the mPFC with the dorsal striatum (dStr) results in reduced performance on the 5-CSRTT and impaired inhibitory control (Christakou, Robbins and Everitt 2001). Excitotoxic lesions of the medial striatum also reduce discriminatory responding on the 5-CSRTT and increase the number of premature responses (Rogers et al., 2001). A more recent study using optogenetics and electrophysiology examined a dorsal PFC to dorsomedial striatum (DMS) circuit and highlighted its importance in regulating impulse control (Terra et al., 2020). Silencing of the circuit produced increased premature responses, with the amplitude and onset of activity related to inhibitory control, thus demonstrating a potential proactive inhibitory control circuit (Terra et al., 2020). These results suggest the key neural nodes in mediating premature responses on the 5-CSRTT include projections from the PFC to the striatum, and hippocampus. However, the circuit may also encompass other key regions, as other reports have highlighted the role of the basolateral amygdala (BLA) (Yin et al., 2019) and the subthalamic nucleus (STN) (Nishioka et al., 2020) in mediating impulsive action.

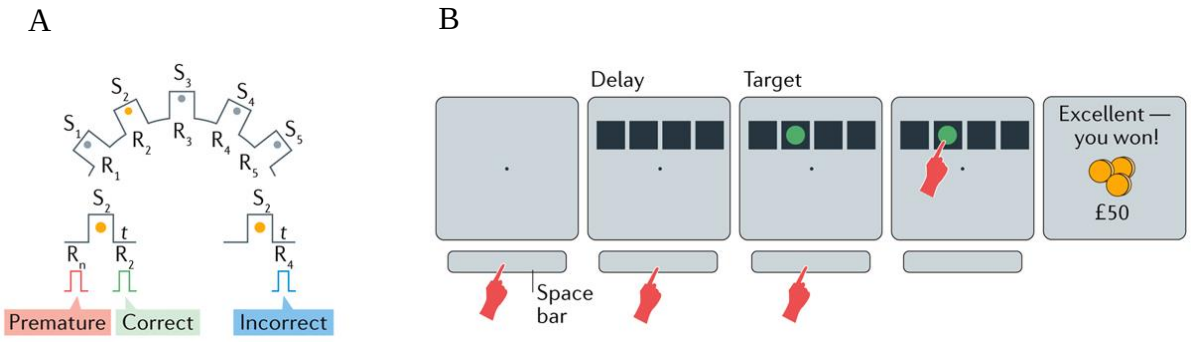


Fig1. Schematic overview of the **(A)** rodent 5-choice serial reaction time task and **(B)** human 4-choice serial reaction time task. **(A)** Rats are trained to nosepoke (R_2) in an aperture that is illuminated (S_2), deemed a correct response. Responses prior to illumination after a trial has started are deemed premature response (R_n). Incorrect responses also occur if a nosepoke is registered in an aperture that was not previously illuminated (e.g., R_1). **(B)** In the analogous task in humans, early space bar release after a trial has been initiated is deemed a premature response. Adapted from Dalley and Robbins 2017.

The 5-CSRTT has been modified and adapted for use in humans (**Fig1B.**) (Voon et al., 2014). The 4-CSRTT, specifically designed to measure premature responding, consists of a touchscreen visual display where four boxes appear on the screen to the subject. The appearance of the four boxes on the screen signals the initiation of the trial after which the

subject must press their dominant index finger to the space bar of the computer. Once the space bar has been pressed this indicates the cue onset time. After a waiting period, known as the cue-target interval, a green circular image appears briefly in one of the four square boxes on the touchscreen display in a random fashion. The subject must then release the spacebar and touch the box in which the green circular image was displayed (Voon et al., 2014). The primary measure of the 4-CSRTT is premature responding, indexed through early release of the spacebar by a subject during the trial and has clear and obvious parallels to premature responses as recorded in the rodent version of the task.

The neural networks subserving response inhibition on the 4-CSRTT have been mapped through the utilisation of human brain imaging (Morris et al., 2016). Using functional magnetic resonance imaging (fMRI), Morris et al., evaluated the neural networks that underlie inhibitory responses on the 4-CSRTT, as well as the SSRTT in healthy controls, binge drinkers and subjects with alcohol use disorder. Relative to healthy volunteers, binge drinking participants showed higher levels of premature responses on the 4-CSRTT. Using an *a priori* hypothesis, greater premature responding on the 4-CSRTT was negatively correlated with connectivity between the subgenual anterior cingulate cortex (sACC) (considered to be homologous to the IL cortex in the rat (Ongur and Price 2000)) and the STN. In addition, reduced connectivity between the right vStr and STN negatively correlated with impulsive responding. Comparatively, response inhibition in the SSRTT, as assessed through stop signal reaction time (SSRT) did not correlate with connectivity between the STN and sACC or vStr. This study offers the first insight into the neural mechanisms of premature responding on the 4-CSRTT, a novel human analogue of the rodent 5-CSRTT, and are consistent with previous reports in rodents implicating the IL cortex, vStr and STN (see above) as nodes in a central network underlying response inhibition on the 5-CSRTT. The observation that both forms of impulsivity assessed in this study, namely the SSRTT and 4-CSRTT, were non-overlapping both at the behavioural and neural level, draws obvious parallels with the rodent literature. Thus, HI animals on the 5-CSRTT do not differ in their SSRT (Robinson et al., 2008), suggesting a distinction between the two forms of impulsivity and reinforcing the notion of a multifaceted impulsivity construct (Dalley and Robbins 2017).

Another variant of the rodent 5-CSRTT has been developed by researchers at the University of Sussex, namely the Sussex 5-CSRTT (sus5-CSRTT). This is a direct analogue of the rodent 5-CSRTT task designed for use in humans (Sanchez-Roige et al., 2014). In the

simplified version of the task with fixed ITIs binge-drinkers displayed higher levels of premature responses when compared to non-binge drinkers. In the same study, increased premature responding was also seen in mice with a history of elevated alcohol consumption, thus highlighting the possible translational utility of the sus5-CSRTT in understanding impulse control deficits across species, and their relationship to drug use (Sanchez-Roige et al., 2014). Although the binge drinking group showed higher levels of premature responding on the sus5-CSRTT, no group differences were observed across any measure of the SSRTT, including SSRT, findings broadly like that observed in Morris et al., 2016.

1.5.2 Stop signal reaction time task

The SSRTT has been used extensively within the human and rodent literature to understand the relationship between impulsive action and substance dependence. Briefly, the SSRTT consists of a 'Go' signal and a 'Stop signal', temporally separated. In rodents, the basic task set up consists of two levers, adjacent to a central food magazine. The task is initiated by a nosepoke in the food well. One (either left or right) lever will then be presented for a short amount time. Contingent responses on this lever represent the 'Go' phase of the trial. After the lever has been pressed it is then retracted and the opposite lever is presented for a protracted amount of time (the limited hold period). A lever press on the second protracted lever will then lead to a successful completion of the run, and the rat is rewarded with a food reward. However, on several trials (commonly 20%), a tone is played during the presentation of the second lever. This tone is the 'Stop' signal and rats must refrain from responding on the second lever to gain a reward. If a rat responds on the second lever during the 'Stop' signal this is deemed an incorrect response and results in a time-out period, after which a new trial can be initiated (**Fig2A.**) (Eagle et al., 2008; Bari et al., 2011).

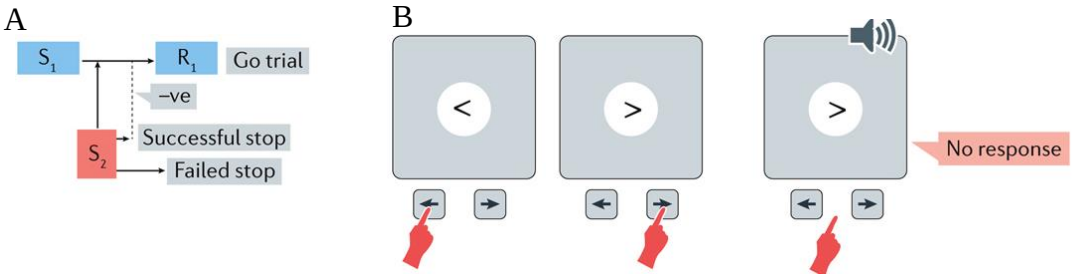


Fig2. Schematic overview of the **(A)** rodent and **(B)** human stop signal task. After the signal (S_1) the trial is initiated, and the subject is required to rapidly respond ($R_{1)}$. However, on a small number of trials, commonly 20%, after the trial has started a stop signal will occur (S_2). The subject will then have to abort their response to successfully complete the stop trial. The stop signal reaction time is then measured and used as index of impulse control. Adapted from Dalley and Robbins 2017.

The DMS, STN and orbitofrontal cortex (OFC) have been identified as key structures mediating impulse control on the SSRTT in rodents (Eagle et al., 2008; Bari et al 2011). Pharmacological inactivation of the DMS (Eagle et al., 2003), OFC (Eagle et al., 2008), Prelimbic cortex (PrL) and ACC (Bari et al., 2011) all increase reaction time. In contrast to premature responding on the 5-CSRTT, lesions of the AcbC and IL cortex had no effect on reaction time (Eagle and Robbins 2003; Eagle 2008), once again reinforcing the dichotomy between different neural circuits subserving non-overlapping forms of impulsivity. More recent evidence further supports the role of the OFC in mediating inhibitory control on the SSRTT. Using an optogenetic approach, Hardung et al., inhibited activity in the ventral OFC, among other regions, and found that animals displayed a significant impairment in their ability to respond rapidly to predictive cues, significantly increasing late responses during the short-delay trials (Hardung et al., 2017). In summary, the mPFC, and OFC play a pivotal role in mediating reaction time on the SSRTT, potentially through their connections with thalamic nuclei, and through various reciprocal connections with other midbrain regions such as the globus pallidus, substantia nigra and ventral pallidum (Izquierdo et al., 2017). Thus, impulsive responding may represent an imbalance in the relevant timing of signals between the STN, pallidum and substantia nigra pars reticulate (Schmidt et al., 2013), which may in turn be modulated by prefrontal cortical areas (Aron et al., 2016).

In humans, the basic task structure of the SSRTT is very similar to that of the rodent paradigm (**Fig2B.**). There are many variants of the task design, but commonly subjects must respond on either the left or right arrow of a keyboard to two distinct visual cues such as a triangle and a circle. For example, presentation of a triangle would necessitate a response on the left arrow, and conversely presentation of a circle on the right arrow. These trials occur around 75% of the total trial count, are known as the ‘Go’ trials, and require the subject to respond rapidly and accurately to the visual cue (Thakkar et al., 2013). The remaining 25% of the trials are known as the ‘Stop’ trials. After presentation of the stimulus a short tone is presented immediately signalling the ‘Stop’ trial instructing the subject to withhold their

response. In general, the SSRT measures the speed at which a subject can withhold an already initiated and engaged motoric response, and therefore differs from other forms of motoric impulsivity, such as anticipatory responding on the 4-CSRTT (Logan et al., 2014).

Impulse control on the SSRTT may be measured and calculated in a number of different ways. Classically, the independent race model has been successfully used to understand and analyze data from the task, capturing the main features of performance (Logan and Cowan 1984). The independent race model mainly concerns two processes; a go process, beginning when the go signal is presented and a stop process, beginning when the stop signal is presented. The race between the two processes (go and stop) is then analyzed. If the stop process finishes first this results in the participant stopping successfully (e.g., no response). If the go process finishes first this results in an unsuccessful attempt to stop and inhibition is not observed. The race model provides the theoretical framework to estimate the SSRT and has been successfully used to understand response inhibition. However, recent evidence calls into question the validity of the model under certain conditions (Bissett et al., 2021), with small changes in experimental design having a critical impact on the correct interpretation of the results. A recent consensus paper (Verbruggen et al., 2019) outlined several important caveats to consider when calculating SSRT. Both the mean and integration method of analysis were evaluated. Broadly, the integration method was more reliable and produced the least biased estimates of SSRT. Several important considerations must be met prior to analysis with either the mean or integration methods. For example, independence of the two processes (go and stop) must first be confirmed. Following this, calculation of SSRT is recommended to be carried out in individuals where p(response|signal) is between 0.25 and 0.75, with the level of go omissions capped at a certain level, with individuals over this level excluded (for more details see Verbruggen et al., 2019). Thus, the careful examination of response parameters and experimental set-up are critical factors that must be taken into account prior to any formal calculation of the SSRT and before any interpretation of the overall effect on impulse control.

The neural circuitry of stop signal response inhibition has been mapped using various non-invasive imaging techniques including magnetic resonance imaging (MRI) (Aron and Poldrack 2006; Aron, Robbins and Poldrack 2014), transcranial magnetic stimulation (TMS) (Chambers et al., 2006) and electroencephalography (EEG) (Huster et al., 2013). Using fMRI, Aron and Poldrack mapped the blood-oxygen level dependent (BOLD) signal in

healthy subjects undergoing the SSRTT. Stopping on the task significantly activated the right STN, the right inferior frontal cortex (IFG), right globus pallidus pars interna and right pre-supplementary motor area (pre-SMA) (Aron and Poldrack 2006). The role of the IFG in mediating stop-signal performance is a robust finding having been replicated across several studies using a number of different imaging modalities and study techniques including resting-state functional connectivity, structural imaging and in subjects with frontal lobe lesions (Aron, Robbins and Poldrack 2014; Swick, Ashley and Turken 2008; Sharp et al., 2010; Whelan et al., 2012; Aron et al., 2003). Although the exact rodent homologue of the IFG is unknown, a potential candidate may be the anterior rhinal cortex (Aron, Robbins and Poldrack 2014), a region situated just behind the lateral OFC (LatOFC). However, limited studies have interrogated this region with circuit mapping techniques, although, lesions of the OFC have shown to lead to longer SSRT (above), suggesting potential cross-species convergence.

1.5.3 Go-No/Go task

The GNGT has obvious parallels with the SSRTT. However, wherein the SSRTT the go stimulus is always shown first, with a stop signal presented on a number of trials after a short delay (the stop signal delay), in the GNGT a no-go signal is presented in place of a go signal on a defined percentage of trials. Therefore, the main difference in the tasks is that the inhibition signal is presented at different temporal scales relative to the go signal (**Fig3.**) (Raud et al., 2020).

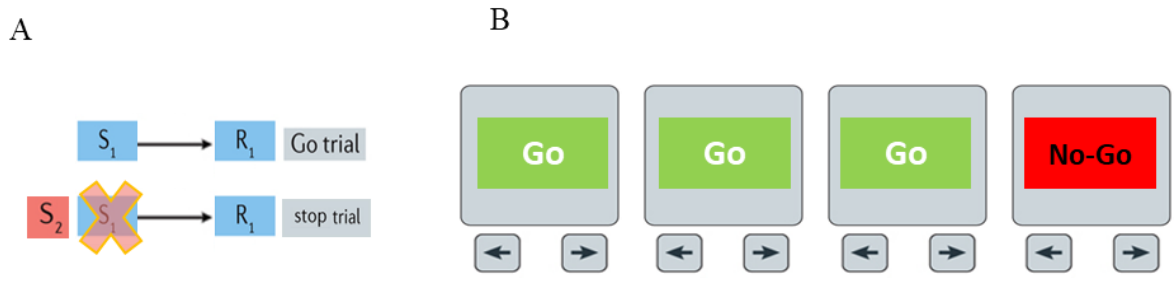


Fig3. Schematic overview of the **(A)** rodent and **(B)** human go-no/go task. After **Go** the signal (S_1) the trial is initiated, and the subject is required to rapidly respond ($R_{1)}$. However, on a small number of trials, commonly 20%, after a No/Go signal will occur (S_2). The subject will then have to abort their response to successfully complete the No/Go trial. A key difference in the go-no/task is that the No/Go signal is provided prior to the start of a trial. Therefore, subjects are not inhibiting an already initiated motor response as seen on the stop signal task. Adapted from Dalley and Robbins 2017.

Compared to the SSRTT, the GNGT has been less utilised in rodents to study action restraint and impulsive action. Nonetheless, several studies have highlighted overlapping and divergent neural constructs between the two behaviours. Thus, individual differences in DA transporter function in the OFC is related to increased impulsive action on the GNGT (Yates et al., 2016). Using deep brain stimulation in rodents, Anderson, Sheppard and Dorval showed that stimulation of the STN in control rats increased impulsive behaviour in the GNGT, as well as an alternative go/stop task (Anderson, Sheppard and Dorval 2020). Moreover, PFC-striatal connectivity increased directly prior to failures to respond to reward-paired tones in the GNGT, suggesting a possible top-down influence from the PFC to the striatum in action selection during ongoing behaviour on this task (Stubbendorff et al., 2019). In humans, event related fMRI has mapped the neural correlates of the go signal, the no-go signal, as well the preparation phases of the task (Watanabe et al., 2002). Activation of the thalamus, primary sensorimotor, ACC and cerebellar anterior lobule are associated with the go phase of the task, whereas activity in the middle frontal gyrus, occipitotemporal, dorsal premotor and posterior intraparietal regions are associated with the no-go phase of the trial (Watanabe et al., 2002). When comparing no-go to go contrasts, Horn and colleagues also found significant frontal lobe activation during response inhibition, with activation in the dorsolateral prefrontal cortex (dlPFC) and cingulate gyrus (Horn et al., 2003). The underlying neural correlates of response inhibition on the GNGT may be influenced by task design, and the complexity of the task. Relative to the 'simple' format of the task, with one go stimulus and a single no-go stimulus (Liddle, Kiehl and Smith 2000), more complex tasks have been used, including tasks with higher working memory load (Mostofsky et al., 2003), and varying complexities of stimuli presentation (Kelly et al., 2004; Garavan et al., 2002). A meta-analysis using activation likelihood estimation techniques investigated the neural circuitry of response inhibition on the GNGT (Simmonds, Pekar and Mostofsky 2008) and revealed a right-lateralised network, with activation in the pre-SMA, right prefrontal gyrus, bilateral inferior parietal, occipital regions, putamen and left premotor area associated with successful inhibition on the No-go trials. Comparing the complex *versus* simple task designs, only one region showed overlap in activation – the pre-SMA, suggesting a key role of this area in response inhibition irrespective of task demand (Simmonds, Pekar and Mostofsky 2008).

1.6 Impulsive choice

1.6.1 Delay discounting

As described above, impulsivity is non-unitary trait and can be separated based on a number of different criteria. One broad classification within the field is that of impulsive action (described above), and impulsive choice. When compared to impulsive action, impulsive choice is thought to predominantly reflect decision-making in situations of differing temporal delays to reward as well as processes under uncertainty of reward, or risk. Impulsive choice is commonly assessed using the DDT, rat gambling task and probability discounting task. Delay-discounting assesses the ability of a subject to choose larger but delayed rewards *versus* smaller but more immediate rewards. Essentially, the task is designed to assess the tolerance for delayed gratification and the ability to exert self-control over responding to reward at early temporal horizons (Winstanley, Eagle and Robbins 2006). In rodents, the task design often consists of two levers in an operant box, responding on one provides a small but immediate reward (e.g., 1 reward pellet), while the other lever delivers a larger food reward (e.g., 4 pellets), but only when pressed after a certain temporal delay (**Fig4.**). The temporal delay is increased throughout the session and the mean choice for the large reward, an index of impulsive choice, is calculated (Winstanley et al., 2006). Delay discounting curves can be generated from the DDT, and mainly reflect a hyperbolic function, with steeper discounting reflecting higher levels of impulsive choice.

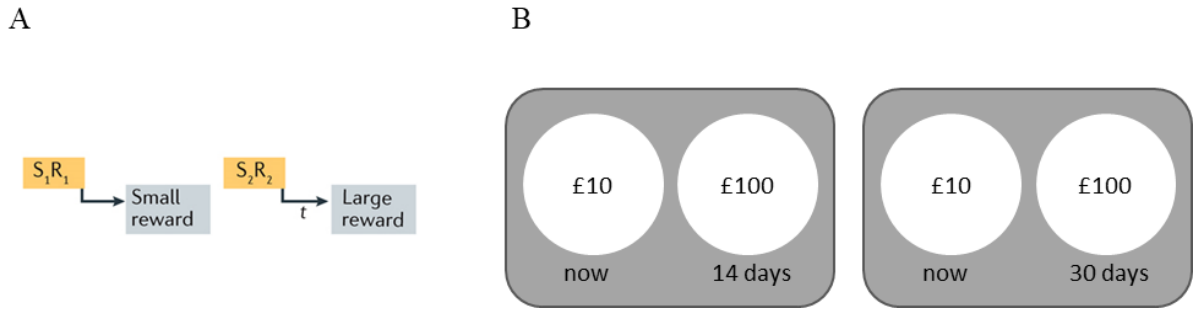


Fig4. Schematic overview of the **(A)** rodent and **(B)** human delayed discounting task. In rodents, only two stimuli ($S_{1,2}$) are presented sequentially in the delay discounting paradigm. In one instance, responding on one lever (R_1) after a short period of time has elapsed after stimulus presentation (S_1) will lead to a small reward (e.g., 1 reward pellet). Conversely, responding (R_2) after a delayed time (t) to an alternative stimulus (S_2) will result in a large reward (e.g., 2 reward pellets). In humans, monetary reinforcers are often combined with monetary reinforcers in a hypothetical setting. Adapted from Dalley and Robbins 2017.

Like measures of impulsive action, lesions of the AcbC have been shown to modulate impulsive choice. Lesions of the AcbC facilitated impulsive choice in rats, decreasing the preference for the larger, delayed reward (Cardinal et al., 2001). Much focus of neuroanatomical investigations on the DDT has fallen on the OFC. Thus, OFC lesions broadly increase impulsive choice in the discounting procedure (Mobini et al., 2002), however this may be dependent on the exact location of the lesion (Mar et al., 2011). Other regions, including the ACC (Cardinal et al., 2001; Rudebeck et al., 2006) and mPFC (PrL and IL cortex) (Cardinal et al., 2001) have no effect on discounting rates when lesioned. Other regions such as the hippocampus, and BLA have also been shown to influence discounting rate when lesioned (Winstanley et al., 2004; Cheung and Cardinal 2005), suggesting a putative circuit involving the OFC, striatum, amygdala, and hippocampus in mediating the propensity to choose delayed rewards in rodents.

The neural networks of impulsive choice have been mapped in humans through the advent of non-invasive imaging. Overall, activation of the PFC has been highlighted in several different human imaging studies evaluating choice impulsivity (Noda et al., 2020; Hamilton et al., 2015). Specifically, activation of the ventrolateral PFC (vlPFC), and dlPFC has been associated with choice for larger delays (McClure et al., 2004). The regional specificity of activation relative to delayed response gratification is further supported by the finding that disruption of the lateral PFC using TMS increased the choice for immediate rewards (Figner et al., 2010). Age-related behavioural changes in temporal discounting seen in development correspond, at the neural level, to increased activation of fronto-striato-parietal networks (Rubia et al., 2000; Christakou, Brammer and Rubia 2011) and further reinforce the important contribution of these regions in impulsive choice. As seen in rodents, ventral striatal activation also plays an important role in impulsive choice in humans (Hariri et al., 2006). However, preference for smaller, immediate rewards over larger delayed rewards was associated with increased activation of the vStr (Hariri et al., 2006), whereas AcbC lesioned animals increased their impulsive choices (Cardinal et al., 2001). However, the vast difference in the two techniques used to understand functional recruitment and the neuroanatomical basis of impulsive choice (e.g., brain imaging and excitotoxic lesions) are very different, and caution is advised when comparing the two reports. Nonetheless, in humans, impulsive choice is regulated through interaction between striatal and PFC regions, with other notable areas also being shown to play a role in discounting, such as the insula (Sellitto et al., 2015) and anterior cingulate (Frost and McNaughton 2017). OFC-striatal

circuits have also been implicated in the choice of immediate rewards, with significant activity in the LatOFC predictive of immediate reward (Tanaka et al., 2004), with obvious parallels to that of the rodent literature (Mar et al., 2011, but see Jo et al., 2013). A schematic overview of the key nodes in the neural networks across multiple forms of impulse response control in rodents and humans is provided in **Fig5.**

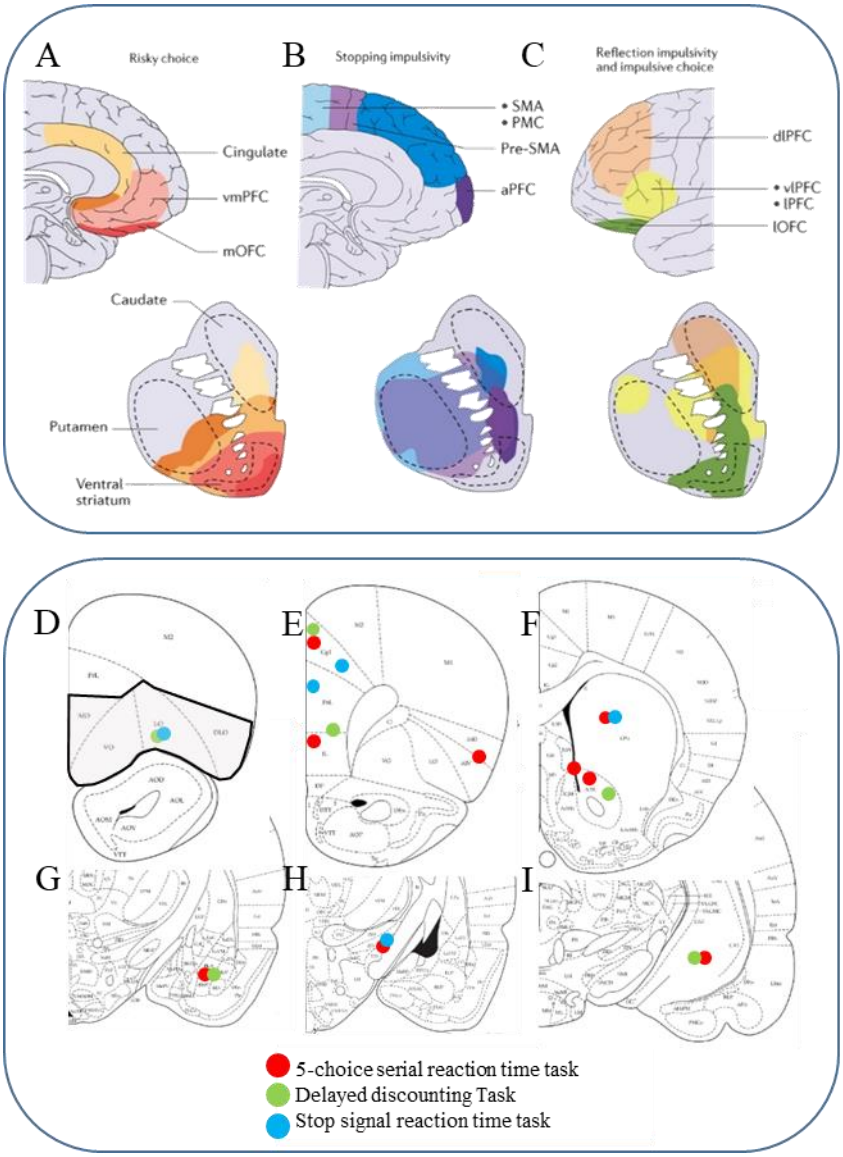


Fig5. Schematic overview of the **(A-C)** human and **(D-I)** rat cortical and subcortical circuitry and associated impulsivity constructs. **(A-C)** Overlapping prefrontal and striatal colour maps represent functional connectivity between regions. Topographic organization is observed between the prefrontal cortex and striatum for risky choice, stopping impulsivity, impulsive choice and reflection impulsivity in humans. **(D-I)** In rodents, the orbitofrontal cortex **(D)**, medial prefrontal cortex **(E)**, striatum **(F)**, basolateral amygdala **(G)**, subthalamic nucleus **(H)**, and hippocampus **(I)** have all been implicated across all three sub-dimensions of impulsivity. Adapted from Voon and Dalley 2016.

1.7 Attribution of incentive salience

Motivational cues within the environment that are associated with rewards can influence and exert control over behaviour, guiding an organism away from harm and towards rewarding outcomes such as food rewards. One of the most well-known examples of the ability of a CS in the environment to evoke a conditioned response (CR) is the case of Pavlov's dogs, and their salivation upon hearing the ringing of a bell (Pavlov 1902). Since this seminal discovery, research on the way cues in the environment guide behaviour and the role conditioned stimuli have in evoking complex motivational and emotional states, a process known as incentive sensitisation/salience, has been carried out (Robinson and Berridge 2001). The neural correlates of incentive salience and the sign-tracking phenotype have been mapped extensively using c-fos activation. In ST, higher levels of c-fos expression were observed in circuits encompassing the midline thalamus, amygdala, striatum and lateral habenula (Yager et al., 2015; Haight and Flagel 2014). Moreover, correlation between c-fos activity in the paraventricular thalamus (PVT) and the AcbS was also observed in ST, but not GT (Flagel et al., 2011). Thus, it appears that the level of c-fos activity is differentially modulated across ST and GT. For example, increased c-fos activation was observed in afferents from the amygdala and hypothalamus to the PVT in ST, whilst inputs from the PrL to the PVT showed greater activation in GT (Haight et al., 2017). Further evidence supports the role of the PVT as a central node in regulating conditioned responses to motivational cues. After phenotyping (ST/GT), lesions of the PVT increased sign-tracking responses only in GT, with no effect observed in animals classified as ST (Haight et al., 2015). More recent circuit mapping techniques using chemogenetics have shown that stimulation of the PrL to PVT pathway decreases conditioned approach in ST, whereas inhibition of the same circuit increased sign-tracking behaviour in GT (Campus et al., 2019). These results suggest that the PVT is critical in cue-motivated behaviours. One hypothesis put forward is that the PVT regulates attribution of cue salience in ST through its sub-cortical connections including that of the vStr and thalamus, whereas goal-tracking behaviour is regulating by top-down cortical control mechanisms of the mPFC including the PrL and IL cortices, and their connections to the PVT (Haight and Flagel 2014; Flagel and Robinson 2017). In short, aberrant attribution of incentive salience is thought to reflect an imbalance of bottom-up *versus* top-down control (Campus et al., 2019; Flagel and Robinson 2017).

Measuring the attribution of incentive salience, or appetitive conditioning in humans can be achieved through the utilisation of secondary reinforcers such as money (Austin and Duka 2010; Garofalo and Pellegrino 2015), or sexual imagery (Klucken et al., 2009). Another approach in humans is to use food (e.g., sweets or chocolate) (Burger and Stice 2014; Meyer et al., 2015). Importantly, the variation in task design and the specific cue used in the various reports make direct comparisons across studies challenging, as differences in neuronal activation have been observed dependent on primary or secondary rewards (Sescousse et al., 2013). Commonly, self-report questionnaires are used to assess the valence of the stimuli after the conditioning has taken place (van den Bosch et al., 2015). However, other studies have measured psychological responses such as heart rate, skin conductance, pupil size and pupil gaze (Wardle, Lopez-Gamundi and Flagel 2018). Turning to the underlying brain mechanisms, neuronal signalling in the striatum has been shown to play an important role in appetitive conditioning. Thus, signalling in the caudate nucleus is progressively increased after exposure to cues predicting milkshake reward and may reflect activation responsible for cue-reward learning (Burger and Stice 2014). In the same study, activation of the putamen and ventral pallidum was inversely related to repeated reward exposure. One recent study combined the evaluation of sign- and goal-tracking responses *via* eye-tracking with neuroimaging and behavioural modelling (Schad et al., 2019). Using eye-tracking and pupillometry, Schad and colleagues assessed the gaze response of healthy male subjects in a Pavlovian conditioning task with visual and auditory conditioned stimuli, paired with monetary reinforcers. As seen in the rodent task (Flagel et al., 2010), individual differences in sign- and goal-tracking responses were observed, with some participants showing a dominance in gaze towards one of the five conditioned stimuli (fractal-like pictures and tones) – a ST, *versus* gazing towards the US (in this case a picture of coins indicating the monetary win or loss) – a GT. In addition, ST showed increased instrumental approach responses on a Pavlovian instrumental transfer task, a finding with direct translation to that seen in the rodent literature (Robinson and Flagel 2009). In using computational modelling of the behavioural and eye responses, GT appeared to rely more on model-based learning, whereas ST appeared to rely more on model-free learning. Model-free learning is thought to underpin habit-based learning mechanisms and has been shown previously to be important in addiction (Groman et al., 2019). Conversely, model-based learning is thought to represent goal-directed learning and is more flexible. Model-based learning relies on complex internal states that depict how and in which way the environment may change allowing for a much wider repertoire of behavioural outcomes. In the study by Schad et al., ST relied more heavily

on model-free reward prediction errors signals for learning. The model-free prediction errors (computed as the trial-by-trial temporal difference in reward prediction error for conditioned and unconditioned stimuli) strongly predicted activation in the vStr, more specifically the right Acb in ST but not GT (Schad et al., 2019). These results parallel similar reports in rodents (Gillis and Morrison 2019) and provide evidence for an overlapping, translationally relevant neural substrate (Acb) of sign-tracking in humans and rodents. Moreover, these findings open important considerations between the hypothesised link of bottom-up *versus* top-down influences, and model-free *versus* model-based behavioural control in PavCA, with a possible neural locus of this effect being the vStr in ST.

1.8 Novelty/sensation seeking

Novelty/sensation seeking is defined as a tendency to pursue intense emotional sensations and novel experiences and refers to individuals who have exacerbated desires to experience novel stimuli and desire intense sensations. It is a multifaceted trait encompassing novelty-seeking, novelty-preference, harm avoidance and excessive risk-taking (Zuckerman, 1974; Cloninger et al., 1993; Carmen Arenas et al., 2016). In rodents, sensation-seeking is commonly assessed by the locomotor response of animals placed in a novel, inescapable environment (Dellu et al., 1996). Commonly, HR animals are separated from their LR counterparts by the amount of distance travelled within the test environment over the course of 2-hours (Piazza et al., 1989). Alternatively, the free-choice NPP paradigm has been employed to assess the choice between spending time in a novel, unfamiliar environment with that of a familiar, previously experienced environment (Belin et al., 2011), and represents a dissociable behavioural trait, when compared to sensation-seeking (Hughson et al., 2019). The first example of the importance of the HR phenotype in relation to drug SA was shown by Piazza and colleagues in 1989. Based on interindividual differences in LocR, HR animals showed a greater propensity to acquire psychostimulant SA (Piazza et al., 1989). At the neural level, convergent evidence points towards a dysregulated stress-response system in HR rats, implicating the hypothalamo-pituitary-adrenal (HPA) axis (Blanchard, Mendelsohn and Stamp 2009). Although at baseline levels, prior to locomotor assessment, no differences in corticosterone levels are observed between HR and LR animals, repeated cocaine administration and exposure to a novel environment (during the test) differentially modulated the endocrine response, with higher corticosterone responses observed in HR when compared to LR animals (Piazza et al., 1990; Dellu et al., 1996). These results, and

others (Piazza and Le Moal, 1998) support the view that HR and LR animals differ in their stress response, with HR animals showing increased magnitude and duration of endocrine release in response to novelty. The endocrine system, including the HPA axis, as well as the central amygdala (CeA) and hippocampus have an important role in modulating DA within the mesolimbic system (Ortiz et al., 1996; Bardo, Neisewander and Kelly 2013). The complex interplay between the mesolimbic DA system, the HPA axis, and exposure to novelty is yet to be fully elucidated, but evidence points towards a dysregulated DA system in HR animals, that may be modulated by the stress system, which as a result may be responsible for the differential rate of psychostimulant administration seen in these animals (Rouge-Pont al., 1998). Thus, modulation of DA signalling in the vStr, dStr, mPFC and ventral tegmental area (VTA) have all been implicated in the neural mechanisms responsible for mediating the HR phenotype (Flagel et al., 2014; Carmen Arenas et al., 2016). Evidence for the neural substrates of NPP have been less well studied. However, there is evidence to suggest that this behaviour relies on processing in the hippocampus, presumably due to the strong contextual (differences in colour/texture and openness) and spatial (different chambers) parts of the task (Mumby et al., 2002). In the PrL, overexpression of D1 receptors on cortical glutamatergic neurons increases the preference for the novel side in a novelty preference task but has no effect on locomotor activity (Sonntag et al., 2014). In line with this, DA uptake in the PFC is positively and negatively correlated with NPP and LocR, respectively (Zhu et al., 2007).

In humans, large-scale imaging studies have evaluated the underlying neural substrates of novelty seeking in adolescents (Qi et al., 2021). As part of the IMAGEN consortium, a large-scale project examining how psychological, biological and environmental factors during adolescence may influence brain development, Qi and colleagues investigated several networks in reward processing, impulsivity and novelty seeking in early adolescent subjects and asked whether these networks would predict the symptoms of a wide range of neuropsychiatric disorders. As early as 14 years old, multimodal markers of both structural MRI and fMRI were found in relation to novelty seeking, with differences seen in key areas of the reward networks such as the PFC, striatum, amygdala and hippocampus (Qi et al., 2021). Both within the same cohort, and in an independent sample, these network features significantly predicted a number of other disorders including attention-deficit hyperactivity disorder (ADHD), major depressive disorder (MDD) and schizophrenia, with a high degree of accuracy (Qi et al., 2021), highlighting the role of novelty-seeking, and the underlying

reward network as a transdiagnostic risk marker. In line with this network, another study showed that novelty seeking was associated with activation in the right striatum, supplementary motor area, and right posterior insula (Wang et al., 2015). Although novelty seeking was positively correlated with risk preference in this study, only right posterior insula activation significantly correlated with novelty seeking after controlling for the effect of risk preference. Moreover, right posterior insula and right striatal connectivity negatively correlated with novelty seeking. Although at the behavioural level it is clear that novelty seeking and risk-taking share common processes, it appears, at least at the neural level, that these constructs are dissociable (Wang et al., 2015). As in rodent studies, the striatum appears to be a central node in the network controlling response to novelty. Thus, decreased connectivity to the vStr from the posterior cingulate cortex (PCC) is related to higher response to novelty in humans (Zhang et al., 2017), and increased volume of the bilateral caudate has been shown to be associated with higher novelty seeking scores (Laricchiuta et al., 2014). The AI and dorsal anterior cingulate both form key nodes of the salience network (Seeley et al., 2007). The salience network is thought to be responsible for the integration of highly salient stimuli within the environment, integrating sensory data with interoceptive autonomic processing and mediating appropriate responses to external stimuli. The dysregulation of signalling in these regions has been linked to several psychiatric disorders (Uddin 2015) and more recently to novelty seeking and salience expectancy (Li et al., 2017). Using a salience expectancy task, Li and colleagues investigated functional connectivity differences in relation to high or low salience expectancy across novelty seeking. They found reduced functional connectivity between the right AI and right middle cingulate cortex was related to increased preference for novelty, thus shedding light on the potential important role of the salience network in mediating preference for novelty (Li et al., 2017). More recently, evidence for a role of the salience network in rodents has been established (Rohan et al., 2021). Thus, female rats with overexpression of D1 receptors in the PrL showed deactivation of the BOLD responses related to novelty preference within the insula cortex. However, novelty scores were not correlated with activation in control rats (Rohan et al., 2021), and the deactivation was dependent on the precise cortical location (seen in the dorsal agranular insular cortex and not the ventral insular cortex). Nonetheless, this study does provide tentative cross-species evidence to support the role of the salience network and associated structures in novelty seeking, although further research is needed at this stage.

1.9 Cognitive flexibility

Cognitive flexibility has commonly been assessed in rodents and humans using deterministic and probabilistic RL, among other paradigms. In short, RL involves the repeated pairing of an action with an outcome (Nilsson et al., 2015), e.g., physical interaction (right hand side lever press or touch of a screen in relation to a specific visual cue), with a food reward. After repeated training sessions and when an appropriate accuracy criterion is met the reversal phase is implemented. During reversal the previous action no longer delivers the expected outcome, and the subject must flexibly respond to the changing condition, e.g., physical interaction (left hand side lever press or altered visual cue) with a food reward. Optimal RL requires the ability of the subject to withhold previously trained responses and adapt to changes in reward contingency quickly and effectively. Typically, cognitive flexibility is assessed in a test session with several reversals and changes in reward contingency. Cognitive flexibility is widely accepted to depend upon the PFC, more specifically the OFC, and the caudate nucleus (Izquierdo et al., 2017).

Although there is a paucity of studies that have used MRI to evaluate the underlying neural correlates of RL in rodents, a wide range of studies have used pharmacological manipulation and excitotoxic techniques to evaluate the importance of multiple brain regions in successful RL performance (Izquierdo et al., 2017). More recently, circuit mapping techniques such as optogenetics and chemogenetics have also been used to probe the underlying pathway-specific contribution of certain prefrontal, amygdala, and striatal regions to RL performance in rodents (Groman et al., 2019). Broadly, research in rodents has implicated the OFC as a critical and important brain region necessary for task performance. Lesions of the OFC consistently impair task performance (Schoenbaum et al., 2002; McAlonan and Brown 2003; Bissonette et al., 2008). The OFC has been attributed to being involved in many different functions including response inhibition (Izquierdo and Jentsch 2012), economic value expectancies (Padoa-Schioppa and Assad 2006), encoding expected or predicted outcomes (Gottfried, O'Doherty and Dolan 2003) and the utilisation of value information, of which it tracks across RL. More recently, the idea that the OFC may represent a cognitive map of task space has been put forward (Wilson et al., 2014). Under this theory, the OFC represents an abstraction of currently available information of a task and integrates task relevant states that may be inferred and depend on functions that are not externally available to the agent such as explicit sensory information and working memory (Niv 2019). A recent study paired an odour-guided choice task with different action-outcome contingencies, and therefore different

task states, with single-unit recording in the LatOFC to understand the neural correlates of different state representation within this region (Stalnaker, Raheja and Schoenbaum 2021). The authors found that neural firing within the LatOFC could be successfully decoded and significantly represented information about the task. Moreover, these neural representations were seen across the entire trial, and the integration of the signal across states predicted how quickly the rat adapted to the new state suggesting that the OFC, and the representations within it, are important in decision-making and guiding appropriate action (Stalnaker, Raheja and Schoenbaum 2021). Due to its extensive set of connections, the OFC is well placed to allow the seamless integration of a wide range of inputs, including sensory information, learning and memory information. Moreover, the OFC may modulate actions through its reciprocal connections with the striatum and amygdala, regions critical for the representation of reward and motor output. Groman et al., used a pathway specific modulation technique and investigated the role of LatOFC to amygdala, or LatOFC to Acb circuits on RL in rats (Groman et al., 2019). Using a reinforcement learning model to evaluate the computational dynamics of the rats' choice in the task, ablation of the OFC-Acb and BLA-OFC pathway disrupted learning from negative and positive feedback, respectively (Groman et al., 2019). Other evidence points towards the role of the DMS in controlling flexible behaviour on the RL task. Loss-of-function studies using lesion techniques show that the DMS plays a pivotal role in cognitive flexibility (Ragozzino 2007; Castane, Theobald and Robbins 2010), with inactivation of the region impairing performance. The medium spiny neurons in the DMS can largely be separated into those that form functional units of the direct and indirect pathways. Subpopulations of MSNs in specific pathways can be interrogated using cell-specific and pathway-specific optogenetic and chemogenetic tools. Thus, optogenetic stimulation of the indirect pathway increases the probability of switching after a loss, whereas activation of the direct pathway increases the probability of selecting the same action after a rewarded trial (Nonomura et al., 2018). Moreover, in a two-choice RL task optogenetic inhibition of indirect striatal neurons in the DMS impaired response flexibility (Peak et al., 2020). The DMS and dorsolateral striatum (DLS) have been suggested to be critical nodes responsible for goal-direct and habit-based systems, respectively. In other terms, the DMS system reflects a model-based system, with the DLS reflecting model-free control. The balance between the two systems, whether biased towards more goal-directed or more habitual responding is thought to reflect different strategies of behavioural control, where behaviour can be biased towards one system (Khamassi and Humphries 2012; Lucantonio, Caprioli and Schoenbaum 2014). It is thought that both systems operate in parallel, and the balance between the two

systems is mediated through experience. Thus, whilst the balance towards the DMS and goal direct system dominates early on in learning, eventually this dominance shifts towards the DLS after extended training. Recent evidence has indeed suggested a dynamic interaction between the two regions, with DLS (in)activity also present during early stages of behavioural training. Using *in-vivo* single-unit recording, Bergstrom et al., recorded neural activity in the DLS across changes in stimulus-reward contingencies in a touchscreen-based reversal task in mice (Bergstrom et al., 2020). Prior to a choice being made, global activity in the DLS shifted from excitation to inhibition, with a large shift indicative of improved reversal. This was observed at early stages of the task, prior to reversal so is less likely to reflect a suppression of an old contingency (as none would have formed yet) but may reflect a dynamic balance of activity typifying a weakened contribution of DLS control to behavioural output, perhaps with a dominance towards the DMS when behaviour is still presumably under goal-directed control at early stages of training (Bergstrom et al., 2018; 2020). Evidence supporting the role of the ventral areas of the striatum, including the AcbC and AcbS, in cognitive flexibility is equivocal, with lesions of the vStr showing no effect on flexibility (Schoenbaum and Setlow 2003; Castane, Theobald and Robbins 2010), and pharmacological inactivation of the region disrupting spatial discrimination (Annett, McGregor and Robbins 1989). The different subregions of the Acb may account for the disparate effects observed. Indeed, inactivation of the AcbS impaired reversal performance in a probabilistic RL paradigm, whereas performance was unaffected in rats with AcbC inactivation (Dalton, Phillips and Floresco 2014). The IL cortex has dense connections with the AcbS (Vertes 2004), however, its involvement and more generally the precise involvement of the mPFC in RL remains open (Hamilton and Brigman 2015). The mPFC has been suggested to play an important role in controlling stimulus detection, outcome monitoring and action timing (Laubach, Caetano and Narayanan 2015). The ACC has been recently shown to be important in guiding choice through action-state transitions (Akam et al., 2021), akin to that observed in the OFC (Wilson et al., 2014). Combining a novel two-step decision-making task in mice with optogenetics and calcium imaging, Akam et al., showed that the ACC is a critical node in model-based, goal-directed control. Moreover, optogenetic inhibition of the ACC decreased the degree to which action-state transitions guided behaviour, irrespective of the reinforcing properties of reward (Akam et al., 2021). There is evidence to suggest that functional connectivity between the ACC and OFC is relevant for value-based decision-making (Fatahi et al., 2018), suggesting that the OFC (Wilson et al., 2014), and ACC (Akam et al., 2021) may both play an important role in the representation of latent task structure,

potentially with their interaction with the hippocampus (Miller, Botvinick and Brody 2017), however the precise mechanisms by which these structures interact to guide behaviour are still unknown.

In humans, several methods have been used to understand the neuroanatomical basis of cognitive flexibility. One method for understanding brain function is through the evaluation of task performance in subjects with focal brain lesions, or subjects with traumatic brain injury. Dependent on the location of the lesion or damage site, investigators can probe the underlying contribution of this region to atypical flexibility. Through these methods, a broad role of the frontal cortex, including the mPFC and OFC, as well as striatal regions have been implicated in controlling cognitive flexibility (Hornak et al., 2004; Cools, Ivry and D'Esposito 2006; Fellows and Farah 2003). An alternative avenue to investigate cognitive flexibility is by mapping the BOLD signal in participants during performance of a RL task, assessing relative activity, through which reverse inference to the neuroanatomical importance of these regions in relation to behavioural control may be made. Although many different task designs have been used, each emphasising distinct parts of the decision-making process, activity within the vlPFC, OFC and striatal areas have largely correlated with performance on task-based fMRI studies involving cognitive flexibility (Highgate and Schenk 2020). Using a probabilistic RL paradigm, Cools et al., showed that activation of the right vlPFC and vStr was associated with switching after reward contingencies were changed (Cools et al., 2002). Similarly, using a facial expression matching reversal task, Kringelbach and Rolls showed that the OFC, ACC and the vStr were activated at the time of reversal (Kringelbach and Rolls 2003), and Leber and colleagues showed that cognitive flexibility was significantly predicted by activation of the ACC, PFC, posterior parietal cortex and basal ganglia (Leber, Turk-Browne and Chun 2008). As in the rodent literature, both the PFC and striatum are key nodes in a cortico-striatal network governing cognitive flexibility. Indeed, this notion was investigated by perturbing the frontal cortex using a TMS protocol and measuring the BOLD response in subjects performing a cognitive switching task (van Schouwenburg et al., 2012). The authors showed that pertubations of the PFC, which has been shown to influence dopaminergic signalling in the striatum (Strafella et al., 2003), decreased functional connectivity between the PFC and putamen, reducing putamen activity, and modulating task performance (Schouwenburg et al., 2012). This study, and others (Nagano-Saito et al., 2008) suggest that the integrity of the cortico-striatal circuitry is critical for flexible behaviour, possibly through dopaminergic mechanisms. Although the precise role

of the PFC is still debated, in alignment with rodent studies, the OFC (Schuck et al., 2016), ACC (Heilbronner and Hayden 2016), along with the hippocampus (Garvert, Dolan and Behrens 2017) have been shown to independently represent abstract representations of task space across a number of different cognitive function tasks and are both necessary and critical for the control of cognitive flexibility and appropriate motor response in humans. It has been suggested (Wilson et al., 2014) that the OFC may be one structure in the part of a network governing cognitive control, including the ventral and dorsal striatum, VTA and the substantia nigra pars compacta. It would be reasonable from the above evidence to also include the ACC and hippocampus in this network. The hippocampus encodes navigation strategies through the generation of cognitive maps of spatially tuned cells such as place and grid cells (O'Keefe and Nadel 1978). The notion that the hippocampus may also be important for the generation of abstract, domain-general cognitive maps has also been put forward (Cohen and Eichenbaum 1993; Eichenbaum 2004; Rolls and Kesner 2006). Park et al., showed that the hippocampal-entorhinal region in humans, as well as the ventromedial PFC (vmPFC) and OFC is important for the integration of separately learnt cognitive schemata into a unified, multi-dimensional representation. This representation is important in allowing one to make transitive inferences and consequently, is important in adaptive decision-making and novel inference (Park et al., 2020). However, the precise mechanisms by which grid cells in the hippocampus encode representation of higher-order task structure is still an active area of research (Bellmund et al., 2018). In summary, cortico-striatal connections from the PFC and ACC play an important role in guiding flexible actions. Moreover, the hippocampus, as well as the amygdala and wider basal ganglia circuitry also likely contribute to RL performance.

1.10 Modelling drug addiction in rodents

Modelling a complex neuropsychiatric disorder such as addiction in experimental animals is extremely challenging. Whilst an animal model can never fully recapitulate the complex environment of the human condition, experimental approaches in rodents have become increasingly sophisticated to model the multifaceted, progressive nature of addiction. In the 1960's, Weeks first reported the use of the SA paradigm in which unrestrained, freely moving rats were implanted with an intravenous catheter in which morphine was delivered on a fixed-ratio 1 (FR-1) schedule (1 lever press = 1 infusion) of reinforcement (Weeks 1962). This seminal work showed that rodents will readily self-administer drugs of abuse commonly

abused by humans and laid the foundations for later studies assessing the neural substrates of drug-taking behaviour and aberrant drug reinforcement mechanisms in experimental animals.

1.10.1 Escalation

Loss of control of drug use is a central feature that emerges over the development of drug dependence (Wee, Specio and Koob 2007). In rodents, loss of control, or escalation, is typically measured by varying the time in which animals are allowed to self-administer the drug and examining the number of infusions taken in this extended time period. Commonly, this is done under a FR-1 schedule of reinforcement with sessions consisting of a short access period (ShA) (e.g., 1-hour) and a long access period (LgA) (e.g., 6- or 12-hours) (Rotge et al., 2017; Ahmed and Koob 1998). Extended exposure has been shown to steadily increase the rates of responding under these conditions. Thus, LgA rats increase cocaine SA across several sessions, and importantly, when compared to ShA rats have higher rates of responding during the first hour of the extended session (Ahmed and Koob 1998). Even though the escalation procedure only captures one aspect of drug addiction – a dramatic increase in drug consumption - it has been associated with other diagnostically relevant addiction-like behaviours, although there does appear to be some inconsistencies within the literature. For example, escalation of cocaine has been associated with increased motivation to self-administer drugs, with increased rates of cocaine intake related to higher breakpoints for cocaine under a progressive ratio schedule of reinforcement (Paterson and Markou 2003; Ducret et al., 2016), whilst other studies have observed no relationship between escalation and breakpoint (Liu, Roberts and Morgan 2005). Moreover, a sub-population of rats with extended access to cocaine show increased resistance to footshock punishment (Pelloux, Everitt and Dickinson 2007). However, this effect was observed in a subgroup of rats with escalation experience (i.e., not all animals that escalated use subsequently developed compulsive cocaine seeking), therefore suggesting that escalation was not the only factor driving compulsive use in this study. Indeed, other studies have shown that under punishment conditions both LgA and ShA groups show similar suppression of responding (Ducret et al., 2016), thus suggesting that escalation alone is not sufficient for the development of sustained drug use under punishment. The escalation paradigm has been used to probe the relationship between pre-existing vulnerability traits and increased drug consumption. Thus, animals classified as HI on the DDT show increased levels of cocaine consumption under LgA conditions (Anker et al., 2009). Moreover, using an intermittent access and withdrawal paradigm, Dalley et al., showed that HI animal, as assessed through the 5-CSRTT, showed

escalation of cocaine self-administration compared with LI animals (Dalley et al., 2007). Together, these studies implicate impulsivity as a vulnerability factor for the escalation of cocaine intake. Several studies have evaluated the underlying neural correlates of stimulant escalation largely implicating the insula (Rotge et al., 2017; Joshi et al., 2020), amygdala (Schmeichel et al., 2017), thalamus (Pelloux et al., 2018) and Acb (Guillem, Ahmed and Peoples 2014). In addition, bilateral excitotoxic lesions of the AI differentially modulate the propensity to escalate cocaine intake during extended access, with pre-behavioural lesions exacerbating cocaine intake (Rotge et al., 2017).

1.10.2 Relapse

Maintaining abstinence from illicit drugs once dependent is extremely difficult, with low retention and completion rates observed in treatment programmes (Simpson et al., 2002). Even after extended periods of abstinence, experiences of stress, negative feelings and interpersonal conflicts can trigger relapse (Yang et al., 2015). In the laboratory, one of the most common models used to assess relapse is the extinction-reinstatement paradigm (de Wit and Stewart 1981; Shaham et al., 2003). Although many versions of the task have been developed, with varying procedural differences, most commonly rats are trained to self-administer cocaine under a fixed ratio (FR) schedule of reinforcement (commonly FR-1) for a number of weeks, where drug delivery is explicitly paired with a stimulus (commonly a lever press paired with a light cue). Once the rats have reached appropriate levels of responding for the drug during the SA procedure, they then undergo extinction training. During extinction training responses on the previously reinforced lever no longer result in drug infusion. Under these conditions, rats progressively decrease their levels of responding, resulting in low levels of active lever presses (ALP), until a minimum (experimenter-controlled) criterion is reached. Reinstatement of the instrumental response can then be investigated and is commonly done so after 1) administration of a priming dose of the drug, 2) in relation to drug-paired CS presentation, 3) administration of stress-inducing compounds or 4) introduction of contextual cues (Bossert et al., 2013; Marchant, Li and Shaham 2013). The neural networks involved in reinstatement have been largely delineated, with circuits broadly forming the PFC, striatum, amygdala, lateral septum and the hippocampus (Farrell, Schoch and Mahler 2018; Dong et al., 2017; Kalivas and McFarland 2003).

1.10.3 3-criteria model of addiction

The 3-criteria model is a preclinical model of addiction adapted and operationalised from the DSM criteria. The 3-criteria model includes clinically relevant addiction-like behaviours such as 1) loss of control over drug-seeking or an inability to refrain from drug-seeking, assessed by measuring active lever presses for drug during an explicitly signalled no-drug period, 2) continued use despite punishment, assessed through the contingent presentation of footshock punishment during drug-taking and 3) increased motivation to obtain the drug, assessed through the use of a progressive ratio schedule of reinforcement (Deroche-Gamonet et al., 2004; Belin et al., 2008). The 3-criteria model is a chronic SA model designed to assess drug-taking behaviour, and not drug-seeking, and is commonly carried out over the course of several months. Upon completion, animals are scored independently on each addiction-like behaviour (above), and the highest percentile (33% in the case of Deroche-Gamonet et al., 2004) is used to classify animals in to 4 categories (0, 1, 2, 3 criteria). Animals with high scores across each addiction-like behaviour are classified as 3-criteria and are thought to best represent the behaviours observed in the human condition. On the other hand, rats classified as 0-criteria have low scores across each addiction-like behaviour and are typically classified as being resilient to addiction (Belin et al., 2008). Importantly, Belin and colleagues have used the 3-criteria model to investigate and tease apart the underlying contribution of various vulnerability traits to the development of addiction-like behaviour in the rat. Thus, pre-existing high levels of impulsivity, as assessed through the 5-CSRTT, as well as pre-existing high levels of novelty preference, as assessed through a NPP procedure, both predict the development of cocaine addiction-like behaviour (Belin et al., 2008; 2011). Using this model, high spontaneous levels of activity in response to novelty predicted the acquisition of cocaine SA but was not related to the propensity to develop addiction-like behaviour (Belin et al., 2008), further shedding light on the individual contribution of different behavioural endophenotypes to distinct stages of addiction. Due to the length of the experiment and the complexity of catheter patency over long durations relatively few studies have evaluated the underlying neural substrates of 3-criteria rats. Nonetheless, using this model, Kasanetz et al., evaluated synaptic plasticity mechanisms in the Acb after short- and long-term cocaine SA in 3- *versus* 0-criteria animals. During early cocaine exposure, NMDA receptor (NMDAR)-dependent long-term depression (LTD) was supressed in the AcbC in all animals. However, after prolonged exposure to cocaine, 3-criteria rats showed persistent impairment in NMDAR-dependent LTD, whereas the ability of 0-criteria rats to generate NMDAR-

dependent LTD was progressively recovered (Kasanetz et al., 2010). Utilising the same paradigm, 3-criteria rats show impairments in $mGluR_{2/3}$-dependent LTD mechanisms in the dorsomedial prefrontal cortex and reduced $mGluR_{2/3}$ protein expression. Furthermore, AMPA/NMDA ratio, a measure of synaptic strength, is increased in 3-criteria rats in the PrL (Kasanetz et al., 2013). These studies suggest an important role for glutamatergic signalling in mediating addiction-like behaviours in rodents through dysregulated synaptic plasticity mechanisms (Kalivas 2009). Importantly, these mechanisms take place in key nodes of the cortico-striatal network, potentially mediating addiction-like behaviour through top-down cognitive control mechanisms from the PFC to the striatum through glutamatergic mechanisms.

1.10.4 Seeking-taking chain schedule

The heterogeneous seeking-taking chain schedule has been successfully used to assess drug-seeking behaviour. Under this schedule, animals seek drugs by pressing on a 'seeking' lever under a random interval (RI) schedule of reinforcement in order to gain access to a 'taking' lever, pressing on which (usually under an FR-1 schedule of reinforcement) results in the administration of the drug (Olmstead et al., 2000). Because of the two levers, the act of drug-seeking and drug-taking is divorced and are clearly separated into two distinct behaviours (Olmstead et al., 2000). Without the introduction of direct or indirect punishment, chain schedules can be used to evaluate the effects of prolonged exposure to cocaine on the development of habitual drug-seeking behaviour. Thus, Zapata and colleagues showed that extended training in the chain link schedule rendered animals less sensitive to extinction of the drug-taking link (Zapata, Minney and Shippenberg 2010), suggesting that after prolonged exposure cocaine seeking had come under control of the habit-based system. This notion was further supported in the study by Zapata and colleagues as transient inactivation of the DLS, a region known to be critically involved in habit formation (Yin, Knowlton and Balleine 2006), rendered extensively trained animals sensitive to the extinction of the taking lever and significantly reduced their seeking response for cocaine (Zapata, Minney and Shippenberg 2010). Modelling compulsive drug-seeking can be carried out using the seeking-taking schedule by the introduction of environmental adversity and negative consequences in the form of intermittent and unpredictable mild punishment, with responses that meet the RI requirement in the seeking link resulting in probabilistic delivery of footshock and a time-out period (Pelloux, Everitt and Dickinson 2007; Pelloux et al., 2012). Other investigations have used the presentation of a shock-paired CS as a way of probing the ability of conditioned

punishment signals to suppress compulsive cocaine seeking (Vanderschuren and Everitt 2004). In both contexts, resistance to punishment develops over the course of extended training and access to cocaine where it is thought that under these conditions drug-seeking behaviour is dominated by stimulus-response associations and is under habitual control. Only a small proportion of rats (~20%) trained under this schedule show resistance to punishment, suggesting the important role that drugs of abuse may have in interacting with pre-existing vulnerability endophenotypes to drive compulsion, and highlights the utility of this paradigm in investigating interindividual differences in the vulnerability to develop addiction-like behaviour. The underlying neurobiological substrates of compulsive drug-seeking have largely implicated regions including the PFC (Chen et al., 2013), striatum (Giuliano, Belin and Everitt 2019) and the amygdala (Sun and Yuill 2020; Xue, Steketee and Sun 2012). Similar to that observed in 3-criteria rats (Kasanetz et al., 2013), aberrant synaptic plasticity in the PFC, more specifically PrL cortical hypoactivity is also observed in rats who compulsively seek cocaine in the seeking-taking task (Chen et al., 2013). More recently, it has been shown that alcohol-seeking, but not taking, during delivery of probabilistic foot shock punishment in the seeking-taking chain schedule paradigm becomes progressively dependent on the anterior dorsolateral striatum (aDLS) after extended training (Giuliano et al., 2019). Furthermore, individual differences in the development of compulsive alcohol-seeking appear to be dependent on the susceptibility to aDLS DA receptor blockade. Animals that showed a higher reliance on aDLS dopaminergic mechanisms were more vulnerable to develop compulsivity. The functional transition to drug-seeking habits and punishment resistance may, in part, depend on the recruitment of the aDLS *via* interactions in neuronal signalling in the amygdala and Acb (Murray et al., 2015), as inhibition of the CeA has been shown to impair the ability of probabilistic foot shock to suppress cocaine seeking (Xue, Steketee and Sun 2012).

1.10.5 Second order schedule of reinforcement

Drug-seeking behaviour can also be assessed using a second order schedule of reinforcement (^{2nd}SOR). In a ^{2nd}SOR, delivery of the drug is prevented until a certain time has elapsed - the fixed interval (FI) (usually 15 minutes). During the 15-minute interval, responding on the active lever every nth time (the FR schedule) results in a presentation of a drug associated CS, usually a light cue. Once the 15-minute interval has elapsed, nth lever presses will result in delivery of the drug. The interval is then reset, and the animal is forced to wait another 15

minutes before further drug administration (Everitt & Robbins 2000). During the prolonged drug-free periods (the FI) the drug-associated CS takes a pivotal role in maintaining and invigorating drug-seeking responses, with levels of seeking dramatically contingent on stimuli presentation (Belin and Everitt 2008; Arroyo et al., 1998). Importantly, the distinction between instrumental responding during the first drug-free interval, and subsequent intervals is made, the former behaviour being directly under control of the conditioned reinforcing properties of the drug CS and represents drug-seeking in a drug-free state, the latter behaviour influenced also by the rate-altering effect of the drug such as increased locomotor sensitisation after psychostimulant administration. At the neural circuit level, cocaine seeking after extended training is associated with a progressive shift in control of behaviour from the ventral to dorsal striatum. Spiralling pathways between midbrain DA neurons and striatal subregions (Haber et al., 2000; Ikemoto 2007) are hypothesised to underlie the shift in behavioural control over drug-seeking (Everitt et al., 2008) with established seeking responses eventually transferring to the dStr and a habit-based system (Belin et al., 2013). Thus, disconnecting intra-striatal connectivity through specific lesions of the AcbC and DLS infusions of the DA receptor antagonist flupenthixol decreased cocaine seeking in rats extensively trained under a ^{2nd}SOR (Belin and Everitt 2008). Consistent with this shift, earlier seminal studies remarkably demonstrated that neurochemical and metabolic markers in the dStr were affected by chronic, but not acute, cocaine SA in non-human primates (Letchworth et al., 2001; Porrino et al., 2004). Moreover, phasic DA release decreased in the ventromedial striatum (VMS), and increased in the DLS after several weeks of cocaine exposure (Willuhn et al., 2012), while DA release in the dStr, but not the vStr, was evoked by response-contingent, drug-associated stimuli, during well-established cocaine seeking (Ito et al., 2002).

1.10.6 Compulsive cocaine seeking – a novel paradigm

Between 2014 - 2017 Dr. Maxime Fouyssac and colleagues from the Belin Laboratory embarked on designing a novel behavioural paradigm to assess compulsive heroin seeking (see Chapter 3, section 3.7 for details). The model successfully builds on the framework of the ^{2nd}SOR paradigm (above), implementing a deterministic punishment schedule during seeking to assess compulsivity. Unlike the multi-symptomatic 3-criteria model, the paradigm is uniquely positioned to assess the maintenance of drug-seeking in the face of negative consequences. Due to the analgesic properties of heroin and the confounding effect this may have on the interpretation of responding during foot shock presentation, the paradigm

consists of distinct punished and unpunished seeking periods within one interval period. Thus, within the first 8 minutes of the interval seeking responses are not punished, whereas during the last 7 minutes of the schedule seeking is punished with the advent of electric foot shock. In collaboration with the Belin laboratory, this model was used to assess the relationship between several behavioural traits previously linked to addiction-like behaviour and the development of compulsive cocaine seeking.

1.11 Vulnerability to drug addiction – neuroimaging in humans

In humans, compulsive drug use may arise from both pre-existing and drug-induced abnormalities in brain function. To what extent brain function is altered prior to drug exposure or is a result of the neurotoxic effects of the substance is still an open question. Most human neuroimaging studies in stimulant-dependent individuals have evaluated brain structure and function after the transition to addiction has occurred (i.e., in dependent individuals *versus* healthy controls). As a result, it is difficult to discern whether the changes in brain function are a result of long-term exposure to the substance, or are pre-existing deficits, which thus reflect a vulnerability to the development of addiction (Ersche et al., 2013). One way to circumvent the apparent circularity is to study first-degree relatives of stimulant-dependent individuals. Due to the strong genetic (Agrawal et al., 2012; Goldman, Oroszi and Ducci 2005) component of addiction and the notion that brain function is highly heritable (Thompson et al., 2001), this approach has revealed several candidate brain markers of vulnerability to stimulant use, irrespective of exposure to the drug. An alternative approach is to study children who have been exposed to stimulants prenatally (Derauf et al., 2009), therefore being affected by the disorder indirectly but having no prior exposure to the substance themselves. Longitudinal studies investigating within-subject changes in behaviour and brain function across multiple timepoints also offer an alternative method in which to understand and evaluate the role of the developing brain to later problematic drug use, although these studies are complicated by operational demand and are costly to carry out. However, in combination with novel prediction models and machine learning methods longitudinal studies do offer a powerful way of examining the predictive capacity of several imaging and behavioural markers in at risk populations (Whelan et al., 2014; Buchel et al., 2017). Additionally, studying changes in brain function longitudinally in previously drug-dependent individuals who have successfully abstained from using the substance (Garavan et al., 2013) also offers an alternative perspective on the compensatory brain mechanisms involved in the control of drug use.

To address the question of vulnerability, Ersche and colleagues evaluated brain structure in stimulant-dependent individuals and their non-dependent siblings, as well as healthy controls (Ersche et al., 2012). Relative to the healthy control group, both siblings (stimulant-dependent and non-dependent) showed enlargement of the amygdala and putamen, and reduction of the postcentral gyrus, superior temporal gyrus and posterior insula (Ersche et al., 2012). In addition, both siblings showed elevated levels of impulsivity on the SSRTT relative to control subjects, with lower fractional anisotropy, a proxy measure of the degree of myelination, axonal integrity, and axonal fibre arrangement (Alba-Ferrara and Erausquin 2013), related to reduced reaction times. In combination, these findings suggest that vulnerability to stimulant dependence may be conferred through dysregulated inhibitory control mechanisms (Ersche et al., 2010), as seen behaviourally, and related to abnormalities at the neural level in key structures implicated in response control. Using the same sibling-pair dataset (Ersche et al., 2012), Morein-Zamir et al., investigated the functional correlates of inhibitory control in relation to substance dependence. Not surprisingly, stopping was impaired on the SSRTT for stimulant-dependent individuals. Using both brain wide mapping, and region-of-interest (ROI) analysis, hypoactivity in the ACC was associated with unsuccessful stopping attempts on the SSRTT. Interestingly, non-dependent siblings showed a hyperactivation in the same region during the trial, suggesting a possible compensatory mechanism and alluding to increased resilience in this subpopulation, although this remains to be tested fully (Morein-Zamir et al., 2013). The notion of resilience in addiction is less understood and relatively understudied in comparison to at risk and vulnerable populations (Rose et al., 2019). However, potentially just as important are the neural adaptations, either pre-existing, or in response to recreational use, that prevent the development of problematic use. Understanding the significance of the underlying neural systems responsible for resilience and how they may be recruited to prevent harmful use may provide additional avenues in understanding the development of addiction in vulnerable individuals. Under this line of questioning, Ersche et al., recently evaluated functional connectivity between several striatal seed-regions of interest in stimulant-dependent individuals and their non-dependent siblings, recreational users, and healthy controls (Ersche et al., 2020). Familial vulnerability, as assessed through the first-degree non-dependent relative subgroup, was associated with hypoconnectivity between the ventromedial caudate nucleus and the OFC, mPFC and rostral ACC. Subjects with either a familial risk but no stimulant use, or stimulant use but no familial risk (deemed the ‘resilient’ group in this study) showed hyperconnectivity in two circuits. Hyperconnectivity was seen between the ventromedial caudate seed and the inferior

frontal and middle frontal gyrus, and between the dorsolateral putamen seed and insula, middle cingulate, superior medial frontal, supplementary motor area and central opercular cortex. Increased functional connectivity between these regions may reflect an increased coupling between regions important in cognitive control and executive function, with resilient individuals exerting increased inhibitory control as a means to control behaviour (Ersche et al., 2020). Longitudinal studies in at risk populations have shown that activation in reward-related regions including the vStr, and PFC, are associated with and can predict greater substance use in adolescence (Urosevic et al., 2015). One study from the IMAGEN consortium has shown that diminished neuronal activity in the vStr, as well as the PFC, during a monetary incentive delay task at age 14 subsequently predicted problematic drug use at age 16, a finding related to levels of novelty seeking (Buchel et al., 2017). However, other studies have shown that greater Acb activation is associated with future binge drinking in adolescence (Morales et al., 2018), whilst others have shown no differences in activation (Just et al., 2019). Differences in task design and study implementation make cross comparisons difficult in this instance. More recently, a systematic meta-analysis using multi-level kernel density analysis examined whole-brain task-based MRI activation across 22 studies of vulnerability to substance use (Tervo-Clemmens et al., 2020). In vulnerable individuals, either because of a family history of use or through retrospective prediction of substance use onset, ventral and dorsal striatal activation was most closely associated with vulnerability during adolescence (Tervo-Clemmens et al., 2020). The results from this analysis support the view that vulnerability to addiction may be driven, in part, through excessive motivational drive modulated in the striatum. Along these lines, Kwon et al., reported that a family history of substance use was associated with negative connectivity between the right middle occipital gyrus and the left Acb, a finding suggestive of less top-down control from the PFC to the striatum (Kwon et al., 2021). These findings suggest that increased vulnerability to substance use may be caused by pre-existing differences in cortico-striatal circuits known to be involved in reward processing and executive function.

1.12 Rodents to humans – brain homologues and translational relevance

Although precise measurements are not available it is estimated on average that the human brain has over 420 times the number of neurons of a rat brain and weighs roughly 830 times as heavy (Herculano-Houzel et al., 2006; Azevedo et al., 2009; Herculano-Houzel 2009).

Early work evaluating cross-species homology focused upon the mediodorsal nucleus of the thalamus and its reciprocal connections with cortical areas to define prefrontal cortical homologues across different species (Rose and Woolsey 1948). Over several decades, viral tracing studies including more advanced anterograde and retrograde techniques have refined this perspective suggesting that the strength or relative number of connections between the mediodorsal thalamus and the mPFC may be the defining factor in mapping the PFC across species (Uylings, Groenewegen and Kolb 2003). However, what regions constitute the rodent PFC, and in indeed whether there is a rodent PFC, is not without controversy (Preuss 1995; Laubach et al., 2018), and the precise cross-species homologues are still debated (Schaeffer et al., 2020). Nonetheless, a consensus has tentatively emerged suggesting that the rodent IL cortex is homologous to area Broadmann's area (BA) 25 (subcallosal cingulate gyrus), the rodent PrL is homologous to area BA32 (dorsal anterior cingulate region), whilst the rodent OFC is considered homologous to the human OFC, although the exact subregion comparison is lacking (Wise 2008; Bicks et al., 2015). Additionally, the rodent cingulate cortex is thought to be homologous to area BA24, however, the exact nomenclature and way in which the cingulate is subdivided in rodents (e.g., ventral-dorsal or caudal-rostral) may impact this comparison (van Huekelum et al., 2020) **(Fig6.)**. Although it is tempting to make direct comparisons across species, caution must be taken, especially in regard to comparisons of the PFC. An alternative approach to mapping connections and using tracing studies has been to evaluate cross-species functional similarities or 'functional homologies' by evaluating how behavioural function is modulated in comparative regions across species. In this vein, several converging cognitive tasks developed in experimental animals and humans have provided valuable insight into homologous function (Eagle and Baunez 2010; Eagle, Bari and Robbins 2008). Relative to the PFC, a relatively late structure to emerge in evolution (Kaas 2013), basal ganglia circuits are well preserved across most species (Milardi et al., 2019), allowing for more direct comparisons of structure and functional differences. For example, the DLS is critical for the emergence of habitual control in rats (Yin et al., 2004), and functional activation of the homologous posterior putamen in humans is associated with habitual responding after devaluation (Tricomi, Balleine and O'Doherty 2009). Moreover, activation of the anterior caudate nucleus and mPFC are associated with goal-directed behaviour in humans (Tanaka, Balleine and O'Doherty 2008). This observation is strikingly similar to that seen in rodents, with the DMS, potentially representative of the caudate nucleus in humans, and its connections with the PrL critically involved in goal-directed learning (Yin et al., 2005). In summary, strong similarities exist both at the functional and anatomical level

between rodents, primates, and humans. Although there is considerable heterogeneity between the rodent and human PFC, correspondence has been observed through specific pharmacological manipulation or excitotoxic lesion techniques in studies utilising analogous cognitive tasks in experimental animals (Robbins 1998; 2007). In addition, overlap between phylogenetically conserved areas such as the striatum allow less tentative links to be drawn, with considerable functional overlap seen in these regions. For example, habitual and goal-directed learning systems (above) (Balleine and O'Doherty 2010) and recent studies showing the functional homology between neural nodes of action impulsivity on the rodent 5-CSRTT and the human 4-CSRTT (Morris et al., 2016).

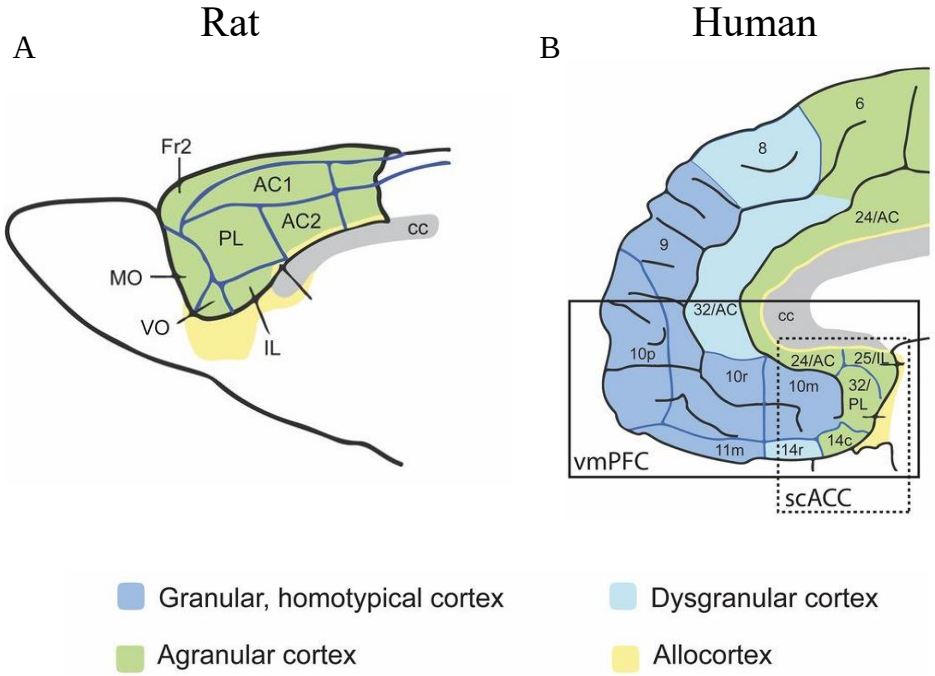


Fig6. Schematic overview of **(A)** rat and **(B)** human ventromedial prefrontal cortex and subgenual anterior cingulate cortex. Although exact homologies are imprecise, broadly it is thought that Areas 25, 32 and 24 in humans corresponds to the infralimbic cortex, prelimbic cortex, anterior cingulate cortex, respectively. Whilst the orbitofrontal cortex is not shown here it is considered to be homologous to the rodent orbitofrontal cortex, although direct subdivision comparisons are difficult and lacking. Adapted from Roberts and Clarke 2019.

1.13 Addiction-like behavioural endophenotypes and their interrelationships

In summary, addiction-like behaviour in rodents is predicted by several behavioural endophenotypes (discussed above). A large majority of studies often address how a particular or single behavioural trait may predispose an animal to a certain aspect of addiction-like behaviour. However, often is the case that several of these traits are co-expressed and may, in combination, represent a unified construct for addiction liability. For instance, ST, who have a higher propensity to attribute incentive salience to reward cues, have also been shown to be express the HI phenotype on the 2-choice serial reaction time task (2-CSRTT), a modified version of the 5-CSRTT (Lovic et al., 2011). Somewhat surprisingly, in the same study, ST also showed a higher preference for a delayed, larger reward on the DDT, a measure of sustained impulse control behaviour (Lovic et al., 2011). In an analogous choice reaction time task, ST were also more likely to respond prematurely, and lever contacts during the final day of PavCA procedure were positively correlated with impulsivity (King et al., 2016). However, using the 5-CSRTT, Robinson and colleagues reported no differences in PavCA in high *versus* low-impulsive animals (Robinson et al., 2009), a finding replicated by Pena-Oliver et al., 2015. Other evidence suggests that ST discount rewards more readily than GT (Tomie et al., 1998), and HR rats are also more likely to be ST (Flagel et al., 2010). A more recent study reported a dissociation between sign-tracking, NPP, and LocR (Hughson et al., 2019), demonstrating that these were non-overlapping traits, a finding in line with other reports (Vanhille et al., 2014; Belin et al., 2011, however see Beckmann et al., 2011). Similarly, HI animals on the 5-CSRTT show no obvious relationship with novelty preference (Molander et al., 2011; Dalley et al., 2007; Lukkes et al., 2016). To what extent individual risk to addiction is predicted by pre-existing behavioural vulnerability traits, their interactions, and how drug-induced neural plasticity mechanisms hijack cortico-striatal circuits to drive the pathophysiology of addiction is still unknown. To address these questions, the relationship between a wide range of antecedent vulnerability traits and their role in predicting vulnerability to the subsequent development of compulsive cocaine seeking was assessed. Furthermore, evaluation of the underlying structural and functional neural substrates of these risk traits and compulsive cocaine seeking was assessed through non-

1.14 Scientific predictions and overarching hypothesise

Compulsivity is a fundamental feature of SUD and has been described as persistent drug-seeking and use in the face of significant adverse or detrimental consequences, typified by rigid, stimulus-bound patterns of behaviour possibly reflecting reduced top-down control mechanisms over goal-directed behaviour. Of the individuals who take drugs of abuse, only a small subset will eventually lose control over their intake and transition towards compulsive use. Several antecedent behavioural traits and perturbations in brain networks underlying impulse control and reward-related behaviours have been shown to heighten risk of this transition. In rodents, the traits of impulsivity, novelty-/sensation-seeking, incentive salience attribution and cognitive flexibility have been linked with specific aspects of addiction-like behaviour. However, to date, the relationship between these risk traits, their underlying structural and functional brain networks, and compulsive cocaine seeking has yet to be investigated.

The aims of this thesis were to answer these three questions: 1) What are the dimensional relationships between several behavioural endophenotypes (risk traits) of addiction? 2) What is the relationship between these pre-existing endophenotypes and the subsequent risk to develop compulsive cocaine seeking? 3) What are the functional and structural brain substrates of drug-naïve animals who display these risk traits, and who are retrospectively classified and destined to develop compulsive cocaine seeking? Thus, the overarching aim of this thesis was to address this question - What mediates increased risk to the development of future compulsive drug use? Based on previous human and animal data the hypothesis put forward in this thesis is that pre-existing deficits in goal-directed behavioural control and response inhibition contribute towards enhanced vulnerability to compulsive cocaine seeking through reduced top-down cortico-striatal cognitive control of goal-directed and reward-motivated behaviours.

To address the first question, a longitudinal behavioural testing procedure was performed to evaluate the interrelationships between 5 behavioural traits previously linked with addiction-like behaviour including impulsivity (5-CSRTT), novelty preference (NPP), sensation seeking (LocR), incentive salience attribution (PavCa) and cognitive flexibility (deterministic RL). The results of this analysis are presented in Chapter4.

Following behavioural testing, rats were trained to self-administer cocaine with compulsive cocaine seeking subsequently assessed using a translationally-relevant concurrent punishment- drug-seeking procedure (Chapter 5). Following cocaine exposure, a retrospective analysis was used to determine whether specific behavioural endophenotypes subsequently predicted compulsive cocaine seeking.

Finally, structural and functional MRI was implemented to investigate the underlying neural substrates of future compulsive cocaine seeking and several identified risk traits (Chapter 6). Voxel-based morphometry was used to analyse structural abnormalities and region-to-region connectivity analysis was used to understand functional perturbations in cortico-striatal circuits linked previously to addiction-like behaviour.

Chapter 2

Magnetic resonance imaging: methodology

2.1 Introduction

The first early evidence of MRI can be traced back to the work of Paul Lauterbur and Peter Mansfield (Lauterbur 1973; Mansfield and Grannell 1975; Morris 2021). The groundbreaking demonstration that magnetic resonance (MR) relaxation times could be used to create an image paved the way forward for future studies in medical imaging. Early work in clinical imaging demonstrated that MRI could be used to differentiate between cancerous and non-cancerous cells (Damadian 1971), and since then its utility in clinical and preclinical medicine has flourished. Briefly, MRI works by utilising the inherent 'magnetic' properties of hydrogen atoms which when combined with oxygen make up H_20 – water. Hydrogen atoms are made up of a single proton which is positively charged and are abundant in the body in water and fat. At resting state, hydrogen atoms spin or precess in a random fashion relative to each other. However, when put in an MRI scanner, and subjected to an external magnetic field (B0 field), the randomly spinning hydrogen protons realign with this field. Most hydrogen atoms realign in the same direction (parallel state), although a minority of atoms do align in the opposite direction (anti-parallel state). The strength of the main magnetic field, referred to as the B0 field, will dictate the speed of the hydrogen atom's precession. The field strength is tightly related to spin precession, with the frequency of precession defined based on the Larmor equation (Berger et al., 2002; Currie et al., 2013). Once the protons are in B0 field and aligned, an electromagnetic radiofrequency (RF) pulse is used to disturb or flip the protons out of alignment. The energy from the RF pulse, when

applied at a specific frequency (the Larmor Frequency), can be absorbed by the hydrogen protons. This causes the protons alignment to rotate away from the B0 field. At the same time, a secondary magnetic field is applied (B1) that varies in strength across the body. This causes different parts of the body, and therefore the hydrogen atoms within, to experience different magnetic fields and precess at slightly different speeds. The RF pulse is used to excite and flip the protons can then be differentially applied to target a particular area of the body. When the RF pulse is turned off the hydrogen protons will return to their ground state and begin to precess in a random fashion once more, giving off energy as they do (for overview see **Fig1.**). The way in which this energy is dissipated, commonly known as the relaxation rate, very much depends on the local environment of the hydrogen atoms and can therefore be used to generate different contrasts in images.

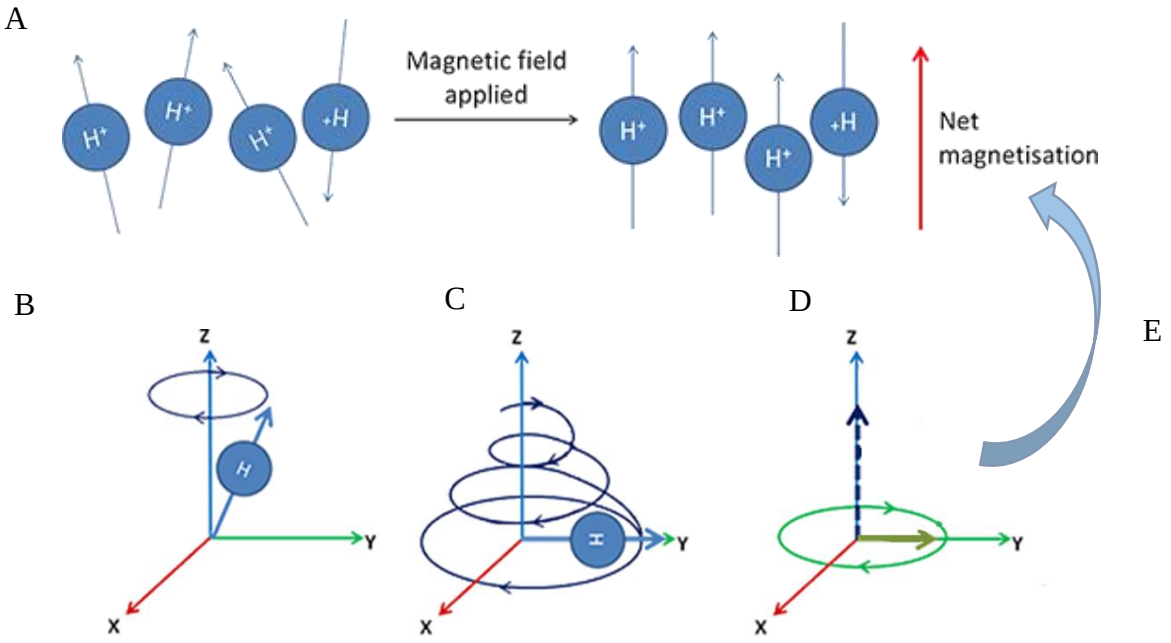


Fig1. Overview of magnetic resonance imaging principles. **(A, B)** Upon application of an external magnetic field (B0) the randomly precessing hydrogen nuclei will align and precess in a parallel state to the applied field at the Larmor frequency, with some precessing in an anti-parallel state. **(C)** Application of an RF pulse is used to excite and flip the precessing protons into the transverse plane, resulting in net magnetisation in the transverse plane (dark green arrow) **(D)**. **(E)** As the RF pulse is turned off the hydrogen atoms relax back to their resting state and once more are aligned with the B0 field. Adapted from www.radiologycafe.com.

Remarkable technological advances over the last several decades and ever more sophisticated methods have been employed to facilitate the understanding of the developing brain and

cognition (Morita, Asada and Naito 2016). MRI is a highly versatile imaging technique, and in the case of brain imaging, has been used to understand grey and white matter structure, brain activity *via* fMRI, as well as blood perfusion (Veldsman and Egorova 2017). Traditional MRI images are largely based on the acquisition of so-called weighted images, for example T1-weighted (T1-W) and T2-weighted images (T2-W). Weighted images are generated by adjusting acquisition parameters such as the repetition time (TR) - the amount of time between successive RF pulse applications, and echo time (TE) - the time between the RF pulse and the recording of the echo signal (the energy the hydrogen atoms give off after being excited by the RF pulse). Changes in these parameters will change the relative weighting of the images and will determine the contrast and brightness of different tissue classes in the brain. However, it is important to note that the signal acquired in T1-W or T2-W images may not be specific to only that weighting and may be influenced by additional tissue parameters (i.e., in a T1-W image you can have a high degree of T2-W signal (Yokoo et al., 2010)). Moreover, other tissue parameters also comprise the signal of T1-W and T2-W images, for example, spin density (the concentration of hydrogen atoms precessing at the Larmor frequency in any given region) is frequently present during both conventional T1-W and T2-W scans (Elster 1998; Yokoo et al., 2010). Nonetheless, both sequences are routinely used by radiologists and clinicians, along with non-clinical researchers, to assess disease progression and evaluate structural morphology (Nazir et al., 2016; Iwata et al., 2012; Trip and Miller 2005).

2.2 Cross-species comparison and vertical translation

One important advantage of non-invasive imaging is that it can be used across multiple timepoints to assess longitudinal changes in brain structure and function across development and time (Vernon et al., 2011; Hu et al., 2019; Mengler et al., 2014). Moreover, using translationally relevant imaging procedures in experimental animals provides the unique ability to carefully control and manipulate experimental conditions and investigate pre-existing brain states prior to any causal manipulation. One particular advantage of using MRI is that it can be used to map and evaluate whole brain function in a relatively short space of time. Going beyond conventional circuit mapping techniques, MRI can be used in investigations of whole-brain connectomics and long-distance networks (Bullmore and Sporns 2009; Hagmann et al., 2007). MRI is one of the safest non-invasive techniques used to repeatedly map brain structure and function without damaging brain tissue and unlike other

non-invasive methods no radioactive isotopes are used (e.g., PET). Finally, MRI techniques can be used similarly in rodents, primates and humans displaying its translational potential. With the advancement of ever more standardised analytical tools (Esteban et al., 2019; Glasser et al., 2013; Grandjean et al., 2020), comparative neuroscience investigations of brain function across species are increasingly common. In addition, multimodal investigations in a given subject are becoming more commonplace, with acquisition of differentially weighted structural scans, spectroscopy, functional imaging as well as diffusion tensor imaging (DTI) often carried out in a single scanning session. To a certain degree, scan time is not a limiting factor in studies employing MRI techniques in rodents, which improves image quality and allows for more detailed and thorough investigations of brain function (through multi-modal imaging as discussed above). A recent review by Mars et al., highlighted two distinct translations between and within experimental animals and humans (Mars et al., 2021). Thus, within or vertical translation can occur within the same species but across different MRI modalities and timescales as well as across alternative techniques, for example combining brain imaging with genome sequencing (Fulcher et al 2019; Cong et al., 2016). In addition, horizontal or between translation is the comparative analysis across species using the same technique, such as comparisons made between primate and human (Rilling et al., 2014), rodent to primate (Balsters et al., 2020), or indeed rodent to human (Barron et al., 2020). Vertical translation in experimental animals offers the unique advantage to compare histological or neuronal tracer data with MRI measures. Whereas horizontal translation allows for the comparison of potentially invasive methods in experimental animals (which should be validated by successful vertical translation within species using, for example, histological and MRI methods, e.g., Vernon et al., 2010) with humans. Utilising invasive methods not available in humans to directly map connections in the brain of experimental animals to generate novel predictions about cortical mapping and network properties in humans is evidenced through the work of the Allen mouse brain connectivity atlas (Oh et al., 2014). Although many studies have evaluated specific region-to-region connectivity, Oh and colleagues were the first to map whole-brain projection patterns across the entire mouse brain. This seminal work demonstrated specific network integration and global networks at a mesoscale in mice and is just one example where work in experimental animals has opened important avenues for future cross-species investigations.

2.3 Structural Imaging

The notion that the brain can be defined based on a series of distinct cytoarchitectonic maps is not new. Perhaps the most influential work in this area is that of Brodmann, von Economo and others (Zilles and Amunts 2010). In the early 19th century, seminal work from the aforementioned authors provided an incredibly detailed anatomical map and description of the cytoarchitecture of the human brain. These pioneering efforts, and the emerging notion of an intimate relationship between brain structure and function, laid the foundations for the notion that individual brain regions may control different cognitive functions. This notion was further supported by the works of Broca and Wernicke/Lichtheim who discovered the link between cortical damage and speech processing (Broca 1861; Wernicke 1874; Lichtheim 1885). As a direct result of this work many studies have evaluated structural brain mapping in relation to brain function over the last century. Structural neuroimaging refers to the visualisation and statistical comparison of the anatomical properties of the brain. As the name suggests, structural imaging refers to the structural properties of the brain. However, 'structure' is a very broad term and depending on the particular MRI acquisition sequence and analysis pipeline used can mean many different things. For example, structure could represent 1) grey matter volume as assessed through voxel-based morphometry (VBM); 2) volume assessed through ROI analysis – which can either be a) manual delineated or b) delineated through a probabilistic atlas, or 3) the analysis of the thickness of the cortical mantle (i.e., cortical thickness). Therefore, the chosen analytical method is an important factor to take into account when assessing brain 'structure'.

2.3.1 Voxel-based morphometry and region-of-interest analysis

VBM is a computational neuroimaging analysis technique that measures differences in the local concentrations of grey matter at each voxel (a voxel can be thought of as a 3D pixel or small cube, in which the brain is partitioned – e.g., the brain is built up of many 3D cubes of brain tissue). VBM is a brain-wide technique, employing a mass univariate approach across the whole brain. Thus, at each voxel the difference between grey matter concentration is tested statistically (Ashburner and Friston 2000; 2001; Mechelli et al., 2005). VBM represents one of the most widely used techniques to characterise structural brain differences and has been used to characterise the neural correlates of a wide range of neuropsychiatric disorders (Scarpazza and De Simone 2016). Voxel-based morphometric methods have been successfully used to assess structural differences across many different species including, but

not limited to mice (Sawiak et al., 2009), rats (Jia et al., 2018), primates (Sawiak, Picq and Dhenain 2014; McLaren et al., 2010) and humans (Mechelli et al., 2005). Unlike earlier manual segmentation, VBM is an automated analytical pipeline that allows detection of group differences after appropriate pre-processing steps have been carried out (for more details see Chapter 3, section 3.9). In preclinical studies, several reports have employed VBM analysis to study neuroanatomical differences in murine models of Huntington's disease (Sawiak et al., 2009), Parkinson's disease (Westphal et al., 2016) and in relation to behavioural endophenotypes of addiction (Caprioli et al., 2014). In several notable utilisations of the VBM pipeline, Sawiak and colleagues were able to demonstrate the utility of non-subjective VBM over that of manual 2D morphometry (Sawiak et al., 2009). Using the R6/2 transgenic mouse model of Huntington's disease, Sawiak et al., manually delineated several ROI, including the striatum, hippocampus, and amygdala, among other regions, on a slice-by-slice basis. Relative to wild-type controls, several differences in volume were observable in the R6/2 mouse line. In the accompanying paper, Sawiak et al., found that when taking a whole-brain approach (*via* VBM) structural differences that were not observed *via* manual morphometry were revealed (Sawiak et al., 2009). Importantly, volumes measured *via* MRI and *ex-vivo* histology were strongly related, suggesting that volume measured non-invasively through imaging techniques relates well with ground truth histological measures, at least in this instance (Sawiak et al., 2009). The above findings do not necessarily mean that one technique is 'better' than the other. Both manual ROI analysis and VBM provide slightly different analysis of structural differences. In one case, VBM reflects a voxel-by-voxel analysis of grey matter, which is independent of any ROI, includes the whole brain and has a greater sensitivity to detect subtle changes than a ROI analysis. This increased sensitivity is a result of the fact that the technique is voxel-by-voxel, and unlike a ROI analysis, does not suffer from a loss of sensitivity as region size increases (Voormolen et al., 2010) (as the ROI increases, if an effect is focal, this effect will be averaged out). However, it is important to note that due to the mass univariate technique employed in a VBM analysis appropriate thresholding and multiple comparison correction must be considered. Therefore, in some instances a more conservative correction in the VBM analysis may reduce the power to detect an effect. This is not the case for the ROI analysis, unless a very large number of ROI's are used. Evaluating the effect of spatial scale on the detection of anatomical differences in a simulated dataset, Voormolen and colleagues showed that increasing ROI size decreased the ability of manually segmented ROI analysis to detect an effect, whereas the sensitivity of VBM to detect the effect remained relatively unchanged

(Voormolen et al., 2010). When compared to VBM, manual ROI tracing has several obvious disadvantages (Astrakas and Argyropoulou 2010). For example, manual tracing relies on the ability of an experimenter to accurately discern the boundaries of the ROI, sometimes problematic due to the inherent spatial resolution limits of the MR images. In addition, if multiple investigators delineate different regions, or the same investigator delineates identical regions across subjects, appropriate controls must be in place, or tested for statistically. Hence, inter- and intra-rater reliability should always be assessed. One other key limitation of manual tracing is the use of these methods longitudinally. It is often difficult to replicate the exact location and geometry of a region across each subject over multiple timepoints. A final caveat to manual tracing techniques is that they are very time consuming, and are thus unsuitable for large-scale studies. Nonetheless, manual tracing has been used successfully to detect anatomical differences between disease groups in rodents (Sawiak et al., 2009). An alternative to manual tracing, but still based on ROI analysis principals, is the use of a probabilistic atlas. This method can involve manually delineating several ROIs and combining the ROIs to generate an atlas, a procedure often carried out in the space of the study template in which the native images will be registered. Conversely, several published and readily available atlases are available and can be appropriately registered to delineate and extract within-subject information. In humans, perhaps the two most common template spaces and atlases are the Montreal Neurological Institute (MNI) template (Mazziotta et al., 1995), and the Talairach space and template (Talairach and Tournoux 1988). In addition, several other publications have generated highly detailed and expansive ROI maps that cover the entire cortex such as the Desikan-Killiany atlas (Desikan et al., 2006), whilst other atlases have focussed almost exclusively on white matter anatomy (Wakana et al., 2004).

Atlas-based techniques are widespread. One key reason is that these techniques provide a reference framework for each ROI within defined coordinates. This increases reliability as ROIs are no longer operator-dependent and related to the skill and experience of the experimenter. As a direct result this increases comparability across studies. Once the atlas has been made, delineating structures is far less time-consuming than manually drawn ROI analysis techniques and in addition atlas-based approaches allow consistency in labelling across time and therefore are appropriate for use in longitudinal studies. However, it is important to be aware of the derivation of the atlas used. For example, the Talairach atlas was derived from a single female brain, and therefore may not generalise to a wider population (Dickie et al., 2017). Additionally, specific atlases may not be appropriate for studying

certain populations, such as young children (Van Phan et al., 2018). Nonetheless, atlas-based techniques are highly versatile and given the relative advantages over manual delineation, with appropriate validation within a study, offer a robust way in which to evaluate local structural and functional differences. Like humans, there have been a number of published atlases in rodents, although substantially less than those available for human studies. Based on the stereotaxic coordinates of Paxinos and Watson (P&W) (Paxinos and Watson 2006), the Tohoku atlas by Valdes-Hernandez, Kawashima and colleagues is constructed from in-vivo imaging of 30 rats of the Wistar strain and provides 96 lateralised ROIs (**Fig2.**) (Valdes-Hernandez et al., 2011). However, the PFC and its subdivisions (e.g., IL *versus* PrL*)* are not delineated in this atlas, therefore decreasing its utility. Based on the Sprague Dawley strain of rat, the Waxholm Space Atlas provides an alternative atlas-based parcellation. Combining ex-vivo structural imaging with high resolution DTI, Papp et al., delineated 76 ROIs, with a focus on sub-cortical delineation in areas such as the hippocampus (Papp et al., 2014). However, this atlas was formed from imaging a single rat, therefore the atlas represents a necessary starting point but should be further validated with additional animals to enhance its robustness. The SIGMA rat brain atlas is a more recent addition (Barriere et al., 2019). The Sigma atlas was generated from 47 male Wistar rats. The Sigma atlas parcellation represents the combination of the aforementioned Waxholm and Tohoku atlases, and therefore features both cortical and sub-cortical delineations. Thus, the Sigma parcellation is very expansive, and has 'full brain coverage' with 246 structures (124 from the Tohoku atlas, and 122 from the Waxholm atlas) across both hemispheres (123 per hemisphere). Other notable atlases are available (Nie et al., 2013; Calabrese et al., 2013; Schwarz et al., 2006) with some focusing on anatomical structures during development stages (Calabrese et al., 2013). Although many regions are based on discrete anatomical landmarks and in reference to the P&W defined space, careful evaluation of the spatial extent of each ROI is important. As already mentioned, most of the available atlases do not separate the mPFC into its constituent sub-divisions. In addition, the process of warping the atlas to a study specific template may introduce biases in the anatomical locations due to misalignment during registration. Moreover, using different rat strains (e.g., Sprague Dawley, Wistar, Lister Hooded etc.) may lead to discrepancies in anatomical location of the atlas after registration. Thus, careful quality control is necessary, and evaluation of registration in relation to histological sections (Paxinos and Watson 2006) at the subject level is a critically important step in any preclinical MRI investigation. Thus, atlas-based ROI analysis continues to be an appropriate and well

used technique to analyse anatomical differences, so long as appropriate quality control has been carried out.

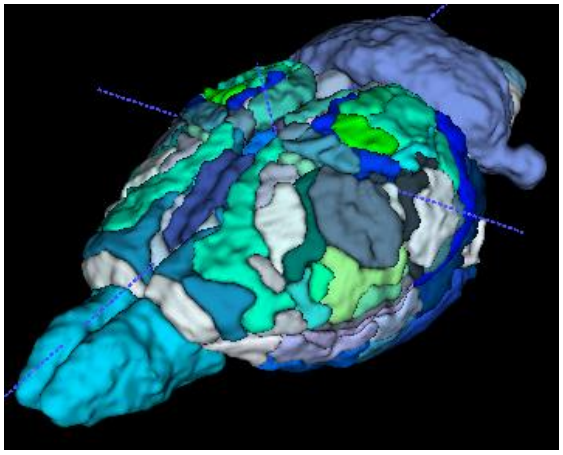


Fig2. Example of 3-dimensional representation of region-of-interest parcellation of the rat cortex. Parcellation based on regions specific in the Tohoku University atlas (Valdes-Hernandez et al., 2011). Parcellation includes 96 regions of interest.

2.4 Strutural covariance networks

Structural covariance analysis is an analysis technique that determines the inter-relationship of distinct morphological regions within a group of subjects. In the first stage of structural covariance mapping the brain is parcellated into a number of pre-defined sub regions **Fig3A.).** Morphological information (e.g., volume, cortical thickness, surface area) is then extracted for each ROI, for each subject (**Fig3B, C.**). A correlation is then calculated for each ROI-ROI pair across subjects (**Fig3D.**). This is then done for all ROIs (**Fig3E.**), forming a correlation matrix, which represents the structural covariance network across the group of subjects (Carmon et al., 2020; Alexander-Bloch, Giedd and Bullmore 2013). Structural covariance network analysis has been used to understand the relationship between impulsivity and brain morphology in humans (Pehlivanova et al., 2018). In rodents, structural covariance network analysis is less common but recent reports have shown the predictive relationship between morphological networks and behavioural outcomes (Mueller et al., 2021). Whilst VBM analysis was carried out to understand the relationship between brain morphology and behavioural outcomes in this thesis, structural covariance network mapping, and indeed analysis of ROI volumetric information, offer another window into understand the underlying relationship between brain morphology and vulnerability to compulsive cocaine seeking above and beyond that evaluated through VBM and will be considered for future analysis.

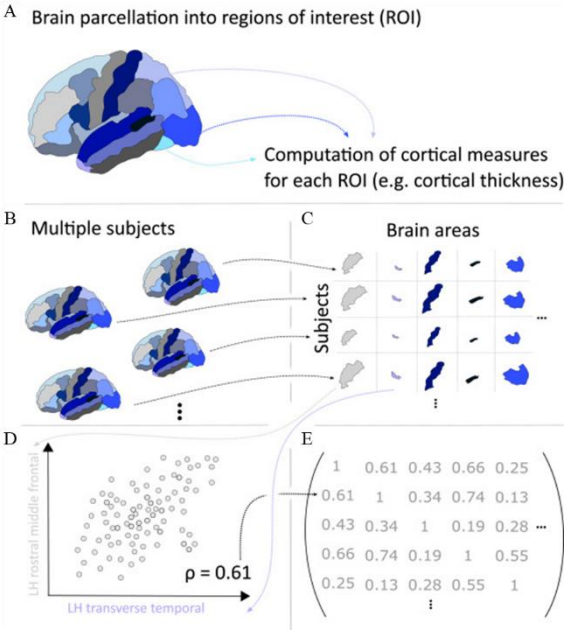


Fig3A-E. Structural covariance mapping analysis pipeline. (A) The brain is parcellated into several pre-defined region of interests (ROI) and a single cortical measure is extracted from each ROI. (B) This process is carried out for multiple subjects. (C) A matrix is formed consisting of data from each brain region for each subject. (D) For each region, across all subjects, a correlation is computed. (E) This is done for each brain region forming a structural covariance matrix for each group of subjects. Adapted from Carmon et al., 2020

2.5 Functional neuroimaging analysis

In the early 1990's several laboratories across the world visualised dynamic changes in brain function through fMRI and demonstrated the underlying functional relationship between specific brain regions and scanner-related activity or tasks (Bandettini 2012). Neurovascular coupling describes neuronal activity within the brain and the corresponding change in cortical blood flow. When a set of neurons fire microvessels in the brain dilate and changes in local blood perfusion are observed – with an increase in blood flow to the site of activation. This increase in blood flow supplies glucose and oxygen, key substrates used in energetic and metabolic processes, to provide energy for the increased activation, which is mainly driven by the energy demanding process of pumping ions across the membrane for action potential propagation and neuronal firing (Attwell and Laughlin 2001; Attwell and Iadecola 2002). The underlying principles of fMRI *via* BOLD imaging are embedded in the magnetic properties of oxygen present in haemoglobin (Ogawa et al., 1990; Kwong et al., 1992), and more specifically the difference in magnetism between oxygenated and deoxygenated blood. As neuronal activity occurs oxygen consumption increases and neurons draw oxygen from the vasculature network resulting in a local increase in deoxygenated haemoglobin, which due to differences in the magnetic properties, leads to a decrease in the BOLD signal – termed the 'initial dip' (Yacoub et al., 2001). To maintain oxygen supply, cerebral blood flow is augmented leading to a large increase in oxygenated haemoglobin being delivered to the site

of neuronal activation. At this point, decreases in deoxygenated haemoglobin and increases in oxygenated haemoglobin result in changes in the magnetic resonance intensity and thus blood flow. This then changes the BOLD signal which is therefore used as a surrogate marker of neuronal activity. MRI is an indirect measure and the exact mechanisms by which the signal is generated are still unknown. However, several studies have used various invasive methods to understand and clarify the relationship between the BOLD signal and underlying neural activity.

Logothetis and colleagues studied the underlying neuronal signatures of the BOLD response by combining electrophysiology with simultaneous fMRI recording in monkeys (Logothetis et al., 2001). In their study, both BOLD activation and electrical signals from the visual cortex of monkeys viewing a rotating chequerboard pattern were measured. The authors analysed local neuronal network dynamics through several different signals including single- and multi-unit recording as well as local field potentials (LFP). They observed a high degree of overlap between single- and multi-unit neuronal activity, activity thought to reflect the output of neuronal populations close to the recording electrode and represent the firing or spiking of local neurons. Conversely, the neuronal activity measured through LFP is low frequency and averaged over a larger neuronal population, with the LFP signal derived from synchronised dendrosomatic activity of synaptic signals, among other mechanisms (Berens et al., 2008). When evaluating the two signals (LFP *versus* multi-unit recording), Logothetis et al., found that the BOLD signal was more closely related to LFP, which significantly predicted the haemodynamic response (Logothetis et al., 2001). Thus, one interpretation leading from this work is that the BOLD signal reflects the underlying integration of signal processing from a large neuronal population (as indexed through LFP) and less likely reflects specific excitatory action potentials generated in that region (as indexed through the electrical activity recorded through multi-unit electrodes) (Raichle 2001). However, the precise mechanisms by which the BOLD response is derived are still unclear, and even further complicated by the ambiguous composition of the LFP signal (Logothetis 2008). Thus, the output from the LFP signal likely reflects multiple integrative processes within excitatory-inhibitory microcircuits (Douglas and Martin 2004), including, not only post-synaptic potentials, but soma-dendritic processes, membrane oscillations and spike afterpotentials (Logothetis 2008). In short, LFP, which is tightly linked to the BOLD response, is likely generated by a complex feedback, feedforward, and modulatory activity of both inhibitory

and excitatory neurons within discreet microcircuits. The effect of local inhibition on the BOLD response is an important factor that must also be considered. For example, GABA-ergic interneurons play an essential role in the inhibitory network modulating excitatory output in the cortex. GABA-ergic neurons may act as modulators to synaptic output, thus being a critical determinant in mediating synaptic response gain, neuronal firing, and the adjoining metabolic demand of cortical excitatory neurons. Indeed, more recently, increased levels of GABA were linked to a decrease in the BOLD signal (Frangou, Correia and Kourtzi 2018).

One way to causally investigate the underlying contribution of cell-specific and circuit-specific neuronal populations to the generation of the BOLD signal is by utilising optogenetic methods in combination with fMRI (Lee et al., 2017; Albers et al., 2018). In this vein, Lee et al., 2010 measured the BOLD response after the direct activation of a selected population of neurons *via* optogenetic stimulation. Briefly, the authors injected channelrhodopsin (ion channels activated by specific wave lengths of light) into the primary motor cortex (M1) under the CaMK11 promotor, therefore transfecting a certain subset of excitatory principal cortical neurons and not glial cells or GABA-ergic neurons. Direct activation of these neurons with specific light pulses evoked robust BOLD signals in the M1, whereas control animals (animals injected with saline and not the light-activated opsin) showed no BOLD response after light stimulation (Lee et al., 2010). Furthermore, the dynamics of the BOLD response generated through optical stimulation corresponded well with previous observations in both rodents and humans (Buxton et al., 1998; Donahue et al., 2006). This important study is the first to causally demonstrate the contribution of a defined class of neurons to the generation of the underlying BOLD response. However, it is important to proceed with caution as it is likely that the optogenetic activation of specific cortical neurons also activated peripheral microcircuits. Moreover, the observed response also likely reflects astrocytic processes and downstream neuronal activations. Nonetheless, activation of specific subclasses of excitatory neurons was sufficient to drive a complex BOLD response, reminiscent of that seen previously (Lee et al., 2010). As a result, this report demonstrated the utility of the two methods for mapping causal connectivity in rodents (Palmer 2010), and revealed, in part, the underlying mechanisms of the BOLD response. An important step forward in understanding the relationship between fMRI BOLD responses and neuronal activity.

2.5.1 Resting-state functional magnetic resonance imaging

When compared to task-related fMRI, resting-state fMRI (rs-fMRI) describes the spontaneous correlated activity between different regions within the brain at rest (Biswal et al., 1995; Lowe et al., 1998). That is, certain regions within the brain form networks at rest (so-called resting-state networks) that have a high degree of correlated activity (sometimes referred to as functional connectivity). The phenomena of resting-state MRI can be traced back to the 1990's with the seminal work of Bharat Biswal and colleagues (Biswal et al., 1995), for historical perspective see (Biswal 2012). Using a block design incorporating finger tapping and rest periods, Biswal et al., reported low frequency signals with a high degree of temporal synchronicity in bilateral sensorimotor cortices in subjects under wakeful, resting conditions (Biswal et al., 1995). These signals were present even after the removal of noise signals from cardiac and respiratory sources. Moreover, these signals converged with those seen during the task-based activation paradigm incorporating finger movement, suggesting that the resting-state signal was linked to functional connectivity or information processing between the two somatosensory regions. In addition, other homologous functional relationships were observed. Thus, co-ordinated bilateral temporal activity of the BOLD signal was seen in the motor cortex in both hemispheres of the brain suggesting that at rest connections between functionally homologous regions reflect important aspects of the brain's functional organisation. Since this ground-breaking study, the field of rs-fMRI has grown rapidly. A Pubmed search of 'resting-state fMRI' shows a dramatic increase in the number of studies published with this term, with over 2,500 papers published in 2020 alone (PubMed) and importantly, publications across different species including human, primates, and rodents. Functional connectivity, defined as the 'statistical dependencies among remote neurophysiological events' (Friston 2011), best represents the relationship between the BOLD signal of two separate and independent brain regions, commonly assessed through correlation analysis, and thus reflects the level of functional correspondence between specific brain regions and across local networks (van den Heuvel and Hulshoff Pol 2010). It is thought that the functional link between different brain regions play an important role in cognition and executive functions (Reineberg et al., 2018; van den Heuvel and Hulshoff Pol 2010). Early work focussed on the coupling between two regions (Biswall 1995; Lowe et al., 1998), whereas Raichle et al., in 2001 were the first to pioneer the idea of resting-state networks, and more specifically the default mode network (DMN) (Raichle et al., 2001). The

DMN consists of a series of integrated brain regions with response dynamics in these regions closely aligned to the dynamics observed during task-based activations (Smith et al., 2009). For example, during states of goal-directed action, or non-passive states, activity within the DMN was reduced, with the converse, an increase in activity, observed during states of rest, suggesting an attenuation of default mode activity during executive functioning (Raichle et al., 2001; Gusnard et al., 2001; Gusnard and Raichle 2001; Greicius et al., 2003). To date, multiple putative resting-state networks have been reported within the literature (Yeo et al., 2011; van den Heuvel and Hulshoff Pol 2010; Beckmann et al., 2005; Laird et al., 2011). However, it should be noted that many different analysis techniques have been used to identify such networks, such as independent component analysis (ICA) or seed-based correlation analysis (SBCA) to evaluate resting-state networks (highlighted below). This has given rise to many putative networks, but perhaps two of the most well studied are the DMN (above) and the salience network (Seeley et al., 2007), both of which have been reported in rats (Lu et al., 2012; Tsai et al., 2020).

2.5.2 Independent component analysis

ICA is a hypothesis-free method used to separate brain-wide BOLD signal into spatially independent clusters of activation – or nodes in a network (McKeown, Hansen and Sejknowski 2003; McKeown et al., 1998; Kiviniemi et al., 2003). ICA is a data-driven method that can be performed without explicitly stating an *a priori* seed location, avoiding any prior spatial assumptions. Although the exact methodology is often embedded in the software used, ICA-based methods extract network information by decomposing the neuroimaging data into maximally independent signals in space (spatial location) and time (BOLD time course) – the number of components. ICA operates brain-wide, investigating the statistical dependence of thousands of voxel-voxel BOLD signals across distinct regions within the brain, and as a result, ICA represents a powerful tool to evaluate resting-state networks across species. These methods have been used to study network-based measures in humans, and more recently in rats. Using ICA, Lu et al., investigated rs-fMRI networks in anaesthetised rats. Initially the decomposition *via* the ICA was restricted to 30 components; under these conditions several networks were found including bilateral somatosensory and insular networks. Of particular importance, networks containing regions that broadly overlap with the human DMN were also observed (Buckner et al., 2008) (**Fig4.**). Thus, significant

clusters of correlated activity were found in several prefrontal cortical regions, auditory and temporal associative cortices, and the retrosplenial cortex (Lu et al., 2012). Although replication is not exact due to cross-species homologue differences, the authors demonstrate the convergence of the network across both rodents, primates and humans and thus suggest that the DMN is evolutionary conserved across the different species (Lu et al., 2012).

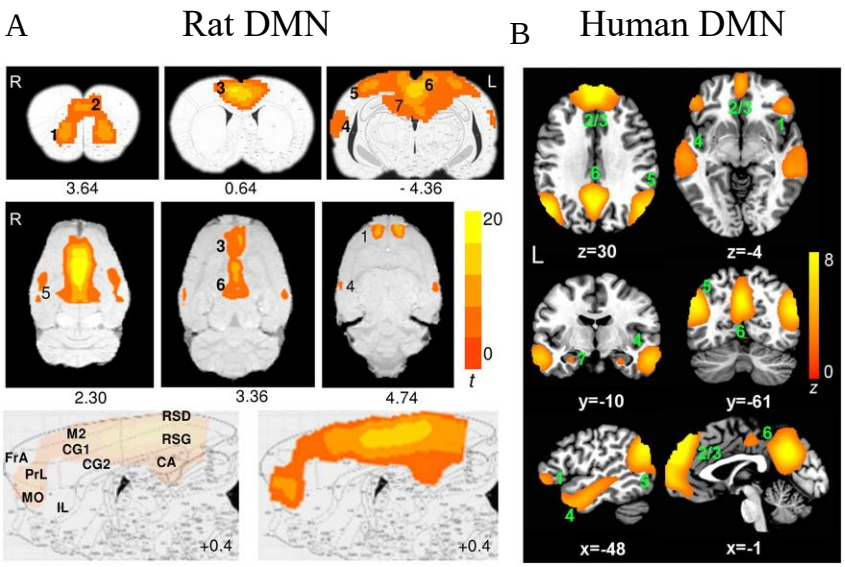


Fig4. Independent component analysis classification of the default mode network (DMN) across **(A)** rat and **(B)** human. In the rat, the DMN has been shown to constitute the (1) orbital cortex, (2) prelimbic cortex, (3) cingulate cortex, (4) auditory/temporal, (5) posterior parietal, (6) retrosplenial cortex and (7) hippocampus. Correspondingly, in humans, the DMN is thought to constitute the (1) orbital frontal cortex, (2/3) medial prefrontal cortex/anterior cingulate cortex, (4) lateral temporal cortex, (5) inferior parietal lobe, (6) posterior cingulate/retrosplenial cortex and (7) hippocampus/ parahippocampal cortex. Adapted from Lu et al., 2012.

More recently, a large multi-centre study incorporating several independent rs-fMRI datasets examined functional connectivity distributions in key networks including the DMN in mice (Grandjean et al., 2020). The study gathered a total of 17 multi-site, raw rs-fMRI datasets acquired in wild-type C57Bl/6j mice with a wide variety of acquisition parameters and anaesthesia protocols and analysed them within a common pre-processing framework with rigorous quality assurance steps (Grandjean et al., 2020). Using ICA-based methods the authors found evidence to support activation in areas representative of the DMN such as the ACC, retrosplenial gyrus and temporal associative area in some, but not all mice. Similarly, using the ACC as a seed region, activation was observed in the PFC and retrosplenial cortex

(Grandjean et al., 2020). Thus, both analysis routes (see SBCA below) converge showing activation in three central nodes of the reported DMN in rats (Lu et al., 2012). Although it must be noted that there are several possible disadvantages or caveats when employing ICA-based methods that must be considered. For example, the number of components of the network must be preselected (i.e., the number of networks must be preselected). There are options to leave this number unconstrained, therefore allowing the model to select the number of components through Bayesian dimensionality estimation techniques (Beckmann and Smith 2004). However, this can lead to a high number of noise components and the robustness of the decomposition in this situation is tightly linked to data quality (Cole, Smith and Beckmann 2010). The number of components selected is arbitrary, and the number may influence the resulting networks, for example, higher order dimensionality reduction may fractionate networks into their constituent parts, or similarly, identify subnetworks within the larger networks commonly found. Thus, model selection is an important factor when carrying out any ICA-based analysis on rs-fMRI data (Abou-Elseoud et al., 2010; Smith et al., 2009).

2.5.3 Seed-based correlation analysis

Unlike ICA, SBCA is a hypothesis driven method which requires the determination of an *a priori* ROI (the seed), which in most cases is based on previous literature and the hypothesised role of the region relative to the study question. After the seed has been selected, the BOLD signal from the ROI is extracted. The extracted BOLD signal is then correlated, brain-wide, with every other single voxel in the brain outside that seed (**Fig5.**). Essentially, SBCA evaluates the connectivity between the seed region of choice and the rest of the brain.

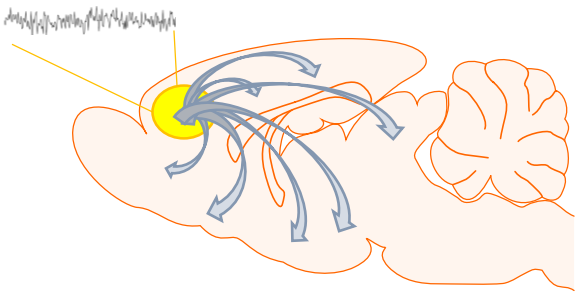


Fig5. Schematic representation of seed-based correlation analysis. The seed region is first chosen (yellow circle). The time-series from this seed region is then extract and correlated with every single other voxel in the brain (denoted by the blue arrows).

SBCA has been used extensively throughout both the human and preclinical literature to investigate rs-fMRI networks, as well as direct region-to-region connectivity (Greicius et al., 2003; Grandjean et al., 2020). A particular advantage of SBCA is that the results are intuitive, in that the generated statistical maps represent correlated activity (brain-wide) with the seed region. Moreover, the network generated is assessed across the whole brain and therefore spatially broad. Thus, negating the seed selection process, SBCA is relatively unconstrained in its spatial extent and can be used to explore brain-wide connectivity patterns. Multiple seeds can be used to classify and reveal connectivity profiles across the brain and studies employing these methods have been useful in defining connectivity patterns in relation to cognitive function (Morris et al., 2016). However, it is important to note that SBCA evaluates the statistical significance between the chosen seed and the rest of the brain, thus disregarding relationships between other nodes within a potential network, as well as disregarding any relationship across multiple nodes. Thus, although SBCA is a brain-wide technique it does impose spatial and statistical limitations on the measurement of network connectivity and network organisation. One important consideration to bear when carrying out SBCA is the exact location of the seed region and the spatial extent of the seed. Critically, the size, shape or location of the seed can lead to very different network connection maps and can lead to changes in the spatial characteristics of the networks generated. For example, taking three slightly different seed positions of a similar region, Cole, Smith and Beckmann show that even small differences in seed location can lead to large variability in the derived connectivity maps and underlying networks generated from the correlation analysis (Cole, Smith and Beckmann 2010). This important notion is further exacerbated in rodent studies where seeds may be manually drawn and the widespread use of defined anatomical seeds *via* appropriate atlases are less common, thus limiting the widespread reproducibility and replicability of these studies in rodents.

2.5.4 Region-to-Region functional correlation analysis

A more data-driven method, and like SBCA, is time-series correlation analysis (although the exact name for this type of analysis is sometime ambiguous (Mehler and Kording 2018 (*preprint*)). In this type of analysis, a probabilistic atlas, or several manually drawn ROIs, are used to extract an average signal from the rs-fMRI data at a defined spatial location. The extracted signal is often represented as the raw time-series signal, averaged across all the voxels in each ROI. Alternatively, a Singular Value Decomposition (SVD) may be carried

out across all voxels within a given ROI. The SVD method is used to provide a latent statistical summary of the signal within the ROI, with the principal component or the first Eigenvariate capturing the maximum signal variance within a ROI and representing the weighted mean of the data. Once the signal has been extracted the temporal synchronicity between the two ROIs is measured, commonly through correlation coefficients (such as a Pearson's or Spearman's coefficient), although other methods are also used (Geerligs, Cam-CAN and Henson 2016). One advantage of this analysis pipeline is that it gives a direct answer to a direct question – how does signal in region A correlate with signal in region B? Thus, the simple interpretability, ease of processing and intuitive results makes this technique a powerful tool for assessing brain dynamics. However, there are several limitations associated with this type of analysis. One clear issue is that of selection bias, only selecting specific regions of interest and negating others. Ideally, pre-registered analysis designs would prevent this from happening, with clear *a priori* regions chosen based on extensive background literature. Moreover, this analysis does not evaluate networks *per se,* as region-to-region connectivity only evaluates the correlation between two regions. In addition, and perhaps a point more generally to all ROI based analysis pipelines, is that important information may be lost by averaging across very large ROIs (see VBM *versus* ROI analysis – structural imaging, above). Finally, simple correlation-based methods that are commonly performed in this type of analysis may fail to capture non-linear relationships between interacting regions, which have been observed previously (Xie, Cao and Weng 2008).

2.6 Resting-state functional magnetic resonance imaging: quality assurance

Rigorous quality assurance checks on fMRI data and careful data curation are important steps prior to any formal statistical analysis (Lu et al., 2019). Although there is no clear consensus on the exact quality assurance procedures one should run, qualitative inspection of the reconstructed image is commonly first carried out followed by assessment of (t)SNR, motion and registration efficacy (Grandjean et al., 2020). Prior to any formal analysis (Chapter 6), careful inspection of each functional image was carried out visually. Subsequently, both SNR and tSNR was assessed and found to be well in line with previously published data for rodent fMRI investigations (Grandjean et al., 2020) (**Fig6A,B.**). For adulthood scans, average tSNR was 36.95 (minimum:27.74; maximum: 43.51; STD: 3.93), and average SNR was 305.44 (minimum:230.50; maximum:354.43; STD:30.89).

Undesirable head movement during scanning is a well-established central issue in data quality. Although many different methods exist to evaluate and remove noise, motion artefacts are commonly measured using FWD (Power et al., 2014). FWD is calculated *via* the sum of 6 motion parameters estimated over time. The distribution of FWD across each scan can then be used to estimate the variability of motion for each subject. This information can subsequently be used to exclude subjects based on an arbitrarily pre-defined movement threshold. In this thesis, all animals showed minimal FWD, where even in the maximum case (0.0744mm) FWD was below the size of half a voxel ($0.15mm^3$) (**Fig6C.**). These values sit in line with other arbitrary thresholds used (Grandjean et al., 2020), and as a result no animals were excluded based on their motion parameters. To further control for the impact of motion on functional connectivity the estimated translation and rotation parameters from the motion correction procedure were used and regressed out of the functional data (see pre-processing – methods). As another quality control, the contributing role of FWD on a number of functional connectivity metrics was evaluated. All three values were correlated with average FWD to reveal any residual relationships between motion and functional connectivity. Edgewise functional connectivity refers to the functional connectivity between each region-to-region correlation value for each animal. A summary of the p-values generated from this correlational analysis are all greater than $p<0.05$, indicating no relationship between edgewise connectivity and motion, and are shown in (**Fig6D.**). Regional connectivity was calculated by averaging the correlation matrix row-wise for each subject. The averaged rows (12 in total) for each subject were then correlated with FWD. A summary of the p-values from this correlation are all greater than $p<0.05$, indicating no relationship between regional connectivity and motion, and are shown in (**Fig6E.**). Global connectivity was calculated for each subject by computing an average value of the entire correlation matrix; a correlation graph showing no relationship between global connectivity and FWD is displayed (**Fig6F.**). Therefore, although movement was observed, as expected, it is unlikely that this had a substantial bearing on the fMRI results discussed. Registration, in lay terms, is the gross alignment of two images. Essentially, registration is performed to successfully align images in individual subject space to that of a reference template so group statistics can be performed. The assessment of registration efficacy is commonly carried out through careful qualitative visual assessment. Although the contrast varies greatly between structural and functional images, several landmarks were used to assess registration in the analysis carried out in the work presented. Following registration, as a first step, gross anterior/posterior,

rostral/caudal and ventral/dorsal positioning was assessed between each functional image and the template image. Subsequently, the corpus callosum, a white matter landmark that is visible on both structural and functional scans, albeit to a lesser degree in the latter, was then identified and successful registration was assessed by the overlap of these structures across images. A representative map of spatial overlap between a rs-fMRI subject scan and the study template (red) is seen for adult and PND-63 scans, respectively (**Fig7A, B.**).

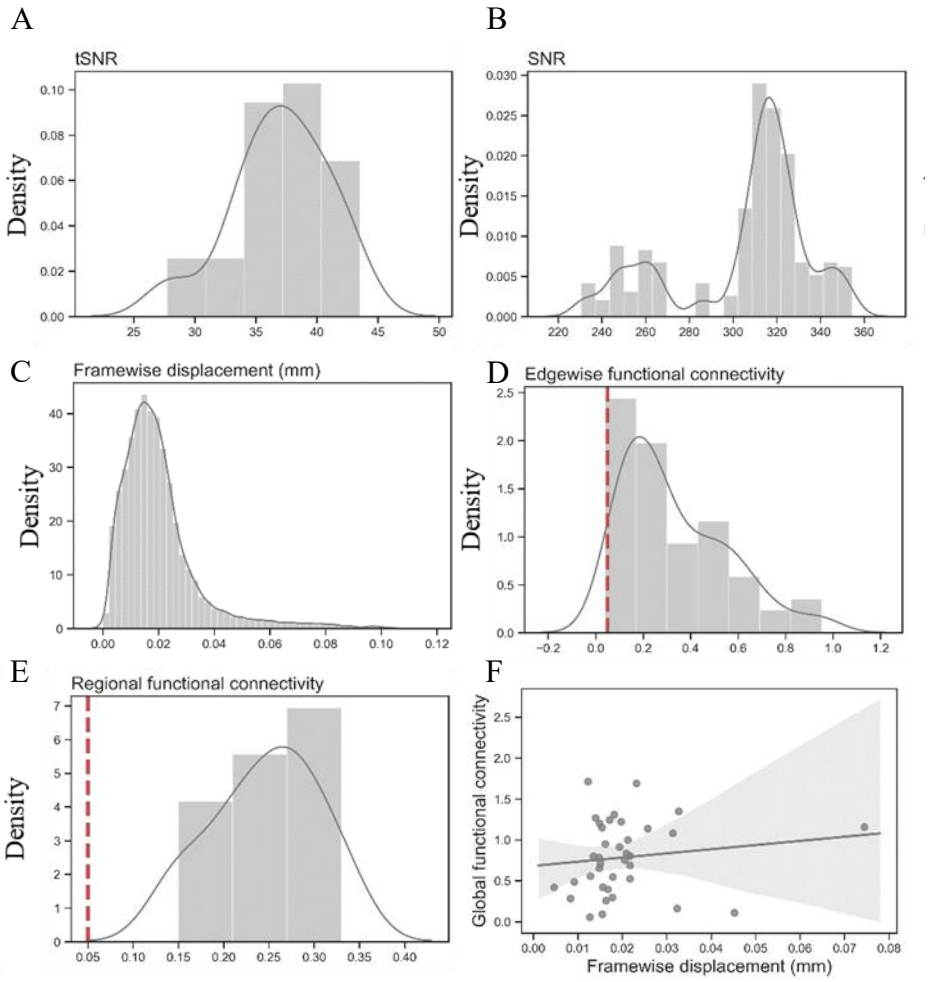


Fig6. Quality control assessment of signal-to-noise, framewise displacement and the evaluation of framewise displacement on functional connectivity. (A) Temporal signal-to-noise (tSNR) was calculated by taking the average blood oxygenated level-dependent (BOLD) signal of all brain voxels, excluding non-brain voxels, across all volumes and dividing it by its standard deviation. Average tSNR was 36.95 (minimum:27.74; maximum: 43.51; STD: 3.93. **(B)** Signal-to-noise (SNR) was calculated by subtracting the background noise signal from the global BOLD signal of several volumes across acquisition). Average SNR was 305.44 (minimum:230.50; maximum:354.43; STD:30.89). **(C)** Framewise displacement (FWD) was used to estimate subject motion. Average FWD was 0.02mm, with maximum FWD 0.11mm. Several functional connectivity metrics were regressed against average FWD to evaluate the residual levels of motion on connectivity. **(D)** Edgewise connectivity describes the region-to-region connectivity between a set of ROIs and showed no relationship with FWD ($p>0.05$). (E) Regional connectivity describes the average row-wise connectivity within the connectivity matrix and also showed no relationship with FWD ($p>0.05$). (F) Global functional connectivity, calculated as the average functional connectivity across each subjects' correlation matrix, did not correlate with FWD ($p>0.05$).

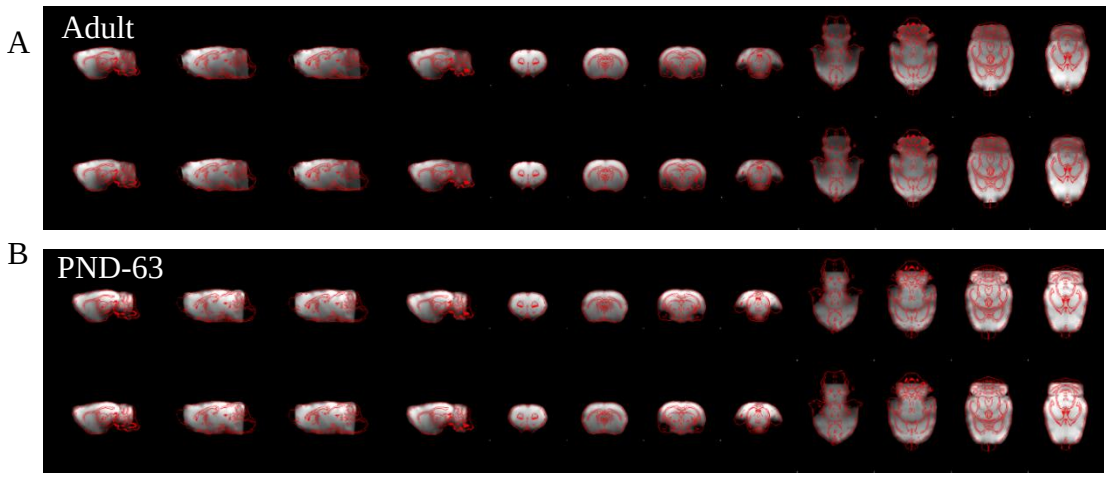


Fig7. Quality assurance of functional magnetic resonance registration efficacy. **(A, B)** Overlap between functional images and study template and registration efficacy was evaluated at adult and PND-63 timepoints, respectively. *Abbreviations:* PND, post-natal day.

As an additional step in assessing functional data quality, the overall functional connectivity pattern between the ROIs at adulthood (**Fig8A.**) and PND-63 (**Fig8B.**) timepoints was assessed. A strikingly consistent pattern of functional connectivity was observed. For example, at both adult and PND-63 timepoints, higher functional connectivity between

closely associated ventral striatal regions (e.g., AcbC and AcbS) and PFC regions (e.g., ACC and PrL) is observed, suggesting that the overall average functional connectivity pattern is relatively conserved across time.

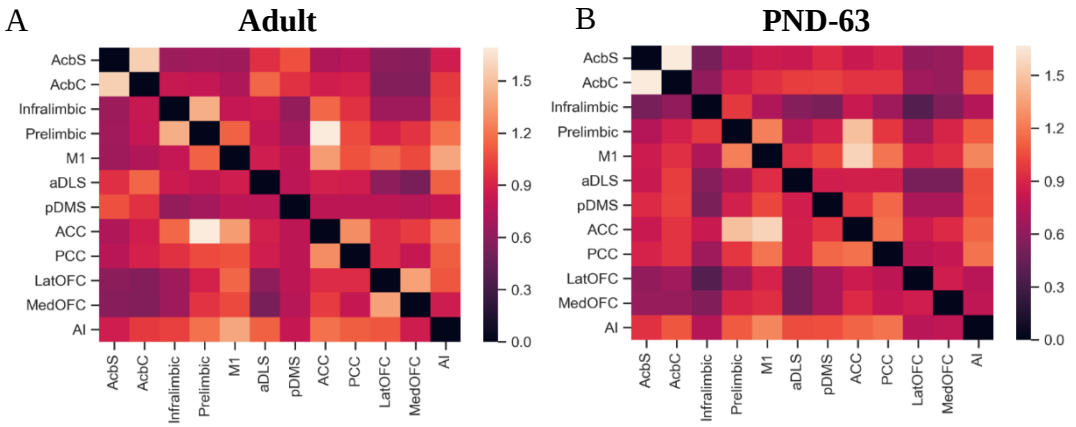


Fig8. Average functional connectivity correlation matrix of all included subjects from both the final adulthood scan **(A)** and the **(B)** pre-behaviour (post-natal day 63) scan. *Abbreviations:* PND, post-natal day. For abbreviations of ROIs please see Chapter 3, section 3.10.3.

2.7 Limitations of magnetic resonance imaging

First, and foremost, MRI is an indirect measure of brain activity, or brain structure. The signal generated is a surrogate measure of brain tissue, as in structural imaging, or neuronal activity, as in functional imaging. This notion must be considered when drawing inferences from MRI data regarding brain function. Nonetheless, good correspondence is observed between brain volume measured *via* histological sections and MRI (Sawiak et al., 2009), and between electrophysiological data and the underlying BOLD response measured in functional imaging (Logothetis 2001). In addition, hardware limitations such as magnetic field strength restrict the spatial resolution of the imaging voxel; with important implications in the ability to define and visualise small brain nuclei (Mulder et al., 2019). However, ultra-high field imaging, with its increased spatial resolution, is becoming more commonplace with the approval of 7 Tesla imaging for clinical use in 2017 in the U.S (Karamat, Darvish-Molla and Santos-Diaz 2016). Thus, it does appear that current spatial resolution limitations may be overcome in the future (Ladd et al., 2018; Tona et al., 2019). Physiological noise can be an important modulator of signal intensity in rs-fMRI. The relatively slow oscillations of rs-fMRI signals (~0.01-0.1 Hz) are sensitive to aliasing (an effect where the two signals become

indistinguishable) of high-frequency cardiac and respiratory signals (van Buuren et al., 2009). Aliasing events can influence the BOLD signal measured, thus appropriate pre-processing techniques such as utilising physiological recordings and removing the estimated cardiac or respiratory signals from the BOLD signal, often *via* linear regression, can improve data quality (Murphy, Birn and Bandettini 2013). Finally, rs-fMRI signals can be influenced severely by head movement during scanning. Head motion can systematically alter and lead to spurious functional connectivity estimations between regions (Maknojia et al., 2019; Power et al., 2014). In rodents, this problem can be reduced by performing the procedure under anaesthesia.

2.8 Anaesthesia

Unlike humans, rodents are often sedated during acquisition of rs-fMRI to minimise head movement, prevent motion artefacts, and reduce stress. However, anaesthesia has been shown to have complex effects on neurovascular coupling, brain metabolism, and neuronal activity (Gao et al., 2017; Masamoto and Kanno 2012). Several different anaesthesia regimes have been used in rodent studies mainly classified based on their site of action. The most used anaesthetic is the $GABA_A$ receptor agonist isoflurane (Mandino et al., 2020). Isoflurane is widely used in rodent studies for its ease of use. Gaseous isoflurane is easy and quick to administer, well controlled and is ubiquitously used for induction regardless of the final anaesthesia protocol used. Furthermore, isoflurane is thought to have relatively low levels of toxicity, with few long-lasting side effects, making it suitable for longitudinal imaging studies, although recent evidence challenges these assumption (Bajwa et al., 2019; Seubert et al., 2013). Other notable anaesthetics used in rodent studies include medetomidine, halothane, alpha-chloralose and ketamine (Mandino et al., 2020). Several studies have investigated the effects of different anaesthetic regimes in both mice (Grandjean et al., 2014) and rats (Paasonen et al., 2018). One particularly relevant study evaluated six different anaesthetic regimes, as well as non-anaesthetised awake imaging, in rats during acquisition of rs-fMRI and evaluated the functional connectivity patterns of cortical and sub-cortical connections under each regime (Paasonen et al., 2018). At an inspired concentration of 1.3% isoflurane, increased connectivity was observed between cortical and striatal regions when compared to a wide range of other anaesthetic regimes, and awake, non-sedated animals. However, this increase was not apparent in sub-cortical regions such as the hypothalamus and thalamus; connectivity decreased in the isoflurane group in these regions (Paasonen et al., 2018).

Similarly, at higher doses of isoflurane (~1.8% isoflurane), increased BOLD signals were also observed across cortical and sub-cortical areas in a separate study (Liu et al., 2013). The reported effect appeared to be a global phenomenon, with widely enhanced BOLD responses distributed spontaneously and intermittently across cortical regions with no obvious spatial pattern (Liu et al., 2013). These reports, and others (Grandjean et al., 2014), suggest that higher gaseous concentrations of isoflurane may lead to increased connectivity in cortico-cortico regions, with decreased connectivity in sub-cortical regions. Thus, lower concentrations of isoflurane (<1%) are recommended to limit the effect of isoflurane on enhancing global cortico-cortico connectivity (Chuang and Nasrallah 2017). Using medetomidine in conjunction with low dose isoflurane (~0.6%) is an alternative and has been shown to modulate cortico-cortico connectivity less when compared to isoflurane alone (Paasonen et al., 2018). However, practical constraints may limit the widespread use of this particular anaesthesia regime. Studies in head-restrained awake rodents offer an alternative assessment of rs-fMRI under 'rest' conditions. Indeed, many groups have evaluated rs-fMRI connectivity in awake rodents, but careful habituation and head-restraint is necessary to minimise stress and movement related artefacts (Stenroos et al., 2018). In awake animals, thalamocortical activity is generally less inhibited when compared to isoflurane (Chang et al., 2016), and functional connectivity strength is increased in awake animals subcortically compared to animals under several different regimes of anaesthesia (Paasonen et al., 2018). However, important considerations including refinement of restraint design (Chang et al., 2016) and longer acclimatisation periods (Harris et al., 2015) must be considered to reduce elevated stress common to restraint procedures associated with awake imaging in rodents. Indeed, it has been shown that restraint can have short- and long-term consequences on sensory and emotional processing (Low et al., 2016) in rodents. In this vein, Low et al., replicated a common restraint procedure performed during rs-fMRI experiments and investigated corticosterone and pain responses to formalin. Restrained animals showed higher levels of corticosterone during and after restraint, and additionally, showed a decreased nociceptive response to a formalin challenge, a finding linked to increased fos-positive cells in the CeA (Low et al., 2016). Combined with the finding that chronic restraint stress (10 days' immobilisation) also alters functional connectivity patterns in somatosensory and DMN-like regions (Henckens et al., 2015), it is very important to control for stress when carrying out awake rodent rs-fMRI studies. In summary, it is important to carefully consider the anaesthesia regime used as they all inherently alter, to a certain extent, functional connectivity.

2.9 Comparison of non-invasive imaging techniques

Several non-invasive imaging techniques exist to understand brain function. For example, MRI, PET, single photon emission computerized tomography (SPECT) and optical imaging have all been used in a pre-clinical setting to map changes in the rodent brain over time. MRI was utilized in this thesis to understand brain structure and function. MRI has several advantages over and beyond other imaging techniques such as PET and SPECT. Both PET and SPECT fall under the category of nuclear medicine procedures. This means that both techniques involve the use of ionizing radiation with the emission of positrons and gamma rays in PET and SPECT, respectively. This is not the case in MRI, where water protons and radiofrequency waves are used to visualize brain states, mitigating any risks associated with radiation exposure. In the case of PET and SPECT, the use of radioactive tracers, their half-life and their production has to be carefully considered. One of the most commonly used radiotracers is the fluorinated glucose molecule – 18^{F}-Fluoro-deoxy-glucose (18^{F}-FDG). This molecule has a half-life of approximately 110 minutes. As a result, access to commercial radiotracer facilities is required, especially in instances where local production is not available. Along these lines, research laboratories carrying out PET and SPECT studies must consider whether production chemists are available and easily accessible, especially in the instances where novel compounds, or compounds using radiotracers with smaller half-lives, are needed. Unlike PET and SPECT, MRI is multimodal and can be used to map both brain structure and function. It can also be used to understand myelin (DTI), and regional metabolite concentration (spectroscopy). These investigations can occur in series, in the same animal, during the same scanning session, greatly enhancing the functionality of MRI when compared to the other nuclear medicine techniques. However, MRI is not without its limitations. As pre-clinical scanners strive for higher field strength (9.4T was used in this thesis) to improve signal-to-noise, enhanced physiological noise and higher magnetic field inhomogeneity is likely to occur. As physiological noise increases this will likely represent a stronger source of aliasing in the fMRI signal. In humans, as field strength increases, specific energy absorption rate will also limit the amount of time in the scanner, thus potentially limiting multimodal investigations of brain function on of the key advantages of MRI over that of PET and SPECT.

2.10 Brain-behaviour associations studies: pros and cons

Linking individual differences in brain function and structure to typical, and indeed atypical, variation in behavioural (endo)phenotypes is a pivotal objective of human neuroscience. Over the last several decades the availability and use of non-invasive imaging, especially MRI, has vastly increased. As a direct result, the evaluation of brain-behaviour associations via non-invasive neuroimaging has increased in parallel over the last several decades. Brain-behaviour association studies have several benefits, not least providing a solid framework in which to probe and evaluate cognitive function, helping to understand and inform cognitive processes and theories.

A central feature of these types of studies is that they localize psychological functions to specific brain regions, identifying brain-behaviour correlations. Localization studies have substantial value, leading investigators to understand the underlying relationship between brain organization and neuropsychological processes. Importantly, these studies can be used to predict abnormal cognitive function along a typical continuum via brain-behavioural correlations. With large-scale data becoming more commonplace, these types of localization studies may also predict future risk factors for a wide range of psychiatric disorders. Moreover, these studies are also important for longitudinal assessment of brain function and structure, given the non-invasive nature of their design. Repeated assessment throughout the lifespan allows the exploration of the association between behaviour and brain development. Through brain-behaviour association studies we can also evaluate and assess the degree of overlap in functional activation maps between different tasks. These studies shine a light on how slight nuances in behavioural task structure may require similar brain activation patterns, and therefore potentially similar underlying cognitive processes. On the other hand, brain-behaviour association studies also reveal differences in brain activation between different tasks, with these studies shining a light on how slight nuances in behavioural task structure may require different brain activation patterns, and therefore potentially divergent cognitive processes.

Through the advent of the human connectome project and other imaging consortiums, access to large-scale brain imaging datasets and behavioural phenotyping information is becoming more commonplace. As a direct result, human brain-association studies using MRI often have a large number of participants (often 1,000+), leading to enhanced statistical power. Other efforts in the human domain involve using similar MRI sequences across different sites, and pooling participants. In substance abuse studies, the numbers of participants are commonly

much lower (<100). Pre-clinically, there are commonly a the wide-range of different scanning sequences used, especially in relation to functional imaging, limiting the possibility of pooling data. In addition, chronic self-administration procedures, like those used in this thesis, are extremely difficult to carry out, therefore limiting the final number of animals available for post-administration brain-behaviour association studies. In addition, large-scale animal studies are not commonplace given the global interest in reducing the use of animals in scientific research, as well as practical constraints, especially in studies assessing longitudinal changes involving multiple scanning time points throughout the lifespan. This is an important point, however, given evidence suggesting that brain-behavioural studies with small sample sizes exhibit reduced statistical power (Marek et al., 2020 pre-print). This concept may not be specific to brain-behaviour associations with low levels of statistical power seen widely across studies in humans within neuroscience and psychology (Button et al., 2013). This is also true in studies in rodents. Studies commonly have group sizes of 8-12 animals and very few studies reach 80% power, the commonly accepted lower limit (Carneiro et al., 2018). Nonetheless, several efforts are being explored to increase statistical power in pre-clinical studies without increasing sample sizes. For example, Bonapersona et al., used control group data from existing studies (Bonapersona et al., 2021). In short, smaller studies should always seek to replicate their findings in future, larger studies where possible. As a starting point, studies should also contribute study data to repositories for future data aggregation.

Chapter 3

General methods

3.1 Subjects

Male Lister Hooded rats (n=52) (Charles River, Kent) were used in this study. Pregnant dams were initially housed in temperature and humidity controlled individually ventilated cages upon arrival. Timed-mating provided staggered offspring groups for discrete post-natal day (PND) developmental imaging. At PND-21 pups underwent their first developmental MRI scan. On completion, offspring of the same dam were housed in groups of four and the dam was sacrificed. All animals were kept in these housing groups and conditions during developmental scanning and behavioural phenotyping. All animals were kept on a reverse light/dark cycle with red light on between 07:30am and 19:30pm and white light on between 19:30pm and 07:30am. Animals were allowed ad libitum access to food and water during developmental scanning. Animals were subsequently kept under food restriction (85% of their free feeding weight) during behavioural training. All animals received the same behavioral training schedule. The specific training schedule was carried out in a fixed format. All animals underwent training first on the autoshaping procedure, then the 5-CSRTT, the RL task, the locomotor reactivity to novelty task and the novelty place preference task, in that order. Upon completion of behavioural phenotyping and intravenous catheter surgery animals were singly housed for the duration of the experiment. **Fig1.** shows an overview of the experimental timeline. Several animals were culled throughout developmental scanning and behavioural training due to unforeseen circumstances including complications with administered anaesthetics during scanning and malocclusion. In addition, several animals were culled throughout the SA process due to complications with catheter patency. Due to scheduling constraints 3 rats were not scanned at PND-63. An overview of the number of subjects at both the PND-63 and adult stages of the study are presented in **Table1.** All

experiments were carried out in accordance with the (U.K Animals) Scientific Procedures act (1986) under the UK Home Office project license (PPL 70/7587) and were approved by the University of Cambridge ethics committee.

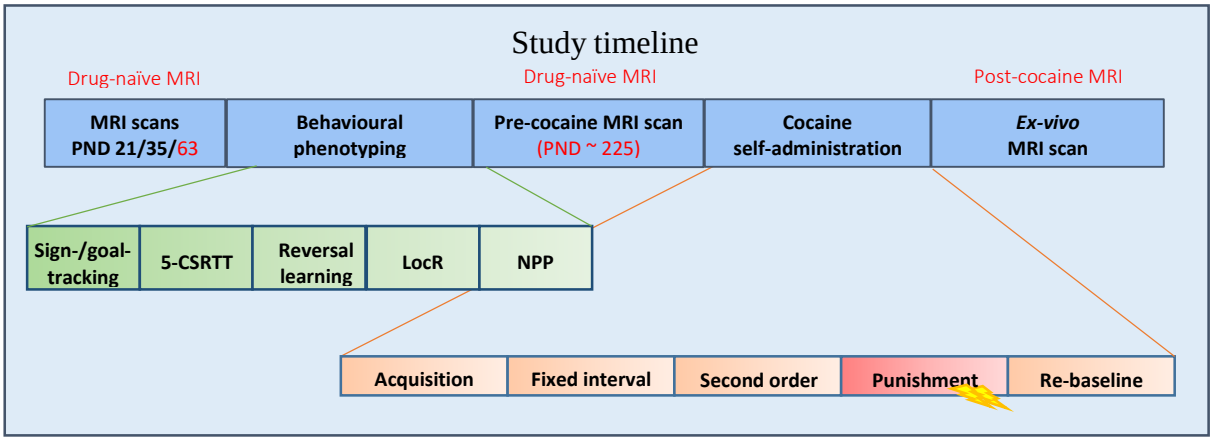


Fig1. Overall timeline of thesis research including developmental magnetic resonance imaging scans, behavioural phenotyping, cocaine self-administration and the evaluation of compulsive cocaine seeking. The PND-63 and post-behaviour MRI scans are assessed in this thesis. *Abbreviations*: MRI, magnetic resonance imaging; PND, post-natal day; 5-CSRTT, 5-choice serial reaction time task; LocR, locomotor reactivity to a novel inescapable environment; NPP, novelty place preference; SA, self-administration.

	Behavioural classification	**SA**	**Structural imaging (Beh)**	**Functional imaging (Beh)**	**Structural imaging (SA)**	**Functional imaging (SA)**
PND-63	48[#]	39	45	31	37	24
Adult	48[#]	39	48	37	39	29

Table1. Overview of the number of animals used in this thesis at PND-63 and adult timepoints. The total group size after behavioural classification was n=48. Due to issues with catheter patency 9 animals were culled during self-administration training (n=39). The differences in number of animals between timepoints and between behaviour and self-administration can be accounted for by 1) attrition during the self-administration procedure, 2) three animals did not receive a PND-63 scan, 3) within-study optimisation prevented 12 rats from being used in the functional analysis, 4) three scans at PND-63 had to be removed due to excessive distortion. The final number for each analysis is presented above. [#] indicates that one rat had to be excluded from impulsivity screening.

3.2 Attribution of incentive salience: sign- and goal-tracking

Twelve operant chambers (31.8cm x 25.4cm x 26.7cm, Med Associates, St Albans, USA) were used for the Pavlovian conditioning experiments. Each chamber was housed in a sound-attenuating outer cabinet, with a constant low-level background noise being provided by a ventilator fan. The chambers were equipped with a pellet receptacle and dispenser in the middle of the front wall in between two retractable levers (at equal height). The levers were set such that they would be deflected by 10 grams of force, resulting in a "lever contact" being recorded. Infrared detectors were fitted inside the food magazine to detect "nosepoke" responses. A cue light was located above each lever and a white house light (which was illuminated for the duration of the training sessions) was located at the top of the opposite wall above the food magazine. Rats were familiarised with the same reward pellets used in the Pavlovian conditioning procedure (45 mg Noyes dustless pellets, Sandown Scientific, UK) in their home-cage for two consecutive days prior to training. The rats were then placed into the operant chambers on the following day for a pre-training session. This involved the delivery of 50 reward pellets into the food magazine under a variable interval (VI) 30 second schedule (with pellets delivered between 0 and 60 seconds). Following the pre-training session, the task was carried out over five consecutive daily sessions. Each session consisted of 25-trials (CS – US pairings), with a single trial involving the insertion of a lever (CS) into the chamber for 8 seconds on a VI 55-second schedule. Upon retraction of this lever, a reward pellet (US) would be delivered into the food magazine (independent of the behavioural response of the animal). The MED Associates software recorded the number of lever contacts and nosepokes (head entries into the food magazine) during the 8 second lever presentation, as well as the latency to the first lever contact and nosepoke upon lever presentation. All variables measured were averaged across the final two sessions. CS responses (lever presses) (Peña-Oliver et al., 2015) were used to define sign-tracking behaviour, whereas nosepokes in the food magazine were used to define goal-tracking behaviour. Alternatively, the PCA-index for each animal was also calculated (Meyer et al, 2012). Briefly, the response bias index (ratio of level presses and head entries into the food magazine relative to total number of responses), probability index (difference in probability between approaching the lever *versus* entering the food magazine) and latency index (difference in latency between approaching the lever and food magazine) were calculated. The Pavlovian condition approach index (PCA-index) was calculated as an average of the three indices, from the last two PCA sessions. Animals were ranked accordingly. A PCA

score of -1 and +1 indicated goal- and sign- tracking behaviour, respectively. A schematic representation of both sign-tracking (**Fig2A.**), goal-tracking (**Fig2B.**) and an overview of the task structure is presented in **Fig2A-C**.

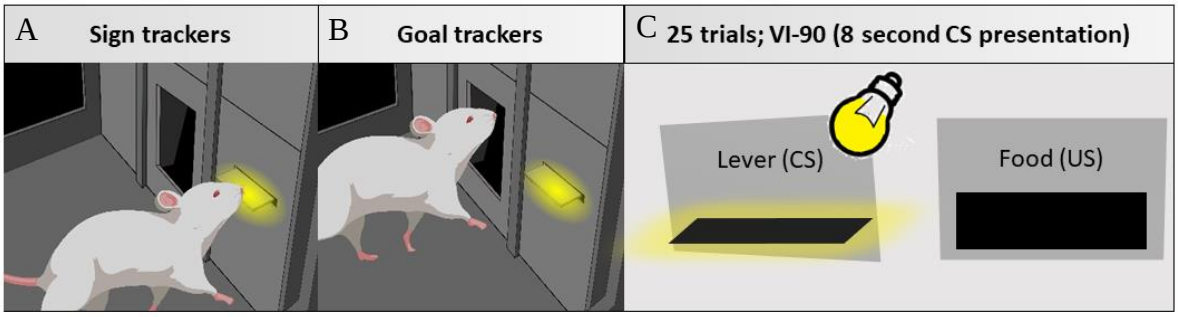


Fig2. Schematic representation of the Pavlovian conditioned approach task, demonstrating **(A)** sign- and **(B)** goal-tracking behaviour. Sign- and goal-tracking animals are categorised based on their approach behaviour to a lever cue. Sign-trackers more actively and preferentially approaching the cue **(A)**, whereas goal-trackers show preferential approach to the food magazine, the site of reward delivery **(B)**, even though no response is required for food delivery **(C)**. Adapted from Campus et al., 2019.

3.3 Impulsivity: 5-choice serial reaction time task

The 5CSRTT has been usd to measure impulsivity and has been previously described (Bari et al., 2008). The apparatus consisted of a sound attenuated, fan assisted chamber (31.8cm x 25.4cm x 26.7cm, Med Associates, St Albans, USA) with a food magazine on the rear wall illuminated by a house light and five equally spaced apertures (2.5cm x 2.5cm) on the front wall. A light-emitting diode was situated at the rear of each aperture. Infrared detection systems were present in both the food magazine and each of the five apertures. The task contingencies were controlled *via* WhiskerServer software (version 2.8) and FiveChoice Client (version 2.6) (Cardinal & Aitken, 2010). Animals were trained on average 6-days a week. To start a trial, the animal first nosepoked in the food magazine releasing a reward pellet (45 mg Noyes dustless pellets, Sandown Scientific, UK). Consequently, a brief visual (light) stimulus was presented in one of the five apertures in a pseudo-random fashion. If an animal responded at the previously illuminated aperture this was deemed a correct trial and the animal was rewarded with a single pellet. Failure to respond within a given timeframe (limited hold), after light presentation was regarded as an omission. If an animal responded in an aperture that was not previously illuminated this was regarded as an incorrect trial. Both

omission and incorrect trials were penalised with no food reward and a brief 5 second time-out period, during which the chamber light was switched off and no food reward was dispensed. Premature responses were recorded if an animal made a response prior to the illumination of the aperture also resulting in no food reward and a time-out period. Animals progressed through various training stages to reach a stable level of responding, with a stimulus duration of 0.7 seconds and ITI (duration between magazine entry and stimulus presentation) and limited hold period (period in which animal must respond after light stimulus) of 5 seconds. A variable short and long ITI schedule was then used to assess impulsivity. All animals underwent three long ITI (7 seconds) schedule sessions, separated by short (5 second) ITI baseline sessions. A schematic overview of the task is provided in **Fig3A.,** with a simulation of behavioural responses and their outcomes seen in **Fig3B**. Premature responses on this task were calculated from the final 2 long ITI sessions. Premature responses were calculated as the percentage of premature response trails relative to total trails, averaged across the final two long ITI sessions.

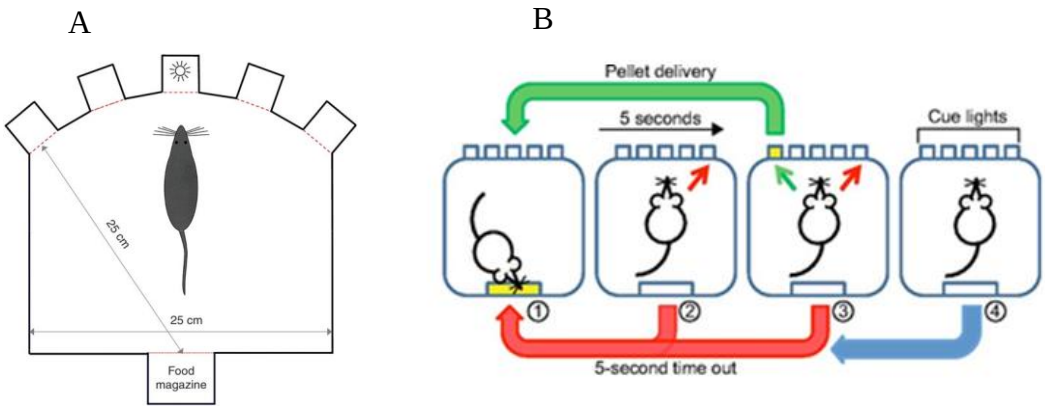


Fig3. Schematic representation of the 5-choice serial reaction time task and cartoon depiction of task parameters and task contingencies. **(A)** Briefly, the testing box for the 5-CSRTT consists of one panel with five nosepoke apertures, which are illuminated in a pseudo random fashion, one at a time. The opposite wall contains a central magazine, where food reward is delivered after a correct response is made. **(B)** A trial is started when the animal nosepokes in the food magazine, after a limited hold period (e.g., 5 seconds) one of the five apertures is lit up for a period of time (stimulus duration), the animal must then make the correct response and nosepoke the illuminated aperture. If this is done, the animal receives a reward. Conversely, if the animal nosepokes any of the five apertures prior to the illumination, but after a trial has been initiated, this is deemed a premature response and is punished with a time-out period. Additionally, a nosepoke in the non-illuminated aperture after aperture illumination is an incorrect response and is also punished with a time-out response. Finally, omission responses occur when an animal fails to nosepoke in any of the apertures after trial initiation. Adapted from Jupp, Caprioli and Dalley 2013; Dalley, Cardinal and Robbins 2004.

3.4 Cognitive flexibility: deterministic reversal learning

The apparatus consisted of a sound attenuated, fan assisted chamber (31.8cm x 25.4cm x 26.7cm, Med Associates, St Albans, USA) with a food magazine on the rear wall illuminated by a house light and five equally spaced apertures (2.5cm x 2.5cm) on the front wall. The middle three apertures were blocked using a metal plate and were not part of the experimental setup. Infrared detection systems were present in both the food magazine and each of the three active apertures. The task contingencies were controlled *via* WhiskerServer software (version 2.8) and FiveChoice Client (version 2.6) (Cardinal & Aitken, 2010). Training began with two days of habituation. The first trial of the session started by an animal making a nosepoke response in the food magazine. Consequently, this triggered the illumination of a cue light in each aperture (both left and right). A response in either aperture was rewarded with a single reward pellet (45 mg Noyes dustless pellets, Sandown Scientific, UK). Failure to respond within 30 seconds following a nose poke in the food magazine (the initiation of the trial) was regarded as an omission and was followed by a 5 second time-out, where all lights were switched off. The difficulty of the task was increased with only one aperture being reinforced under consecutive FR-1, FR-2 and FR-3 schedules of reinforcement. A criterion of 50 correct trials was required to proceed to each level. Upon stable level of responding at FR-3 the ITI was increased gradually to 5 seconds. Following stable responding the animals were then tested in the spatial discrimination task. In this task, light within each aperture was presented during successive trials however only one of the apertures was rewarded. Rewarded and non-rewarded apertures were counterbalanced across subjects. During the first session animals were given 1-hour to complete the discrimination task by achieving 9 out of 10 correct trials. The following day animals underwent a retention test whereby the same aperture was rewarded as the previous day. Following 9 out of 10 correct trials during this session the contingency was reversed. A nose poke in the previously rewarded aperture resulted in no reward delivery and the rats were required to respond in the other previously unrewarded aperture to obtain reward. Animals could complete up to three reversals during the 1-hour session. A schematic representation of the task structure is provided in **Fig4**.

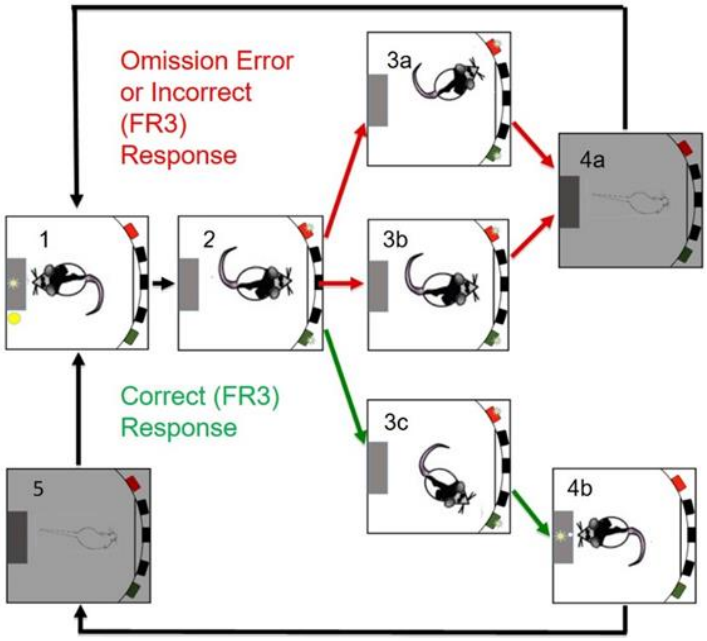


Fig4. Schematic representation of the spatial-discrimination reversal learning task. Briefly, an initial nosepoke in the food magazine initiates a trial. Subsequently, nosepoke responses in the rewarding aperture under a fixed ratio 3 schedule of reinforcement (i.e., 3 nosepokes in the correct aperture) resulted in reward delivery. If a nosepoke response is made in the incorrect aperture, or no nosepokes are made (omission trials), then a 5 second time-out is also delivered. Following 9/10 correct responses the contingencies are reversed and the previously non-rewarded aperture is now rewarded. Conversely, the previously rewarded aperture is now punished with a time-out. All animals were given either 1-hour to complete the task or up until three reversals were completed. All animals completed three reversals in the given timeframe. Adapted from Zhukovsky et al., 2017.

3.4.1 Computational modelling

A Q-learning model with three parameters (alpha (α), beta (β), kappa / stickiness (κ)) was fitted to each animal's RL data as previously described (Zhukovsky et al., 2019). Throughout this thesis, kappa and its descriptive equivalent 'stickiness' will be used interchangeably. Stickiness (or 'sticky') is used to describe animals showing kappa values close to 1. Briefly, the Q-learning model is equivalent to Rescorla-Wagner learning (Rescorla & Wagner 1972) where, at each trial t, an animal assigns an expected value to each choice available (left or right aperture). The expected Q-value is then updated based on the reward prediction error, which is the difference between the reward expected and the reward received. The extended

model (Zhukovsky et al., 2019; model 3) includes three model parameters. Alpha is the Rescorla-Wagner learning rate and determines how quickly the model is updated based on positive or negative feedback. Animals that show a high alpha value more readily increase (or decrease) the expected Q-value placed on that response if the subsequent response is followed by a reward (or not). Beta is the inverse softmax temperature parameter and broadly determines an animal's exploration or exploitation. A low beta leads to an animal relying heavily on the expected Q-values of the response, leading to exploitation. Conversely, a high beta value would lead to exploration and under some circumstances increased rewarded outcomes. However, in the present deterministic task, a high beta would result in fewer rewards. Kappa is a measure of 'stickiness' or the tendency for a rat to choose the same previous choice regardless of reward outcome. A kappa parameter close to 1 indicates an animal 'sticking' to its previous response whilst kappa values close to -1 reflect choice alternation.

Whilst Bayesian methods are currently favoured when modelling reversal learning in humans (e.g., Kanen et al., 2019; Bartolo and Averbeck 2020) and rats (Wilson et al., 2014), the choice to utilize a q-learning approach in this thesis was two-fold. First, the q-learning algorithm used in this thesis had been previously utilized in our laboratory to understand choice behaviour on the serial deterministic reversal learning task in relation to cocaine reinforcement. These results showed that escalation of cocaine modulated several important parameters of this model; for example, high levels of cocaine escalation were related to enhanced stickiness (Zhukovsky et al., 2019). This relationship, along with the establishment of the algorithms in the laboratory, strongly influenced model choice in this thesis. Second, recent work has utilized similar Q-learning models to understand the relationship between psychostimulant use, reversal learning performance and how parameters within these models are modulated by discreet prefrontal subregions in the rat (Groman et al., 2017; Verharen et al., 2020), therefore showing the utility of these models in pre-clinical investigations of cognitive flexibility and addiction-like behaviour. For more details on the computational models used please see Zhukovsky et al., 2019.

3.5 Novelty place preference

NPP was assessed using two equally sized rectangular chambers partitioned by a third, central chamber (**Fig5A, B.**). Both chambers were accessible from the central chamber using a sliding door. Of the two compartments, one had black walls and a smooth floor, whilst the other had white walls and a grid floor, thus providing distinct visual and tactile cues for the animal. To start the session, animals were placed in the central chamber for 5 minutes (**Fig5C.**). After 5 minutes, one of the sliding doors was removed (the familiar chamber). Animals were allowed to explore this chamber for 25 minutes (**Fig5D.**). Animals were then placed back in the central chamber (5 seconds) (**Fig5E.**) and both doors removed. Animals were then allowed to freely explore both chambers (novel and familiar) for 15 minutes (**Fig5F.**). Light intensity in the central corridor was 15 Lux, whereas light intensity in both compartments was 0-0.2 Lux. The initial familiar compartment was counterbalanced across different testing arenas (white *versus* black / smooth *versus* rough grid floor). The time spent in each compartment was recorded using automated video-tracking software (ViewPoint

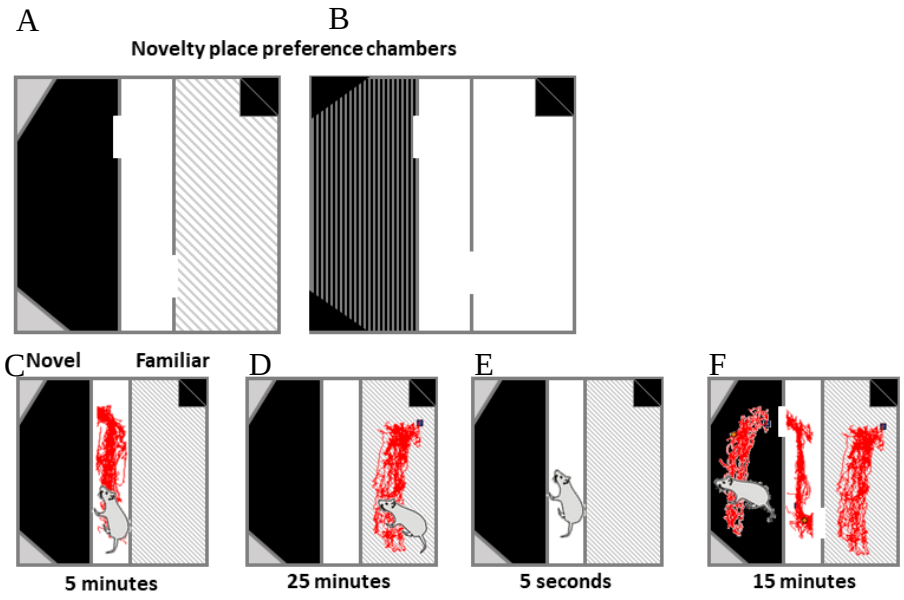


Fig5. Schematic overview of the novelty place preference (NPP) chamber and task parameters with contextual modification. Both NPP chambers contain different visual and tactile cues between the familiar, central, and unfamiliar chambers **(A, B)**. In the NPP procedure rats are first put in the central chamber for five minutes **(C)**, they are then allowed to explore the familiar compartment for 25 minutes **(D)**. Subsequently, rats are placed back in the central chamber for five seconds **(E)**, after which both access to the familiar and novel chambers is granted for 15 minutes **(F)**. NPP is measured in this time window, with high novelty preferring animals showing spatial bias to the novel compartment over that of the familiar compartment.

Behaviour Technology, Lyon, France). NPP was calculated via (time spent in novel compartment / (time spent in novel + familiar compartments) x 100.

3.6 Sensation seeking: locomotor reactivity to novelty

LocR was measured in four open fields (50cm x 50cm x 50cm) using an automated behavioural tracking software (ViewPoint Behaviour Technology, Lyon, France). Animals were placed in the arena for 120 minutes between 9am and 4pm under a light intensity similar to that of the holding rooms (~550 Lux at the center of the open field). Animals were not habituated to the activity chambers prior to the test. Locomotor activity was recorded, and distance travelled measured. An overview of the task and the tracking output is provided in **Fig6**.

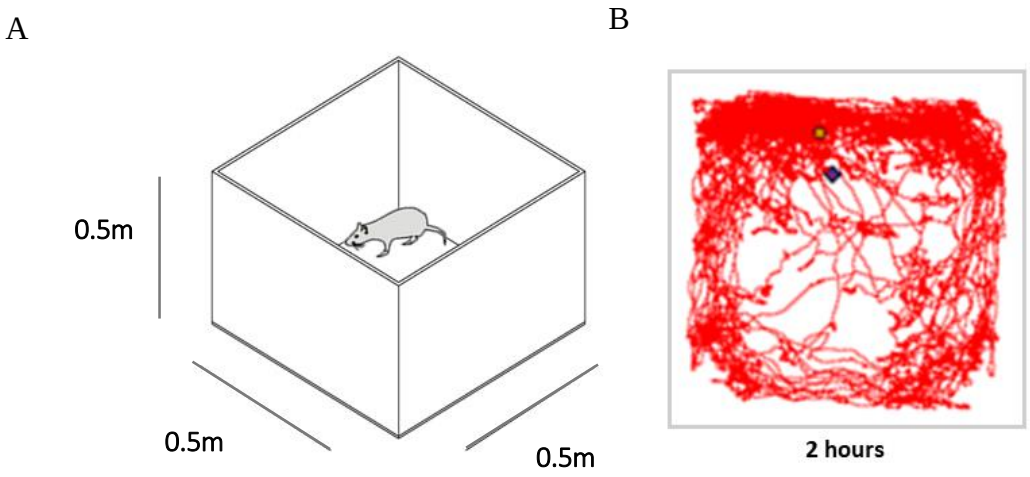


Fig6. Schematic overview of the locomotor reactivity to novelty chamber and representative tracking output from viewpoint systems. **(A)** Locomotor reactivity to a novel inescapable environment reflects the distance travelled by a rodent placed in a 0.5m x 0.5m x 0.5m open field chamber over the course of a 2-hour duration. **(B)** A representative tracing map of movement over the 2-hour timeframe.

3.7 Rational of behavioural endophenotypes assessed

Whilst several paradigms exist to assess impulsivity in rodents, the 5-CSRTT was used in this thesis due to the strong link between high impulsivity on this task and compulsive cocaine-taking behaviour as assessed through the 3-criteria model of addiction (Belin et al., 2008). High impulsivity on the 5-CSRTT has also been linked to other compulsive behaviours

including greater susceptibility towards the development of SIP (Belin-Rauscent et al., 2016). High impulsive animals on the 5-CSRTT also show differential rates of cocaine intake following intermittent exposure (Dalley et al., 2007). Autoshaping was assessed given the evidence linking sign-tracking and addiction-like behavior (Tomie and Morrow 2018), the association between sign-tracking and motoric forms of impulsivity, e.g., the 2-CSRTT (Lovic et al., 2011), and the recent evidence suggesting a possible link between sign-tracking and resistance to footshock-induced punishment of cocaine taking (Pohorla et al., 2021). Spatial deterministic reversal learning was specifically chosen building off previous work in our laboratory linking cocaine reinforcement and escalation to changes in performance on the task (Zhukovsky et al., 2019), and wider studies showing the modulatory effect of cocaine on reversal learning performance (Groman et al., 2020; Calu et al., 2007). LocR was assessed given its strong historical links to stimulant self-administration (Piazza et al., 1989), whilst NPP was assessed given its link to compulsive cocaine taking in the 3-criteria model of addiction (Belin et al., 2011).

3.8 Cocaine self-administration

3.8.1 Intra-jugular surgery

Rats were implanted with a home-made indwelling catheter into their right jugular vein under isoflurane anaesthesia (O_2 carrier gas; 2 L/min; 5% for induction and 2-3% for maintenance) and analgesia (Metacam, 1mg/kg, sc., Boehringer Ingelheim) as previously described Belin et al., 2008. Following surgery, rats received daily oral treatment with the analgesic for three days and an antibiotic (Baytril, 10mg/kg, Bayer) for one week. Catheters were flushed with 0.1 ml of heparinized saline (50 U/ml, Wockhardt®) in sterile 0.9% NaCl every other day after surgery and then before and after each daily SA session.

3.8.2 Self-administration apparatus

Experiments were conducted using twenty-four standard operant conditioning chambers (Med Associates, St. Albans, VT, USA) enclosed within a sound-attenuating box containing a fan to eliminate background noise. Each chamber was equipped with two retractable levers (4 cm wide, 12 cm apart, and 8 cm from the grid floor), a cue light (2.5 W, 24 V) above each

lever, and a white house light (2.5 W, 24 V) at the back of the chamber, in front of the levers. Silastic tubing shielded with a metal spring extended from each animal's IV catheter to a liquid swivel (Stoelting, Wood Dale, IL, USA) mounted on an arm fixed outside of the operant chamber. Tygon tubing extended from the swivel to a Razel infusion pump (Semat Technical, Herts, UK) located adjacent to the external chamber. Lever presses, presentation of light stimuli, reward delivery, and data collection were controlled by a PC running MED-PC4.

3.8.3 Self-administration training

Rats were trained to acquire cocaine SA (0.25 mg/100µl/5.7sec/infusion) under continuous reinforcement over 4 daily, 2-hour sessions. Under this schedule, each active lever press resulted in drug infusion initiated concurrently with a 20 sec time-out that included onset of a 20 s illumination of a cue light positioned above the active lever (CS), offset of the house light and retraction of both levers. Inactive lever pressing was recorded but had no scheduled consequence. Active and inactive lever assignment was counterbalanced, and a maximum of 30 infusions was available for each session at this stage. Following 4 daily sessions under FR-1 reinforcement, the daily schedule of reinforcement was changed to a FI schedule of reinforcement. In a FI schedule of reinforcement responding on the lever during the interval results in no drug-delivery. The rat must wait until the interval is complete before responses on the active lever are rewarded with the administration of cocaine. After drug infusion, the interval is reset and any responses on the active lever before the interval is once more complete results in no drug-delivery. The interval was reset 5 times in this procedure, resulting in a maximum number of 5 cocaine infusions during FI and ^{2nd}SOR training. The FI was increased daily across training sessions from 1 min (fixed interval 1 min, FI-1) to FI-2, FI-4, FI-8, FI-10 and eventually FI-15. After three sessions under an FI-15 schedule of reinforcement rats were trained to seek cocaine under the control of the drug-paired CS for 30 sessions under a FI15(FR10:S) ^{2nd}SOR. The ^{2nd}SOR employed here is nearly identical to that of the FI-15 schedule, however, on the ^{2nd}SOR every ten lever presses, regardless of where in the schedule, resulted in the response-contingent presentation of a cue-light. For more details on ^{2nd}SOR see Chapter 1, section 1.10.5.

3.8.4 Compulsive cocaine seeking

For five daily sessions under ^{2nd}SOR (sessions 21-25), drug-seeking behaviour was punished when rats were actively engaged in foraging for the drug, namely during the last 7 minutes of each 15-minute interval. During the last 7 min of each interval, mild electric foot shocks (1 sec duration, 0.25-0.45 mA) dispensed by a scrambler (Med Associate St. Albans, USA) connected to the grid floor in the operant boxes was delivered on every 16th lever press. Punishing responding only during the second part of each interval also offered the advantage that animals did not receive an electric foot shock immediately after a drug infusion, which could result in counterconditioning and a decrease in the aversiveness of the shock. Each aversive stimulation was paired with a cue light stimulus located on the top middle region of the wall (independent from those paired with drug infusions) and rats received one shock every 16th lever press to minimise the probability of co-occurrence of CSs and shocks and to avoid a total extinction of their drug-seeking behaviour. Following the punished sessions, rats were re-exposed to five baseline ^{2nd}SOR sessions to investigate their ability to recover their initial drug-seeking behaviour or whether they would display long-term behavioural adaptations to successive punishment sessions. An overview of the SA timeline is provided in **Fig7**.

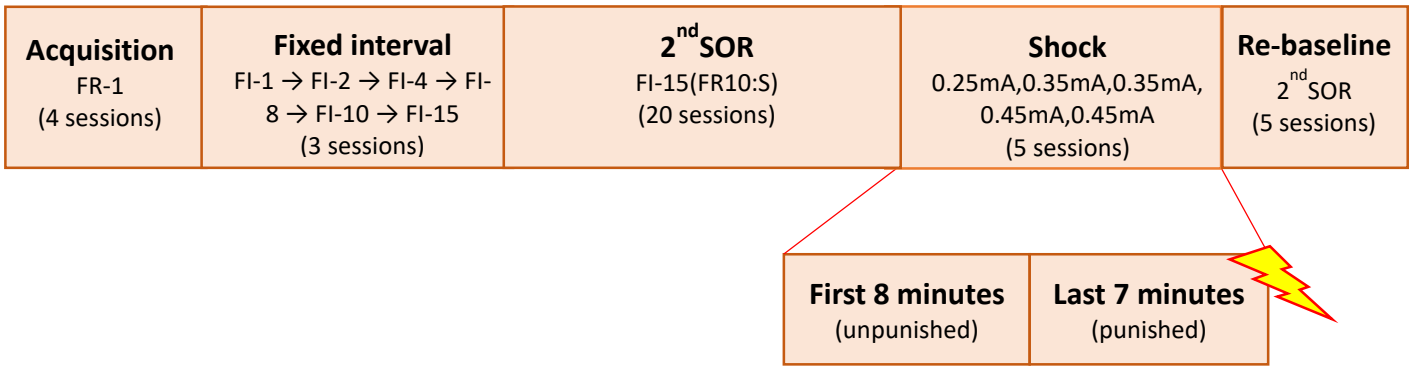


Fig7. Overview of the self-administration procedure. Briefly, acquisition of self-administration is carried out by initial Fixed ratio (FR)-1 sessions where 1 lever press leads to 1 infusion of cocaine. Following acquisition, increasing fixed-interval (FI) schedules are incorporated (8 sessions in total), resulting in appropriate levels of responding under an FI-15 schedule of reinforcement, repeated over three days. Rats were then trained on a second order schedule of reinforcement for 20 sessions prior to the introduction of mild electric foot shock. Analysis of compulsive cocaine seeking occurred over 5 days where foot shock was administered with increasing amplitude (day(d)1 – 0.25mA, d2/3 – 0.35mA, d4/5 – 0.45mA). Following five days of shock sessions animals were re-baselined on a second order schedule of reinforcement for a further 5 sessions. *Abbreviations*: FR, fixed-ratio; FI, fixed-interval; ^{2nd}SOR, second order schedule of reinforcement; mA, milliamps.

3.8.5 Pain sensitivity assessment

To ensure that potential differences in resistance to punishment were not attributable to a differential pain threshold, high and low compulsive rats from an independent cohort were subjected to a hot plate test prior to the valuation of compulsive cocaine seeking. Six hours following the 17th drug SA session under ^{2nd}SOR, rats were placed on a hot plate (Ugo Basile, Gemonio, Italy), calibrated to remain at a stable temperature of 52ºC. The time elapsed before the appearance of pain-associated behaviours, including paw-licking, and jumping, was measured, and considered a direct indicator of pain threshold. Rats were then immediately removed from the hot plate and returned to their home cage (**Appendix1C-D.**).

3.9 Magnetic resonance imaging

3.9.1 Acquisition

High resolution structural MRI was performed on a 9.4 T horizontal bore MRI system (Bruker BioSpec 94/20 Bruker Ltd. Coventry UK). Images were acquired using the manufacturer-supplied rat brain array coil with the rat in a prone position. Structural images were obtained based on a three-dimensional multi-gradient echo sequence (TR/TE 25/2.4ms with 6 echo images spaced by 2.1ms, flip angle 6° with RF spoiling of 117°). The field of view was 30.72×25.6×20.48 mm^3 with a matrix of 192×160×160 yielding isotropic resolution of 150µm with a total scan time of 6m 36s with zero-filling acceleration (25% in the readout direction; 20% in each phase encoding direction). Magnetisation transfer pulses (10µT, 2kHz off-resonance) were applied within each repetition to enhance grey-white matter contrast. Post-reconstruction, images from each echo were averaged after weighting each by its mean signal. A series of rs-fMRI scans were carried out using a single-shot gradient-echo EPI sequence. Scan parameters were: matrix size = 64x48x40, echo time = 15ms and repetition time = 1.832 seconds. A total of 450 volumes of images were collected, with three echoes acquired for each volume and a slice thickness of 0.5mm. Throughout all scans, rats were inducted with isoflurane (1-2% in 1l/min O_2:air 1:4). During rs-fMRI acquisition isoflurane was reduced to 0.8%. Respiratory rate and pulse oximetry (SA instruments; Stony Brook, NY) were measured and anaesthetic dose rates were adjusted keeping these in physiological range. Body temperature was measured with a rectal probe and a heated water system adjusted to maintain 36-37°C. As a result of within-study optimisation, 12 animals were

scanned using a different acquisition sequence with voxel dimensions 0.35x0.35x0.35mm, matrix size = 64x64x48, echo time = 15ms and repetition time of 3 seconds.

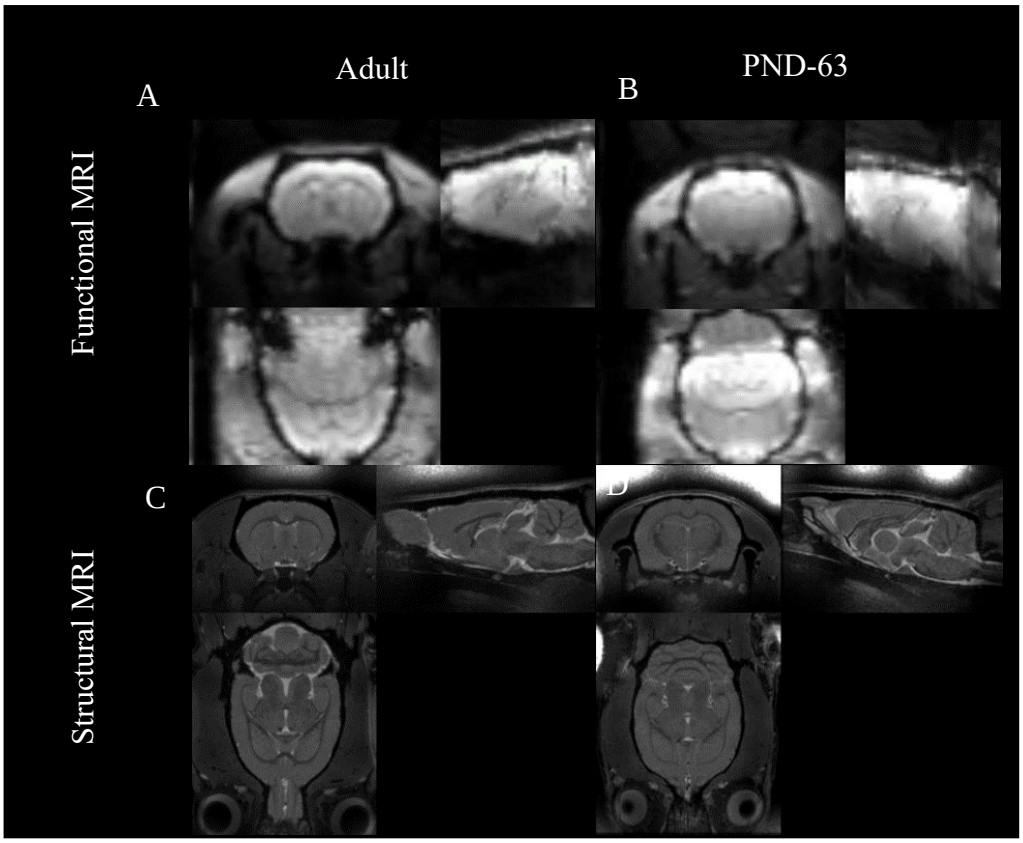


Fig8. Representative functional **(A, B)** and structural **(C, D)** magnetic resonance scans at both post-behaviour (adult) **(A, C)** and pre-behaviour (post-natal day 63) **(B, D)** timepoints. Adult scans, representing **(A, C)**, and pre-behaviour scans at PND-63 **(B, D)**. *Abbreviations:* MRI: magnetic resonance imaging; PND: post-natal day.

Representative images of both structural (Magnetisation transfer (MT)-weighted) and functional imaging scans are presented in **Fig8**. Three PND-63 scans were excluded from functional connectivity analysis due to excess distortion when scanning (See **Table1.**, (**Fig9.**)). In addition, signal drop-out in deep brain regions, including the amygdala, was observed excluding these regions from further functional connectivity analysis (**Fig10**). Magnetic susceptibility artefacts in the aural cavity are more common at high field strength and are a common cause of signal drop-out. In rodents, several methods have been proposed to mitigate against signal drop-out including reducing slice thickness and filling the cavity with toothpaste or other materials (Li et al., 2015). To reduce susceptibility artefacts in this

region a slice thickness of 0.5mm was used. However, in this thesis all scanning took place on a high-field 9.4T system and thus may be one reason for the apparent signal drop-out.

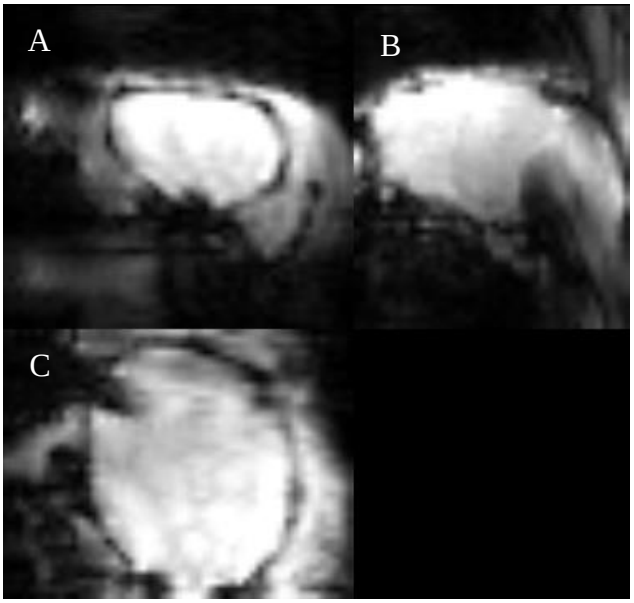


Fig9. Representative image of excess distortion seen at PND-63. Three scans of this type were excluded from functional connectivity analysis at PND-63. Shown in the **(A)** coronal, **(B)**, sagittal and **(C)** horizontal planes.

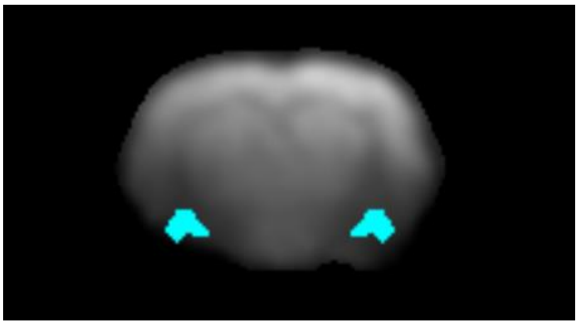


Fig10. Representative signal dropout observed in pre-processed scan at adult timepoint in the posterior portion of the brain, near the amygdala (blue mask). Presented in the coronal plane.

3.10 Structural Imaging analysis

3. 10.1 Voxel-based morphometry

Voxel-based morphometry was performed to assess grey matter volume differences. Images were first manually reoriented in 6 directions (pitch, yaw, roll, x, y, z) to match the orientation of a reference template image (provided by Dr. Steve Sawiak) using the bulk manual registration tool in the Statistical Parametric Mapping (SPM) 8 (Wellcome Trust

Centre for Neuroimaging, University College London, UK) toolbox SPMMouse (SPMMouse, Wolfson Brain Imaging Centre, University of Cambridge (Sawiak et al., 2009)). After correspondence was achieved structural images were bias corrected and segmented into three different tissue classes (grey, white, cerebrospinal fluid). Tissue class images were then rigidly co-registered to a reference template image. Non-linear registration was achieved with the diffeomorphic anatomical registration using exponentiated lie algebra (DARTEL) procedure (Ashburner 2007). Using the warp fields from the DARTEL procedure images were then warped and modulated to match the newly generated template images (**Fig11.**). All images were manually checked for accurate registration and the modulated grey matter maps were then smoothed with an isotropic Gaussian kernel filter of 0.45mm^3**.** Although there exists no consensus on the exact required level of smoothing, a balance between reduced spatial sensitivity and enhanced power must be considered (Sawiak et al., 2013). Commonly, in preclinical studies smoothing values range from 2-6 times the voxel size (Sawiak et al., 2009; 2012, Cannella et al., 2017; Meyer et al., 2017). In view of this, both structural and functional data was smoothed by 3x the voxel size in order to improve SNR whilst reducing the potential decrease in spatial sensitivity observed by smoothing at higher spatial extents.

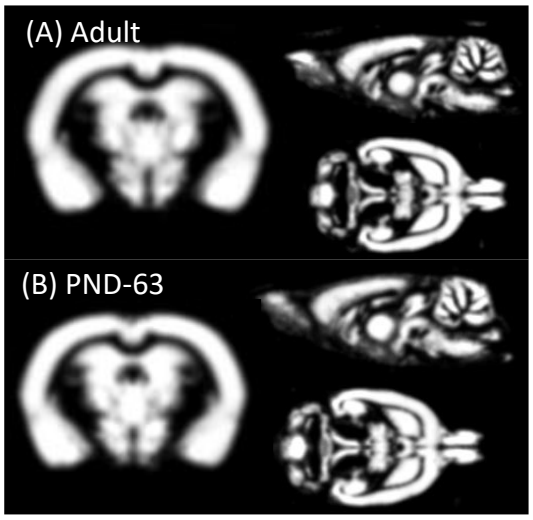


Fig11. Representative smoothed, modulated and warped grey matter segmentation used for evaluation of grey matter differences *via* voxel-based morphometry at the **(A)** post-behaviour (adult) scan timepoint, as well as the **(B)** pre-behaviour timepoint at post-natal day 63.

VBM was chosen over DBM for structural analysis in this thesis. Deformation based morphometry is another automated technique used for whole brain analysis of the rat brain (Gaser et al., 2012). Similar to VBM, DBM is a voxel-wise technique that detects structural differences. DBM detects structural differences via analysis of the deformation fields that map a subject-specific brain to a reference template by nonlinear registration at the voxel level (Gaser et al., 1999, Ashburner et al., 1998). Unlike VBM, DBM does not require the

automatic segmentation of the rodent brain into its different tissue classes (e.g., grey matter, white matter, and cerebrospinal fluid). This may be an important consideration in images acquired on low field strength scanners that have lower levels of SNR and contrast between the different tissue types. In this instance, DBM may be preferable as generating high contrast scans may require very long scanning times. In this thesis, a 9.4T MRI system was used. In addition, several different structural scans were used including PD-, T1- and MT-weighted scans. The MT-weighted scans that were used in the structural analysis performed provide excellent contrast between the different tissue boundaries, resulting in improved segmentation. Moreover, this contrast could be achieved in a relatively small timeframe (less than 20 minutes). Thus, the two most commonly cited limitations of the use of VBM, and indeed the preference for DBM, were not observed due to the system used. In addition, previous work in our laboratory using VBM has linked differences in grey matter to behavioral endophenotypes important in addiction-like behavior (Caprioli et al., 2014). Thus, considering this, and the improved contrast due to the system used to acquire the images, VBM was chosen over DBM in this instance. An overview of the structural pre-processing pipeline is provided in **Fig12** (below)

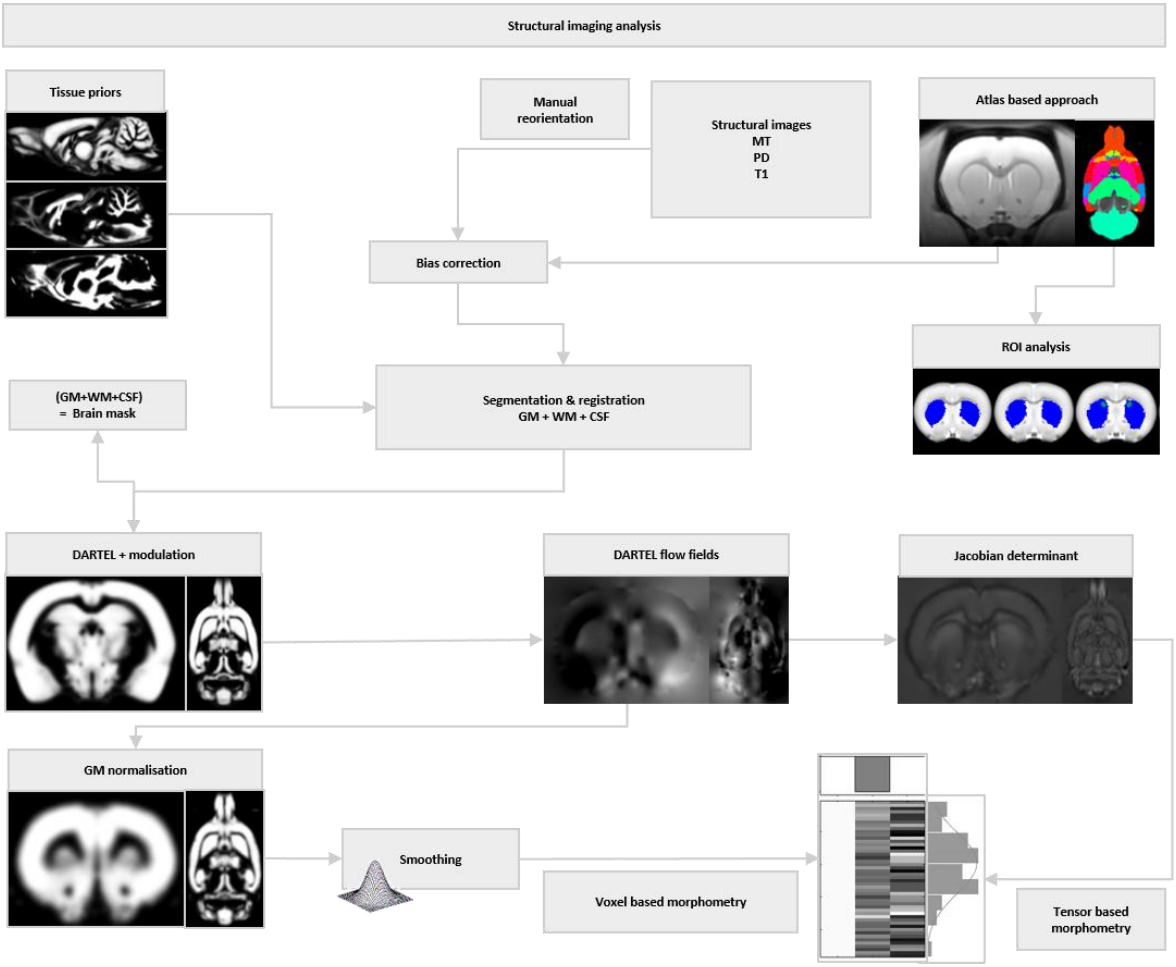


Fig12. Overview of structural imaging pre-processing pipeline. Structural images (T1-*weighted,* MT-*weighted* and PD-*weighted)* were first manually orientated to a reference template provided by Dr. Steve Sawiak using SPMmouse (SPMmouse; Sawiak et al., 2009). The aligned images were then bias corrected to correct for artefacts and enhance uniform intensities across the image. Following this, grey matter, white matter, and cerebrospinal fluid priors are used to segment the structural scans into the three different tissue classes. Subsequently, nonlinear image registration using diffeomorphic anatomical registration through exponentiated lie algebra (DARTEL) is performed. The output of which is a set of flow fields that map each voxel of the native structural scan to the study specific template. Grey matter native structural scans are then warped to the template space *via* the DARTEL flow fields, whilst simultaneously modulated by the Jacobian determinant, a process known as normalisation. The output images - subject specific grey matter scans in a common reference space (or template space) - are then smoothed with a Gaussian kernel of full width at half maximum of $0.45mm^3$ or 3x voxel size. Statistical analysis on these images (voxel-based morphometry) is then carried out. Alternatively, a Jacobian determinant image can be calculated from the DARTEL warp fields prior to the normalisation procedure and can be used to understand relative shape change across time, in an analysis pipeline called Tensor-based morphometry. Similarly, using the inverse of the DARTEL warp fields a probabilistic atlas can be warped back to native space and volumetric information extracted. For more information, see future directions.

3.11 Functional imaging analysis

3.11.1 Functional connectivity analysis

Prior to functional connectivity analysis voxels in both the structural (MT-weighted) and functional images were scaled by x10 to equate for differences in processing between rodents and humans. Pre-processing steps included 1) averaging of echo times, 2) removal of the first ten volumes, 3) brain extraction, 4) registration, 5) despiking, 6) motion correction, 7) spatial smoothing and 8) bandpass filtering (Grandjean et al., 2020) (**Fig13.**). Images were first manually reoriented to match a reference template (as previously described). For each volume of the rs-fMRI time-series the three echo times were averaged (3dcalc, Analysis of Functional Neuroimages, AFNI_16.2.07, https://afni.nimh.nih.gov) (Cox 1996) and the first ten volumes discarded, to reach steady state (fslmaths). To improve registration non-brain voxels were removed from both structural and functional images. The study specific DARTEL template containing grey matter, white matter and cerebrospinal tissue segmentations from the structural analysis were combined and an arbitrary threshold was used to obtain a mask of non-brain voxels. The inverse warps of the DARTEL procedure were then used to backwards register the study mask to subject space for each rat. This resulted in a subject-specific mask for each rat which excluded non-brain voxels. This mask

was subsequently used for brain extraction. Due to the enhanced contrast of the study specific template used as the reference image in which the functional images were registered (not the DARTEL template from the VBM analysis), the brain-extraction tool in FSL was used to extract any non-brain voxels from this image (Smith et al., 2002). Following brain extraction, registration of functional images to a reference template was performed using a two-step registration approach. First, the brain-extracted functional image was registered linearly (6 degrees of freedom (DOF)) to the brain-extracted structural (MT-weighted) image (FLIRT (FMRIB Linear Image Registration Tool; Jenkinson et al., 2002)). Subsequently the structural image was registered non-linearly to the template image (FNIRT (FMRIB Nonlinear Image Registration Tool; Smith et al., 2004)). Both the linear transformation matrix and the nonlinear warp-fields were combined to transform the functional image into template space in a one-step process to minimise interpolation effects (Mahmoudzadeh and Kashou 2013). Registration accuracy was manually check for each image. Temporal spikes were then removed (3dDespike) followed by motion correction (3dvolreg). Excess motion was calculated through relative framewise displacement (FWD) (Power et al., 2012) (for more details see Chapter 2, section 2.5). Next, the motion parameter outputs from motion correction were regressed out of the functional images (fsl_regfilt). Spatial smoothing was then applied using an isotropic 4.5mm^3 kernel (3dBlurInMask) and bandpass filtering was applied (0.01-0.1 HZ) (3dBandpass). SNR was estimated from multiple volume timepoints across acquisition. Mean signal was extracted from a cortical mask containing the whole isocortex, as well as from the edge of each scan (representing the noise component). Signal-to-noise was then evaluated by subtracting the background/noise signal from the global BOLD signal. Temporal SNR (tSNR) was calculated by taking the average temporal signal of the entire rs-fMRI acquisition, using the same global cortical mask as above, and dividing it by the standard deviation of the BOLD signal from the same mask. Region-to-region correlation analysis was then performed on fully pre-processed functional data in template space. Using the probabilistic atlas (below), as well as several manually drawn regions (JAJ/SS), the first Eigenvariate of the timeseries was extracted for each ROI (fslmeants) and correlation between each ROI per animal was assessed through Spearman's rank coefficient. Correlation matrices were subsequently used to address the relationship between connectivity and behaviour. All correlation values were corrected for multiple comparisons *via* false discovery rate (q = 0.05) (Genovese et al., 2002).

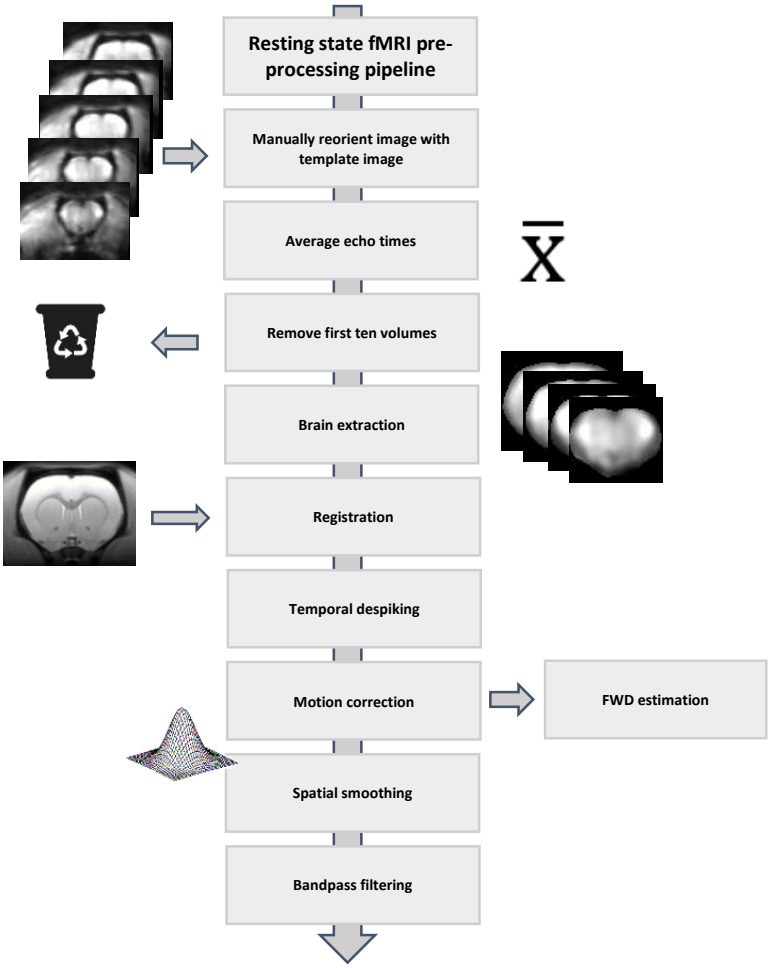


Fig13. Overview of functional imaging pre-processing pipeline. Voxels in the native resting-state functional magnetic resonance imaging (rs-fMRI) scans are first enlarged by a factor of 10 to account for differences in processing between rodents and humans. Subsequently, scans are manually aligned to a reference template provided by Dr. Steve Sawiak using SPMMouse (Sawiak et al., 2009). Once the images are aligned the multi echo sequence is averaged and the first ten volumes are removed to facilitate steady state. Following this, brain extraction is performed, and the functional scans are registered to a template. Once the images are in template space, temporal despiking and motion correction is carried out. Following this, spatial smoothing ($0.45mm^3$) and bandpass filtering (0.01-0.1 Hz) is then carried out. The output of this pipeline is fully pre-processed rs-fMRI scans that have been registered to a common template space.

3.11.2 Global signal regression

Global signal regression (GSR) is the removal of the global mean time course from the underlying timeseries of each voxel through linear regression. The main aim of the technique is to remove the effects of global variations in non-neuronal signal that may potentially contribute to region-specific changes in brain activity. As a result, GSR is primarily used to remove artefacts driven by respiration and motion (Li et al., 2019). However, the use of GSR is not without controversy (Murphy and Fox 2017; Liu, Nalci and Falahpur 2017). Some argue that GSR may remove valuable information within the underlying data. In support of this, the GSR signal has been related to neural activity in anaesthetised monkeys (Scholvnick et al., 2010) and has been shown to obscure group comparisons of fMRI connectivity (Gotts et al., 2013). In preclinical studies, GSR is less common. In a number of recent publications GSR was not included as a pre-processing step in rs-fMRI data analysis (Lu et al., 2012; Tsai et al., 2020, Hu et al., 2019). In line with this, and due to the notion that GSR may be related to the underlying region-specific signal (above), GSR was not used in this thesis.

3.11.3 Region-of-interest delineation

All ROIs used in this thesis were delineated based on P&W coordinates (Paxinos & Watson, 2011). Of the selected number of regions investigated, one ROI (M1) was used based on a previously published atlas (Valdes-Hernandez et al., 2011). Of the regions investigated several were manually drawn by Dr. Stephen Sawiak (AcbC/AcbS/IL cortex/PrL) based on P&W coordinates. All remaining parcellations were manually defined by JAJ. Coronal sections of all 14 ROIs and their sagittal extent are presented in (**Fig14.**). The specific selection of the ROIs was based on extensive previous literature implicating the OFC, PFC, striatum, amygdala, and motor cortex in a wide range of impulsive, compulsive and behavioural decision-making processes (Dalley, Everitt and Robbins 2011; Koob and Volkow 2010; Cardinal et al., 2002; Dalley, Cardinal and Robins 2004; Jentsch and Taylor 1999; Kalivas and Volkow 2005).

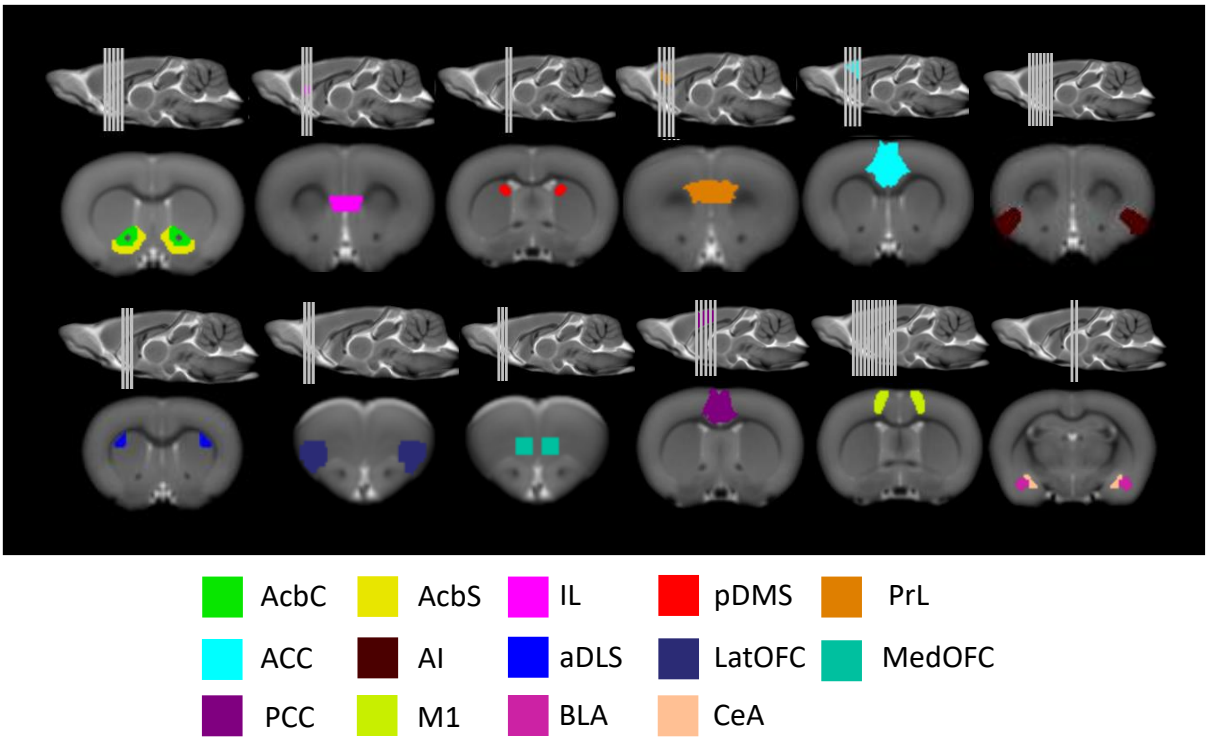


Fig14. Region-of-interest parcellation. All brain regions, excluding M1, were delineated by Dr. Stephen Sawiak and JAJ. All were based on Paxinos and Watson histological sections (Paxinos and Watson 2006). M1 was delineated bsed on the Tohoku University atlas parcellation (Valdes-Hernandez et al., 2011). *Abbreviations*: AcbC, nucleus accumbens core; AcbS, nucleus accumbens shell; IL, infralimbic cortex; pDMS, posterior dorsomedial striatum; PrL, prelimbic cortex; ACC, anterior cingulate cortex; AI, anterior insula; aDLS, anterior dorsolateral striatum; LatOFC, lateral orbitofrontal cortex; MedOFC, medial orbitofrontal cortex; PCC, posterior cingulate cortex; M1, primary motor cortex; BLA, basolateral amygdala; CeA, central nucleus of the amygdala.

Chapter 4

Interrelationships of behavioural endophenotypes linked to addiction-like behaviour

4.1 Introduction

Humans express a wide range of complex behavioural traits that can differ substantially at the individual level (i.e., impulsivity (Chamorro et al., 2012)). Over the last several decades, researchers have searched for an 'addictive personality' to isolate and develop a mechanistic explanation for why only certain individuals who take drugs of abuse eventually go on to increase their intake and develop SUD. However, the notion that there is a singular 'addiction personality' is potentially too narrow a view. Perhaps more cogent is the notion of a multifaceted construct consisting of several overlapping behavioural risk traits that reflect and contribute towards an increase in vulnerability to the development of specific aspects of substance abuse. However, it should be noted that SUD is a highly heterogeneous disorder, therefore, some people with a diagnosis of SUD may present several, a few, or indeed, no so-called risk traits (Wong and Schumann 2008; Zilberman et al., 2018). In rodents, a wide range of translationally relevant behavioural paradigms have been developed to evaluate the expression of behavioural traits linked with the co-expression of SUD, such as impulsivity (Dalley and Robbins 2017) and cognitive flexibility (Izquierdo et al., 2017), among others discussed in Chapter 1. Of the behavioural risk factors operationalised in rodents to understand addiction risk, the motoric form of impulsivity assessed *via* the 5-CSRTT has emerged as an important endophenotype for the development of compulsive cocaine use (Belin et al., 2008). However, traditional training on the 5-CSRTT typically takes up to

several months (Bari, Dalley and Robbins 2008). This labour-intensive training regime limits training efficiency and restricts high-throughput testing (although see Bruinsma et al., 2019 for details on new automated home-cage based methods). This has resulted in many studies in rodents evaluating how the expression of a singular behavioural trait (e.g., solely impulsivity) relates to addiction-like behaviour (Dalley et al., 2005; 2007). Although, studies assessing the relationship between several behavioural risk traits and addiction-like behaviour are emerging (e.g., (Belin et al., 2008; Molander et al., 2015; Vanhille et al., 2015)). On the other hand, previous studies have investigated the interrelationships of several vulnerability traits within a single subject without explicitly linking this to differential rates of drug reinforcement or drug-related behaviours. These studies commonly address the question - to what extent do multidimensional behavioural risk traits overlap? And how are they related? For example, one study evaluated LocR, NPP and attribution of incentive salience and found no interrelationships between the behavioural traits (Hughson et al., 2019), suggesting that these three traits are independent and non-overlapping. Of particular interest is that there is a large degree of variation in the expression of all three behavioural traits in rodents, lending support to the notion, that like humans, rats show heterogeneous expression of translationally relevant behavioural traits linked to addiction-like behaviour. Unlike both sensation seeking and novelty preference, there is evidence to suggest that the traits of impulsivity and incentive salience attribution overlap. HI rodents assessed by the 2-CSRTT, a variant on the 5-CSRTT (Robbins et al., 2002), show preferential approach behaviour to the CS+ lever and are more likely to be ST (Lovic et al., 2011). Using an analogous choice reaction time task, King et al., also showed that impulsivity was related to levels of sign-tracking behaviour, with CS+ lever presses (an index of sign-tracking) being positively correlated with premature responding (King et al., 2016). However, it should be noted that other studies have found no correlation between premature responding on the 5-CSRTT and sign-tracking (Pena-Oliver et al., 2015; Robinson et al., 2009). To what degree premature responding on the 5-CSRTT relates to cognitive flexibility as assessed through RL is still very much an open question. To my knowledge, there isn't a single rodent study that has assessed both behavioural constructs in the same experiment, and indeed within the same rat. Nonetheless, there is evidence to suggest an overlap between the two behaviours in humans. Thus, deficits in behavioural decision-making on a wide variety of tasks including the Iowa Gambling Task, Rogers Decision-Making Task and probabilistic RL task are all related to high levels of impulsivity in humans (Franken et al., 2008). However, impulsivity assessment was questionnaire-based in this study, therefore this relationship may differ if laboratory measures of impulsivity are

used. In summary, several studies have evaluated the interrelationship and overlap between several different behavioural traits suggested to play an important role in conferring risk to SUD. Several studies (highlighted above) have addressed the underlying relationships across different recognised vulnerability traits yet have fallen short in addressing the relationship between impulsivity and RL, as well to which degree these constructs relate to LocR, NPP and the attribution of incentive salience within a single subject. To address this issue, the interrelationships between impulsivity, cognitive flexibility, incentive salience attribution, NPP and LocR were assessed in a serial behavioural testing platform.

Based on previous literature I predicted that:

[1] Impulsivity, as indexed by % premature responses on the 5-CSRTT, would be correlated positively with sign-tracking, as measured through the number of CS+ lever contacts during PavCA;

[2] Conventional perseverative measures of RL (i.e., perseverative errors) would be closely related to kappa or 'stickiness', a relatively new parameter derived from reinforcement learning algorithmic approaches that represents the tendency to repeat an action regardless of whether the action is reinforced (i.e., if it is rewarded or not);

[3] NPP, incentive salience attribution and sensation seeking would be uncorrelated and independent behavioural constructs;

[4] Due to the tentative link between RL performance and impulsivity in humans, RL performance and % premature responses on the 5-CSRTT would be related.

4.2 Materials and methods

Timeline of behavioural phenotyping

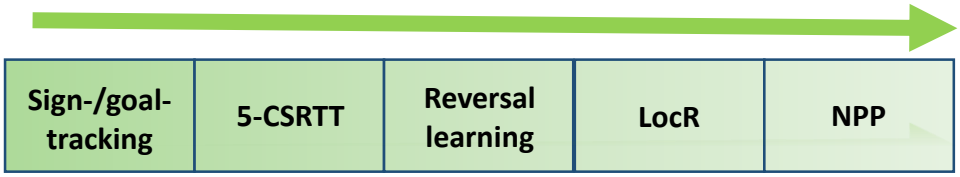


Fig1. Behavioural phenotyping timeline. After final developmental magnetic resonance imaging on post-natal day 63, rats were first phenotyped for sign- or goal-tracking behaviour on the Pavlovian conditioning task. Subsequently, impulsivity was assessed through the 5-choice serial reaction time task (5-CSRTT), and cognitive flexibility, in the form of deterministic spatial reversal learning was then assessed. Next, locomotor reactivity to a novel inescapable environment (LocR) was tested, and finally novelty place preference (NPP) was assessed. *Abbreviations:* 5-CSRTT, 5-choice serial reaction time task; LocR, locomotor reactivity to a novel inescapable environment; NPP, novelty place preference.

Subjects

Male Lister Hooded rats (n=48) (Charles River, Kent) were used in this study. For further details on housing, and more specific details on housing conditions prior to PND-63 please see Chapter 3, section 3.1. Briefly, all behavioural training started after the imaging session at PND-63; for overview of phenotyping timeline see **Fig1**. All rats were housed in groups of four, with rats housed with their littermates. For full details on the number of animals used see Chapter3, section 3.1. Rats were kept under food restriction to 85% of their free feeding weight during behavioural training. All experiments were carried out in accordance with the (U.K Animals) Scientific Procedures act (1986) under the UK Home Office project license (PPL 70/7587) and were approved by the University of Cambridge Animal Welfare and Ethical Review Body.

Attribution of incentive salience: sign- and goal-tracking

See Chapter 3, section 3.2 for more details.

Impulsivity: 5-choice serial reaction time task

Several measures above and beyond impulsivity were assessed on the 5-CSRTT. Response perseveration on the task was assessed by evaluating the number of perseverative nosepokes in the previously illuminated port (5C NP SP). Although this is a gross measure of perseveration (calculated as an overall measure across the total session (average of the last two long ITI sessions)) it was used for comparative purposes with other metrics of perseveration across different tasks (e.g., RL). A surrogate measure of goal-tracking behaviour was also assessed *via* the number of perseverative panel pushes of the food magazine throughout the task (5C PPP). See Chapter 3, section 3.3 for more details.

Cognitive flexibility: deterministic reversal learning

Several measures were evaluated from the RL task. These included 1) errors to criterion, 2) perseverative errors, 3) probability of win-stay, 4) probability of lose-shift and three computational parameters of performance (alpha, beta, kappa). Briefly, errors to criterion were calculated as the number of errors made until a subject achieved criterion. Perseverative errors (RL: PE) were calculated as 7 incorrect trials in a 10-trial window as previously described (Barlow et al., 2015). Probability of win-stay was calculated as the probability of making a correct choice after a correct trial, whilst probability of lose-shift was calculated as the probability of making a correct choice after an incorrect trial (Alsio et al., 2019). Computational analysis of RL *via* trial-by-trial Q-learning generated three measures including alpha (the learning rate), beta (inverse temperature) and kappa (stickiness). An overview of the models employed to derive these parameters is found in Chapter 3, section 3.4.1. For a more comprehensive description of the derivation of these parameters see Zhukovsky et al., 2019. For more details on task parameters see Chapter 3.4.

Novelty place preference

See Chapter 3, section 3.5 for more details.

Sensation seeking: locomotor reactivity to novelty

See Chapter 3, section 3.6 for more details.

Overview of behavioural trait classification

Table 1 gives a key summary of the behavioural dimensions, behavioural tests and associated behavioural measures of the reported behavioural endophenotypes assessed. Key behavioural

measures shown previously to be related to several different aspects of drug reinforcement and addiction-like behaviour in rodents are presented.

Behaviour dimension	Behavioural test	Behavioural measure
Impulsivity	5-CSRTT	% premature responses (last two long ITI sessions)
Sign- and goal-tracking	Autoshaping in operant chamber	Number of lever presses during 8s CS presentation
Cognitive flexibility	Deterministic spatial reversal learning	Win-Stay (P(Win|Stay)) Lose-Shift (P(Lose|Shift)) Errors to criterion Perseverative errors Alpha (learning rate) Beta (exploration/exploitation) Kappa (autocorrelation/stickiness)
Novelty preference	Novelty place preference	% time spent in novel *versus* familiar compartment
Sensation seeking	Locomotor reactivity to novelty	Total distance travelled in an open field

Table1. Table outlining several selected behavioural endophenotypes assessed. The behavioural dimension, the associated tests used to evaluate the behavioural dimension and the measures evaluated are reported. *Abbreviations:* 5-CSRTT, 5-choice serial reaction time task; ITI, inter-trial interval; CS, conditioned stimulus.

Statistical analysis

All behavioural measures were assessed for normality *via* the Shapiro-Wilk test. If normality was violated, data were transformed according to several well-known transformations (e.g., square root, log, reciprocal, exponential transformations). Following transformations, normality was assessed again using the Shapiro-Wilk test. If normality was not met, then a non-parametric Spearman's rank correlation coefficient was used to evaluate the relationship between behaviours. Unless otherwise stated all correlation coefficients represent Spearman's *rho*. All statistical analysis was performed using custom scripts in python (Python 3.7), or SPSS (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.). Significance was set at $p<0.05$ *(uncorrected).*

Multiple comparison correction

It is acknowledged that without multiple comparison correction the risk of inflating type 1 errors is increased. However, there are reports suggesting that not correcting for multiple comparisons is not uncommon within several behavioural neuroscience investigations (White et al., 2020 – see reviewers' comments on this manuscript). Nevertheless, the number of behavioural measures that were included in the correlational analysis was limited to the most important metrics from each task that have been shown previously to be linked with addiction-like behaviour (Belin et al., 2016) to limit the impact of type 1 errors. Prior to the start of the experimental work an *a priori* power calculation was conducted based upon previous results within the Dalley laboratory to determine appropriate group sizes. Power calculations suggested an overall sample size of 48 animals was sufficient to minimise the number of animals used in this study whilst also minimising potential type 2 errors.

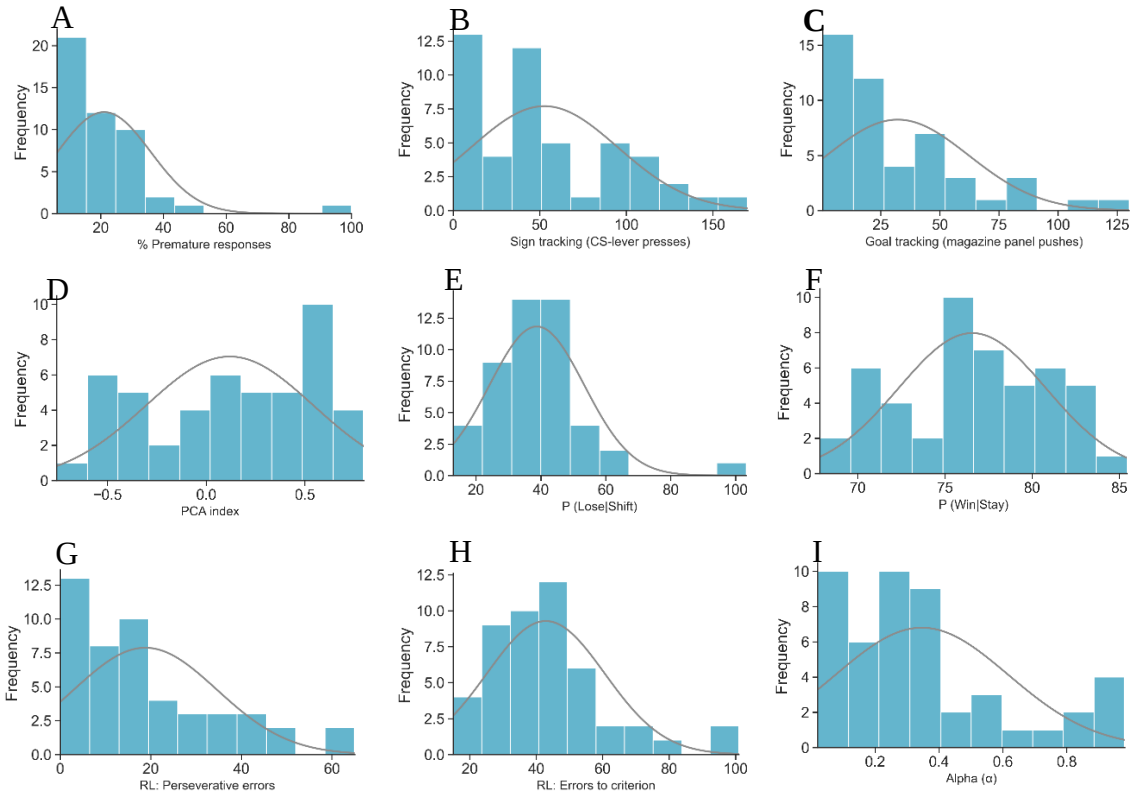
Principal component analysis

To understand the underlying latent relationship between the selected behavioural measures a principal component analysis (PCA) was undertaken. Prior to the PCA, a correlational analysis was performed to assess collinearity. A high degree of correlation between several 'conventional' metrics of the RL task and the computational parameters (alpha, beta, kappa) was observed. Due to the links between kappa and SUD (Kanen et al., 2019; Zhukovsky et al., 2019) it was decided that only the RL parameters derived from the computational analysis would be included in the overall PCA analysis. Along the same lines, of the three computational parameters, alpha and beta were highly correlated ($p<0.05$), whereas kappa did not show any relationship to either alpha or beta ($p>0.05$). To avoid a high degree of collinearity (between alpha and beta) a separate PCA was run with the three computational parameters. This resulted in two factors, one primarily representing alpha and beta, with the other factor representing kappa. The former factor, subsequently termed ($\alpha\beta$) was used in the overall PCA. The other behavioural measures used were premature responses on the 5-CSRTT, kappa, CS+ lever responses (sign-tracking), distance travelled in the LocR test and % time spent in the novel compartment in the NPP test. All factors were determined with a minimum eigenvalue of 1, and were factor rotated using a normalised Varimax rotation.

4.3 Results

Behavioural trait classification

The distribution of several selected behavioural measures recorded from each independent behavioural test are reported in **Fig2**. All behavioural measures in **Fig2.** are reported in their raw, untransformed format for ease of comparison. The distribution of each behavioural measure was assessed using the Shapiro-Wilk test, with normal data classified as having a p-value > 0.05. Of the 15 behaviours tested only one (1/15) was normally distributed. The remaining 14 of the 15 (14/15) behaviours were non-normally distributed, with p-values < 0.05 (Shapiro-Wilk test). Non-normal data is not a new concept and is common within scientific research (Smith 2017; Bono et al., 2017). Several methods have been used to adjust non-normal data, such as log, square root, arcsine, and reciprocal transforms, among others. All non-normally distributed behavioural measures observed in **Fig2.** were transformed according to one of these methods. After transformation, if the measure of interest was still non-normally distributed, non-parametric correlational analysis (e.g., Spearman's rank correlation coefficient) was performed.



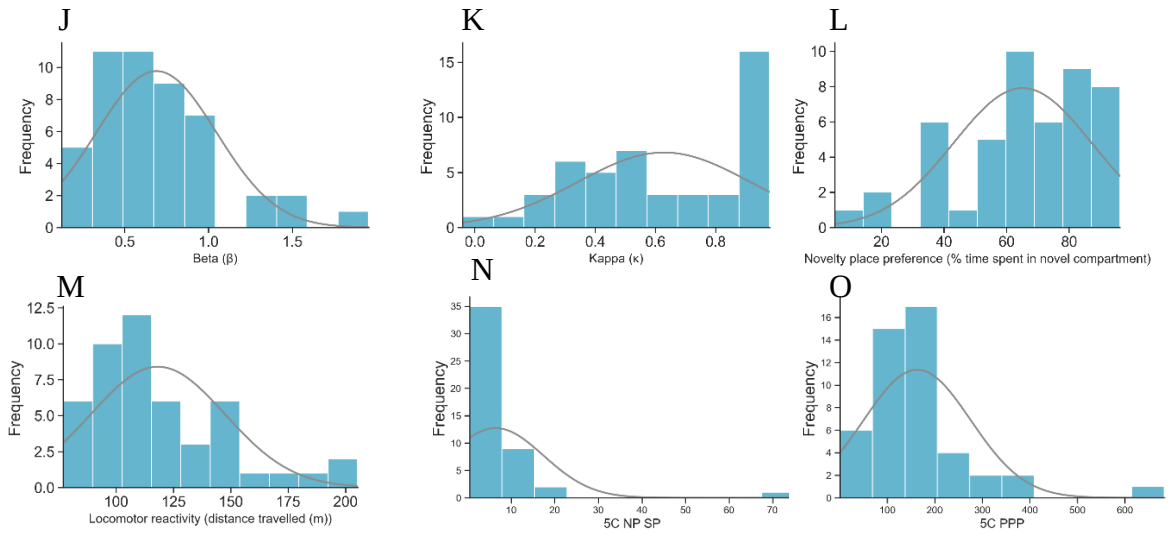


Fig2. Distribution of several selected behavioural endophenotypes **(A)** Percentage premature responses, **(B)** sign-tracking, **(C)** goal-tracking, **(D)** PCA-index, **(E)** probability lose-shift, **(F)** probability win-stay, **(G)** perseverative errors (reversal learning), **(H)** errors to criterion (reversal learning), **(I)** alpha, **(J)** beta, **(K)** kappa, **(L)** novelty place preference, **(M)** locomotor reactivity to a novel inescapable environment, **(N)** 5-choice serial reaction time task (5-CSRTT) nose pokes in same port (5C NP SP) and **(O)** 5-CSRTT perseverative panel pushes (5C PPP). All values are in their raw format for ease of comparison. *Abbreviations:* RL, reversal learning; CS, conditioned stimulus; OA, open arm; 5-CSRTT, 5-choice serial reaction time task; NP, nosepoke; SP, same port; PPP, perseverative panel push.

To evaluate the underlying interrelationships between several behavioural traits shown previously to predict distinct aspects of addiction-like behaviour a correlation matrix was calculated (**Fig3.**) (Spearman's correlation coefficient). Several interrelationships were observed, with variation in the strength of correlation across different behavioural traits as well as within different behavioural performance measures derived from the same task. Specifically, different performance measures of RL were highly correlated. For example, errors to criterion and perseverative errors were highly correlated (*rho*=0.723, p=0.000). In addition, correlation analysis revealed key interrelationships between several independent behaviours. A summary of which is provided in **Fig3.**, with key relationships addressed in **Figs 4-6, 8, and 9**.

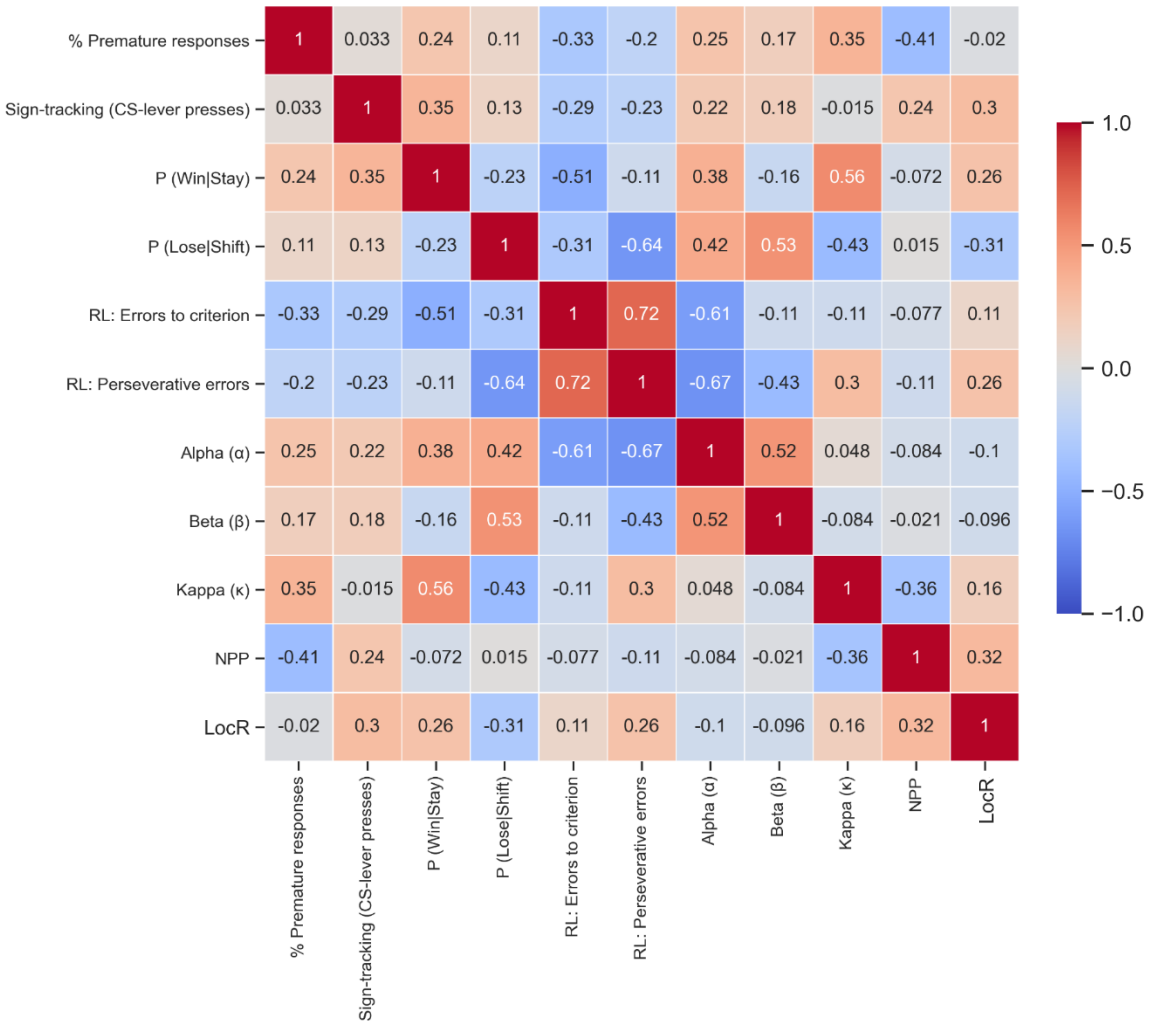


Fig3. Correlation matrix highlighting interrelationships between a selected number of behavioural dimensions. Evaluation of the relationship between variables measured from the same task showed high degrees of correlation. Alpha, beta and kappa, the reversal learning parameters derived from a reinforcement learning algorithm evaluating trial-by-trial performance, were highly correlated with either P(Win|Stay), or P(Lose|Shift). Similarly, errors to criterion and perseverative errors, both measures of reversal learning performance, were highly correlated. Between tasks, high levels of premature responses predicted higher stickiness (kappa), and lower novelty preference in the NPP task. Both measures of novelty, namely locomotor reactivity to novelty and novelty place preference were also positively correlated. *Abbreviations:* NPP, novelty place preference; LocR, locomotor reactivity to a novel inescapable environment; RL, reversal learning.

Dimensional analysis of behavioural traits

Fig4. outlines the relationship between sign-tracking and impulsivity. Sign-tracking behaviour indicated by high levels of CS+ lever responses did not correlate with premature responses measured on the 5-CSRTT (*rho*=0.033, p=0.827) (**Fig4A.**). Additionally, no relationship was seen between the PCA-index and impulsivity (*rho*=0.057, p=0.704) (**Fig4B.**). Perseverative responding in the food magazine during 5-CSRTT testing, a surrogate measure of goal-tracking behaviour, did not correlated with premature responses measured on the same task (*rho*=-0.0164, p=0.9129*)* (**Fig4C.**). Similarly, goal-tracking, as measured by the number of magazine responses during the PCA task, did not correlate with premature responses on the 5-CSRTT (*rho=-0.0002,* p=0.9985) (**Fig4D.**), thus indicating that attribution of incentive salience is an unrelated and independent dimension to impulsivity, as measured by premature responding on the 5-CSRTT.

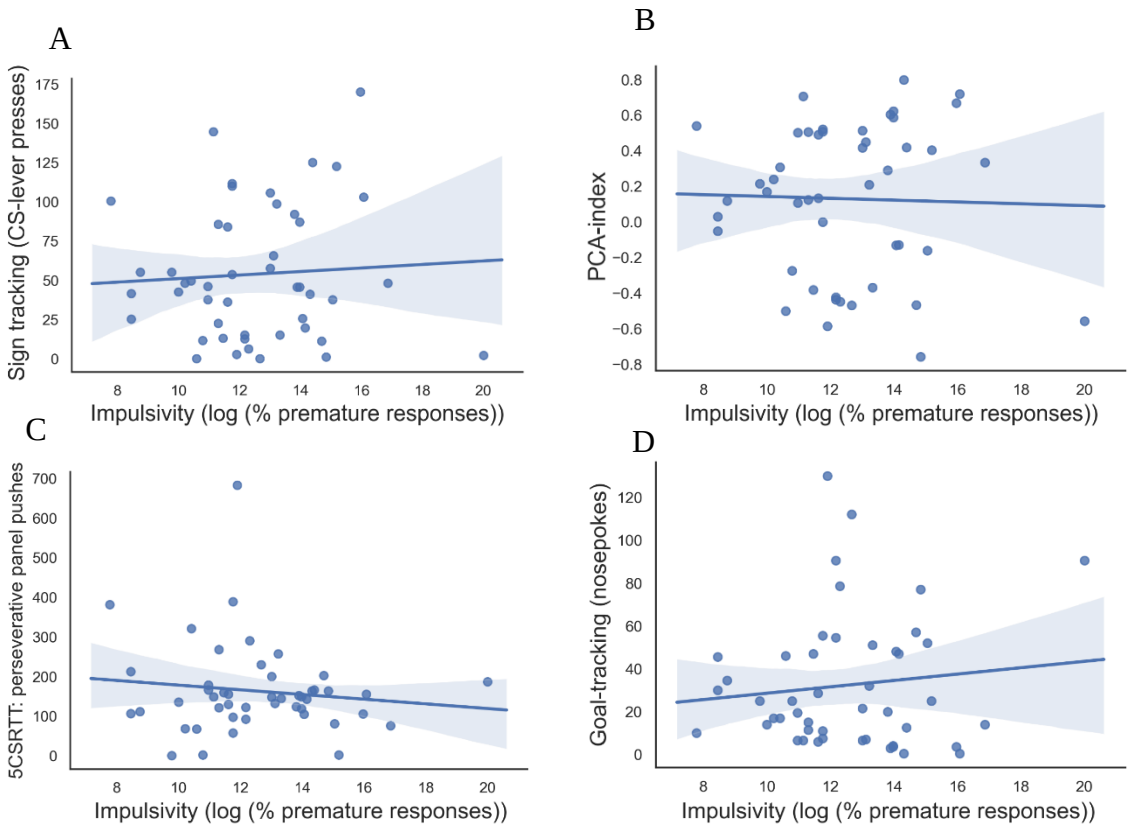


Fig4. Lack of a relationship between impulsivity and the attribution of incentive salience. **(A)** sign-tracking, as measured through the number of lever contacts during the 8 second CS+ presentation, did not strongly correlate with impulsivity, as measured through percentage of premature responses on the 5-CSRTT (*rho*=0.033, p=0.827*)*. **(B)** An alternative measure of incentive salience attribution, the PCA-index (where 1 and -1 represent sign-tracking and goal-tracking, respectively), did not strongly correlate with premature responding on the 5-CSRTT (*rho*=0.057, p=0.704). **(C)** A surrogate marker of goal-tracking activity, the number of magazine panel pushes on the 5-CSRTT, also did not correlate with premature responding on the 5-CSRTT (*rho*=-0.0164, p=0.9129*)*. **(D)** Goal-tracking, as measured by the number of panel pushes in the food magazine during the 8 second presentation of the CS+ response during an autoshaping task did not correlate with premature responses on the 5-CSRTT (*rho=-0.0002*, p=0.9985). *Abbreviations:* CS, conditioned stimulus; 5-CSRTT, 5-choice serial reaction time task; PCA-index, Pavlovian conditioned approach index.

Correlation analysis also revealed relationships between sign-tracking and sensation seeking (**Fig5.**). Thus, sign-tracking (measured *via* PCA-index and CS+ lever responses) was positively correlated with LocR (rho=0.301, p=0.037; rho=0.3, p=0.038, respectively) (**Fig5A, B.**). Similarly, sign-tracking (PCA-index) was somewhat positively related to preference for the novel compartment during a free-choice NPP paradigm (rho=0.261, p=0.073) (**Fig5C.**). However, CS+ lever responses did not correlate with NPP (rho=0.239, p=0.101) (**Fig5D.**), suggesting that the PCA-index, which reflects a composite measure of performance, may capture distinct behavioural features not captured in the CS+ lever response measure.

Evaluation of both sensation seeking and novelty seeking revealed a positive relationship, in such that rats who travelled more within a 2-hour period in a novel inescapable environment also favoured the unfamiliar compartment when presented with a choice between familiar and unfamiliar areas during the NPP paradigm (*rho*=0.325, p=0.024*)* (**Fig6.**).

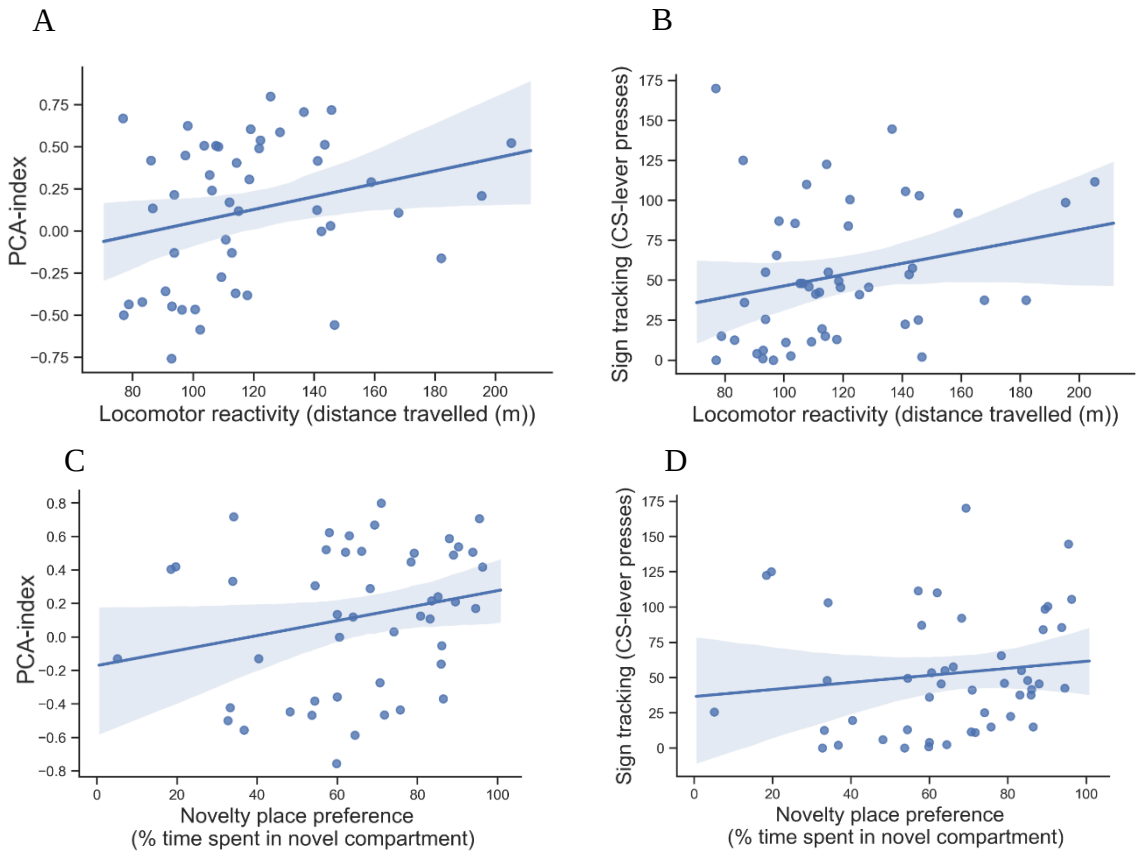


Fig5. Relationship between locomotor reactivity to novelty, novelty place preference and the attribution of incentive salience. **(A)** Locomotor reactivity was positively correlated with the PCA-index (*rho=0.301,* p=0.037). **(B)** Locomotor reactivity positively correlared with CS+ level presse (*rho*=0.3, p=0.038*)* **(C)** Novelty place preference was somewhat positively related to the PCA-index, with sign-trackers (closer to 1) showing increased preference for the novel *versus* familiar compartment of the novelty place preference test (*rho=0.261,* p=0.073*).* **(D)** Sign-tracking, as indexed through CS+ responses, did not positively correlate with novelty place preference (*rho*=0.239, p=0.101*). Abbreviations*: PCA index-index, Pavlovian conditioned approach index; CS, conditioned stimulus.

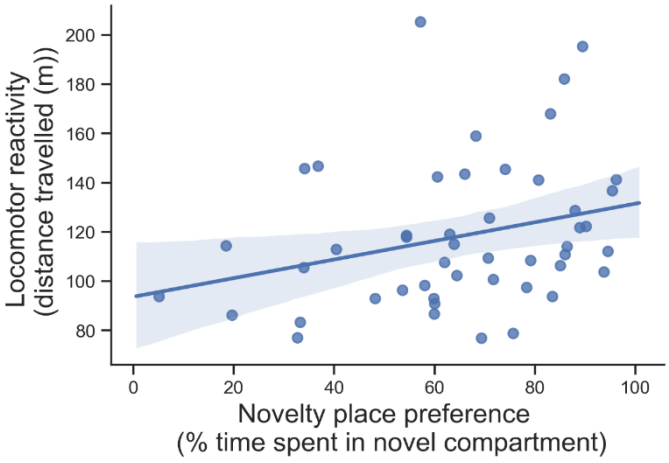


Fig6. Evaluation of the interrelationship between novelty place preference and locomotor reactivity to novelty. Locomotor reactivity to a novel inescapable environment was positively related to the preference to explore a novel *versus* familiar compartment in the novelty place preference paradigm. (*rho*=0.325, p=0.024*).*

To evaluate to what extent several possibly distinct but interrelated forms of perseveration overlap or diverge a correlation matrix was performed on several behavioural measures thought to represent distinct forms of perseverative responding across a wide range of different behavioural paradigms. Perseverative nosepokes in the same aperture that was previously rewarded on the 5-CSRTT (5C NP SP) was not related to perseveration on the RL task (**Fig7.**).

Fig7. Overview of several measures of perseveration across different behavioural dimensions. Perseverative responses in the previously rewarded apertures were evaluated by measuring the number of nosepokes in the same aperture (5C NP SP). Perseverative panel pushes (i.e., panel pushes of the food magazine receptacle) were also recorded, as a proxy measure of goal-tracking behaviour (5C PPP). Perseverative errors on the reversal learning task (RL: PE) and kappa, the stickiness parameter was measured. No interrelationship was observed between the 5-CSRTT and RL paradigm, across any variable measured. Kappa positively correlated with conventional perseverative measures on the RL task. Numbers in cells and colour bar represent Spearman's rho correlation coefficient. *Abbreviations:* 5-CSRTT, 5-choice serial reaction time task; 5C NP, five choice nosepoke; 5C NP SP, five choice nosepoke same port; RL: PE, reversal learning perseverative error; RL: Kappa, reversal learning kappa (stickiness); RL, reversal learning.

However, stickiness positively correlated with the more conventional, non-computational measure of perseverative responding in RL - perseverative errors (*rho*=0.302, p=0.037*)* (**Fig8.**).

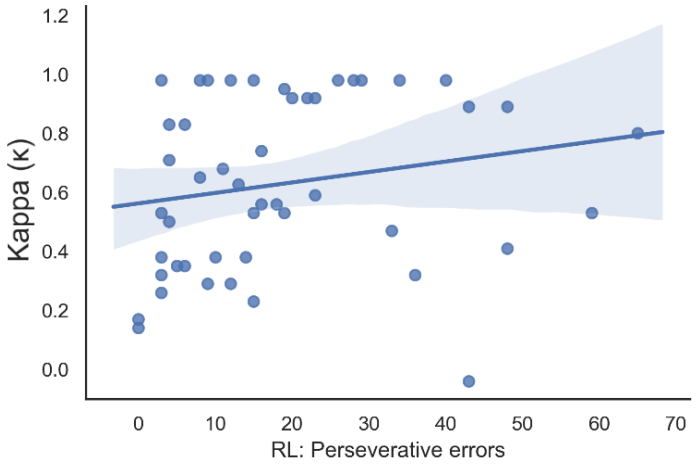


Fig8. Relationship between conventional measures of perseveration on the reversal learning task with computational measures of proposed perseverative-like responses. Perseverative errors positively correlated with increased stickiness (*rho*=0.302, p=0.037*).* *Abbreviations:* RL, reversal learning.

In addition, high levels of impulsivity were also related to stickiness. Thus, animals that show a higher degree of anticipatory or premature responding on the 5-CSRTT are more likely to choose the same response (in this case either left or right lever press) regardless of whether the response is reinforced by a food reward or not (*rho*=0.353, p=0.015*)* (**Fig9.**).

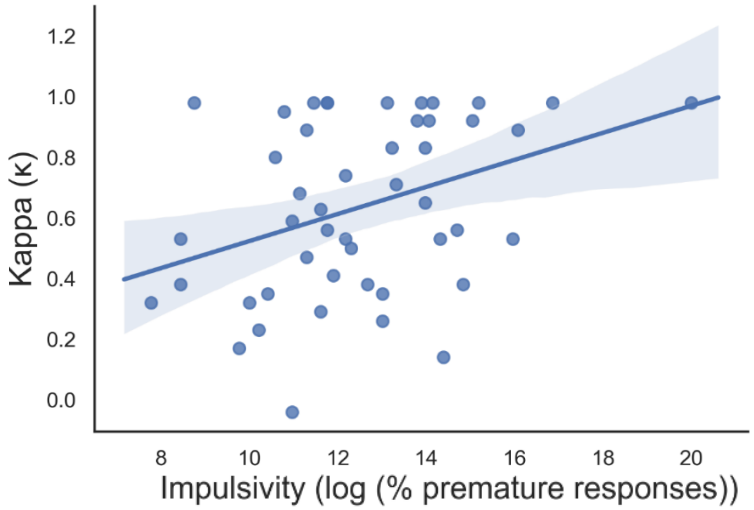


Fig9. Relationship between the reversal learning parameter kappa (stickiness) and impulsivity. Higher levels of stickiness, indicative of a higher likelihood to choose a similar response to the previously chosen response regardless of reinforcement, is positively correlated with premature responding on the 5-CSRTT (*rho*=0.353, p=0.015).

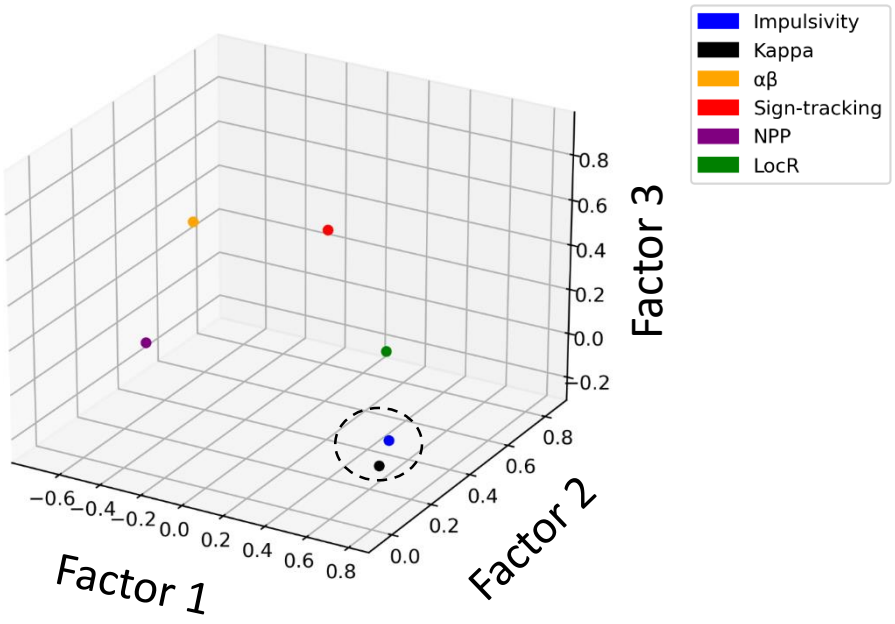


Fig10. Principal component analysis of addiction-relevant behavioural endophenotypes. Factor 1 primarily loaded impulsivity, kappa and NPP and explained 29.393% of the total variance. Factor 2 primarily loaded LocR and explained 21.802% of the total variance. Finally, factor 3 primarily loaded the αβ component and explained 17.888% of the total variance. Impulsivity and stickiness component highlighted outlined with dashed circle *Abbreviations:* LocR: Locomotor reactivity to a novel inescapable environment; NPP: novelty place preference.

Finally, a PCA was performed to evaluate whether the behavioural traits measured may be reduced to higher-order constructs that better represent and explain the underlying relationships. Prior to the main PCA being run, the three RL computational variables were run through a separate PCA analysis (see methods). Both alpha and beta loaded significantly on to a single factor – termed αβ, whereas kappa loaded on to the second factor. In the second PCA, the latent variable representing the shared variance of αβ was used, as well as kappa, minimising collinearity in the matrix prior to analysis (see methods). The main principal component analysis (PCA) revealed three factors accounting for 69.083% of the total variance (**Fig10.**). Impulsivity, kappa, and NPP loaded on factor 1 and explained 29.393% of the variance. Conditioned approach to a reward-predictive stimulus (sign-tracking) and LocR loaded on factor 2 and explained 21.802% of the variance. Factor 3 loaded on the αβ component and explained 17.888% of the total variance. A summary of the loadings, and explained variance is observed in **Table2-3.**, respectively.

Component	Total	% of variance	Cumulative %
1	1.764	29.393	29.393
2	1.308	21.802	51.195
3	1.073	17.888	69.083

Table2. Total variance of principal component analysis. Factor 1 accounted for 29.393% of the total variance. Factor 2 accounted for 21.802%, and factor 3 accounted for 17.888% of the total variance. Cumulatively, 69.083% of the total variance was explained by these 3 factors.

	Component		
Behavioural variable	**1**	**2**	**3**
Impulsivity	0.786	0.099	0.025
Sign-tracking	-0.035	0.625	0.432
αβ	0.003	-0.050	0.906
Kappa	0.755	0.084	-0.086
NPP	-0.724	0.433	-0.090
LocR	0.068	0.850	-0.233

Table3. Principal component scores from principal component analysis of behavioural endophenotypes shown previously to predict distinct aspects of addiction-like behaviour. Factor 1 was primarily loaded by impulsivity, kappa and novelty place preference, factor 2 by locomotor reactivity to novelty and sign-tracking, with factor 3 primarily loaded by the αβ component. *Abbreviations:* LocR: Locomotor reactivity to a novel inescapable environment; NPP: novelty place preference.

4.4 Discussion

To address the first hypothesis, the relationship between impulsive responding on the 5-CSRTT and aberrant attribution of cue salience to reward predictive cues, as assessed by the PavCA procedure, was evaluated. No relationship between impulsivity and sign-tracking was found, findings in alignment with some (Pena-Oliver et al., 2015; Robinson et al., 2009), but not other (Lovic et al., 2012; King et al., 2016) reports. The relationship between attentional control over behavior *via* reward-related cues (sign-tracking) and impulse control is yet to be fully elucidated. Sign-tracking animals are characterized by their increased conditioned approach behavior to a CS and can be separated from GT based on their aberrant allocation of incentive salience to reward cues (Flagel, Akil and Robinson 2009). In essence, incentive salience reflects hypersensitivity of motivational circuitry, and is associated with an increase in 'wanting' of drugs thought to represent a form of neural sensitisation (Robinson and Berridge 2001). In the case of the PavCA procedure, sign-tracking animals, therefore, display enhanced motivational engagement and approach to the CS, in this case a light cue. In these animals, it is thought that approach behavior towards the light is reflective of an aberrant cue-controlled motivational effect. The overlap between impulsivity and sign-tracking may emerge from this phenomenon. For example, impulsive animals who are also ST (Lovic et al., 2012) imbue the CS, in both these cases a light cue, with greater incentive value. Thus, ST more readily instigate approach behavior and as a result are more likely to engage prematurely with the noseport/aperture in choice reaction time tasks. It is thought that this drive may be mediated by an imbalance between 'bottom up' subcortical mechanisms of dopaminergic activity in regions such as the VTA and striatum and deficits in top-down behavioural control, mediated by prefrontal cortical projections (Tomie and Morrow 2018). The relationship between sign-tracking and impulsivity may relate to a shared deficit in inhibition, possibly mediated by increased incentive sensitisation mechanisms and reduced top-down control. Thus, increased bottom-up, and decreased top-down control may ultimately facilitate sign-tracking, and indeed impulsivity. Taking this into account, the observation that sign-tracking and impulsivity exhibited no relationship was somewhat surprising. However, as outlined, there is evidence to suggest that these traits are dissociable. Using a procedurally analogous task, Robinson et al., found no differences in cue approach behavior (i.e., sign-tracking) in HI *versus* LI rats (Robinson et al., 2009), demonstrating alternative evidence suggesting that the two behaviours are dissociable. These findings are

consonant with those of the PCA analysis reported herein which demonstrated differential loading of both impulsivity and sign-tracking to different factors. An alternative dimensional relationship between the two behaviours has been put forward suggesting that impulsivity is positively related to goal-tracking (Murray et al., 2014; Pena-Oliver et al., 2015). Initial observations were born out of the report that HI animals transitioned to habitual cue-control cocaine seeking at later stages of training than LI animals (Murray et al., 2014). As a result, it was postulated that HI rats may have increased goal-directed tendencies. In the study by Murray et al., the relationship between goal-tracking and impulsivity was evaluated unconventionally through magazine panel pushes on the 5-CSRTT (an index of approach behavior to the port of food delivery) and premature responses on the 5-CSRTT, respectively. They found a positive relationship (i.e., HI animals made more food magazine panel pushes). This relationship was bolstered by another study showing that perseverative responding in the food magazine on the 5-CSRTT positively correlated with goal-tracking responses on the PavCA procedure (Pena-Oliver et al., 2015). However, no relationship between goal-tracking and impulsivity was observed in this study, nor was any relationship with sign-tracking. Like impulsivity, the relationship between sign-tracking, NPP and LocR within the literature is also inconsistent (Hughson et al., 2019; Beckmann et al., 2011). Sign-tracking has been linked with increased NPP (Beckmann et al., 2011), has been shown to be non-related, or indeed both have been shown to be orthogonal traits (Hughson et al., 2019). In this report, a positive relationship between both LocR and NPP with measures of sign-tracking was observed. However, the relationship between NPP and sign-tracking was weak and not significant. Whilst LocR was related to both CS+ lever presses and the PCA-index, NPP was tentatively related to the PCA-index. These results suggest that unconditioned motoric activity and cue-elicited approach behavior may share a common underlying mechanism. HR rats are characterised based on their reactivity to a novel environment and have increased stress reactivity, with corticosterone secretion higher in HR than LR animals (Piazza and Le Moal 1996). Similarly, there is evidence showing that sign-tracking animals also exhibit elevated stress responses (Tomie et al., 2000). This convergent evidence tentatively suggests that the innate ability of the two tasks to induce differential stress responses in either ST or HR rats, may relate to a common mechanism by which the behaviours are interrelated. Along this line, selective breeding of rats over several generations that show high degrees of LocR, namely bred HR (bHR), differ on their propensity to assign incentive salience to reward cues. Relative to bred LR (bLR), bHR show an enhanced approach behavior towards a lever-CS and are more likely to be ST (Flagel et al., 2011). Although the direct comparison between

selectively bred lines and outbred animals must be done with caution, this does suggest that HR animals may be more conditioned to approach a reward predictive cue, possibly through an increase stress response in these animals. However, one recent noteworthy study assessing these traits in a large heterogeneous outbred population of rats found no overlap between sign-tracking, LocR and NPP (Hughson et al., 2019). There are several experimental and procedural differences that may explain the apparent discrepancy of results from those observed in this thesis. For example, unlike the Lister Hooded strain used in the present thesis, Hughson et al., used a heterogeneous stock population (Solberg Woods and Mott 2017), with different strains possibly having very different innate behavioral characteristics (Clemens et al., 2014). In this thesis, LocR was tested in a $50cm^3$ testing apparatus for a 2-hour duration, unlike the 30 minutes tested by Hughson and colleauges. Moreover, in the Hughson et al., study, NPP was tested in the same apparatus as LocR, and novelty was introduced by changing the tactile nature of one portion of the floor (from a wire mesh to a solid metal plate). Moreover, the two floors (novel and familiar) were not separated or partitioned, as in the case of this thesis, whereby the two chambers were separated spatially as well as visually by partitioned walls either side of the central chamber. Therefore, procedural differences may have accounted for the discrepancy in results observed here. Nonetheless, it was initially hypothesised that these traits would be independent, yet the results presented suggest otherwise, and may either represent procedural differences, or importantly may represent an emerging stress-related phenotype that generalizes across the two behaviours. Were this the case, it may go some way in explaining the relationship that was observed between NPP and LocR (higher LocR related to higher NPP), however, there is limited evidence to suggest that high novelty preferring animals on the NPP show a similar stress response to that shown in HR animals, and the reported relationship in this thesis is slightly at odds with reports showing no relationship between the two measures (Belin et al., 2011). Yet, there is evidence showing HR rats exhibit increased exploratory behavior in the novel arm of a Y-maze (Dellu et al., 1993). Although a Y-maze was not used in this thesis, the results presented do align with this report in the sense that HR rats showed an increased preference for novelty and exhibited enhanced exploratory behaviour of the novel side of the test apparatus in the NPP paradigm.

Both impulsivity and NPP have been shown to predict the propensity to develop compulsive cocaine taking behavior in the 3-criteria model of addiction (Belin et al., 2008; 2011). From the proposed predictive overlap between the two behavioural traits, it was predicted that high

impulsivity on the 5-CSRTT would positively correlate with NPP. In contrast to this prediction, HI animals showed significantly lower exploration of the novel chamber in the NPP paradigm, a finding bolstered by the PCA analysis revealing a positive and negative loading of impulsivity and NPP on factor 1, respectively. To my knowledge, only one previous study has evaluated the co-existence of these two traits within a single subject (Molander et al., 2011). HI animals on the 5-CSRTT showed an increase preference for the unfamiliar/novel chamber on NPP assessment, with an increase in overall mean time spent in the novel chamber relative to the familiar, an effect not observed in LI rats. However, although group differences were observed, the authors reported no dimensional relationship between impulsivity and NPP in a regression analysis (Molander et al., 2011) thus obscuring any clear relationship between the two measures. The precise relationship between impulsivity and sensitivity to novelty is not yet clear, with reports showing no differences in LocR in HI rats (Belin et al., 2008), whilst others have shown that HI rats display lower levels of LocR (Dalley et al., 2007). These findings (Dalley et al., 2007) are somewhat in line with what was observed here albeit for an alternative, and perhaps related (see results section), measure of novelty. However, whilst the HR phenotype has been shown to be unrelated to the sensitivity of punished cocaine SA, animals with high NPP, alongside the HI phenotype, overlap in their predisposition to compulsive use. Several lines of evidence suggest that the psychological and neural mechanisms of drug-taking and drug-seeking are independent (Luscher, Robbins and Everitt 2020), and represent very different underlying process. Therefore, the proposed behavioural relationship between impulsivity and NPP based on their shared risk for the development of compulsive cocaine taking behavior may not generalise to drug-seeking (see Chapter 5).

RL involves adaptive decision-making and the ability to disengage responding to previously rewarded behavior in the face of changes in reward contingencies. In this thesis, RL was evaluated *via* a serial spatial deterministic RL paradigm, as previously described (Zhukovsky et al., 2019). From the task, several different behavioural performance parameters can be measured. For example, errors to criterion reflects how the animal performed the task overall. Calculation of the probability of win-stay and lose-shift has also been used to evaluate the degree in which an animal may learn from positive and negative feedback signals, respectively. Computational analysis including reinforcement learning models, such as Q-learning algorithms (Daw et al., 2009; Zhukovsky et al., 2019), or hierarchical Bayesian models (Alsio et al., 2019) have been used to evaluate RL performance on a trial-by-trial

basis and have been pinned to provide a useful platform in which to compare metrics from translationally relevant tasks across both rodent and human studies. In this thesis, a variant of the Q-learning model was utilized to evaluate RL performance as previously described (Zhukovsky et al., 2019) with three key computational parameters derived to explain behavior including alpha, beta, and kappa (see Chapter 3, section 3.4.1 for more details). Classic measures of response perseveration on the RL paradigm evaluate the extent to which an animal persists in responding to previously rewarded contingencies, indicative of reduced cognitive flexibility, and are termed perseverative errors. Kappa is a computationally derived parameter based on trial-by-trial data from the entire session and represents how likely an animal will perform the same response regardless of whether the outcome is rewarded or not. Descriptively, both measures seem to be interrelated and thus represent similar observations of underlying perseverative behavior. From this, it was hypothesised that animals exhibiting more conventional measures of perseveration would also exhibit increased stickiness. Indeed, this is what was observed. This relationship serves to validate the novel computational approach. However, the correlation that was observed was moderate at best leading to the conclusion that while these behaviours may represent an overarching tendency to perseverate, they may also represent other associative processes. Similar computational models have been used in humans in a series of studies assessing RL performance in clinically relevant populations (Kanen et al., 2019; 2021). Here, the precise relationship between conventional measures of perseverative responding and stickiness is less clear. Examination of stickiness to more conventional measures of perseveration revealed no relationship (Kanen et al., 2021 (*preprint*)), a finding replicated in an independent sample from the same laboratory (Kanen et al., 2021 (*preprint*)). However, another independent sample from the same laboratory found that stimulus stickiness was positively related to the conventional measure of perseveration (number of perseverative errors) on a probabilistic RL task (Kanen et al., 2019). Interestingly, this dimensional relationship was only observed in healthy controls under placebo conditions and was not observed in SUD individuals. However, in the same study, stimulus stickiness was higher in SUD when compared to healthy controls. In the original study (upon which the computational modelling was subsequently based) conventional perseverative errors were also higher in SUD (Ersche et al., 2011). In summary, there appears to be a complex relationship between stickiness and 'classical' forms of perseveration, with no clear salient relationship observed in the literature. This is reflected in the relationship between conventional measures of perseverative responding, stickiness, and learning from either positive or negative feedback signals. In this thesis, perseverative errors were related to lose-

shift only, whereas stickiness was related to both lose-shift and win-stay, albeit in opposite directions. This relationship fits well with how these metrics are derived. For example, conventional perseverative errors are more likely to be experienced after a reversal contingency has changed and therefore it may suggest that these errors are less sensitive to learning from negative feedback. Conversely, due to trial-by-trial analysis, increased stickiness is related to both lose-shift and win-stay, with high sticky animals more likely to stick to a winning choice, and less likely to switch on a losing trial. It is therefore enticing to suggest that the computational modelling parameter may reflect a more nuanced picture of behavioural perseveration, with stickiness reflecting response perseveration regardless of positive or negative feedback. Nonetheless, a positive relationship between stickiness and perseverative errors was observed inviting the view that both measures converge on the central notion of response perseveration.

Similar descriptions may be used when describing certain components of both impulse control deficits and RL performance (i.e., an inability to disengage a response) suggesting, to some extent, that the two behaviours may share similar psychological and neurological mechanisms (Izquierdo and Jentsch 2012). Perhaps the most salient and novel finding was that HI animals showed the highest levels of stickiness (i.e., HI animals were more likely to repeat the same choice regardless of reward outcome on the RL task). The relationship between impulsivity and perseveration or stickiness is not explicitly clear, but there is some evidence to suggest that impulsivity may be related to perseveration in humans (van den Broek and Bradshaw 1993; Morris and Mansell 2018). The notion of perseveration comes under many different guises and within the literature a number of terms have been used to describe similar patterns of behavior (e.g., behavioural rigidity, inflexibility, stickiness, psychological (in)flexibility, cognitive (in)flexibility). The relationship between impulsivity and perseveration is still somewhat emerging with early studies showing that perseveration on the Modified Card Sorting Test was related to impulsivity (van den Broek and Bradshaw 1992). However, in this study, perseveration was only related to impulsiveness as measured by the Matching Familiar Figures Test (Kagan 1966) and not by the more common BIS. In a similar non-clinical sample, Sweitzer et al., found that impulsivity assessed either through the BIS or a delay discounting questionnaire did not predict perseverative errors on the Wisconsin Card Sorting Test (Sweitzer et al., 2008). More relevant to addiction, Fernandez-Serrano and colleagues assessed multiple neuropsychological measures in cocaine-dependent individuals and non-drug using controls (Fernandez-Serrano et al., 2012). The authors found

that, overall, impulsivity was not strongly associated with perseverative responding on a probabilistic RL task, yet negative urgency, one dimension of impulsivity (Cyders and Smith 2008), was positively related to perseveration in cocaine-dependent individuals thus suggesting that certain forms of impulsivity may be linked to increased perseveration. Bolstering this notion, multi-dimensional total BIS scores were related to perseverative errors at trend level ($p=0.01$), whereas the non-planning subdomain of the BIS strongly correlated with perseverative errors on a RL task (Ersche et al., 2008). Taken together, response perseveration and impulsivity appear to be related, but the precise relationship is not entirely clear; whilst some forms of impulsivity, including non-planning components and negative urgency are positively associated with perseveration, others are not (Sweitzer et al., 2008). In addition, to what extent these phenotypes are driven by drug-induced changes is still an open question. Both studies (Ersche et al., 2008; Fernandez-Serrano et al., 2012) evaluated performance in cocaine users *versus* non-drug using controls, therefore the increase in perseveration, the decrease in inhibitory control and their interrelationship could have been driven by duration of use. In this thesis, a positive relationship between a potential form of perseveration – stickiness – and motor impulsivity in drug-naïve rodents who had yet to undergo cocaine SA was observed. As a result, this relationship was not driven by any drug-induced adaptations. To what extent this antecedent relationship in humans is a risk marker for subsequent development of SUD is still an open question. This notion was asessed in a rodent model of compulsive cocaine seeking by asking the question, to what extent do these antecedent behavioural risk traits predict compulsive cocaine seeking? This question is addressed in the following chapter.

Chapter 5

Vulnerability to compulsive cocaine seeking: a behavioural endophenotype perspective

5.1 Introduction

Compulsive drug-seeking despite adverse or negative consequences is a hallmark feature of SUD. Clinically, the compulsive quality of drug-seeking behaviour features prominently in the most recent classification of addiction in the DSM-5 with several criteria largely encompassing negative consequences and the uncontrollable or compulsive nature of drug use. For example, [1] 'Substance use is continued despite the knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by substance use', [2] 'Important social, occupational, or recreational activities are given up or reduced because of substance use' and [3] 'There is a persistent desire or unsuccessful efforts to cut down or control substance use' (Hasin et al., 2013). Thus, clearly, compulsive drug-seeking is a central theme throughout the diagnostic criteria. However, it is widely accepted that only a small number of individuals who take drugs of abuse develop SUD. To what extent pre-existing behavioural traits confer vulnerability or are exacerbated by drug use and contribute to compulsive drug-seeking is still an open question. In humans, this is hard to address due to the 'cause or consequence' phenomenon (i.e., is the behaviour pre-existing or is it caused by drug use?). In rodents, carefully controlled experimental design and analysis of pre-existing translationally relevant behavioural traits prior to any drug exposure circumvent the circulatory issue in humans and provide a platform to answer these questions. Although rodents can never fully recapitulate the complex social and

environmental situations that may increase risk in humans, several SA paradigms have been developed to probe distinct aspects of addiction-like behaviour, with more recently, the development of a ^{2nd}SOR schedule with contingent punishment and the evaluation of compulsive drug-seeking (see Chapter 3, section 3.8) (Belin-Rauscent et al., 2016). The central theme of compulsivity (i.e., negative consequences) can be operationalised and assessed in rodents through several different punishment paradigms such as mild electric foot shock, lithium chloride injections or quinine adulteration (Vanderschuren et al., 2017). Of the behavioural traits investigated pre-clinically, impulsivity and NPP emerge as frontrunners in conferring risk to the development of compulsive cocaine use (Belin et al., 2008; 2011). Thus, both impulsivity, as measured on the 5-CSRTT, and NPP predict cocaine intake in the face of mild electric foot shock in the multisymptomatic 3-criteria model of addiction-like behaviour (Deroche-Gamonet et al., 2004). However, in the three-criteria model foot shocks are delivered during (FR-5) and preceding (FR-4) the drug-taking response (FR-5), therefore the schedule in this model punishes the drug-taking response and not the drug-seeking response. Thus, impulsivity and NPP are important risk factors for drug-taking, but to what extent they model risk to drug-seeking is yet unknown. In marked contrast to impulsivity and NPP, LocR is not associated with compulsive drug use, but is linked with increased acquisition of psychostimulant SA (Piazza et al., 1989). Similarly, aberrant attribution of incentive salience to reward cues predicts increased choice of cocaine over saccharin (Tunstall and Kearns 2015; but see Vanhille et al., 2015) and cue-induced drug-seeking (Everett et al., 2020). Moreover, ST are more sensitive to the motivational effects of drug-paired conditioned stimuli, with the removal of a cocaine associated cue decreasing cocaine intake in ST but not GT (Saunders and Robinson 2010). However, limited evidence supports the notion that sign- or goal-tracking is related to compulsivity. Thus, no differences in cocaine SA are seen between sign- or goal-trackers when an adverse foot shock is delivered in a conflict-based relapse model (Saunders, Yager and Robinson 2013). A more recent study also found that the PCA-index was not dimensionally related to any addiction-like behaviour in the 3-criteria model (Pohorala et al., 2021), however it should be noted that a group analysis (ST *versus* GT) did reveal that ST show a decrease sensitivity to punishment, a finding that requires more investigation. Abnormal decision-making is a key feature of SUD (Clark and Robbins 2002). Several studies have evaluated cognitive flexibility in rodents *via* RL paradigms to investigate the relationship between the antecedent nature of decision-making deficits and cocaine SA. Thus, rats with a history of poor reversal performance show increased cocaine intake after a short history of exposure, relative to high performing rats

(Groman et al., 2020). However, to date, relatively few studies have investigated the relationship between RL performance and compulsive drug-seeking behaviour.

Taken together, there is clear evidence to support the notion that distinct behavioural traits contribute to different aspects of addiction-like behaviour but the relationship of these traits specifically to compulsive cocaine seeking is relatively understudied. Herein, several risk traits (highlighted in Chapter 4) were evaluated prior to the assessment of compulsive cocaine seeking and the relationship between pre-existing risk traits and the development of compulsive cocaine seeking was addressed.

Based on previous literature, primarily related to alternative schedules of punishment (i.e., ^{2nd}SOR, the 3-criteria model and the seeking-taking chain schedule), I predicted that:

[1] The compulsive cocaine seeking phenotype would emerge in a subpopulation of rats (~15%) after prolonged intravenous cocaine SA and response-contingent footshock punishment;

[2] Impulsivity and kappa would positively relate to compulsive cocaine seeking;

[3] Sign-tracking animals would show higher levels of susceptibility to conditioned reinforcement than GT;

[4] LocR would predict the acquisition of cocaine SA.

5.2 Materials and methods

Subjects

Male Lister Hooded rats (Charles River, Kent) were used in this study. For further details on housing, and more specific details on housing conditions prior to SA please see Chapter 3, section 3.1. All SA took place after the MRI scan on PND~228. Upon completion of intravenous catheter surgery all rats were singly housed. Several animals were culled throughout SA training due to lost catheter patency, resulting in n=39 animals that underwent the evaluation of compulsive cocaine seeking. All experiments were carried out in accordance with the (U.K Animals) Scientific Procedures act (1986) under the UK Home Office project license (PPL 70/7587) and were approved by the University of Cambridge Animal Welfare and Ethical Review Body.

Attribution of incentive salience: sign- and goal-tracking

See Chapter 3, section 3.2 for more details.

Impulsivity: 5-choice serial reaction time task

See Chapter 3, section 3.3 for more details.

Cognitive flexibility: deterministic reversal learning

See Chapter 3, section 3.4 for more details.

Novelty place preference

See Chapter 3, section 3.5 for more details.

Sensation seeking: locomotor reactivity to novelty

See Chapter 3, section 3.6 for more details.

Timeline of self-administration

Fig1. Brief overview of self-administration timeline. Acquisition of cocaine self-administration was carried out with 4 sessions under a fixed ratio (FR)-1 schedule of reinforcement. Following successful acquisition, rats were transferred to increasingly delayed fixed-interval training, until fixed interval (FI)-15. Rats were trained for three days on FI-15 before the introduction of the conditioned stimulus. Second order training (^{2nd}SOR) lasted for 20 days (FI15:FR10:S). After 20 days, rats were subjected to mild-electric foot shock for 5 days with increasing foot shock intensity. Following punishment sessions, rats were re-baseline under a ^{2nd}SOR for 5 days.

Self-administration training

Upon completion of the post-behavioural MRI scan at PND~228 all rats were implanted with an indwelling catheter as previously described (Chapter 3, section 3.8.1). Following surgery and post-operative recovery rats were trained to self-administer cocaine according to **Fig1**. (see Chapter 3, section 3.8 for more details). Rats were trained to acquire cocaine SA on a FR-1 schedule of reinforcement for 4 days, with a maximum number of 30 cocaine infusions

a day. Subsequently, rats were trained under FI schedule of reinforcement with increasing intervals (1 minutes (FI-1 – 1 day), 2 minutes (FI-2 – 1 day), 4 minutes (FI-4 – 1 day), 8 minutes (FI-8 – 1 day), 10 minutes (FI-10 – 1 day) and 15 minutes (FI-15 – 3 days). After three sessions under FI-15, a conditioned stimulus was introduced, and rats were trained under a ^{2nd}SOR (FI15:FR10S) for 20 sessions. Following this, rats underwent 5 sessions where drug-seeking was punished with a mild electric foot shock. An overview of the paradigm is provided below (**Fig2.**).

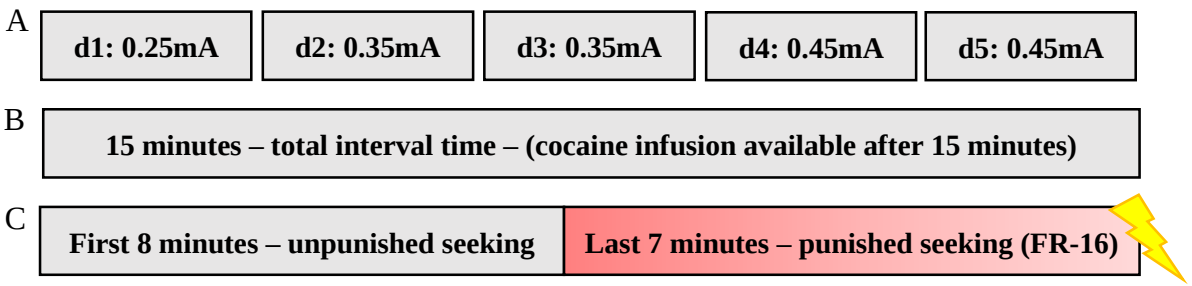


Fig2. Schematic overview of self-administration punishment schedule. Rats were trained for five days in total under punishment conditions, with increasing shock intensities **(A).** **(B)** Rats were trained under a second order schedule of reinforcement (FI15:FR10:S:FR16SHOCK), with 16 lever presses resulting in the presentation of a mild electric foot shock. **(C)** During the 15-min interval only the last 7 min of the interval were punished with foot shock. The first 8 minutes of the interval were unpunished. After completion of the 15-min interval, cocaine was available and upon reaching FR10 an infusion was delivered and in parallel a cue light was illuminated. *Abbreviations:* d, day; FR-16, fixed-ratio 16.

	Upper quartile	Lower quartile	Mid quartiles
Impulsivity	11	10	18
Sign-tracking (CS+)	10	10	19
Sign-tracking (PCA index)	11	9	19
P(Win|Stay)	10	8	21
P(Lose|Shift)	10	10	19
Perseverative responding	9	11	19
Alpha	10	8	21
Beta	11	7	21
Kappa	13	12	14
LocR	10	9	20
NPP	10	9	20

Table1. Relative numbers of rats in upper, lower and middle quarters used in behavioural analysis to assess relationship between pre-existing behaviours and addiction-like behaviour. N=39 (total)**.**

Statistical analysis

The distribution of high, intermediate, and non-compulsive cocaine seeking animals was investigated using a retrospective K-means cluster analysis of the number of foot shocks an animal received in the first drug-free interval of the final two 0.45mA punishment sessions. K-means clustering is a commonly used unbiased approach that searches for commonalities within a dataset and forms multiple clusters that maximises differences between groups within the data. As an unsupervised algorithm, the clustering is unbiased. However, the number of clusters has to be arbitrarily pre-defined. Here, the cluster number was set to 3 based on previous findings indicating that different populations of compulsive drug use may be broadly defined based on high compulsive (HC), intermediate compulsive (IC) and low compulsive (LC) groups (Giuliano et al., 2018; 2019). The HC group consisted of 7 rats and had an average of 3 shocks during the last two days of punishment, the IC group consisted of 16 rats with an average of 1 shock and the LC group also consisted of 16 rats with an average of 0 shocks during the last two days of punishment. Of the 16 rats classified as LC only 7 were used in the formal analysis. The 7 LC rats were randomly selected from the initial 16 and matched for their total cocaine experience relative to the HC group (**Appendix 2A.**). Thus, the final group sizes were HC (n=7) and LC (n=7). The statistical relationship between several measures related to compulsive cocaine seeking were assessed across the different compulsivity phenotype. These included several measures of compulsive cocaine seeking; 1) the number of foot shocks during the first drug-free interval across the five punishment sessions, 2) the number of foot shocks during the total session across the five punishment sessions, and 3) the number of ALP during the first drug-free interval across the five punishment sessions. In addition, the statistical relationship between several pre-existing behavioural risk traits (see Chapter 4) and several SA measures, including 1) compulsive cocaine seeking in a drug-free state - the number of ALP during the first drug-free interval across the five punishment sessions (as above), 2) conditioned reinforcement of drug-paired cues - the number of ALP during the first drug-free interval across the three FI-15 sessions and the first 3 sessions of the ^{2nd}SOR, and 3) acquisition of cocaine SA – the number of ALP across the entire session for the first 4 FR-1 sessions. A repeated measures analysis of variance (ANOVA) was carried out with the factors above used as within-subject factors and either compulsivity (HC *versus* LC) or behavioural risk trait (upper quartile (UQ) *versus* lower quartile (LQ)) used as the between-subject factor (see **Table1.** for details on the number of animals in each group). Homogeneity of variance was assessed by the Mauchly

Sphericity Test and Greenhouse-Geisser correction was used where appropriate. In probing the underlying relationship between impulsivity, kappa and compulsive cocaine seeking an ANOVA was performed with 'vulnerability factor' as a between-subject factor. Vulnerability factor represents the principal component containing primarily stickiness, impulsivity and NPP from Chapter 4 (for more details see Chapter 4, results). All statistical analysis was performed using custom scripts in python (Python 3.7), or SPSS (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.).

Principal component analysis

To understand the relationship between the HC phenotype and several behavioural endophenotypes (discussed in Chapter 4) shown previously to predict distinct aspects of addiction-like behaviour a PCA was undertaken to map the underlying latent variable structure. All factors were determined with a minimum eigenvalue of 1, and were factor rotated using a normalised Varimax rotation. The PCA analysis performed in this chapter is an evolution of that seen in Chapter 4. The difference being that the PCA analysis carried out in this chapter now also contains compulsive cocaine seeking (an average of the ALPs during the first drug-free interval during the final two 0.45mA sessions).

5.3 Results

The distribution of high, intermediate, and non-compulsive cocaine seeking animals was investigated using a k-means cluster analysis of the number of foot shocks an animal received in the first drug-free interval of the final two 0.45mA punishment sessions (**Fig3.**). HC animals represented 18% (n=7) of the sample. The remaining sample comprised of IC animals 41%, (n=16) and LC animals 41% (n=16).

The number of volitional foot shocks was higher in the HC group during the first drug-free interval (HC *versus* LC: $F(2,37) = 3.313$, $p=0.048$ and compulsivity x session interaction: $F(2,37) = 2.152$, $p=0.035$) (**Fig4A.**), an index of drug-free cocaine seeking and the direct conditioned reinforcing properties of the drug-conditioned stimulus. Similarly, the number of volitionally received foot shocks was higher in the HC group throughout the entire session (HC *versus* LC: $F(2,37) = 7.598$, $p=0.002$) (**Fig4B.**).

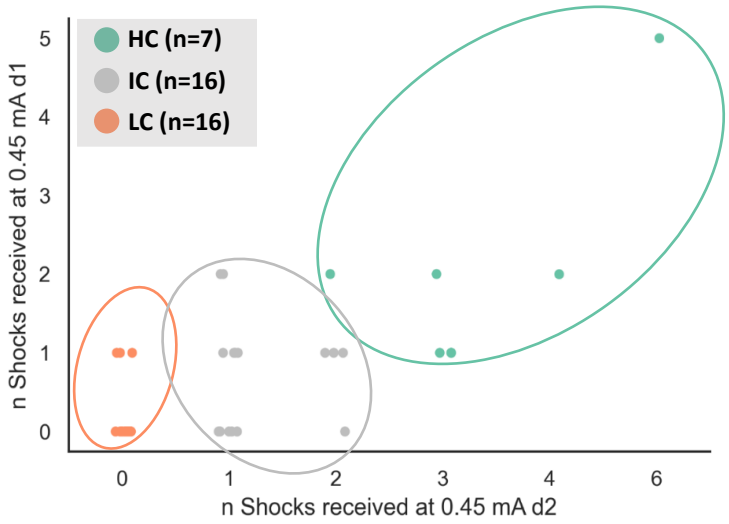


Fig3. Emergence of compulsive cocaine seeking phenotype following history of cocaine self-administration. Distribution of high compulsive (HC) (n=7), intermediate compulsive (IC) (n=16) and low compulsive (LC) (n=16) animals (n=39), separated by the number of shocks on day 1 (d1) and day 2 (d2) of the final 0.45mA shock sessions. *Abbreviations:* n, number; d, day.

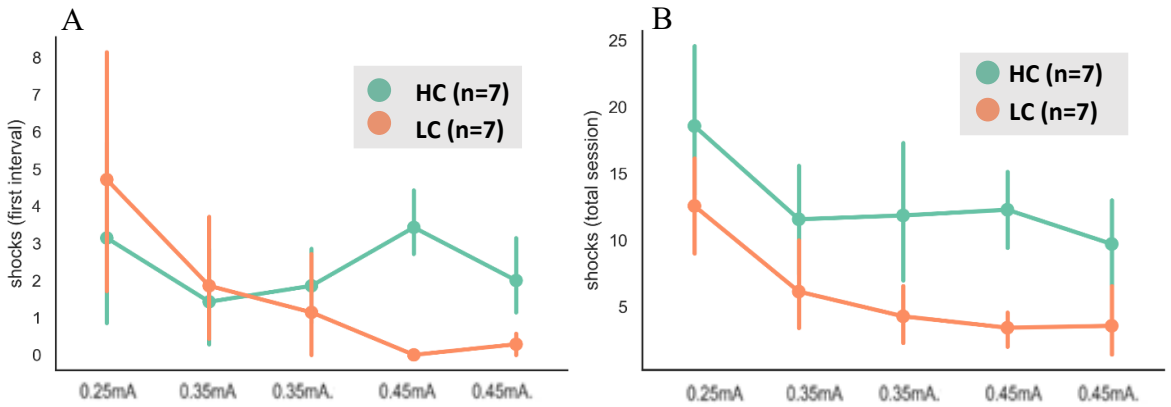


Fig4. Emergence of compulsive cocaine seeking phenotype in subpopulation of rats under punishment conditions. High compulsive (HC) animals volitionally received a higher amount of foot shocks during the **(A)** first interval (HC *versus* LC: $F(2,37) = 3.313$, $p=0.048$ and compulsivity x session interaction: $F(2,37) = 2.152$, $p=0.035$) and **(B)** throughout the entire session (HC *versus* LC: $F(2,37) = 7.598$, $p=0.002$) when compared to low compulsive (LC) animals. Mean data reported with error bars indicating ± 95% CI. *Abbreviations*: mA, milliamps.

Compulsive cocaine seeking during the first drug-free interval was also measured by the number of ALP during the first drug-free interval. HC animals exhibited significantly higher levels of ALPs across the punishment sessions, when compared to LC animals (HC *versus* LC: $F(2,37) = 3.644$, $p=0.036$) (**Fig5.**).

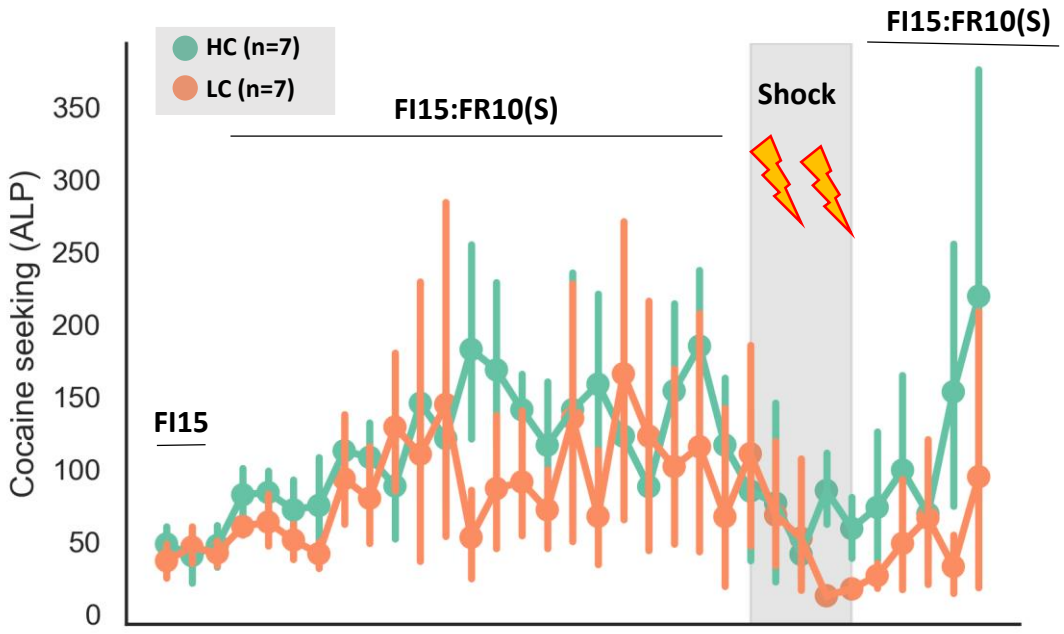


Fig5. High compulsive (HC) animals display higher levels of drug-free cocaine seeking under contingent punishment conditions. Compulsive drug-free cocaine seeking, as indexed by the number of active lever presses during the first drug-free interval, was significantly higher during punishment conditions in HC when compared to low compulsive (LC) rats (HC *versus* LC: $F(2,37) = 3.644$, $p=0.036$). Mean data reported with error bars indicating ± 95% CI. *Abbreviations:* ALP, active lever presses.

A PCA was performed to evaluate the relationship between the HC phenotype with several behavioural phenotypes shown previously to predict distinct aspects of addiction-like behaviour (See Chapter 1 and 4). Like Chapter 4, prior to the PCA being run, the three RL computational variables were run through a PCA analysis. Both alpha and beta loaded significantly on to a single factor, whereas kappa loaded on the second factor of this PCA analysis. In the final PCA, the latent variable representing the shared variance of alpha and beta was used, termed αβ. All other variables in the PCA were unchanged relative to the PCA carried out in Chapter 4. The PCA, now incorporating compulsive cocaine seeking, revealed three factors accounting for 63.759% of the total variance (**Fig6.**). Impulsivity, kappa and compulsivity loaded on factor 1 and explained 30.308% of the variance. NPP negatively and positively loaded on factor 1 and 2, respectively. LocR loaded on factor 2 and explained 17.556% of the variance. Factor 3 loaded on the αβ component, as well as conditioned approach to a reward-predictive stimulus (sign-tracking) and explained 15.931% of the total

variance. A summary of the loadings, and explained variance is observed in **Tables 2.** and **3.**, respectively.

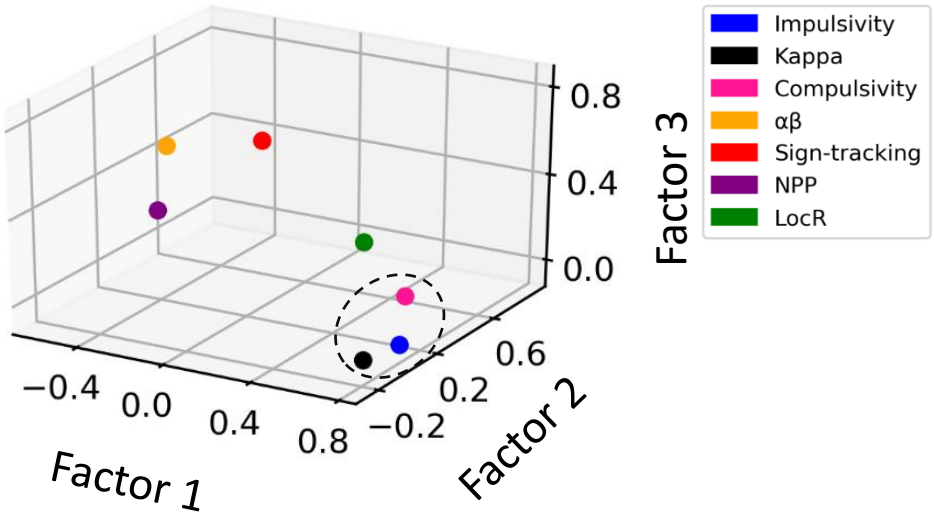


Fig6. Impulsivity and stickiness predict the development of future compulsive cocaine seeking. Principal component analysis including both compulsivity and several relevant behavioural endophenotypes revealed three factors accounting for 63.795% of the total variance. Factor 1 demonstrated a shared construct between compulsive cocaine seeking, impulsivity and stickiness, representing 30.308% of the total variance. Factor 2 was primarily loaded on by locomotor reactivity to novelty (17.556% of total variance). Novelty place preference negatively and positively loaded on factor 1 and 2, respectively. Factor 3 was primarily loaded by the combined αβ component and sign-tracking (15.931% of the total variance). Impulsivity, stickiness and compulsivity component highlighted with dashed circle. *Abbreviations:* LocR: locomotor reactivity to a novel inescapable environment; NPP: novelty place preference.

Component	Total	% of variance	Cumulative %
1	2.122	30.308	30.308
2	1.229	17.556	47.864
3	1.115	15.931	63.795

Table2. Total variance of principal component analysis. Factor 1 accounted for 30.308% of the total variance. Factor 2 accounted for 17.556%, and factor 3 accounted for 15.931% of the total variance. Cumulatively, 63.795% of the total variance was explained by these 3 factors.

	Component / Factor		
Behavioural variable	**1**	**2**	**3**
Impulsivity	0.776	0.094	-0.062
Sign-tracking	-0.013	0.329	0.634
αβ	-0.026	-0.266	0.829
Kappa	0.743	-0.103	-0.058
NPP	-0.599	0.462	0.152
LocR	0.083	0.890	-0.037
Compulsivity	0.760	0.152	0.137

Table3. Principal component scores from principal component analysis evaluating the relationship between compulsive cocaine seeking and behavioural endophenotypes shown previously to predict distinct aspects of addiction-like behaviour. Factor 1 was primarily loaded by compulsivity, impulsivity, kappa and to a certain extent, NPP. Factor 2 was primarily loaded on by LocR. Finally, factor 3 was primarily loaded by the αβ component and to a certain extent, sign-tracking. *Abbreviations:* LocR: locomotor reactivity to a novel inescapable environment; NPP: novelty place preference.

Next, the role in which pre-existing risk traits may later influence several SA measures was evaluated. Thus, animals classified based on their vulnerability traits (Chapter 4) were retrospectively evaluated (after completion of the SA training and evaluation of compulsivity) for 1) compulsive cocaine seeking in a drug-free state (ALPs during the first drug-free interval across the five punishment sessions); 2) susceptibility to the conditioned reinforcing properties of a drug-conditioned stimulus (ALPs during the first drug-free interval across the three FI-15 sessions and the first 3 sessions of the ^{2nd}SOR) and 3) acquisition of cocaine SA (the number of ALPs across the entire session for the first 4 FR-1 sessions). In addition, Factor-1 of the PCA analysis carried out in Chapter 4 (which primarily loaded impulsivity (0.786) and kappa (0.755) and novelty preference (-0.724), accounting for 29.393% of total variance) was used as an additional risk variable (termed vulnerability factor).

Animals classified as HI or sticky (high kappa) did not significantly differ in their active lever responses across the first interval during the punishment sessions when compared to their LI or low sticky counterparts (**Fig8A, I.**), respectively. However, animals classified as vulnerable based on their Factor-1 loading scores (Chapter 4) showed significantly higher

levels of compulsive cocaine seeking during the first interval (compulsivity x session interaction: F(2,37) = 2.902, p=0.017) (**Fig7.**).

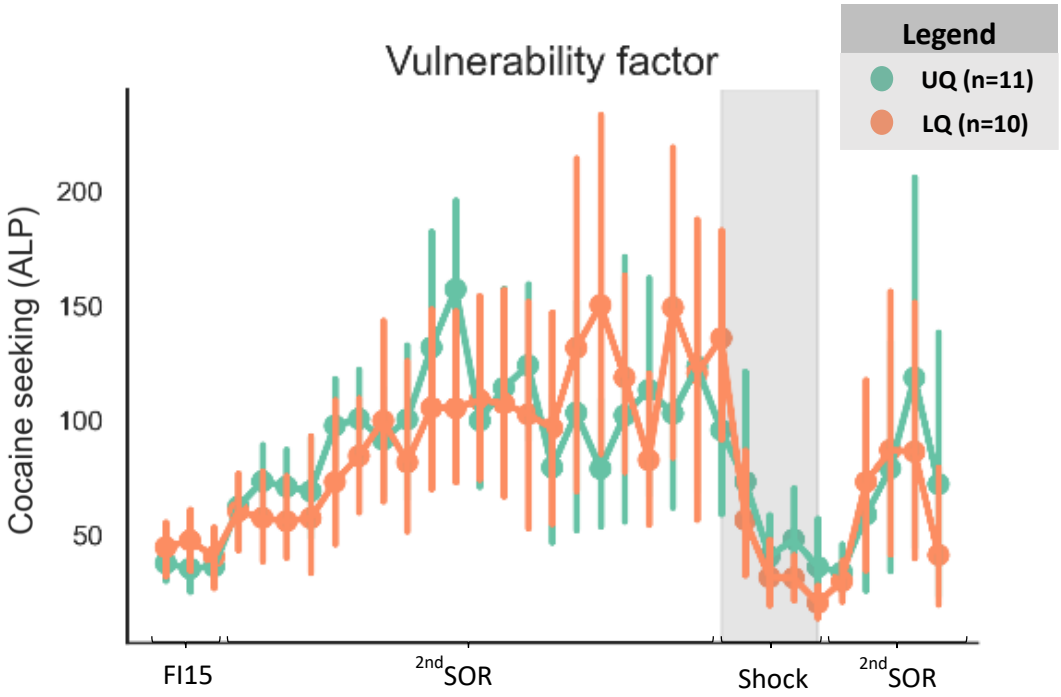


Fig7. Predictive relationship between an underlying risk construct and compulsive cocaine seeking. Animals classified as vulnerable based on their Factor-1 scores from a principal component analysis of several behavioural endophenotypes previously linked to addiction-like behaviour, representing primarily impulsivity (loading score: 0.786), kappa (loading score: 0.755) and novelty place preference (loading score: -0.724), showed increased cocaine seeking under punishment (vulnerability x session interaction: F(2,37) = 2.902, p=0.017). Mean data reported with error bars indicating ± 95% CI. *Abbreviations:* ALP, active lever presses; UQ, upper quartile; LQ, lower quartile

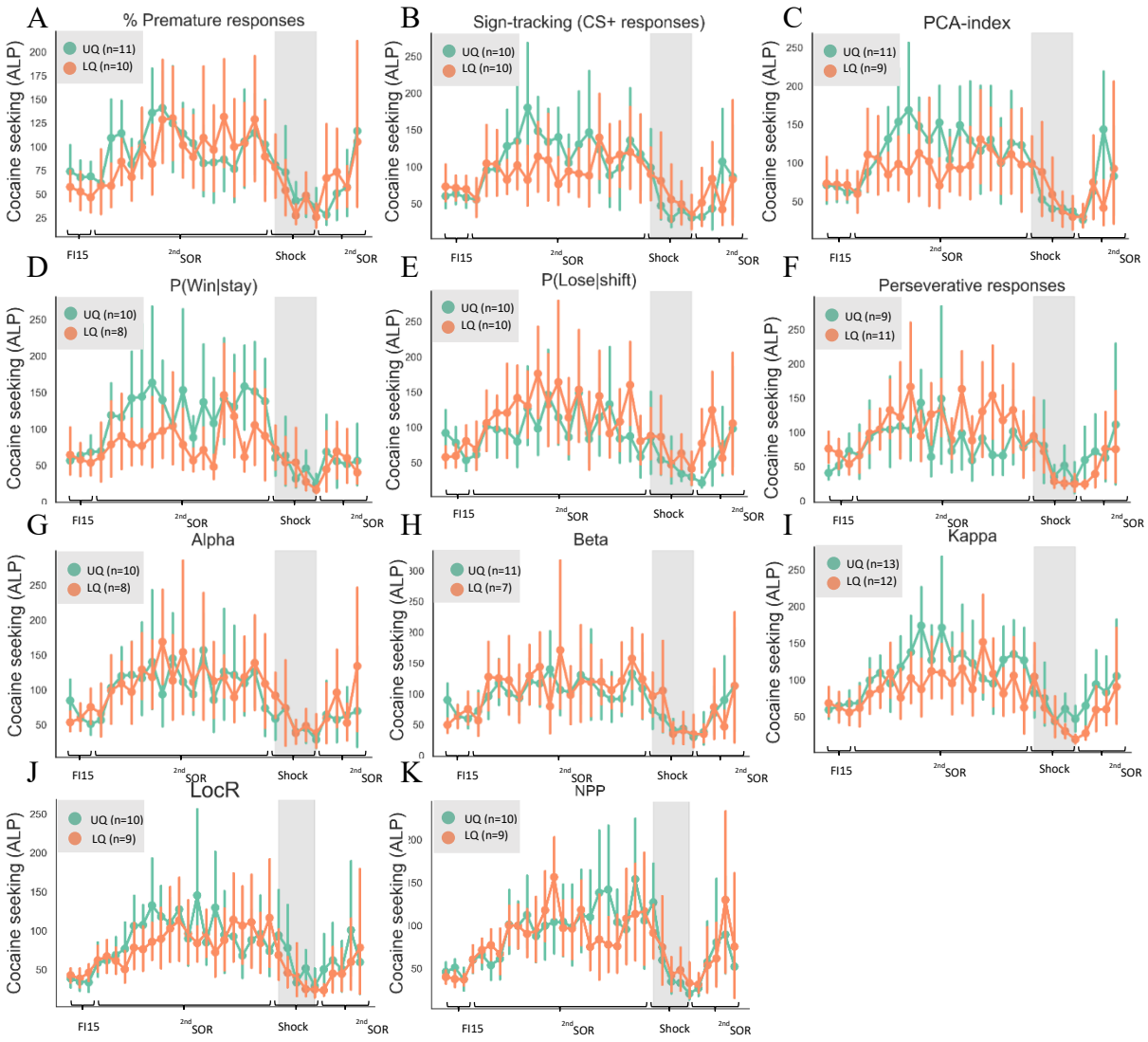


Fig8. Investigation of pre-existing behavioural endophenotypes on the development of compulsive cocaine seeking. No statistical differences were observed in compulsive cocaine seeking under punishment in animals classified based on the upper or lower quartile from several behavioural measures investigated. Mean data reported with error bars indicating ± 95% CI. *Abbreviations:* CS, conditioned stimulus; PCA-index, Pavlovian conditioned approach index; ALP, active lever presses; LocR, locomotor reactivity to a novel inescapable environment; NPP, novelty place preference.

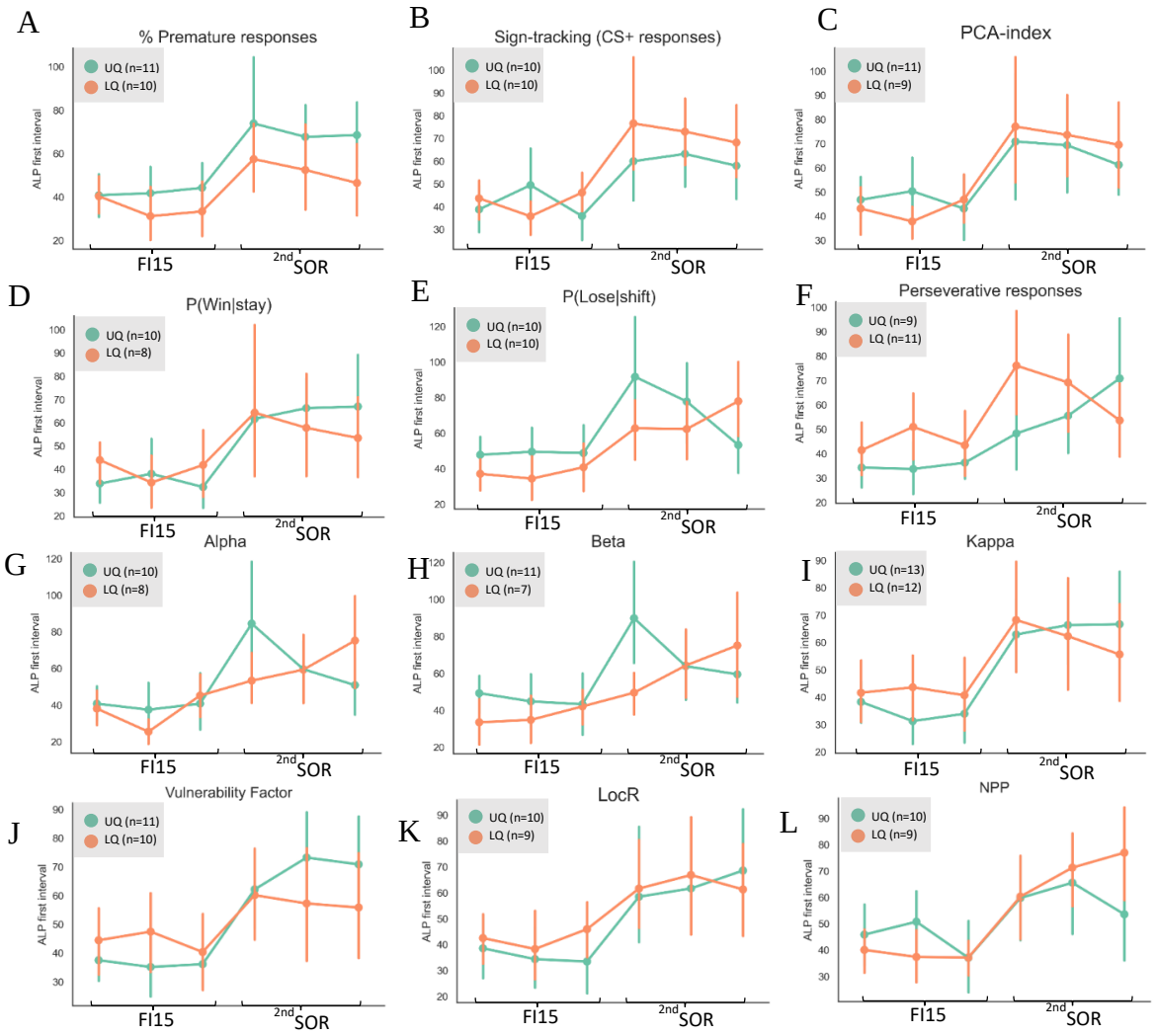


Fig9. Investigation of *pre-existing* behavioural endophenotypes on the sensitivity to reward-predictive conditioned stimuli. No statistical differences were observed in responding during the transition between FI15 and FI15FR10:S schedule of reinforcement in animals classified based on the upper or lower quarter from several behavioural measures investigated. Mean data reported with error bars indicating ± 95% CI. *Abbreviations:* CS, conditioned stimulus; PCA-index, Pavlovian conditioned approach index; ALP, active lever presses; LocR, locomotor reactivity to a novel inescapable environment; NPP, novelty place preference.

Not including the vulnerability factor, none of the 11 behavioural measures were related to, or predicted, drug-free compulsive cocaine seeking (**Fig8.**). To investigate sensitivity to the conditioned reinforcing properties of drug-paired CS, drug-seeking during the first interval was compared between the three FI-15 sessions, and the first three ^{2nd}SOR sessions. Of the 12 measures investigated there were no differences between groups in responding during the transition between FI-15 and ^{2nd}SOR (**Fig9.**).

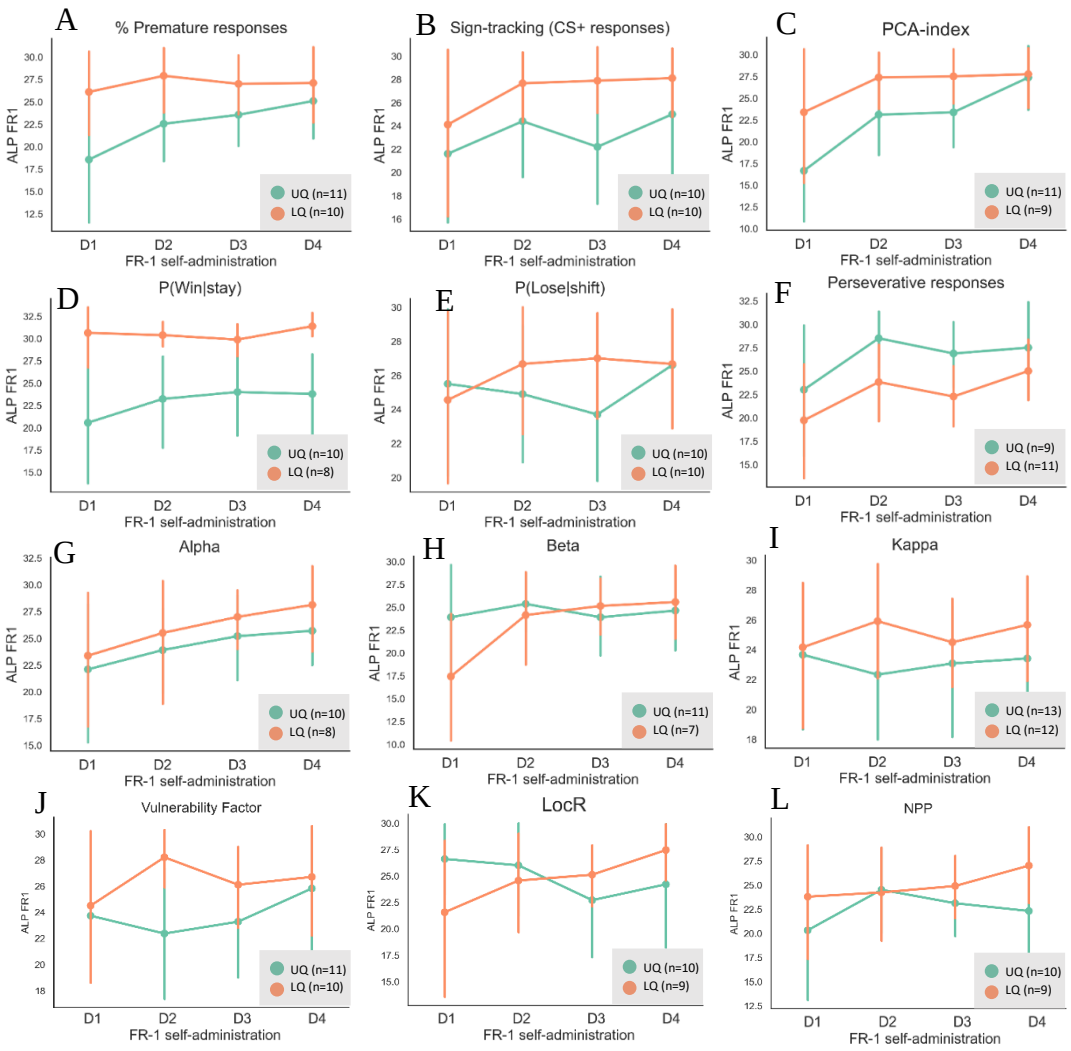


Fig10. Investigation of *pre-existing* behavioural endophenotypes on acquisition of cocaine self-administration. Of the 12 measures evaluated, goal-tracking animals, as evaluated through the PCA-index, had a significantly higher number of active lever presses (ALP) during the acquisition period relative to goal-trackers (Acquisition x PCA interaction: $F(2,37)=3.348$, $p=0.015$). Likewise, animals with low P(Win|Stay) showed increased levels of acquisition when compared to animals showing high P(Win|Stay) (High P(Win|Stay) *versus* Low P(Win|Stay): $F(2,37)=6.595$, $p=0.004$). No differences were observed in the remaining measures investigated. Mean data reported with error bars indicating ± 95% CI. *Abbreviations:* CS, conditioned stimulus; PCA-index, Pavlovian conditioned approach index; ALP, active lever presses; LocR, locomotor reactivity to a novel inescapable environment; NPP, novelty place preference.

Finally, the propensity to acquire cocaine SA across different endophenotypes was investigated. Of the 11 measures evaluated only two significantly differed across groups. Thus, animals that showed lower levels of incentive salience attribution, as indexed *via* the PCA-index (i.e., GT), showed greater levels of ALP during the four FR-1 SA sessions when

compared to sign-trackers (Acquisition x PCA interaction: F(2,37)=3.348, p=0.015) (**Fig10C.**) and animals that showed a lower probability of choosing the same choice after a reward (i.e., low P(Win|Stay)) showed increased acquisition relative to high P(Win|Stay) animals (High P(Win|Stay) *versus* Low P(Win|Stay): F(2,37)=6.595, p=0.004) (**Fig10D.**).

5.4 Discussion

No different to what is observed in humans (Reboussin and Anthony 2006), compulsive cocaine seeking developed only in a small proportion of rats (~18%) exposed to cocaine and response-contingent punishment. This is in alignment with epidemiological studies of human addiction, suggesting that most individuals are resilient to the development of addiction and only a small minority of individuals eventually transition to cocaine dependence following protracted drug use (Ahmed 2010). Moreover, this parallels other rodent studies investigating the compulsive nature of addiction-like behaviour (Belin et al., 2008; 2011; Augier et al., 2018; Giuliano et al., 2019). This small population of punishment-resistant rats also align with studies showing that only a small population of rats (~10%) choose cocaine over an alternative reward (e.g., a sweetened saccharin water solution) in a free choice procedure (Lenoir et al., 2007; Cantin et al., 2009). Therefore, the punishment-resistant rats in this thesis may parallel the small proportion of rats that continue to take cocaine in a free choice procedure and may be homologous to the small proportion of the human population who transition to addiction after protracted drug use. The emergence of compulsive cocaine seeking in this sub-population of rats (Chapter 5) could not be attributed to a differential cocaine-taking history (**Appendix2A-D.**) (Pelloux et al., 2007), as both HC and LC animals showed similar levels of cocaine intake (**Appendix2A.**). Likewise, HC animals did not differ in their ability to acquire cocaine SA as indexed by their comparative levels of responding under four FR-1 sessions when compared to LC animals, respectively (**Appendix2B.**). In addition, responding under both FI and ^{2nd}SOR was similar in both HC and LC groups (**Appendix2C, D.**). Thus, HC animals showed similar levels of responding across ^{2nd}SOR sessions leading up to the assessment of compulsive cocaine seeking. In a separate cohort of animals, compulsive cocaine seeking assessed on the same paradigm was not related to differential sensitivity to pain, as indexed through hotplate tail flick latencies, thus suggesting that differences in punishment resistance assessed in this paradigm were not related to differential pain thresholds (**Appendix1A-D.**). During the final two punishment sessions HC

animals showed increased levels of cocaine seeking during the first drug-free interval. Moreover, HC animals took more shocks in this interval, and across the total session relative to LC animals on both the final two shock days. Thus, the emergence of the compulsive phenotype was not due to differential cocaine experience but was presumably a consequence of an interaction between drug-exposure and antecedent vulnerability traits that caused animals to persist in drug-seeking in the face of contingent foot shock punishment. A PCA analysis was performed to understand the relationship between several pre-existing behavioural risk traits and compulsive cocaine seeking. Like that observed in Chapter 4, impulsivity, stickiness and NPP loaded on to one factor. However, this time, compulsive cocaine seeking also loaded on to the same factor, in the same direction as impulsivity and stickiness. This latent variable structure suggests that these three constructs overlap and in effect suggests that impulsivity and stickiness predict compulsive cocaine seeking. To evaluate the importance of the combined construct of impulsivity and stickiness to the development of compulsive cocaine seeking the latent variable (factor 1 primarily containing impulsivity, stickiness and NPP) from Chapter 4 was subsequently used to separate the animals (into vulnerable and resilient subgroups). Neither stickiness nor impulsivity alone significantly predicted compulsive drug-seeking, whereas in combination (*via* the latent factor) animals deemed vulnerable (showing higher levels of impulsivity and stickiness) maintained their cocaine seeking levels despite foot shock punishment. The importance of the combined impulsivity and stickiness construct was later confirmed by evaluating differences in compulsive cocaine seeking across animals classified based on their expression of several other risk traits; none of the other behavioural traits investigated showed any significant relationship with compulsive cocaine seeking. In addition, a follow up ordinary least square (OLS) analysis confirmed this notion, with impulsivity and stickiness alone significantly predicting compulsive cocaine seeking (**Appendix3.**). Thus, it appears antecedent deficits in impulse control and increased perseverative-like behaviour, in the form of stickiness, are risk markers for the future development of compulsive cocaine seeking.

To what extent impulsivity and compulsivity manifest prior to or are exacerbated by drug use during the transition to dependence is still an open question. Traditionally, it has been suggested that both impulsivity and compulsivity represent two constructs at opposite ends of the same spectrum (Allen, King and Hollander 2003). However, others have suggested that impulsivity and compulsivity may represent orthogonal factors (Fineberg et al., 2010); others have suggested that impulsivity and compulsivity may represent a unitary construct –

'disinhibition'; whilst others have suggested that both represent independent constructs that are highly interrelated. For example, Chamberlain and colleagues evaluated a wide range of personality and psychopathology-based measures and neurocognitive functions in a large sample of healthy adults. These measures encompassed a wide range of metrics related to impulsivity and compulsivity, of which the authors performed a dimension reduction analysis to probe the underlying latent variable structure. They observed two distinct factors with one factor primarily representing impulsivity and the other primarily representing compulsivity, with the two constructs being highly correlated (Chamberlain et al., 2018). Conversely, others have suggested that the development of compulsive drug use reflects a shift from pre-existing deficits in impulse control towards compulsive drug-seeking (Koob and Le Moal 2001; Belin et al., 2008). In line with this, pre-existing deficits in impulse control, alongside stickiness, subsequently predicted future compulsive cocaine seeking. Considering the results of the PCA analysis, the data presented suggests that both impulsivity and compulsivity are overlapping constructs and can be represented (alongside stickiness and NPP) by a shared factor or latent variable which could be deemed 'disinhibition'. These findings contrast with earlier reports showing that both constructs are independent (Chamberlain et al., 2018) or are opposite ends of the same spectrum (Fineberg et al., 2010). However, these results do align with previous reports showing that the transition to compulsive cocaine taking is predicted by pre-existing high levels of impulsivity (Belin et al., 2008), with these findings now extending this to compulsive drug-seeking.

The findings of this chapter demonstrate that LocR does not load on to the same factor as compulsivity, and is not predictive of future compulsive cocaine seeking. In rodents, the sensation seeking trait has been linked to the initial acquisition of psychostimulants (Piazza et al., 1989) (discussed below) but is not a vulnerability marker for the development of compulsive cocaine taking in the 3-criteria model of addiction (Belin et al., 2008). This result has been replicated several times (Belin et al., 2008; 2011), thus high levels of sensation seeking may predispose an individual to initiate drug-taking which is not a factor in the development of addiction *per se*. This parallels research in humans involving drug-dependent and non-dependent sibling pairs showing that sensation seeking is elevated in drug users but not their siblings, suggesting that sensation seeking is exacerbated by drug use and not necessarily a pre-existing risk trait (Ersche et al., 2010). These findings are therefore in alignment with several earlier reports suggesting that the development of compulsive drug use is not related to heightened levels of sensation seeking in rodents.

In contrast to the predictive relationship of impulsivity to compulsive cocaine taking and seeking (Belin et al., 2008; this thesis) NPP was not positively associated with compulsive cocaine seeking. Although it did load on to the same latent variable (discussed above), it loaded in the opposite direction to compulsivity. This is somewhat surprising given the link between NPP and compulsive cocaine taking previously described (Belin et al., 2011). Here, rats who were more likely to choose a novel environment in a free choice procedure were more resistant to foot shock punishment in the 3-criteria model of addiction. It is unlikely that this discrepancy may be explained by procedural differences as, in comparison to Belin et al., 2011, a similar NPP testing design was used. In this thesis, NPP was assessed *via* a two-chamber design with a central alley. In addition, the configuration of several structural inserts was varied across each chamber to create distinct spatial configurations between the familiar and novel compartments, similar to what was previously reported (Belin et al., 2011). Although it cannot be ruled out that there were differences in the specific set up, and conditions were not identical, the general procedure has striking similarities, and therefore these results are unlikely to be a result of technical differences. The observed relationship between impulsivity and NPP may have been a contributing factor here. Whereas previous evidence tentatively suggests an overlap between NPP and impulsivity (Molander et al., 2011), in this report they were inversely related (Chapter 4). Although no predictive relationship between NPP and compulsive cocaine seeking was observed in a separate OLS analysis (**Appendix3.**), the PCA dimensional analysis does align with the correlational analysis performed (Chapter 4). Thus, NPP loaded negatively on the same factor as both impulsivity and compulsivity and NPP was negatively correlated with impulsivity. Therefore, these results suggest that whereas impulsivity is a vulnerability factor for both compulsive drug-taking and drug-seeking, NPP may represent a vulnerability factor for drug-taking only.

As discussed above, LocR has been linked to increased acquisition of cocaine SA (Piazza et al., 2000). However, HR and LR animals did not differ in their number of cocaine taking responses across any of the four initial FR-1 days of SA. The distribution of LocR responses in this thesis (Chapter 4) was in line with previous reports (Vanhille et al., 2015), showing a high degree of interindividual variability, thus ruling out potential suboptimal experimental conditions as a primary factor for the null result observed here. However, there were experimental differences between the work carried out in this thesis and previous reports, in such that assessment of LocR in this thesis was carried out in a square open field and not a circular corridor as previously described (Piazza et al., 1989; 2000, Belin et al., 2008).

However, it is unlikely that this had a large effect on the observed LocR behaviour. Another important point is that LocR was assessed after several months of operant training (Chapter 4 (**Fig1.**)). Although rats had no prior experience of the novel open field prior to testing it is possible that months of handling and prior experience of other testing arenas may have impacted LocR. However, as all animals were handled in a similar fashion and also had similar experiences with operant training and other testing arenas. This impact would be distributed equally throughout, if at all. Although it is now widely accepted that LocR influences acquisition of drug use in rodents (Belin et al., 2016), there is also evidence to the contrary showing that neither HR nor LR rats differ in their acquisition of cocaine SA (Belin et al., 2011). In this study, animals separated based on their locomotor response to a novel circular corridor over a period of 2-hours did not differ in their propensity to acquire cocaine SA at a dose of 0.8 mg/kg. Therefore, it is important to note that the relationship between the HR phenotype and increased acquisition may occur under specific experimental conditions and may reflect an increased propensity for HR animals to acquire SA at lower doses (e.g., Belin et al., 2008), which would be evident after varying the cocaine dose, something not carried out in this thesis. Unlike LocR, animals that showed a lower probability of win-stay displayed higher levels of cocaine taking during early acquisition sessions. The probability of staying after choosing a winning option (i.e., enhanced probability of win-stay) in RL is thought to represent the integration of positive feedback. Thus, animals who are less able to integrate reward signals or have a deficit in their ability to effectively value rewards (Verharen et al., 2018) may take more cocaine initially as a compensatory mechanism. However, this idea is highly speculative and merits further investigation. Nevertheless, one study has shown that cocaine does indeed lower the probability of win-stay behaviour (Verharen et al., 2018), suggesting that cocaine has a modulatory effect on the integration of positive feedback. Whilst this effect appeared to be regulated by cocaine, in this report pre-existing deficits were observed in win-stay behaviour that were predictive of early acquisition suggesting that antecedent differences in reward processing may facilitate the vulnerability to initiate drug use.

^{2nd}SORs are well placed to probe the underlying effects of drug-paired conditioned cues in invigorating drug-seeking responses (Everitt and Robbins 2000). After three sessions of FI-15 a ^{2nd}SOR was introduced. The effect of the conditioned cocaine-paired stimulus on response rates in the drug-free first interval in rats classified based on their expression of a wide range of behavioural traits was then assessed. All animals showed an increase in responding after

the introduction of the cue, as expected. It was hypothesised that the motivational impacts of the conditioned cues would be differentially expressed in ST (who would show increased motivation or 'wanting' towards the drug-paired cue (Robinson and Berridge 2008; Yager and Robinson 2013)) when compared to GT. However, this trend was not observed. Both ST and GT, either defined based on CS+ response or the PCA-index, increased their responding to a similar degree upon introduction of the drug-paired cue. There is evidence to suggest that cocaine cues are more effective conditioned reinforcers in ST than GT (Yager and Robinson 2013; Saunders and Robinson 2010). However, by large, these studies have employed extinction-reinstatement procedures (Shaham et al., 2003), thus, the generalisability of the findings to ^{2nd}SOR are not straight forward. Along these lines, one study implemented a novel cue-removal paradigm during cocaine SA. For ST, responding severely decreased during the sessions when the cocaine cue was removed, whereas responding in GT remained largely unchanged, suggesting that the cue differentially modulated reinforcement across the different phenotypes. However, crucially, the overall rates of responding did not differ between ST and GT during the sessions in which the cue was presented. These results suggest that maximum levels of responding during sessions that included cocaine-paired cues were similar between both groups (Saunders and Robinson 2010) and parallels the data reported in this thesis.

On a technical note, a PCA was performed to understand the latent variable structure of the behavioural measures recorded and determine whether the variance across behaviours could be better explained by fewer dimensions. Studies evaluating the inter-relationship between behavioural endophenotypes of addiction-like behaviour using PCA methods are common (Hughson et al., 2019; Belin et al., 2008; Belin et al., 2011). In particular, an earlier paper by Belin et al., used PCA techniques to understand the relationship between impulsivity and compulsive cocaine use on the 3-criteria model of addiction. Here, impulsivity and 'addiction' loaded on to the same factor within that analysis, thereby representing a shared impulsivity/compulsive cocaine taking construct. Using the same analysis technique, a similar construct was observed here whereby compulsive cocaine seeking loaded positively on to the same factor as impulsivity, and stickiness. The analysis performed here has obvious parallels with the findings from Belin et al., 2008, and show further evidence of an underlying relationship between impulsivity and compulsivity. Whilst PCA was used in this thesis to understand the relationship between risk endopehnotypes and compulsive cocaine seeking, clustering algorithms can also be used to identify and characterize subgroups based

on the inter-relationship between the behavioural variables of interest. K-means clustering, one of the most well-known clustering algorithms, identifies maximally separated centroids (or groups) through an iterative process that minimizes the within-cluster variance. Once k-means has been used and clusters are formed, the underlying importance of the behaviours that form those clusters can be evaluated, i.e., how well that particular behaviour drives the separation of the clusters. One recent study has shown the utility of these methods. Mueller et al., used an optimized k-means approach to evaluate the grouping of offspring of maternal immune activation (MIA) exposed animals, and control animals. Whilst a control versus MIA-exposed group analysis revealed several behavioural differences, a high degree of variability in the MIA-exposed group prompted a k-means clustering analysis in the attempt to reveal sub-group differences. The clustering was performed across a number of different behaviours, ultimately showing that social preference behaviour was the most important behaviour driving the classification and highlighted the utility of this clustering approach to understand subtle nuances in behavioural output (Mueller et al., 2021). Whilst k-means clustering was not carried out in this thesis, it does offer an alternative route to evaluate behaviour, and will be considered in future analysis.

In summary, a novel SA paradigm designed to assess compulsive cocaine seeking was implemented whereby a small subgroup of rats displayed compulsive cocaine seeking behaviour. A retrospective analysis of the predictability of several antecedent risk traits found that impulsivity along with stickiness positively predicted future compulsive cocaine seeking. Thus, animals that displayed impulse control deficits, and who were more likely to repeat the same choice regardless of reward outcome, were more likely to continue seeking for cocaine in the face of contingent punishment.

Chapter 6

Structural and functional brain substrates of behavioural risk endophenotypes and compulsive cocaine seeking

6.1 Introduction

The transition to compulsion is hypothesised to result from a number of different mechanisms including impaired inhibitory response control (Dalley and Robbins 2017), maladaptive perturbations in functional circuits between the PFC and striatum (Ersche et al., 2020), and a progressive shift in control of behaviour from ventral limbic regions of the striatum (i.e., the Acb) to more dorsal associative and sensorimotor areas of the dStr (Porrino et al., 2004). Loss of inhibitory control is thought to reflect one mechanism where maladaptive drug-seeking behaviour is augmented *via* the inability to adequately regulate reward-seeking behaviours (Jentsch and Pennington 2014) and is thought to be mediated by both 'bottom-up' striatal mechanisms as well as 'top-down' cortical control influences (Basar et al., 2010; Dalley, Everitt and Robbins 2011). For example, in rodents, the IL cortex and the vStr form part of an integral circuit that plays a central role in promoting response inhibition (Caprioli et al., 2014; Chudasama et al., 2003; Jupp et al., 2020) and HI animals show lower levels of grey matter in the AcbC, as assessed through VBM (Caprioli et al., 2014). Evidence from preclinical imaging studies also suggests that the AI forms an important node in this network, with premature responding closely related to the thinness of this region (Belin-Rauscent et al., 2014). Not surprisingly, addiction circuits have largely implicated the aforementioned regions suggesting convergent neural substrates underpinning response inhibition and wider drug-

related behaviour. For example, simultaneous inactivation of both the IL cortex and the AcbS promotes the reinstatement of a cocaine seeking response after a period of extinction training (LaLumiere, Smith and Kalivas 2012). Similarly, the AcbC and its connections with the BLA are critical in mediating the conditioned reinforcing properties of drug-paired cues on cocaine seeking (Puaud et al., 2021; Ito et al., 2004), and play an important role in the initiation of cue-controlled cocaine seeking behaviours (Murray et al., 2015). In humans, specific dysfunctions in frontal brain regions have been observed in relation to both impaired response inhibition and addiction (Zilverstand et al., 2018). Impaired response inhibition forms one integral part of the impaired response inhibition and salience attribution (iRISA) (Goldstein and Volkow 2011) model of addiction, with inhibitory control mechanisms primarily being shown to depend upon the IFG (Aron, Robbins and Poldrack 2014), ACC, dlPFC and vlPFC (Goldstein and Volkow 2011). The role of these regions in response inhibition in SUD is highlighted in one functional mapping study showing that activation of the ACC is decreased in cocaine-dependent individuals during the GNGT, a finding related to poor inhibitory control performance under increasing working memory load (Hester and Garavan 2004). Similarly, decreases in grey matter in SUD are broadly observed across the PFC, including the IFG, cingulate cortex, OFC as well as insula regions (Ersche et al., 2012; Hall et al., 2015; Franklin et al., 2002; Matochik et al., 2003). Of note, voxel-based morphometric analysis has revealed that grey matter volume in the ACC as well as the IFG (Ersche et al., 2013), key regions involved in inhibitory control (above), are decreased in cocaine-dependent individuals, with ACC grey matter negatively correlated with duration of cocaine use (Connolly et al., 2013). In short, impulse control deficits, either pre-existing or drug-induced, are robustly associated with dysregulated functional signalling and structural abnormalities in both cortical and subcortical regions that are similarly related to several addiction-like behaviours, lending support to the notion that decreased response inhibition is a key factor in the transition to compulsive drug use.

The recent emergence and utilisation of reinforcement learning algorithms to model cognitive flexibility on a trial-by-trial basis has opened up novel avenues of investigation to understand aberrant behaviour and value-based decision-making in SUD. In particular, response stickiness has been shown to correlate with trait impulsivity (Chapter 4), and also predict compulsive cocaine seeking (Chapter 5). Although the specific neural correlates of stickiness are yet to be determined, one study has shown that stickiness is decreased after excitotoxic lesions of the IL cortex and medial OFC (MedOFC) (Verharen et al., 2020). Moreover,

evidence has shown that stickiness is increased in humans with SUD (Kanen et al., 2019), and is also exacerbated by cocaine SA in rats (Zhukovsky et al., 2019), supporting the notion that enhanced stickiness is associated with drug-induced neural adaptations and the propensity to compulsively seek cocaine (Chapter 5), potentially through interactions between overlapping PFC subregions and their downstream targets.

In combination with decreasing inhibitory control, compulsive drug-seeking is thought to reflect the transition from ventral to dorsal striatal control (Letchworth et al. 2001; Porrino et al. 2004) and the development of a habit-based system (Everitt and Robbins 2016). This notion is supported by reports showing that DA release in the dorsal, but not ventral striatum was associated with well-established cue-controlled cocaine seeking (Ito et al., 2002). Similarly, phasic DA in the vStr has been shown to decrease after prolonged cocaine exposure (Willuhn et al., 2012). Using a disconnection procedure, Belin and Everitt showed that well-established cocaine seeking was dependent on dopaminergic interactions between the AcbC and the DLS (Belin and Everitt 2008), blockade of which decreased cocaine seeking. More generally, the DLS has been proposed to be a critical region involved in mediating habitual control (Yin and Knowlton 2006), whereas the posterior dorsomedial striatum (pDMS) has been linked with goal-directed behavioural control (Yin et al., 2005). Along these lines, the shift from goal-directed to habitual control over cocaine seeking was investigated through selective dopaminergic antagonism of either the pDMS or aDLS following short or long periods of training under a ^{2nd}SOR (Murray et al., 2012). Dopaminergic blockade in the pDMS reduced cue-controlled cocaine seeking during early stages of training, whereas blockade of the aDLS reduced late-stage cocaine seeking, supporting the hypothesis that extended access to cocaine under these conditions facilitates habitual control over behaviour (Murray et al., 2012). The human homologue of the pDMS and aDLS is thought to be the caudate and putamen, respectively (Balleine and O'Doherty 2010). Like rodents, the posterior dorsolateral striatum, along with the pre-motor cortex, has been implicated in habit learning in humans (Tricomi, Balleine and O'Doherty 2009), whereas the caudate nucleus and its connections with the vmPFC (de Wit et al., 2012) has been associated with goal-directed learning (Tanaka, Balleine and O'Doherty 2008). Evaluating the specific neural transition from goal-directed to habitual control over drug seeking is practically very difficult, if not impossible, to achieve in human studies of addiction. Nonetheless, cocaine-dependent individuals show biases towards habitual responses on the slip-of-action test (Ersche et al., 2016), and show less sensitivity to

contingency degradation, with the length of cocaine use correlating with habitual responding (Ersche et al., 2020). In the same study, a spectroscopic analysis revealed a decrease in glutamate and glutamate/glutamine ratio in the putamen in cocaine-dependent individuals; a finding related to the automaticity factor of the creature of habit scale (Ersche et al., 2017) and is consistent with several other studies reporting striatal enlargement in chronic stimulant users (Ersche et al., 2012; Jacobsen et al., 2001; Chang et al., 2005). In combination with the report that a history of addiction impairs the ability to overcome learnt stimulus-response associations (McKim, Bauer and Boettiger 2016), these studies converge on the notion that a history of drug use is associated with enhanced habit formation and a decreased ability to suppress habits, which may facilitate rigid drug-seeking behaviour, a central feature in SUD. However, whether the tendency towards habitual responding lays dormant prior to drug exposure and is exacerbated by drug-induced neural adaptations is another intriguing possibility. Some evidence does suggest that this may be the case, with enhanced putamen volume, speculatively implying an overactive habit-based system, seen in first-degree non-drug using siblings of cocaine-dependent individuals (Ersche et al., 2012). However, many studies have assessed behavioural and neural changes following a history of drug exposure. Consequently, in this chapter, the structural and functional brain markers of drug-naïve rats prior to the emergence of compulsive drug seeking were investigated.

To date, the investigation of the underlying neural correlates of compulsivity, impulsivity, and stickiness *via* translationally relevant imaging in rodents is sparse. In this chapter, both structural and functional imaging was utilised to evaluate the neural correlates of rats phenotyped as impulsive or displaying high levels of stickiness, who were subsequently more vulnerable to develop future compulsive cocaine seeking. In addition, retrospective analysis was carried out to evaluate the structural and functional correlates of compulsive cocaine seeking. All imaging took place prior to cocaine SA and therefore was not biased by any drug-induced neural adaptations. Based on previous literature, I predicted that:
[1] Impulsivity would be associated with decreased grey matter in the AcbC and IL cortex;
[2] Impulsivity would be associated with changes in grey matter in the AI;
[3] Due to the recent evidence linking lesions of the PFC to stickiness (Verharen et al., 2020), grey matter perturbations in the PFC would be associated with stickiness;
[4] Compulsivity would be associated with decreased functional connectivity between the PFC and striatum. Specifically, hypoconnectivity between the PrL and the striatum would be related to compulsive cocaine seeking.

6.2 Materials and Methods

Subjects

Male Lister Hooded rats (Charles River, Kent) were used in this study. For specific details on housing conditions please see Chapter 3, section 3.1. Pre-behaviour imaging took place prior to behavioural training at PND-63. Following behavioural training rats were scanned again at PND~228. All experiments were carried out in accordance with the (U.K Animals) Scientific Procedures act (1986) under the UK Home Office project license (PPL 70/7587) and were approved by the University of Cambridge Animal Welfare and Ethical Review Body.

Impulsivity: 5-choice serial reaction time task

See Chapter 3, section 3.3 for more details.

Cognitive flexibility: deterministic reversal learning

See Chapter 3, section 3.4 for more details.

Magnetic resonance imaging

Acquisition

High resolution structural MRI was performed on a 9.4 T horizontal bore MRI system (Bruker BioSpec 94/20 Bruker Ltd. Coventry UK). As a result of within-study optimisation, 12 animals were scanned using a different acquisition sequence and were subsequently excluded from the functional connectivity analysis. Furthermore, 3 PND-63 scans were also excluded due to excessive distortion. For more details on specific acquisition parameters and anaesthesia protocols see Chapter 3, section 3.9 and 3.9.1.

Voxel-based morphometry

VBM was performed to assess grey matter volume differences. Briefly, images were first reoriented to match a reference template (SPMMouse, Wolfson Brain Imaging Centre, University of Cambridge (Sawiak et al., 2009)) and then corrected for differences in intensity uniformity *via* bias correction. The images were then segmented into three different tissue classes including grey matter, white matter, and cerebrospinal fluid. Subsequently, non-linear normalisation *via* the DARTEL procedure was carried out (Ashburner 2007). Using the warp fields from the DARTEL procedure, segmented grey matter images were warped and

modulated (*via* their Jacobian determinant) to match the newly generated template image (from the DARTEL procedure). All images were manually checked for accurate registration and the modulated and warped grey matter images were then smoothed with an isotropic Gaussian kernel filter of 0.45mm^3. For more details, see Chapter 3, sections 3.10 and 3.10.1.

Functional imaging – pre-processing

To investigate functional connectivity patterns between several cortico-cortico and cortico-striatal regions, a functional connectivity analysis was performed (functional connectivity equated to Spearman's correlation coefficient between the Eigenvariate of two separate BOLD time-series extracted from two ROIs). Briefly, all raw functional images were first manually checked for ghosting, severe motion, distortion and other artefacts. Images were then manually orientated to match a reference template. Both structural and functional images were then scaled by 10 to equate for differences in processing between rodents and humans. Pre-processing steps included 1) averaging of echo times, 2) removal of the first ten volumes, 3) brain extraction, 4) registration, 5) despiking, 6) motion correction, 7) spatial smoothing and 8) bandpass filtering (Grandjean et al., 2020). Brain-extracted functional images (see Chapter 3, sections 3.11 and 3.11.1 for more details on specific brain extraction procedures) were first registered linearly (6 DOF) to the brain-extracted structural (MT-weighted) image (FLIRT (FMRIB Linear Image Registration Tool; Jenkinson et al., 2002)). Subsequently the structural image was registered non-linearly to the template image (FNIRT (FMRIB Nonlinear Image Registration Tool; Smith et al., 2004)). Both the linear transformation matrix (functional to structural) and nonlinear warp-fields (structural to template) were combined to transform the functional image into template space in a one-step procedure to limit interpolation errors (Mahmoudzadeh and Kashou 2013). Registration accuracy was manually check for each image. All pre-processing occurred in template space. Following registration, removal of temporal spikes was carried out through temporal despiking (3dDespike), followed by motion correction (3dvolreg). The motion parameters were then regressed from the functional data using fsl_regfilt. Subsequently, the images were smoothed using an isotropic Gaussian kernel filter of 4.5mm^3 kernel (3dBlurInMask) and bandpass filtering was applied (0.01-0.1 HZ). For more details, see Chapter 3, section 3.11 and 3.11.1.

Functional connectivity analysis (region-to-region analysis)

Region-to-region correlation analysis was performed on fully pre-processed functional data in template space. To extract the BOLD data, several manually defined ROIs based on P&W coordinates were used. The ROIs were defined in the template space of which the functional images were subsequently registered. Additionally, the M1 ROI from a probabilistic atlas was also used (Valdes-Hernandez et al., 2011) (see Chapter 3, section 3.11.3 for more details). Prior to extraction visual inspection of several cortical and sub-cortical ROIs was carried out and their spatial location validated. After this, the first Eigenvariate of the timeseries was extracted for each ROI (fslmeants) and the correlation between the Eigenvariate of each ROI per each animal was assessed through Spearman's rank correlation coefficient. This resulted in a 14x14 correlation matrix representing the degree to which the BOLD signal correlated between all ROIs defined. The correlation matrices were subsequently used to address the relationship between functional connectivity and 1) impulsivity, 2) stickiness and 3) compulsive cocaine seeking. For more details, see Chapter 3.11, section 3.11.1.

Quality assurance

Several quality assurance steps were taken including the assessment of 1) SNR, 2) tSNR and 3) motion estimation *via* FWD (Power et al., 2012) – described in Chapter 2, section 2.6. Briefly, SNR was calculated by extracting an average of the BOLD signal across the entire brain and subtracting the background signal (Chapter2 **– Fig6A, B.**). The background signal was calculated by taking an average signal from the topmost corner of the image. This was done across different volumes spanning the entire scan. Visual inspection of all background signal masks revealed no overlap between rat and background signal mask. Additionally, tSNR was calculated by taking the mean BOLD signal using the same cortical mask as previously described and dividing it by the standard deviation of the signal, across all volumes (Chapter2 – **Fig6A, B.**). FWD was calculated *via* the extracted motion parameters generated from the motion correction procedure during pre-processing ((3dvolreg) see above). These parameters were then used to calculate FWD *via* the method of Power et al., 2012 assuming a rat brain radius of 8mm, as previously described (Chen et al., 2020) (Chapter2 **– Fig6C.**). To assess the impact on motion on connectivity the relationship between average FWD with global, regional and edgewise functional connectivity was evaluated. All three values were correlated with average FWD to reveal any residual relationships between motion and functional connectivity. Edgewise functional connectivity

refers to the functional connectivity between each region-to-region correlation value for each animal. A summary of the p-values generated from this correlational analysis are all greater than $p<0.05$, indicating no relationship between edgewise connectivity and motion, and are shown in (Chapter2 **– Fig6D.**). Regional connectivity was calculated by averaging the correlation matrix row-wise for each subject. The averaged rows (12 in total) for each subject were then correlated with FWD. A summary of the p-values from this correlation are all greater than $p<0.05$, indicating no relationship between regional connectivity and motion, and are shown in (Chapter2 **– Fig6E.**). Global connectivity was calculated for each subject by computing an average value of the entire correlation matrix; a correlation graph showing no relationship between global connectivity and FWD is displayed (Chapter2 **– Fig6F.**). In addition, registration efficacy between the functional and template image was checked visually through the generation of overlayed maps (Chapter2 **– Fig7.**). To visually evaluate the overall functional connectivity profiles across time an average of all region-to-region functional connectivity for all subjects at both timepoints was calculated (Chapter2 **– Fig8.**).

Statistical analysis

The primary objective of the VBM analysis was to understand structural *deficits* in relation to impulsivity, stickiness, and compulsivity. As a result of previous investigations in our laboratory showing reduced grey matter volume in the AcbC of HI animals (Caprioli et al., 2014) and thinning of the AI cortex (Belin-Rauscent et al., 2014) a *one*-way t-test analysis to evaluate structural *deficits* in HI (n=12), relative to LI animals (n=12) – testing for *lower* grey matter volume in HI animals when compared to LI animals – was performed. Due to the observed predictive relationship between impulsivity, stickiness and compulsivity (Chapter 5), the *a priori* hypothesis of structural *deficits* in HI animals put forward, and robust evidence supporting grey matter *decline* in stimulant-dependent individuals (Ersche et al., 2013; Suckling and Nestor 2017) a *one*-way t-test analysis between both HC (n=7) and LC (n=7) with total internal volume (TIV) as a covariate of no interest was carried out to evaluate to what extent grey matter was *lower* in HC *versus* LC. Compulsive cocaine seeking (ALP first interval – averaged across both 0.45mA punishment sessions) was also used as a regressor, alongside TIV as a covariate of no interest, to address the relationship between compulsive cocaine seeking and grey matter volume. A negative regression was carried out to evaluate how *lower* grey matter volume was related to the development of future compulsive cocaine seeking. Additionally, a *one*-way t-test to explore the relationship between grey

matter *deficits* in HK (n=14) *versus* LK (n=14) animals, using TIV as a covariate of no interest was performed. For reasons of completeness the relationship between *increased* grey matter volume and the behavioural variables using opposite contrasts (i.e., HC>LC, compulsivity positive regression, HI>LI, and HK>LK) was also explored. Although simultaneous one-sided t-tests have been carried out extensively within the neuroimaging literature this practice can inflate the type 1 error rate (Chen et al., 2019). As structural *deficits* were of primary interest *one*-way t-tests were performed to increase the sensitivity of detecting an effect in the direction expected based on *a priori* information. However, it is acknowledged that this may have increased the type 1 error rate and the results at this stage should be taken as exploratory. For the VBM analysis an initial cluster-forming threshold of p=0.005 was used with no extent threshold. All maps presented in this chapter result from a chosen extent threshold of k=120 for visualisation purposes. Full brain-wide maps are observed in **Appendix5.** All results are reported as uncorrected cluster-wise p-values. For functional connectivity analysis, the first Eigenvariate of the BOLD time-series from each ROI was correlated against each behaviour of interest (compulsive cocaine seeking, impulsivity and kappa). Functional connectivity is defined as the Spearman's rho correlation coefficient between two extracted Eigenvariate values of the BOLD signal between two ROIs. All correlations between ROI BOLD signal were corrected for multiple comparisons with a false discovery rate set at q=0.05 (Benjamini and Hochberg 1995).

6.3 Results

VBM and rs-fMRI region-to-region analysis was carried out to reveal structural and functional brain markers in drug-naïve rats before the emergence of compulsive drug-seeking. At the adult scan timepoint compulsive drug-seeking animals showed lower grey matter volume in the bilateral IL cortex, left vStr (**Fig1.**), and higher grey matter in the AI cortex (HC *versus* LC: AI cortex: voxels=76, p=0.019 uncorrected) (**Fig2.**). Compulsivity was negatively correlated with grey matter volume in the IL cortex with HC animals showing lower grey matter volume bilaterally in the IL cortex (IL cortex: voxels=132, p=0.013 uncorrected, voxels=130, p=0.014 uncorrected) (**Fig1A-D.**). Similarly, HC animals also showed lower grey matter volume in the left vStr including the AcbC and AcbS (AcbC / ACbS: HC *versus* LC: voxels=135, p=0.003 uncorrected) (**Fig1E-H.**).

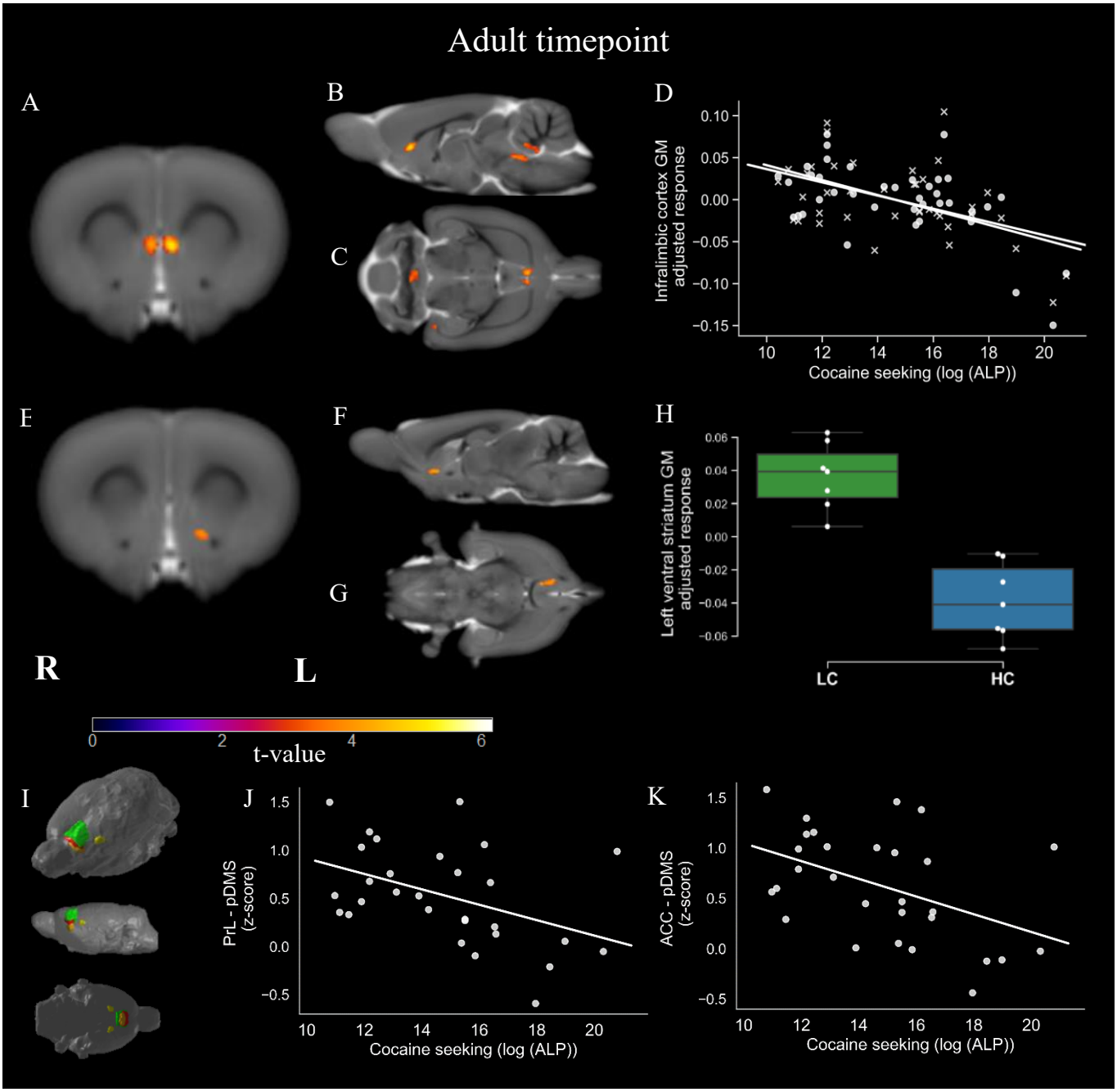


Fig1. Rats destined to seek cocaine compulsively show lower grey matter (GM) volume bilaterally in the infralimbic (IL) cortex, as depicted in the coronal (**A**), sagittal (**B**) and horizontal (**C**) planes. Compulsive seeking regression analysis: IL cortex (left): voxels=132, p=0.013 (uncorrected), IL cortex (right) voxels =130, p=0.014 (uncorrected). **(D)** Regression plot showing the relationship between right IL cortex (x) and left IL cortex (•) grey matter volume and compulsive cocaine seeking. Future HC rats also show lower grey matter volume in the left ventral striatum (HC versus LC: ventral striatum: voxels=135, p=0.003 uncorrected) shown in the coronal (**E**), sagittal (**F**) and horizontal (**G**) planes. **(H)** Box plot showing lower left ventral striatal grey matter volume in LC when compared to HC rats. **(I)** 3D representation of the PFC (anterior cingulate cortex (ACC; green), prelimbic cortex (PrL; red) and IL (IL; orange) and posterior dorsomedial striatum (pDMS; yellow). Cocaine seeking is associated with reduced functional connectivity between the **(J)** PrL and pDMS (r=-0.441, p=0.038 (fdr-corrected)) and between the ACC and pDMS (r=-0.457, p=0.030 (fdr-corrected). R = right hemisphere; L = left hemisphere.

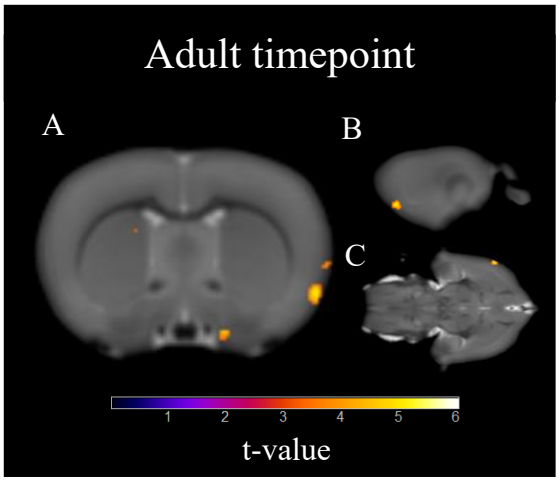


Fig2. High compulsive animals show higher levels of grey matter in the anterior insula cortex when compared to low compulsive animals. (HC *versus* LC: AI cortex: voxels=76, p=0.019 uncorrected), shown in the **(A)** coronal, **(B)** sagittal and **(C)** horizontal planes. R=right hemisphere; L=left hemisphere.

Next, functional coupling between several prefrontal cortical areas and the pDMS, a striatal subregion critical for goal-directed learning in the rat (Yin et al., 2005), was evaluated. A 3D representation of the three prefrontal regions and the pDMS is shown in (**Fig1I.**). Critically, at the adult timepoint, rats destined to seek cocaine compulsively showed reduced connectivity strength between the PrL and the pDMS (r=-0.441, p=0.038 (fdr-corrected)) (**Fig1J.**), as well as the ACC and the pDMS (r=-0.457, p=0.030 (fdr-corrected)) (**Fig1K.**). Of the several region-to-region connections investigated only these two relationships survived multiple comparison correction (**Appendix4.**). Investigation of the relationship between PFC-pDMS connectivity was not significantly correlated with non-punished ^{2nd}SOR cocaine seeking, although this did occur during two sessions **(Fig3.)**.

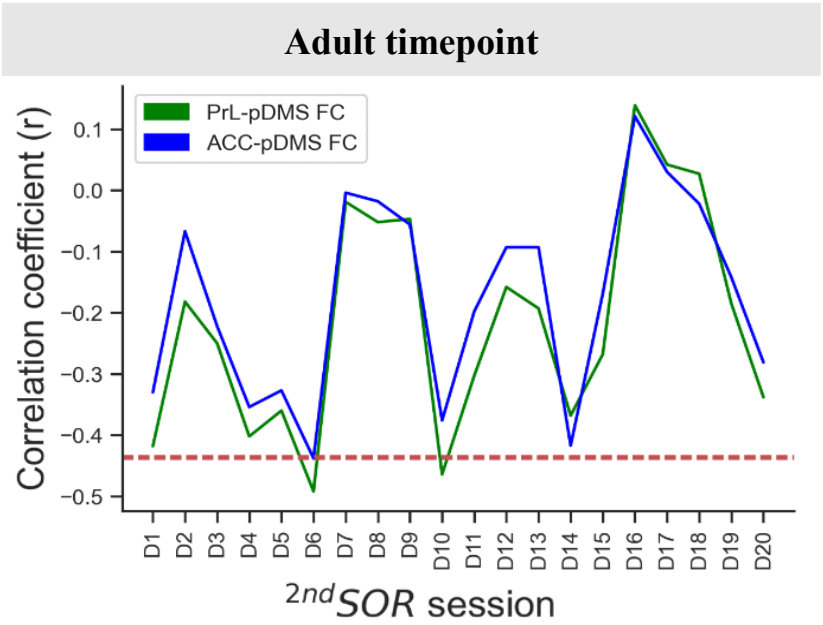


Fig3. Reduced connectivity between the anterior cingulate cortex (ACC) and the prelimbic cortex (PrL) to the posterior dorsomedial striatum (pDMS) is weakly associated with responding under second order schedule of reinforcement (^{2nd}SOR) without explicit punishment. In 10% and 5% of sessions, PrL-pDMS and ACC-pDMS connectivity was associated with responding under ^{2nd}SOR conditions, respectively. Red dotted line indicates correlation value where p<0.05. *Abbreviations:* FC, functional connectivity.

VBM analysis was subsequently used to investigate the underlying structural correlates of impulsivity and stickiness at the adult timepoint. The HI phenotype was characterised by lower levels of grey matter in the IL cortex, vStr, entorhinal cortex and cerebellum, as well as ventricular enlargement (**Fig4., Appendix5.**). HI animals had lower grey matter volume bilaterally in the IL cortex compared to LI animals (HI *versus* LI: IL cortex: voxels=176, p=0.005 uncorrected) (**Fig4A-D.**), in the right vStr, including the AcbC and AcbS (HI *versus* LI: vStr: voxels=397, p=0.000 uncorrected) (**Fig4E-H.**), and the left AI cortex (HI *versus* LI: AI cortex: voxels=154, p=0.008 uncorrected) (**Fig4I-L.**), among other regions (**Fig4A-C, E-G, I-K.**). No notable differences in grey matter were observed in high sticky animals when compared to low sticky animals at the adult timepoint (**Appendix5.**). Following structural analysis, functional imaging correlates of both impulsivity and stickiness were evaluated. Functional connectivity analysis of the BOLD response revealed hypoconnectivity between several cortical areas and the dStr, including hypoconnectivity between the PrL and pDMS (rho=-0.463, p=0.027 (fdr-corrected)) (**Fig5A.**) and the ACC and pDMS (rho=-0.425, p=0.048 (fdr-corrected)) (**Fig5B.**) in rats showing high stickiness but not increased impulsivity (**Fig5C, D.**). Unlike compulsivity, which was only associated with two region-to-region connectivity profiles (PrL-pDMS/ACC-pDMS), several other region-to-region relationships were also observed in high sticky animals within cortico-striatal and insula-striatal regions (**Appendix4.**).

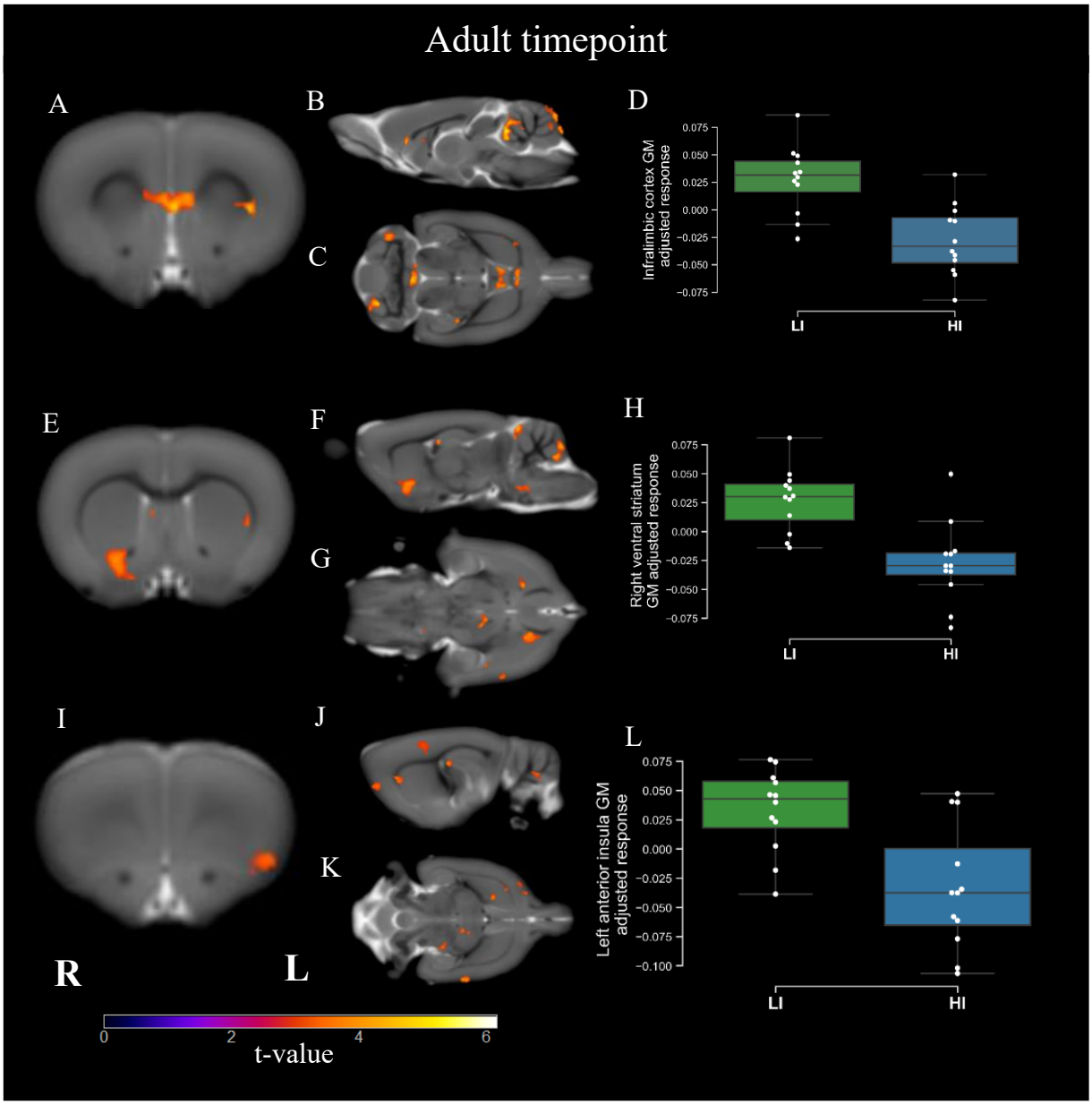


Fig4. High impulsive animals show lower grey matter volume bilaterally in the infralimbic (IL) cortex (HI *versus* LI: IL cortex: voxels=176, p=0.005 uncorrected), shown in **(A)** coronal, **(B)** sagittal and **(C)** horizontal planes. **(D)** Boxplot showing reduced IL cortex grey matter volume in HI when compared to LI animals. HI animals also show lower grey matter volume in the ventral striatum (vStr) (HI *versus* LI: vStr: voxels=397, p=0.000 uncorrected), shown in the coronal **(E)**, sagittal **(F)** and horizontal planes **(G). (H)** Boxplot showing reduced vStr grey matter volume in HI when compared to LI animals. HI animals also show lower grey matter volume in the anterior insula cortex (HI *versus* LI: AI cortex: voxels=154, p=0.008 uncorrected), shown in the coronal **(I)**, sagittal **(J)** and horizontal planes **(K). (L)** Boxplot showing reduced AI cortex grey matter volume in HI when compared to LI animals. R= right hemisphere; L= left hemisphere.

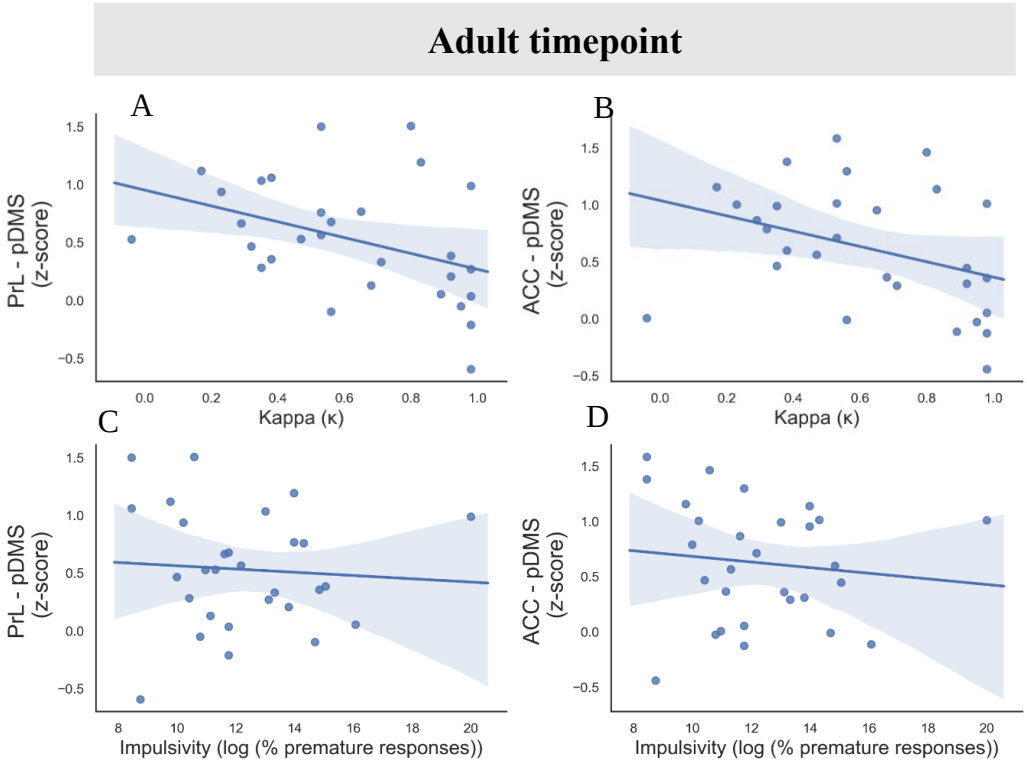


Fig5. Hypoconnectivity between the prelimbic cortex (PrL) and anterior cingulate cortex (ACC) with the posterior dorsomedial striatum (pDMS) predicts stickiness but not impulsivity. **(A)** Decreased connectivity between the PrL-pDMS is reduced in high sticky animals (rho=-0.463, p=0.027 (fdr-corrected)). **(B)** Decreased connectivity between the ACC-pDMS correlated is reduced in high sticky animals (rho=-0.425, p=0.048 (fdr-corrected)). **(C, D)** No relationship was observed between PrL/ACC and pDMS connectivity and impulsivity.

Grey matter correlates of compulsivity, stickiness, and impulsivity at PND-63, prior to behavioural screening, were next evaluated. Implementing an identical VBM analysis, no differences in grey matter in the IL cortex, vStr or AI between HI and LI animals were observed. Additionally, the IL cortex and vStr markers of compulsive cocaine seeking were not evident at PND-63. However, structural differences in sticky animals were observed. Thus, sticky animals showed higher levels of ACC grey matter, although this finding was not replicated at the adult scan timepoint following behaviour. A summary of these findings can be seen in **Appendix5.** The BOLD correlates were also evaluated pre-behaviourally, at PND-63. No relationship between functional connectivity of the PFC and pDMS with compulsivity, impulsivity, or kappa was observed (**Fig6.**).

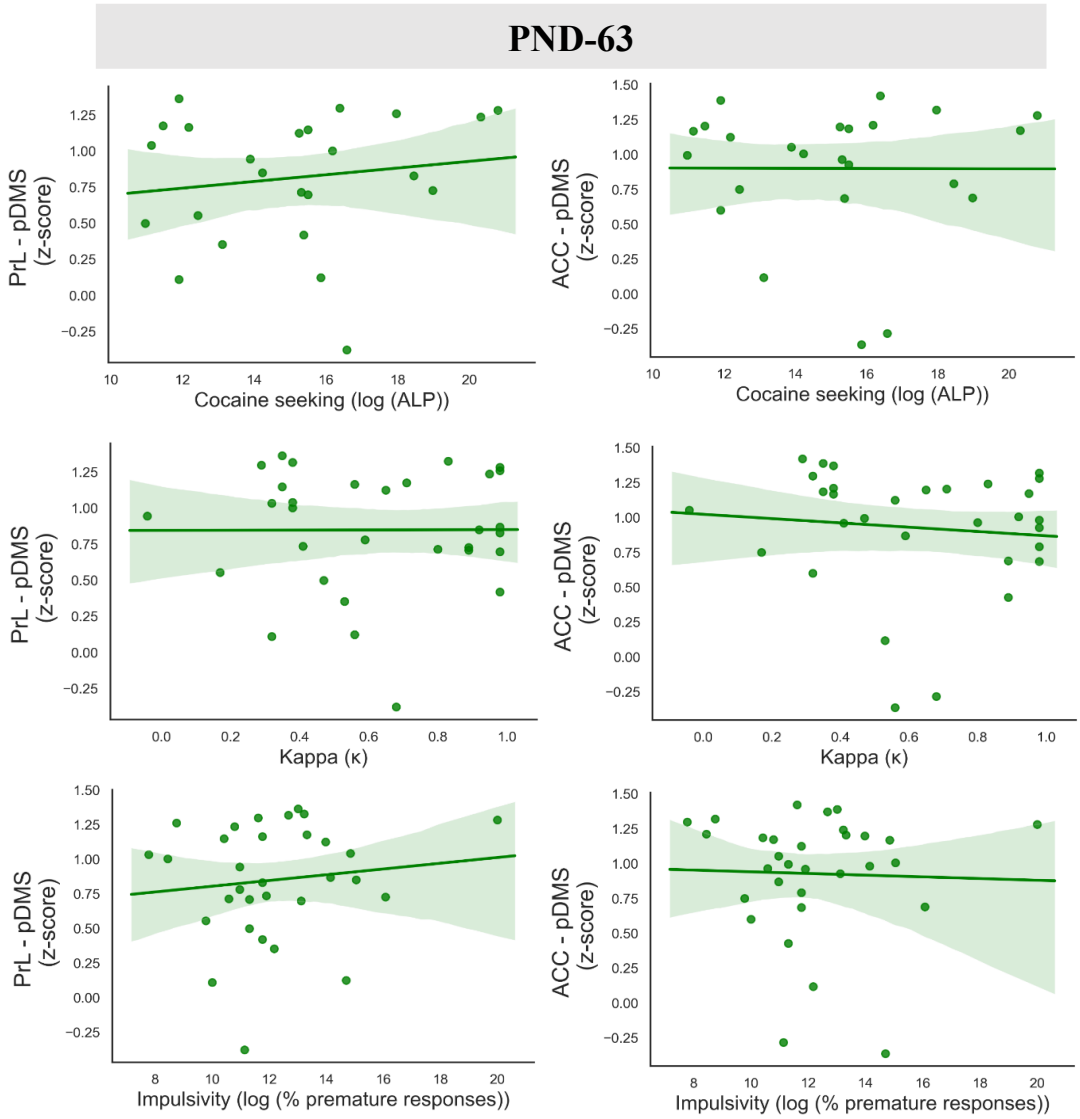


Fig6. Compulsivity, impulsivity, and stickiness are not predicted by hypoconnectivity between the prelimbic cortex and anterior cingulate cortex with the posterior dorsomedial striatum at post-natal day 63.

6.4 Discussion

After carrying out robust quality control procedures described in Chapter 2, section 2.6 the functional and structural precursor brain correlates of compulsive cocaine seeking were investigated. Rats destined to compulsively seek cocaine showed reduced functional connectivity between the PFC (ACC and PrL) and the pDMS. This is a particularly relevant finding given the postulated notion that the development of compulsivity may reflect a dominant engagement of the habit-based system over the goal-directed system (Everitt and

Robbins 2016). Consistent with this hypothesis, addiction vulnerability in humans is also linked with hypoconnectivity of cortico-striatal circuitry (Ersche et al., 2020). In rodents, hypoactivity of the PrL is related to compulsive cocaine seeking, however, it appears that hypoactivity in this region is an emergent property related to prolonged cocaine exposure and the inability to suppress responding under punishment, and not necessarily a pre-existing state (Chen et al., 2013). These results are echoed by a more recent preclinical imaging study using MRI to investigate the underlying neural circuitry of compulsive methamphetamine use. At baseline, prior to any drug exposure, no differences in connectivity were observed between future compulsive and non-compulsive animals. However, only after methamphetamine use in the face of explicit punishment did connectivity differences occur, with future compulsive animals showing a decrease in connectivity between the PrL and vStr, as well as an increase in connectivity between the OFC and medial striatum (Hu et al., 2019). However, it should be noted that a SBCA was carried out in this study with only two seed regions (OFC and PrL), thus, these results do not rule out the possibility that there may have been connectivity differences in other brain regions and networks. Nonetheless, these data fit well with the proposed notion that deficits in top-down control emerge because of prolonged drug exposure (Pelloux et al., 2013; Luscher, Robbins and Everitt 2020). However, more recent studies challenge this notion (Ersche et al., 2020), suggesting that non-drug using individuals characterised as 'vulnerable' to the development of misuse may share similar neural connectivity profiles (i.e., reduced top-down control) to that of individuals with a diagnosis of SUD. The results presented here echo that sentiment, suggesting that pre-existing differences in connectivity can indeed predict future compulsive drug use. However, it should be noted that there was a sparse relationship between ^{2nd}SOR responding and connectivity between the ACC/PrL and pDMS, thus suggesting that this circuit may also influence cue-conditioned drug-seeking alongside compulsivity. Although an exact circuit for drug compulsion has been hard to pin down (van den Heuvel et al., 2016), it is likely that compulsivity is mediated by related neural circuitry governing other motivational, attentional and wider decision-making processes, most likely involving the PFC, insular cortex and the basal ganglia (Dalley, Everitt and Robbins 2011). Indeed, stickiness was also related to a decrease in functional connectivity in these regions. One of the more salient findings being that stickiness is related to decreased functional connectivity between the ACC/PrL and the pDMS, mirroring that observed in this thesis in animals retrospectively classified based on their levels of compulsive cocaine seeking. This bolsters early interpretations, showing that, alongside compulsivity, other decision-making processes appear to be regulated by

overlapping cortico-striatal circuits, and whereas behaviourally stickiness predicts compulsive cocaine seeking, at the neural level, both are predicted by reduced connectivity from the ACC/PrL to the pDMS.

Using VBM, whole-brain structural correlates of future compulsive drug-seeking, impulsivity and stickiness were evaluated. Compulsive drug-seeking was characterised by decreases in grey matter in the IL cortex, as well as the vStr, with a notable decrease observed in the cerebellum of HC animals. Likewise, HI animals also showed a decrease in grey matter in the IL cortex, vStr, AI and cerebellum, among other regions. The PFC and vStr have been widely implicated in controlling response inhibition (Chudasama et al., 2003; Jupp et al., 2020; Caprioli et al., 2014), and drug motivated behaviours (Halladay et al., 2020; Siciliano et al., 2019; Yager et al., 2015), and these findings further support this notion. More recently, the IL cortex and its connections to the AcbS have been shown to be important in regulating alcohol-seeking in the face of punishment (Halladay et al., 2020). Additional reports have also shown a decrease in cocaine seeking behaviour following activation of the IL cortex-Acb circuit (Cameron et al., 2019), as well as others showing that inhibition of this pathway is critically important in augmenting cocaine seeking during extinction-reinstatement procedures (Peters, LaLumiere and Kalivas 2008). These studies, along with the data herein, point towards a central role of the IL cortex and the Acb as key mediators of several drug-related seeking behaviours, and specifically in this thesis – compulsive cocaine seeking. In humans, the sACC (BA 25) is thought to best represent the IL cortex in rats (Morris et al., 2016). However, the sACC is a heterogeneous structure thought to contain various other regions including BA s24, s32, and ventral area 33 (Palomero-Gallagher et al., 2015), making the direct comparison between rodent and human less clear cut. Nonetheless, decreased grey matter in the ACC is widely reported in cocaine-dependent individuals (Franklin et al., 2002; Matochik et al., 2003; Pando-Naude et al., 2021). Moreover, cocaine-dependent individuals show hypoactivation in the ACC during emotionally salient drug-related tasks such as the drug stroop task (Goldstein et al., 2009). Hypoactivation in this area has consistently been related to more frequent cocaine use (Goldstein et al., 2009; Moeller et al., 2014; however, see Marhe et al., 2013), and diminished activity in this region is also predictive of relapse potential (Luo et al., 2013). The ACC projects to both ventral and striatal structures including the Acb, caudate and putamen (Haber 2016). One report has suggested that connectivity between the ACC and vStr is hypothesised to reflect a ‘Stop’ circuit, with connectivity in this circuit compromised in cocaine-dependent individuals (Hu et al., 2015). In this report,

functional connectivity between multiple seeds of interest (including the ACC and OFC, among other regions) was assessed in cocaine-dependent individuals and healthy controls and compared to impulsivity and compulsivity behavioural scores. Of particular note, a decrease in connectivity between the ACC and vStr (superior), combined with an increase in connectivity between the OFC and vStr (inferior), was related to a higher number of DSM-4 criteria, with the total number of these criteria used as a measure of individual severity of loss of control of over drug use in this study (Hu et al., 2015). These results suggest that hypoactivity between the ACC and vStr may be important in driving compulsivity, a finding particularly relevant to the structural deficits and fMRI data observed in relation to HC animals in this thesis. In addition, several studies have shown that hypoactivation in the ACC is linked to decreased response inhibition in cocaine-dependent individuals (Kaufman et al., 2003; Hester and Garavan 2004), thus suggesting that this region may represent a common structure that critically mediates both compulsive drug use and impulse control. A notion bolstered by the convergent grey matter deficits observed between HI and HC rats in this thesis.

Although there were structural differences in the IL cortex and Acb in HC animals, no structural differences in the amygdala were observed in future HC animals. The amygdala, more specifically the BLA, and its interaction with the AcbC, is a critical circuit controlling cue-elicited drug-seeking (Di Ciano and Everitt 2004; Cardinal et al., 2002), along with the pDMS (Murray et al., 2012). A recent study used chemogenetics to causally manipulate this circuit (BLA to AcbC) and found that acquisition of cue-controlled cocaine seeking was indeed dependent on the functional integrity between these two regions (Puaud et al., 2021). This finding is supported by previous excitotoxic lesions studies showing that lesions of the BLA impair the acquisition of cocaine seeking under a ^{2nd}SOR (Whitelaw et al., 1996). However, to date, very few studies, if any, have evaluated the role of the BLA in regulating compulsive cocaine seeking under a ^{2nd}SOR. Although, one study did evaluate cocaine seeking in the face of punishment on the seeking-taking task after selective lesions of the BLA (Pelloux, Murray and Everitt 2013). In this report, no differences between lesion and non-lesion rats were observed under baseline seeking conditions (without punishment, after seeking was established over repeated training sessions), suggesting that BLA lesions did not affect well-established cue-controlled cocaine seeking, a finding in line with the hypothesised transition of control over drug-seeking from the BLA to the CeA after extended training (Murray et al., 2015). However, in the Pelloux study above, lesions of the BLA exacerbated

seeking under punishment conditions, suggesting that under certain conditions amygdala dysfunction may contribute to compulsivity. In humans, reports have shown that amygdala volume is decreased in cocaine-dependent individuals (Makris et al., 2004; Moreno-Lopez et al., 2012) and is also linked with the escalation of stimulant use (Becker et al., 2015). Moreover, years of cocaine use is also negatively associated with amygdala volume (Barros-Loscertales et al., 2011), suggesting that the neurotoxic effects of cocaine may, in part, regulate the decrease in amygdala volume observed. However, other evidence suggests that amygdala volume is increased prior to drug use in vulnerable individuals (Ersche et al., 2012). As a result, it was hypothesised that differences in amygdala grey matter volume would be observable between HC and LC animals. However, no differences were observed (**Appendix5.**). As discussed, the BLA is critically important for the acquisition of ^{2nd}SOR SA (Whitelaw et al., 1996) and no differences in acquisition between HC and LC animals were observed (Chapter 5). In addition, the BLA has been shown to be related to duration of cocaine use (Barros-Loscertales et al., 2011), thus it is possible that drug-induced changes in the amygdala occurred throughout ^{2nd}SOR training prior to punishment sessions and the assessment of compulsivity. Unfortunately, due to the study design and the limitation in scanning at pre-defined timepoints, this could not be tested directly. However, repeated scanning throughout ^{2nd}SOR and during punishment may shed light on the interaction between the neurotoxic effects of cocaine and amygdala volume, an interesting avenue for future studies. As discussed, the amygdala-Acb circuit is an important mediator of cue-controlled drug-seeking. It was envisioned to test this relationship directly using fMRI, however, due to the high degree of signal drop-out experienced (see Chapter 3, section 3.9 and 3.9.1) in posterior regions of the brain near the amygdala – a common occurrence in high field imaging – this analysis could not be carried out.

Initial goal-direct drug-seeking has been shown to be dependent on afferents from the mPFC and OFC to the striatum (Luscher, Robbins and Everitt 2020). Although functional connectivity differences within the PrL and ACC were observed no relationship between OFC-striatal functional connectivity and compulsivity was seen. This was somewhat surprising given the well-established role of the OFC in a wide range of addiction-like behaviours in rodents (Fuchs et al., 2004; Guillem and Ahmed 2017), and human cocaine-dependent individuals (Volkow and Fowler 2000). However, recent reports have suggested that altered OFC connectivity may emerge as a consequence of stimulant use. For example, increased connectivity between the OFC and medial striatum, related to compulsive

methamphetamine use, only emerged in animals that had experienced direct punishment (Hu et al., 2019). Whilst other reports have shown that activity in the OFC and dStr is enhanced after repeated cocaine exposure (Wall et al., 2019). Thus, although deficits were not observed prior to cocaine SA, it cannot be ruled out that future differences in OFC to striatal circuitry emerged as a consequence of drug exposure.

Finally, lower grey matter was seen in the cerebellum of both HC and HI animals. The cerebellum is a relatively understudied region in the context of addiction, even though several lines of research support an important role for this region in regulating drug-related behaviours (Miquel, Gil-Miravet and Guarque-Chabera 2020; Miquel et al., 2016). Whilst a paucity of studies exists evaluating cerebellar activity in addiction models in rodents, in humans, cerebellar grey matter volume is negatively correlated with duration of cocaine use, and is also related to decreased executive function (Sim et al., 2007). Similarly, decreased cerebellar volume and cerebellar dysregulation has been associated widely with impulse control deficits in ADHD (Miquel et al., 2019). In rodents, cerebellar lesions have also been related to disinhibition (Bobee et al., 2000), potentially suggesting cross-species convergence. The results presented in this chapter are in line with these reports, suggesting that both impulse control deficits and compulsive cocaine seeking is predicted by grey matter abnormalities in the cerebellum, a finding potentially related to the proposed role of the cerebellum in regulating cortical and subcortical activity and ongoing motor control (Chen et al., 2014; Watson et al.,2014), as well as mediating habit learning (Miquel et al., 2019).

All the aforementioned structural deficits were observed through VBM, of which several limitations currently exist. Perhaps most crucial of all is the need to correct for multiple comparisons and to control the false positive rate (Bennett, Wolford and Miller 2009; Nichols 2012). A relatively recent paper has come to question the validity and use of parametric statistics including random-field theory and Monte-Carlo simulation in relation to neuroimaging analysis, and the nature in which they control the family-wise error rate (Eklund et al., 2016; Woo et al., 2014). This important report took several pre-existing fMRI data sets and evaluated the false positive rate after controlling for the familywise error rate across several different software platforms. Parametric voxel-wise and cluster-wise inferences were evaluated with several different initial cluster-forming thresholds. Critically, the authors argue that the relatively well published $p<0.01$ initial cluster-forming threshold is too liberal and can substantially inflate the false positive rate (Eklund et al., 2016). However,

additional reanalysis from separate research groups (who maintain the software mentioned as having greatly inflated false positive rates in Eklund et al., 2016) show that at the liberal initial threshold suggested ($p<0.01$) false positive rates are around 20% for most of common software packages used in imaging analysis (e.g., SPM/FSL/AFNI). Importantly, this rate decreases as lower initial cluster-forming thresholds are used ($p=0.001$ in this case), with false positive rates comparable to non-parametric permutation tests (those purported to perform much better than the parametric statistical methods tested (Eklund et al., 2016) at these threshold levels (Cox et al., 2017). To mitigate the occurrence of type 1 errors, whilst providing the best opportunity to avoid type 2 errors, an initial cluster-forming threshold of $p=0.005$ was chosen. However, multiple comparisons were not corrected for, and as such these data should be taken as exploratory. This decision was primarily driven due to the relatively low number of subjects inherent in chronic, long-term SA studies, further exacerbated by the notion that only 10-15% of these subjects will become compulsive (Anthony et al., 1994). Although every attempt possible was made to maximise the number of animals at the outset, due to the complexity of the longitudinal experiment and the chronic SA procedures, in which several animals had to be culled, the final number of animals subjected to the voxel-based analysis was reduced. Nonetheless, in rodents, it is not uncommon for brain-wide voxel-based studies to use uncorrected p-values (Cannella et al., 2017; Jia et al., 2018), although it is important and appropriate that this caveat is addressed. In this report, mitigation against type 1 errors was carried out by using a more stringent cluster-forming threshold (0.005 *versus* 0.01). It is also important to emphasise that the main results of this chapter and the regions in which there are observable differences sit in line with a vast majority of past studies implicating the vStr and the IL cortex in impulse control as well as in drug-related behaviours (discussed throughout this thesis). Additionally, the overlap between the IL cortex is strikingly conserved across both HC and HI animals, and when combined with the additional notion that behaviourally impulsivity predicts compulsive cocaine seeking, it is unlikely that these deficits in grey matter would occur by chance.

Another important point is that differences in functional connectivity were only observed at the adult timepoint and not preceding behavioural training (PND-63). Whilst this may represent the final state of the brain prior to self-administration, and thus may represent most appropriately the vulnerable phenotype, important plasticity mechanisms and early developmental trajectory differences may have played a role in the emergence of the structural and functional markers observed. Brain plasticity - the idea that the brain can ‘re-

wire' or reorganise through experience - is a well-established concept (Kolb and Gibb 2011). Brain plasticity mechanisms can occur relatively early on when learning a new skill or performing certain behaviours. In the context of learning, one popular study involving juggling highlights this phenomenon, with morphometric brain changes observed in subjects learning to juggle after only 7 days of training (Driemeyer et al., 2008). In monkeys, acquisition of skill learning is also related to distinct patterns of neural activity that change over time as learning progresses (Oby et al., 2019). It is possible that operant training over several months induced changes in brain plasticity mechanisms and may be one reason why relationships were observed with behaviour at the adult, but not the PND-63 timepoint. As brain plasticity mechanisms are tightly linked to learning, it was speculated that functional connectivity between the PFC and pDMS may be related to alpha, the learning rate. However, no relationship between PrL–pDMS or ACC-pDMS connectivity with the learning rate at either the PND-63 or at adult timepoints (**Appendix6.**) was observed. Nonetheless, it is possible that brain plasticity mechanisms occurred throughout the operant training process and it is possible that these plasticity mechanisms may have been expressed differentially across the different phenotypes.

Alternatively, differences in brain early developmental trajectories may have also contributed towards the final structura and functional differences observed. However, it is important to note that the animals used were all male Lister hooded rats, and were therefore isogenic. In addition, all animals experience nominally identical environments throughout the entire study. Nonetheless, emergence of individual differences and expression of different behavioural traits naturally occurs in animals that have identical genetic backgrounds. Even when rodents are genetically identical and reared in near-identical environments, individuality and the expression of different behavioural traits is still observed. One method for studying the emergence of phenotypic individuality is through the use of elaborate and complex housing environments. Of particular note, one study exposed 40 inbred mice to a complex multi-chamber environment and monitored behaviour over the course of 3 months. Although the mice were isogenic, having very limited genetic variability, behavioural variability across the mice in the form of environmental exploration developed and increased over time. This variability also correlated with brain development and neurogenesis. Thus, variation in experience within identical environments, in animals with identical genetic backgrounds, can lead to individualized phenotypic differences in behaviour, and brain plasticity and morphology (Freund et al., 2013). This difference could have occurred due to

pre-existing small-scale variation in genetic background (Chebib et al., 2020), or perhaps through epigenetic mechanisms as a cause of differences in behavioural experience and the non-shared environment. In this thesis, differences in behavioural experience in the shared environment may lead to changes in behaviour, as seen in the wide variation across each of the endophenotypes assessed. Moreover, differences in experience may have influenced brain development prior to behavioural testing, leading to the phenotypic variation in impulsivity and compulsive cocaine seeking later observed. Indeed, divergent developmental trajectories in the prefrontal cortex can influence both traits (Ziegler et al., 2019) in humans. Moreover, monozygotic twins raised with near-identical environmental status also exhibit divergent personalities, lending support to the notion that, in humans, the non-shared environment also plays an important role in influencing behavioural output (Torgersen and Janson 2002). Due to time constraints the developmental trajectories of animals at risk to future compulsive cocaine seeking was not evaluated. However, future work will begin to address the notion that individual variation in the rodent brain may occur early in life (Qui et al., 2018), and that divergent developmental trajectories may have played some a role in the later development of vulnerability (see future work).

Whilst the current functional analysis primarily focused on several cortico-cortico, and cortico-striatal ROIs, other analysis pipelines may provide an alternative view of network structure in animals vulnerable to later development of compulsive cocaine seeking. For example, as discussed in Chapter 2, ICA and SBCA could have been used to probe the brain-behaviour relationship. Partial least squares (PLS) is another technique that has been used to characterize distributed patterns of brain structure and activity (McIntosh and Lobaugh 2004). PLS is a multivariate technique that generates a set of latent variables which describe the relationship between both imaging (e.g., structural (voxels), or functional connectivity) and behaviour, in the case of neuroimaging. PLS, therefore, provides a direct means to understand the relationship between distributed patterns of brain activity and measured behavioural data. PLS is a flexible technique that can be used across a wide range of data types, including structural and functional MRI data (e.g., grey matter maps, Jacobian determinant images, voxel-based functional MRI maps). Along these lines, a recent report by Guma et al., evaluated the relationship between behavioural differences related to maternal immune activation and longitudinal structural brain changes using PLS in mice (Guma et al., 2021). In humans, PLS has also been used to map the relationship between behavioural inhibition and brain structure (Romero-Garcia et al., 2021), further showing the utility of this technique.

Future work will explore the utility of PLS in understanding the relationship between behavioural risk traits of compulsive cocaine seeking and brain structure and function.

In summary, both impulsivity and compulsive cocaine seeking is predicted by lower grey matter volume in the IL cortex and vStr. Similarly, compulsive cocaine seeking is predicted by a reduction in functional connectivity between the PFC (PrL and ACC) and the pDMS. A similar pattern of connectivity was also observed behaviourally in 'sticky' animals, who are at risk, alongside HI animals, to develop compulsive cocaine seeking.

Chapter 7

General discussion

7.1 Overview

The work within this thesis has broadly characterised the extent to which a wide range of recognised vulnerability endophenotypes increased the risk of the development of compulsive cocaine seeking. Furthermore, this thesis investigated the underlying neural correlates of two important risk traits for the development of compulsive cocaine seeking along with the brain correlates of future compulsive cocaine seeking itself through the utilisation of translationally relevant non-invasive imaging. The broad scope of this thesis focussed on three fundamental questions; 1) to what extent do several recognised behavioural endophenotypes of addiction-like behaviour relate to each other? 2) how do these antecedent behavioural traits relate to future compulsive cocaine seeking? and 3) what are the functional and structural brain substrates of these risk traits and future compulsive cocaine seeking? Each of these points were addressed in Chapters 4, 5, and 6, respectively. The most salient findings and the wider context of these results will now be discussed.

7.2 Behavioural interrelationships: vulnerability endophenotypes and compulsive cocaine seeking

Perhaps the most critical behavioural findings of this thesis are that impulsivity and stickiness are positively correlated (Chapter 4), and when combined, significantly predict compulsive cocaine seeking (Chapter 5). One question, therefore, is what are the psychological processes that link impulsivity and stickiness with compulsivity? To address this question, compulsivity will first be broken down and the mechanisms that may be driving compulsive drug-seeking will be evaluated (e.g., enhanced habit formation, behavioural (in)flexibility, dysfunctional inhibition / disinhibition). Does impulsivity and stickiness contribute to enhanced habit

formation? Behavioural and neurochemical evidence suggests that after protracted drug exposure long-term cue-controlled drug-seeking is dependent on the habit-based system and behaviour is thought, at this stage, to represent stimulus-bound maladaptive drug-seeking habits (Everitt and Robbins 2016; Belin et al., 2013). However, one study in rodents evaluated the transition to a habit-based aDLS system in HI and LI animals after protracted cocaine seeking showed that impulsve control deficits on the 5-CSRTT was not related to enhanced drug-seeking habit formation (Murray et al., 2014). The authors concluded that impulsivity may not necessarily promote the formation of drug-seeking habits but may in fact contribute to the rigidity of maladaptive drug-seeking habits once they are established. The inter-relationship between maladaptive habit formation and impulse control deficits has yet to be fully elucidated, however, evidence in humans has shown that impulsivity is related to some forms of habitual behaviour but not others (Ersche et al., 2019). For example, impulsivity was positively related to automaticity, but negatively related to the performance of routine behaviours. The question, therefore, arises - how do impulsive individuals develop rigid maladaptive stimulus-bound habits if they show more sporadic behaviour, less routine, and cannot repeat the same behaviour frequently? One hypothesis is that high impulsivity does not necessarily promote the initiation of habits (e.g., through more routine), but contributes to rigid stimulus-bound drug-seeking behaviour once the habit has formed. For example, in the Ersche et al., study HI individuals showed higher levels of automaticity (e.g., a lack of control or awareness) over their behaviour (Ersche et al., 2019), with the authors suggesting that impulsivity may elicit automatic stimulus-bound behaviour and promote an imbalance between the goal-directed and habit-based systems. This notion also fits with the rodent data above (Murray et al., 2014). Other studies in humans have also revealed relationships between impulsivity and habit-based behaviours. For example, outcome-specific devaluation (a measure of goal-directed choice) was reduced in regular smokers with higher levels of trait impulsivity, although this relationship was only observed when subjects were characterised based on their motor impulsivity dimension (Hogarth et al., 2011), and higher negative urgency scores are also related to decreased devaluation (higher habitual tendencies) (Hinojosa-Aguayo and Gonzalez 2019). Thus, it does appear that in humans there is some relationship between impulsivity and the tendency to perform habit-like responses. However, this does appear to be specifically related to the precise sub-type of impulsivity assessed.

To what extent does cognitive (in)flexibility contribute to compulsive drug-seeking? In this thesis, pre-existing deficits in stickiness were related to compulsivity (Chapter 5). However, a

recent study in humans has questioned the extent to which psychostimulant use disorder is mediated by cognitive flexibility or 'dysregulated reinforcement learning' processes (Robinson et al., 2021). In this report, RL performance, analysed both by conventional measures and non-compultaional trial-by-trial approaches, was assessed in methamphetamine-dependent individuals and healthy control subjects. Here, the authors defined inflexibility by analysing switching after reward, after 1 loss, 2, or 3 consecutive losses. In this report, methamphetamine-dependent individuals showed a higher chance of changing their response after experiencing either 1 reward, or after experiencing 1 loss. No differences were observed between groups after experiencing 2 or 3 consecutive losses. The authors argue against an account of differences in inflexibility due to the fact that methamphetamine-dependent subjects were not more likely to perseverate after repeated punishments (2 or 3 consecutive losses) and were more likely to switch their choices after experiencing either 1 reward or 1 loss when compared to control subjects. Reinforcement learning in this report was modelled based on trial-by-trial accuracy scores, and was defined by the percentage of correct responses across the session. Using a mixed-effects model, the authors found a significant group x trial interaction, with the authors suggesting that this may represent a deficit in learning of action-outcome associations throughout the entire task, or as the authors defined a 'reinforcement learning deficit' (Verdejo-Garcia and Chong 2021; Robinson et al., 2021). It is important to note that the trial-by-trial analysis used in Robinson et al., did not employ computational models such as those used in this thesis, and other studies that have used Bayesian computational techniques to understand cognitive flexibility via probabilistic reversal learning (e.g., Kanen et al., 2019). Along this point, Robinson et al., defined reinforcement learning deficits via a trial-by-trial accuracy score. However, other computational models of reinforcement learning incorporate a wider range of parameters that may better capture the underlying psychological processes of cognitive flexibility (Kanen et al., 2019; Daw et a., 2011). Thus, cognitive flexibility has been described through reinforcement learning theory as being driven by reward prediction error signals, with alpha the commonly used learning rate parameter, playing a central role in mapping learning from positive and negative feedback and providing a more mechanistic and quantitative way of informing behaviour and cognitive flexibility (Hauser et al., 2015; Metha et al., 2020). Alongside alpha, other basic parameters such as beta (inverse temperature) and kappa (stickiness) are also important for modelling choice behaviour on the RL task, given their incorporation in to reinforcement learning models has been shown to improve overall model fit (Metha et al., 2020; Zhukovsky et al., 2019). Thus, differences in reinforcement learning

parameters, such as those mentioned above, offer a quantitative way of modelling cognitive flexibility and choice behaviour on RL. These methods have been successfully used to map behaviour in rodents on similar tasks. Chronic methamphetamine exposure in rodents results in decreased levels of cognitive flexibility, increased perseveration and was not related to the ability to learn the initial discrimination in a similar probabilistic RL task (Groman et al., 2018), findings slightly at odds with the relationship reported in humans (Robinson et al.,2019). In this thesis, the notion that learning deficits may contribute to compulsivity was evaluated by analysing the relationship of several behavioural variables with alpha, the learning rate (See Chapter 3, section 3.4 and 3.4.1 for more details). Compulsivity was not related to the learning rate (**Appendix7.**) thus suggesting that inter-individual differences in learning, as assessed through trial-by-trial computational analysis, had little effect on future compulsive behaviour. Additionally, trials to criterion, a rather coarse measure of learning and performance on the RL task was also not related to compulsivity (**Appendix7.**) further supporting the view that deficits in learning do not relate to future compulsive drug-seeking. Thus, learning efficiency on the RL task appears to show little relationship with future drug-seeking, suggesting against the proposed notion that a deficit in learning is a primary driver in compulsive drug use (Robinson et al., 2021). However, whilst computational approaches play an important role in comparing RL performance across species, it is important to understand the underlying theoretical framework in which the parameters are derived from such models. For example, in this thesis, stickiness is the trial-by-trial computation parameter that describes the rats disposition to repeat a similar choice regardless of its previous choice. However, this modelling approach does not take in to account several psychological processes commonly evaluated in human RL studies. In humans, spontaneous errors occur when subjects switch from a correct stimulus to an incorrect stimulus following positive feedback, or reward. In other words, subjects switch from a winning action to a losing action without receiving misleading negative feedback (Ersche et al., 2011; Kanen et al., 2019). Spontaneous errors can be derived using non-computational measures of analysis and have been shown to be higher in stimulant users (Ersche et al., 2011) and cocaine dependent individuals (Kanen et al., 2019). Whilst in this thesis stickiness was the primary meausure of interest, the lack of integration of spontaneous errors captured in this measure should be considered when drawing parallels across rodent and human studies of RL.

Nonetheless, the PCA analysis (Chapter 5) revealed an impulsivity, stickiness and compulsivity construct which was explained by a shared latent variable, and compulsivity was significantly predicted by impulsivity and stickiness in the OLS model. This shared

construct may represent dysfunctional inhibition in these animals, that leads from impulsive actions to ultimately compulsive cocaine seeking. Therefore, the data presented in this thesis suggests that it is not enhanced habit formation or diminished learning rates that act as risk traits for the later development of compulsive cocaine seeking, but increased risk is driven by an interaction between decreased cognitive flexibility and enhanced disinhibition (Chapter 5).

It is important to note that the presented work has focused specifically on vulnerability endophenotypes of compulsive cocaine seeking. Whilst the work presented herein may be relate to wider psychostimulants vulnerability, it is unlikely that is generalizes to all drug classes. For example, impulsivity on the 5-CSRTT is vulnerability marker for compulsive cocaine seeking (this thesis) and escalated cocaine intake (Dalley et al.,2007) but is not a vulnerability marker for the escalation of heroin (McNamara et al., 2010). Along these lines, impulsive rats as assessed through a delayed reward task did not show any differences in acquisition, motivation or extinction to take heroin (Schippers et al., 2012), thus suggesting that pre-existing levels of impulsivity may not represent a vulnerability marker for opiate use. Therefore, the results presented herein may not generalize and caution is advised when comparing these results across drug classes, especially opiates.

7.3 Compulsivity, impulsivity and stickiness - converging neural circuits

7.3.1 Impulsivity

Burgeoning evidence suggests that the IL cortex as well as the vStr are critical neural nodes in the regulation of motor impulsivity on the 5-CSRTT in rodents (Dalley, Everitt and Robbins 2011; Basar et al., 2010). Convergent evidence supports a critical role of the vStr, more specifically the AcbC and AcbS, in regulating impulse control on the 5-CSRTT (Caprioli et al., 2014; Murphy et al., 2008). The functional integration of the two regions is emerging to be an important factor in the regulation of behavioural control. A wide range of findings have suggested dissociable mechanisms of DA receptor signalling in the two subregions (Jupp et al., 2013; Pattij et al., 2007; Besson et al., 2010), additionally supported by the findings showing that lesions of the AcbC exacerbated whereas lesions of the AcbS

attenuated impulsivity during an amphetamine challenge (Murphy et al., 2008). One hypothesis is that the AcbC may regulate impulse control via its inhibitory influence over the AcbS, a finding supported by deep brain stimulation studies showing that stimulation of the AcbS increased impulsivity whereas stimulation of the AcbC decreased impulsivity (Sesia et al., 2008). Additional neurochemical evidence also shows that DA release is decreased in the AcbC and increased in the AcbS of HI animals (Diergaarde et al., 2008), lending further support to this hypothesis. In summary, dysregulated signalling in both the IL cortex and vStr are critically important in the regulation of behavioural inhibition. One core aim of this thesis was to characterise the structural and functional neural correlates of impulsivity through non-invasive imaging (Chapter 6). Based on previous evidence (above), as well as wider reports (Chapter 1), it was hypothesised that deficits in impulse control would be linked to reduced grey matter in the IL cortex, as well as the vStr. In line with this hypothesis (Chapter 6), a reduction in IL cortex grey matter was observed bilaterally in HI animals. Reduced 'top-down' control is often posited as one neural mechanism responsible for regulating impulsive responding (Dalley, Robbins and Everitt 2011), yet the precise mechanisms by which this occurs is relatively unknown. In view of this, the relationship of the BOLD coherence between PFC and striatal regions, indicative of top-down control mechanisms, and impulsivity was evaluated. Thus, the relationship between impulsivity and IL cortex – AcbS connectivity was evaluated. Furthermore, as the AcbC is a central mediator in impulse control (Caprioli et al., 2014), BOLD strength between the PrL and AcbC was also evaluated. Contrary to expectation, neither the correlation strength between the IL cortex – AcbS (**Appendix8A.**) or the PrL – AcbC (**Appendix8B.**) significantly predicted levels of impulsivity. These findings do not complement previous evidence showing that pharmacological disconnection of the IL cortex to the AcbS pathway increases impulsivity (Feja and Koch 2014). However, it should be noted that very different techniques were used in this thesis (MRI) relative to the above study, therefore direct comparison is difficult and cross comparison should be done with caution. Nonetheless, a lower grey matter volume in the vStr of HI animals was observed. These findings are the first evidence to report parallel structural changes in the IL cortex, the AcbS and further confirm earlier reports showing a reduction in grey matter in the AcbC of HI animals (Caprioli et al., 2014). An important caveat here is that both a reduction in grey matter in the AcbC, as well as the AcbS, was seen therefore complicating any straightforward interpretation, as put forward in Caprioli et al., 2014 (where they observed grey matter decreases only in the AcbC), relating to the hypothesis of reduced AcbS control via the AcbC. Nonetheless, these results are consistent

with the notion that the vStr is a critical mediator of behavioural inhibitory control and are in agreement with a limited number of human imaging studies reporting functional, as well as structural grey matter differences in the vStr in relation to impulsivity (Basar et al., 2010; Cho et al., 2013; however, see Tschernegg et al., 2015). Although it must be noted that the human imaging literature implicating the Acb in impulsive responding is anything but clear, and caution is advised when drawing parallels between the rodent and human literature. To investigate the proposed role of the AcbC as a key mediator in AcbS output in impulsivity, the BOLD signal coherence between the two subregions was assessed and compared to premature response measures on the 5-CSRTT. As highlighted above, it was hypothesised that a reduction in BOLD coherence between the AcbC, and the AcbS would negatively correlate with impulsivity (i.e., if the coherence between the two structures was lower this would suggest less functional integration and therefore less regulation of the AcbS by the AcbC). However, this proposed correlational relationship was not observed. Indeed, there was no relationship between functional connectivity between the AcbC and AcbS and impulsive responding (**Appendix8C.**). There are several reasons why this may be the case. First, the AcbS region borders the AcbC and as a result both structures are anatomically close together within the brain. Spatial smoothing is an important step in fMRI pre-processing for improving SNR but reduces spatial sensitivity. As a result of the smoothing process, spatial specificity of the signal may have been lowered, and both regions may have contributed to the sampled signal. However, this is unlikely to be the case because both regions were relatively well defined and smoothing by 3 voxels (full-width half maximum of the kernel was 3x the voxel size) would have only affected a small number of voxels within the masks of the regions. Secondly, the BOLD signal is a surrogate measurement of neuronal activity, which may have not been sensitive enough to pick up subtle neuronal signals in the AcbC that guide action in the AcbS, therefore, future electrophysiological studies may be better placed to evaluate these mechanisms. In addition, lower AI grey matter volume was observed in HI animals (Chapter 6). The insula cortex is thought to be a key mediator in the control of affective or emotional states (Bechara et al., 2005) and has been linked to decision-making and impulse control (Bechara et al., 2004; Hester and Garavan 2004; Chambers, Garavan and Bellgrove 2009). In rodents, one imaging study of particular significance evaluated the thinness of the AI in relation to impulsivity. Here, they showed that high levels of impulsivity are associated with the thinness of the AI cortex (Belin-Rauscent et al., 2014). The reported findings of reduced AI cortical grey matter in this thesis complement these reports and sit in alignment with human imaging studies showing reduced AI grey matter in individuals with

impulse control deficits (Ersche et al., 2011; Moreno-Lopez et al., 2012; Verdejo-Garcia and Albein-Urios 2021). Taken together, these data (Chapter 6) are consistent with the proposed role of the AI cortex in mediating value-based decision-making processes and impulse control *via* the regulation of emotional states and interoceptive mechanisms (Bechara and Damasio 2005).

7.3.2 Stickiness

We next turn to kappa, the stickiness parameter generated from trial-by-trial reinforcement learning algorithmic analysis of RL data and its underlying neural correlates. In rodents, there is limited evidence regarding the neural substrates of stickiness when compared to more conventional measures of RL performance. Nonetheless, a recent report evaluated stickiness in rodents after inactivation of several prefrontal cortical regions. Inactivation of the ACC, PrL, LatOFC was without effect and did not alter stickiness. Inactivation of the IL cortex, as well as the MedOFC decreased stickiness, with a stronger effect observed in the IL cortex (Verharen et al., 2020). Considering these findings, and the well reported role of the OFC in guiding value-based decisions (Wilson et al., 2014), as well as the proposed role of the IL cortex in the development of habitual behaviour (Killcross and Coutureau 2003), it was hypothesised that differences in grey matter in these regions may relate to stickiness. However, this was not the case. At the post-behaviour adult scan timepoint no differences were observed in grey matter in any region of the brain between animals displaying high levels of stickiness when compared to animals with low stickiness (Chapter 6). However, higher levels of grey matter volume in the ACC of sticky animals were observed prior to behavioural testing, at PND-63 (**Appendix5.**). Although this is at odds with the Verharen et al., study ((above); no difference in stickiness after lesioning this region), and even though structural differences were only observed at the pre-behavioural screening timepoint, this result does sit with what was observed functionally. As in, grey matter differences were observed in the ACC pre-behaviourally with functional differences also observed in this area (ACC – pDMS was decreased in sticky animals (see below)) supporting the view that the ACC is an important mediator in response perseveration. Stickiness was also related to differences in functional connectivity between several cortico-striatal and insula-striatal networks. Kappa was associated functionally with a decrease in connectivity between the AI and the striatum including the vStr (AcbC and AcbS) and the dStr (aDLS and pDMS). Thus, it does appear that uncoupling of the AI cortex to the striatum, non-specifically, relates to

increased response stickiness. The insula cortex has been shown to project to each individual subregion of the striatum in humans, including the putamen, caudate and Acb (Ghaziri et al., 2018). Of particular interest, one study reported a decrease in insula-striatal circuit output in cocaine-dependent individuals (McHugh et al., 2013), a finding closely related to the potential to relapse from a residential treatment programme (i.e., individuals more likely to relapse showed lower connectivity between the insular cortex and the putamen) (McHugh et al., 2013). Decreased insular activation is also seen when healthy individuals are more likely to repeat the same choice on a Rock Paper Scissors game, speculatively indicating a role for the insula in response perseveration and action selection (Paulus et al., 2005). Along these lines, insular activation is also blunted in stimulant users in the same task, a finding related to problematic stimulant use (Stewart et al., 2017). More generally, activation of the insular cortex is also related to accuracy on the RL task after the reversal phase has occurred (Ghahremani et al., 2010), and increased insula activity is also observed during RL trials immediately preceding a switch in contingencies (O'Doherty et al., 2003). These reports suggest that activity in the insular cortex, and indeed its connectivity with striatal regions, is an important marker for regulating action selection and therefore, may relate to the insula-striatal connectivity profiles seen in high sticky animals in this thesis. Activity between the PrL and the dStr (aDLS/pDMS) was also related to kappa, and so was activity between the ACC and the pDMS (Chapter 6). Reduced top-down control, either to the pDMS or aDLS in sticky animals may allude to changes in the goal-directed or habit-based systems, respectively, and the fine balance of parallel control between the two systems in regulating behavioural output (Bergstrom et al., 2018). To what extent kappa represents response perseveration or habitual control is still an open question. To my knowledge, the evaluation of whether stickiness relates specially to habitual control has not been investigated. However, stickiness, by definition, is related to the tendency to repeat the same action regardless of outcome and thus speaks, in its most basic form, to perseveration and habitual-like responding. The underlying neural correlates of stickiness also align with this notion, with cortico-striatal projections from the PFC to the pDMS having previously been shown to govern goal-directed learning (Hart et al., 2018) with the silencing of these projections reducing operant behaviour early in training, presumably when behaviour is under goal-directed control (Shipman et al., 2019). In short, reduced connectivity between the PFC and more specifically the PrL, to the pDMS (explicitly linked in goal-directed behaviour) may reflect a shift in dominance towards habitual forms of behaviour and thus response perseveration.

7.3.3 Compulsive drug-seeking

Overall, the functional connectivity analysis (Chapter 6) revealed two relationships which survived multiple comparison correction when tested for their relationship to drug-free compulsive cocaine seeking. Similar to that observed above for kappa, PrL-pDMS and ACC-pDMS connectivity was negatively associated with compulsivity (i.e., the animals that had higher rates of cocaine seeking under punishment showed reduced connectivity between these regions). The transition to compulsive cue-controlled cocaine seeking has been neurochemically shown to depend on the transition between dopaminergic mechanisms in the ventral to dorsal striatum (Ito et al., 2002), with well-established seeking eventually dependent on dopaminergic mechanisms in the aDLS, the central node in the habit-based system (Giuliano et al., 2019). To what extent top-down control signals from the PFC to the striatum are dysregulated and coincide with the shift between ventral to dorsal striatal systems is yet to be fully elucidated. In this vein, one noteworthy study combined electrophysiology and optogenetics with a cocaine seeking-taking chain schedule task with punishment to evaluate prefrontal cortical mechanisms of compulsive drug-seeking (Chen et al., 2013). Similar to what was observed in this thesis (Chapter 5), in that report only a small proportion of animals persisted in drug-seeking behaviour during contingent delivery of punishment in the form of mild electric foot shock. In punishment-resistant rats the PrL became profoundly hypoactive, displaying impairments in neuronal firing and compromised excitability. Furthermore, optogenetic activation of the hypoactive PrL in punishment-resistant rats causally reversed their ability to withstand punishment, decreasing their seeking behaviour. The converse was true for shock-sensitive rats; inactivation of their PrL caused enhanced compulsive cocaine seeking (Chen et al., 2013). The PrL projects to several key structures including the amygdala, Acb and dStr (Sesack et al., 1989; Vertes 2004). Importantly, PrL-pDMS connections appear to play a critical role in governing goal-directed action (Corbit 2018; Hart et al., 2018). The hypothesis put forward in the context of the data observed in this thesis is that pre-existing deficits in control over the goal-directed system from the PrL, and indeed the ACC, to the pDMS may bias an individual to engage the habit-based aDLS system and thus may be more susceptible to the engagement of cocaine seeking habits and compulsive cocaine seeking under punishment after protracted use. Although this remains to be fully tested, it does sit in line with evidence suggesting a reduction in 'top-down' control over the striatum from the PFC in human drug-dependent individuals (Goldstein and Volkow 2011), and is in accord with recent evidence showing a reduction in

top-down control over the striatum during drug-seeking (Hu et al., 2019), and more broadly, evidence showing the important role of the PrL to AcbC in regulating cocaine seeking after forced-abstinence (McFarland and Kalivas 2001). Lastly, these findings also are in alignment with recent neuroimaging evidence from non-drug using siblings of drug-dependent individuals, who show reduced correlational activity between the PFC and the caudate nucleus (Ersche et al., 2020). However, it is important to note that goal-directed versus habitual responding for cocaine was not specifically tested in this thesis. Whilst there is strong evidence to suggest the formation of habitual responding after protracted exposure to cocaine under a ^{2nd}SOR (Vanderschuren et al., 2004; Murray et al., 2015; Belin et al., 2008), these studies have not specifically tested habit formation behaviorally. Testing the formation of habitual intravenous drug self-administration in rodents has yet to be fully elucidated given the practical complexities of devaluing intravenous reward (for more details see Everitt 2014). However, neurochemical evidence does support the transition of dopaminergic signaling to the dorsolateral striatum, a structure known to be important in habitual behavior, after prolonged administration on the schedule used in this thesis (Murray et al., 2015). Nonetheless, it is important to note that neither goal-directed or habitual behavior was explicitly tested in the work presented herein, and appropriate comparisons should always take this into account.

Structurally, HC animals, similar to HI animals, had reduced grey matter volume in the IL cortex as well as the vStr. Behaviourally, an overlap was observed between impulsivity and compulsivity (Chapter 5), with high impulsivity (along with stickiness) predicting high compulsivity. Reduced grey matter volume in the IL cortex of HC animals may represent a decreased inhibition in these animals, as a similar neural marker is seen in animals classified on their levels of inhibitory control (i.e., HI animals). In addition, the IL cortex has been linked to drug-seeking behaviour (Peters, LaLumiere and Kalivas 2008), and more recently it has been shown that inactivation of the IL cortex, among other regions, decreases punishment avoidance in the pursuit of a food reward (Verharen et al., 2019). A more recent study evaluated the role of mPFC circuitry in mice self-administering alcohol under punishment conditions (Halladay et al., 2020). Importantly, a dissociation between the ventral and dorsal subregions of the mPFC was apparent, with electrophysiological evidence revealing time-locked activity in the more ventral portion of the vmPFC, with activity related to suppressing volitional engagement of drug-seeking in the face of punishment. To evaluate the casual role of this region and its downstream targets, the authors used optogenetics to causally inhibit the

downstream AcbS output of the vmPFC, with inhibition causing an increase in lever pressing under punishment (Halladay et al., 2020). These findings provide alternative evidence to suggest that dysregulated neuronal activity in the vmPFC, presumably the IL cortex, and its projection site the AcbS (Vertes 2004), is important in mediating action response under punishment. In line with this, lower grey matter in these structures in animals destined to be HC (i.e., seek cocaine at higher rates under foot shock punishment) were also reported in this thesis, thus suggesting that pre-existing aberrant control mechanisms in circuitry between the vmPFC and striatum may precede drug use and act as a risk marker for the development of compulsive cocaine seeking (Chapter 6), a hypothesis that aligns with human studies in individuals at risk for SUD (Ersche et al., 2020). However, it is thought that a reduction in top-down control and loss of function of the PFC occurs progressively with increasing drug exposure and use (Koob and Volkow 2010). One such study in rodents is consistent with this view. Pelloux et al., selectively lesioned several prefrontal cortical regions, as well as the insular cortex, prior to the evaluation of compulsive cocaine seeking on the seeking-taking chain schedule (Pelloux et al., 2013). Lesions of these regions did not subsequently affect the development of compulsive cocaine seeking and animals with PFC lesions successfully withheld their seeking responses under unpredictable punishment (Pelloux et al., 2013). Thus, it does appear that in this context deficits in prefrontal regions did not influence compulsivity, alluding to the notion that dysregulated top-down control may emerge as drug use occurs. This finding, combined with the evidence showing the robust negative relationships between duration of substance use and grey matter reduction in the PFC (Connolly et al., 2013; Smith et al., 2013), is slightly at odds with the demonstration of pre-existing deficits observed in Chapter 6. However, excitotoxic lesions (as in the Pelloux et al., study above) involve 'taking offline' an entire structure and may not accurately represent subtle neuronal changes that occur within the structure that guide behaviour. In addition, compensatory mechanisms from adjacent non-lesions sites may also affect overall behavioural output, therefore, care should be taken when comparing lesion studies with studies employing non-invasive imaging, such as those used in this thesis (Chapter 6). Pre-existing structural abnormalities in the insula of HC animals were also observed. HC animals showed higher grey matter volume in the insula, a finding potentially related to the important role of the insula in mediating interoceptive states. In humans, a wide range of studies have associated structural abnormalities in the insula with psychostimulant dependence (Mackey and Paulus 2013). In general, reduced insula volume is associated with risk (Ersche et al., 2012), dependence (Franklin et al., 2002) and duration of use (Ersche et al., 2011). In rats, pre-behavioural and post-behavioural lesions

of the AI increase or decrease escalation of cocaine intake, respectively (Rotge et al., 2017), suggesting a more nuanced role of the insula in integrating and regulating SA dependent on prior cocaine experience. Somewhat opposite to these findings, inactivation of the insula reduced cocaine seeking behaviour during a cue-induced reinstatement test (Cosme, Gutman and LaLumiere 2015). In regard to compulsivity, pre-lesions of the insula do not affect future compulsive cocaine seeking (Pelloux et al., 2013). Thus, it appears that the insula may regulate SA dependent on specific aspects of drug-related behaviour. Taken together, these results (Chapter 6) suggest that grey matter differences, and presumably aberrant signalling in the insula, is an important marker for future compulsive cocaine seeking. Although the exact role of the insula in mediating compulsive cocaine seeking is still yet to be fully elucidated, the VBM results reported in this thesis do align with the well-established finding that insula damage can lead to cessation of smoking (Naqvi et al., 2007). Although this is a very different class of drug (nicotine *versus* cocaine) and comparisons should be made with caution, it does offer some evidence to suggest that increased insula grey matter is related to compulsive drug-related behaviours. The IL cortex, and its downstream connections with the AbcS, has not only been linked with drug-seeking (above), but has also critically been linked to habit formation (Killcross and Coutureau 2003). Thus, lesions of the IL cortex cause rats to remain sensitive to reward contingencies, effectively demonstrating goal-directed behaviour, even after extended periods of training. Inactivation of the IL cortex in over-trained animals also renders them more sensitive to reward devaluation through satiety (Coutureau and Killcross 2003). The transition towards compulsion has been hypothesised to represent, in part, the evolution of aberrant and maladaptive habitual responding. Thus, one may ask the question of how a decrease in grey matter volume in the IL cortex in HI, or future compulsive animals (Chapter 6), a finding related to increased goal-directed behaviour (above), relates to the development and formation of maladaptive cocaine seeking habits? To answer this, Murray et al., evaluated the transition to DLS control over cocaine seeking in HI and LI rats. During intermediate or transition stages of training, where seeking becomes reliant on DLS dopaminergic mechanisms and habitual responding begins to develop, dopaminergic blockade in the DLS resulted in decreased seeking in LI but not HI animals (Murray et al., 2014). This study invites the hypothesis that reduced inhibitory control, and dorsal striatal habit formation may represent two overlapping mechanisms by which compulsive drug-seeking develops, and not necessarily a serial interaction between impulse control deficits and increased DLS control. The work presented in Chapter 6 also aligns with this study (Murray et al., 2014), as well as other work showing that lesions of the IL cortex block the

effect of cocaine on habit facilitation (Schmitzer-Torbet et al., 2015). Thus inviting the hypothesis that GM reduction in the IL cortex of HI and future HC animals may represent a vulnerability marker for the development of compulsive drug-seeking through impaired cortico-striatal inhibitory control mechanisms, and not necessarily through the facilitation of DLS habit-based responding during protracted cocaine seeking.

7.4 Future work

The imaging analysis presented in this thesis has focused primarily on the two imaging timepoints prior to behavioural endophenotyping at PND-63 and again prior to cocaine SA. Future work will begin to address the developmental aetiology of the behavioural endophenotypes investigated. Along these lines, an important avenue for future research will be in investigating myelin trajectories across development in animals classified as vulnerable to later development of compulsive cocaine seeking (i.e., impulsive and sticky animals). Recent evidence has supported the view that altered cortico-striatal myelin trajectories are related to impulsive and compulsive phenotypes in humans (Ziegler et al., 2019), and decreased myelin in the ventral putamen is related to high levels of impulsivity (Nord et al., 2019). Whilst the exact sequences used to evaluate myelin differ between the two studies, both converge on the important notion that differential myelin may contribute to the underlying phenotype, a hypothesis that will be tested in future investigations. Along this point, there are currently multiple sequences used to evaluate myelin, and debate around which MRI sequence best reflects and measures myelin is still ongoing (Mancini et al., 2020). For example, a recent meta-analysis sought to clarify the relationship between various MRI sequences used to define myelin and their relationship to histological ground truth data. Diffusion-weighted imaging, magnetization transfer, T2 relaxometry, and T1 relaxometry were all used in the comparison. Apart from diffusion-weighted imaging, magnetization transfer was the most commonly used sequence to identify myelin, followed by T2-weighted imaging. The remaining sequences, including T1-weighted imaging, were then used across a wide range of studies (Mancini et al., 2020). T1-weighted imaging has been linked previously to reflect the loss of myelin and increased gliosis in mice exposed to cuprizone (Wood et al., 2016), thus providing evidence to support the utility of T1-weighted imaging in understanding underlying myelin composition. As a starting point, future work will utilize T1-weighted, T2-weighted and MT-weighted sequences to understand potential myelin differences in animals who are phenotyped retrospectively as at-risk to the development of

compulsive cocaine seeking, or indeed phenotyped as future compulsive animals. Due to the rich, multimodal data obtained there are a wide range of analysis pipelines that can be used to investigate and characterise the underlying neural markers of behavioural vulnerability enophenptyes and compulsive cocaine seeking. TBM involves deriving the Jacobian determinants from the DARTEL warp fields generated via the VBM analysis and can be used to understand changes in regional structural composition across time. TBM is the statistical evaluation of the local expansion/contraction of tissue from an image to a reference template. The Jacobian determinant between the pre-/-post behavioural endophenotype assessment scans encodes the relative shape change across the brain between those time-points. TBM can thus be used to understand whether behavioural training differentially affected brain morphology in different populations of animals (e.g., HI vs. LI, or HC vs. LC). This type of analysis may be able to shine a light on the neural plasticity changes seen across training (see Chapter 6). Although this analysis focuses on morphological changes across two time-points, animals in this thesis underwent two developmental scans at PND-21 and PND-35 (along with PND-63 and ~PND-240). Importantly, phenotypic differences may be explained by divergent developmental trajectories early on in development. Future work will utilize mixed-effects modelling or repeated measures ANOVA to understand potential differences in morphological developmental trajectories across populations (e.g., Mengler et al., 2014; Piontkewitz, Arad and Weiner 2011). This approach may be used either at a voxel level, or a ROI level. Using an ROI approach, different structural scanning sequences may be used (e.g., MT-weighted, T1-weighted) to allow for the evaluation of different images with different underlying histological correlates and thus provide a greater understanding of brain development in animals at risk for future development of compulsive cocaine seeking. Additionally, future analysis of the DTI data will shed a light on white matter tract abnormalities. Likewise, broadening the analysis of the fMRI data by using other analysis techniques such as SBCA or ICA will offer other avenues in which to explore network dynamics of rats vulnerable to compulsive cocaine seeking. Finally, higher-order graph theory metrics may also be employed on either a) the functional data or b) across modalities as in morphometric similarity networks (Seidlitz et al., 2018) to assess network properties across the different behavioural phenotypes. The future work proposed represent a small selection of possible alternate analysis pipelines and is by no means exhaustive.

7.4 Potential implications and translation

Whilst pre-clinical work utilizing novel technologies in basic neuroscience edge us closer to understanding addiction-like behaviour, a paucity of effective treatments currently exist. Nonetheless, findings from the use of novel non-invasive technology in research animals has subsequently informed specific clinical hypothesise and research in humans. For example, Terraneo et al., used a technique called transcranial magnetic stimulation to stimulate the DLPFC in CUD subjects and monitored drug-use and craving over several months (Terraneo et al., 2016). This work was directly influenced by research in rodents showing hypoactivity in the PrL of compulsive rats on the seeking-taking chain schedule (Chen et al., 2013). In the rodent study, stimulation and inhibition of the PrL region (thought to represent human DLPFC) through optogenetics reduced and increased compulsive cocaine use in high and low compulsive rats, respectively. In this thesis, future development of compulsive cocaine seeking was related to hypoactivity between the PrL, ACC and the pDMS. As a direct result, this work may inform future clinical studies targeting these hypoactive regions through the use of TMS to reduce drug-seeking behaviour. Another key finding in this thesis was that impulsivity was a vulnerability marker for compulsive cocaine seeking. Atomoxetine is commonly used to treat ADHD and has been shown to reduce impulsive symptoms (Michelson et al., 2003). It is therefore not surprising that atomoxetine has been tested for its utility to reduce cocaine use in humans. However, results from an initial clinical trial evaluating atomoxetine efficacy in reducing cocaine use revealed no effect (Walsh et al., 2013). However, more recent evidence has shown that atomoxetine is effective in reducing attentional bias to drug related cues in cocaine-dependent individuals (Passamonti et al., 2017). Whilst atomoxetine may not modulate overall cocaine consumption it may go some way in reducing other relevant drug-related behaviour such as drug-seeking, a finding bolstered by work in rodent research showing a reduction in drug-seeking after atomoxetine treatment (Economidou et al., 2011). Finally, it is proposed that addiction may be caused by a dominance of the habit-based system over that of the goal-directed system. Whilst this was not tested directly in this thesis, the functional imaging findings (Chapter 6) allude to this notion. Along this theme, work by Ersche and colleagues suggest that goal-narrowing and a bias towards habitual responding in cocaine-dependent individuals could represent one target for future intervention (Breedon et al., 2021; Ersche et al., 2016), with the authors suggesting that advantageous and non-maladaptive habits could be developed and trained over and above the existing maladaptive aberrant drug-seeking habitual behaviour seen in dependent

individuals. The results in this thesis align with these studies and suggest that this may indeed be an important avenue to explore for future treatment.

The use of similar non-invasive imaging techniques offers unique avenues to translate findings between rodents and humans. However, as previously discussed, the large-scale use of rodents in imaging studies is less common due to practical and ethical constraints. In humans, large-scale data consortiums have taken off in the last decade, evidenced through the human connectome project (Elam et al., 2021), the ENIGMA consortium (Hibar et al., 2015), the IMAGEN study (Schumann et al., 2010) and the ABCD study (Casey et al., 2018). Commonly, these consortiums pool a vast amount of imaging data together, and when combined with behavioral assessment, allow for more powerful statistical comparisons to be made. Along these lines, these datasets could be leveraged to further understand the work presented in this thesis. In particular, the ABCD study tracks adolescent brain development with MRI whilst simultaneously capturing a rich set of behavioural data, in particular, data relevant to substance abuse. Whilst there are a multitude of possible research questions and studies that could be carried out given the large multi-modal data set available through the ABCD study, one such analysis may evaluate the extent to which changes in prefrontal-striatal connectivity evolves throughout adolescence and how that relates to differential exposure to substance of abuse. This is one possible study which would utilize the vast consortium resources to specifically test some of the findings presented within this thesis.

7.6 Conclusion

The work within this thesis investigated the role of several risk traits for the development of compulsive cocaine seeking and evaluated the underlying neural markers of these traits, and future compulsive cocaine seeking. This thesis shows, for the first time, that impulsivity, alongside stickiness, is a vulnerability marker for future compulsive cocaine seeking, supporting earlier observations that impulsivity is a risk endophenotype for compulsive cocaine taking (Belin et al., 2008). In addition to impulsivity, stickiness also predicts future compulsivity, suggesting that stickiness may represent an additional translational marker of risk for compulsive drug use. Moreover, through non-invasive imaging this thesis shows that HI and HC animals have lower grey matter volume in the IL cortex and vStr, and functionally, compulsivity was related putatively to reduced top-down control *via* decreased

connectivity between the PFC and the pDMS, a similar pattern of connectivity was also observed in high sticky animals. Collectively, these findings demonstrate that future resistance to punishment, a hallmark feature of addiction, is predicted by structural and functional abnormalities in cortico-striatal circuitry contributing to impulse control and flexible, goal-directed behaviour. These results closely align with widely reported human imaging studies showing deficits in inhibitory control in drug-dependent individuals (Goldstein and Volkow 2011) and more recent evidence showing reduced cortico-striatal connectivity in drug-vulnerable individuals (Ersche et al., 2020). This thesis also shows that high impulsivity, along with stickiness, predicted future compulsive drug-seeking thus adding to the large body of literature supporting the important role of impulsivity as a vulnerability marker for addiction (Verdejo-Garcia et al., 2021). In summary, the findings presented in this thesis suggest that vulnerability to addiction may arise from pre-existing deficits in behavioural control mechanisms subserved by cortico-striatal circuitry.

Appendix

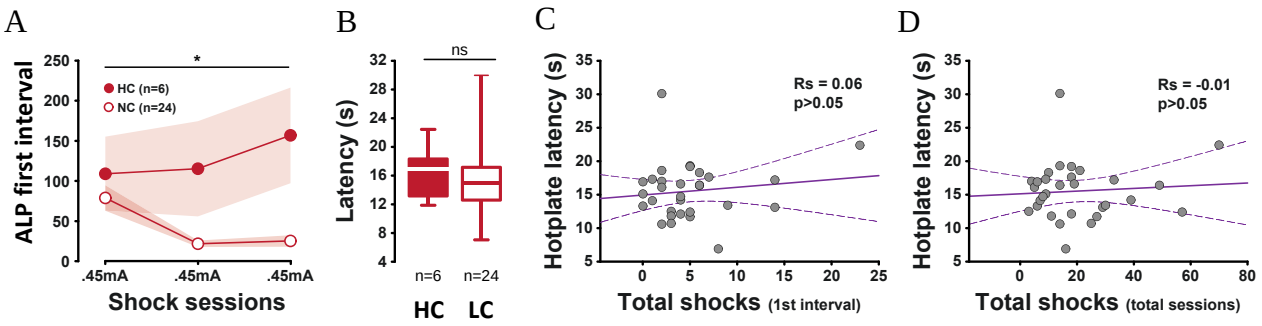


Fig1. High compulsive animals do not express different pain thresholds. High compulsive animals **(A)** did not differ in their hotplate latency **(B).** Hotplate latency did not correlate with the number of shocks in the first interval **(C)** or across the entire session **(D)** in an independent cohort of rats trained under the same schedule used in this thesis. *Abbreviations:* ALP, active level presses.

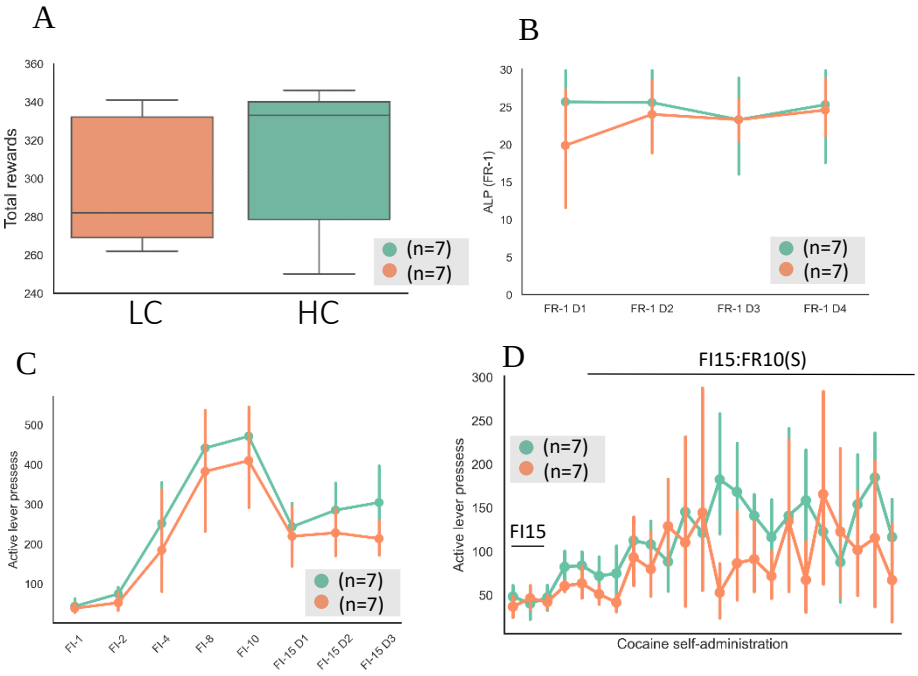
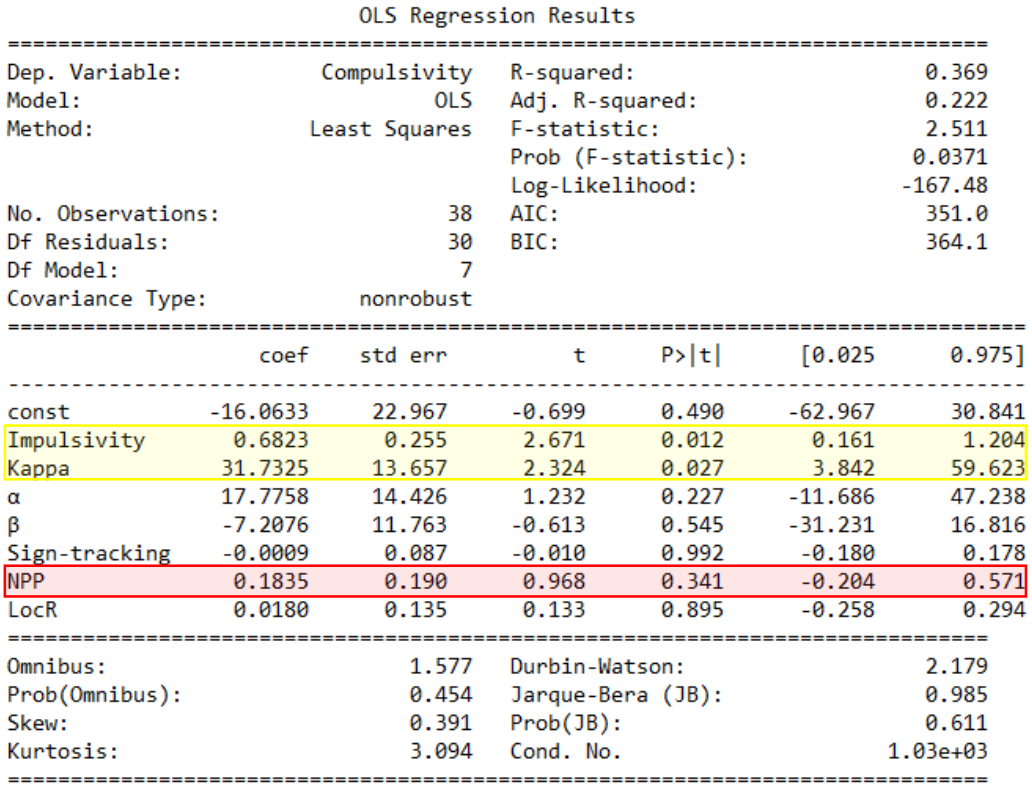


Fig2. High compulsive animals did not differ in their **(A)** their total cocaine intake across all second-order schedule of reinforcement sessions (20 sessions), **(B)** their acquisition of cocaine self-administration, **(C)** their responding under several fixed-interval schedules of reinforcement, **(D)** their responding under a second-order schedule of reinforcement. Mean data reported with error bars indicating ± 95% CI.



OLS Regression Results

Dep. Variable:	Compulsivity	R-squared:	0.369
Model:	OLS	Adj. R-squared:	0.222
Method:	Least Squares	F-statistic:	2.511
		Prob (F-statistic):	0.0371
		Log-Likelihood:	-167.48
No. Observations:	38	AIC:	351.0
Df Residuals:	30	BIC:	364.1
Df Model:	7		
Covariance Type:	nonrobust		

	coef	std err	t	P>|t|	[0.025	0.975]
const	-16.0633	22.967	-0.699	0.490	-62.967	30.841
Impulsivity	0.6823	0.255	2.671	0.012	0.161	1.204
Kappa	31.7325	13.657	2.324	0.027	3.842	59.623
α	17.7758	14.426	1.232	0.227	-11.686	47.238
β	-7.2076	11.763	-0.613	0.545	-31.231	16.816
Sign-tracking	-0.0009	0.087	-0.010	0.992	-0.180	0.178
NPP	0.1835	0.190	0.968	0.341	-0.204	0.571
LocR	0.0180	0.135	0.133	0.895	-0.258	0.294

Omnibus:	1.577	Durbin-Watson:	2.179
Prob(Omnibus):	0.454	Jarque-Bera (JB):	0.985
Skew:	0.391	Prob(JB):	0.611
Kurtosis:	3.094	Cond. No.	1.03e+03

Fig3. Ordinary least squares analysis showing predictive relationship between impulsivity and kappa (yellow shade) with compulsivity No relationship was observed between novelty place preference and compulsivity (red shade).

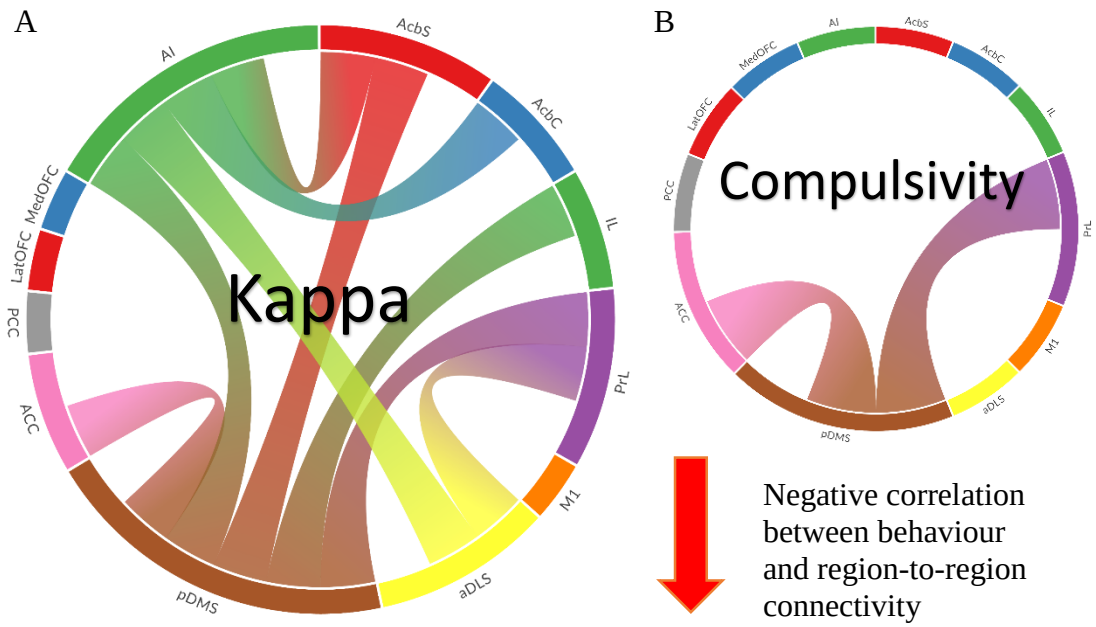


Fig4. Diagrammatic representation of region-to-region functional connectivity, defined based on Spearman's' rho correlation coefficient between two BOLD timeseries (first Eigenvariate values) that **negatively** correlated with either **(A)** kappa or **(B)** compulsive cocaine seeking. All arrows represent region-to-region functional connectivity that negatively correlated with either behaviour. Multiple negative relationships were observed in relation to kappa. Compulsivity was only related to decreased functional between the anterior cingulate cortex (ACC) and the prelimbic cortex (PrL) to the dorsomedial striatum (pDMS). A negative relationship between kappa was also observed with the connectivity between these areas, among other regions. All Spearman's' rho correlations were corrected *via* fdr-correction (q=0.05). For abbreviations of ROIs, please see Chapter 3, section 3.10.3.

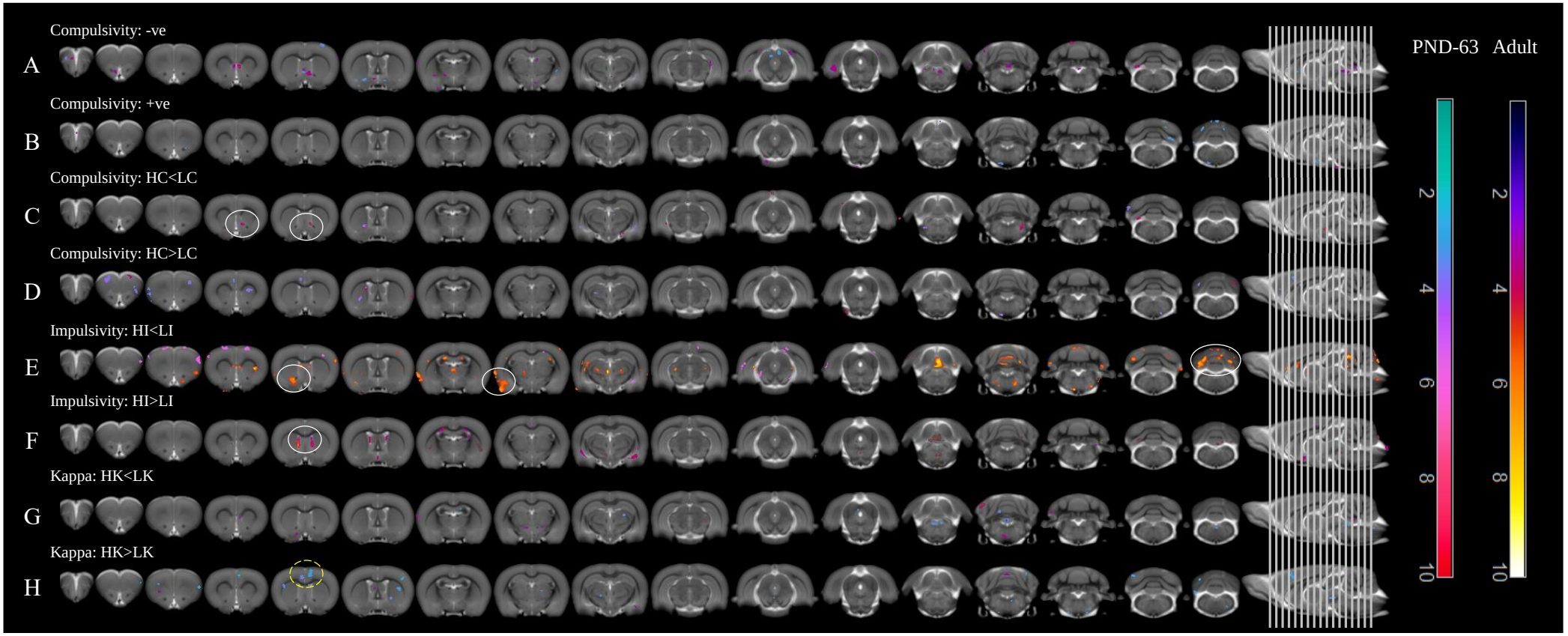


Fig5. Voxel-based morphometric analysis of impulsivity, stickiness, and compulsivity. **(A)** Regression (positive) analysis with compulsive cocaine seeking. **(B)** Regression (negative) analysis with compulsive cocaine seeking. **(C)** Compulsivity: high compulsive less than low compulsive contrast. **(D)** Compulsivity: high compulsive greater than low compulsive contrast. **(E)** Impulsivity: high impulsive less than low impulsive contrast. **(F)** Impulsivity: high impulsive greater than low impulsive contrast. **(G)** Kappa: high kappa (sticky) less than low kappa contrast. **(H)** Kappa: high kappa (sticky) greater than low kappa contrast. All maps presented result from a chosen extent threshold of k=15. Initial cluster-forming threshold of p=0.005, uncorrected. All highlighted circles reflect differences observed at the adult timepoint. Dashed and yellow lines indicate differences observed at PND-63.

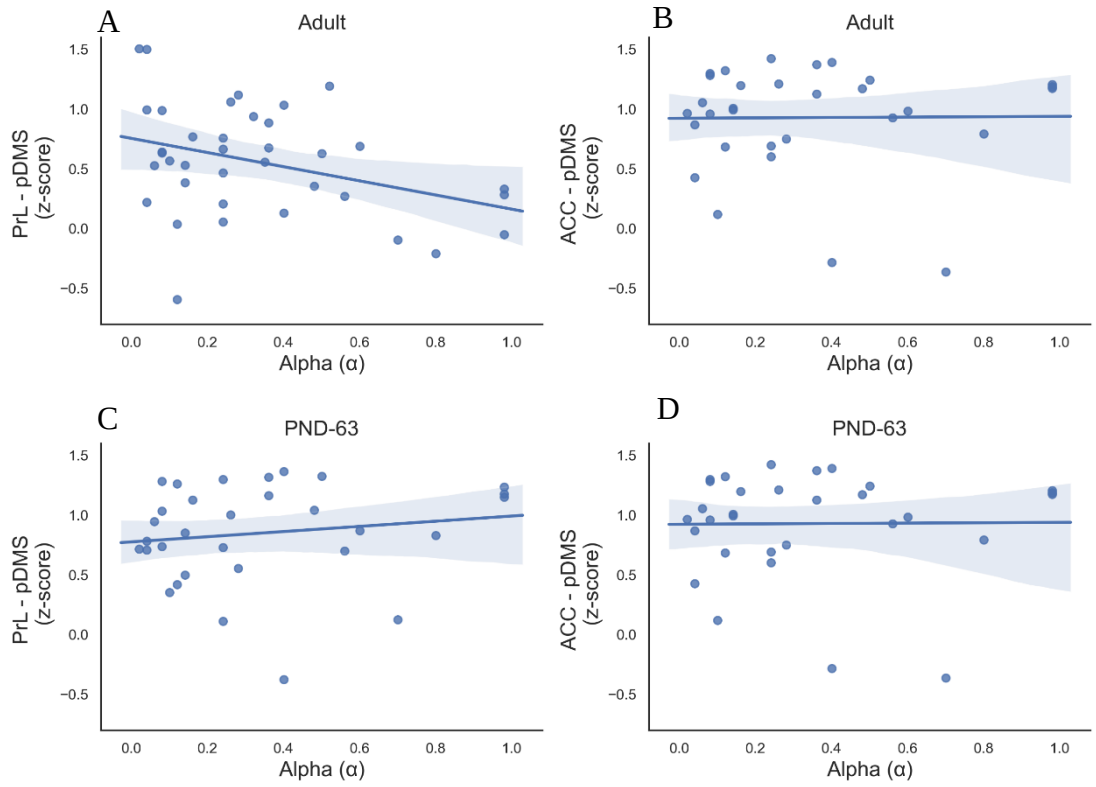


Fig6. The alpha the learning rate is not related to cortico-striatal connectivity at the **(A, B)** adult or **(C, D)** PND-63 timepoint. *Abbreviations*: PND, post-natal day.

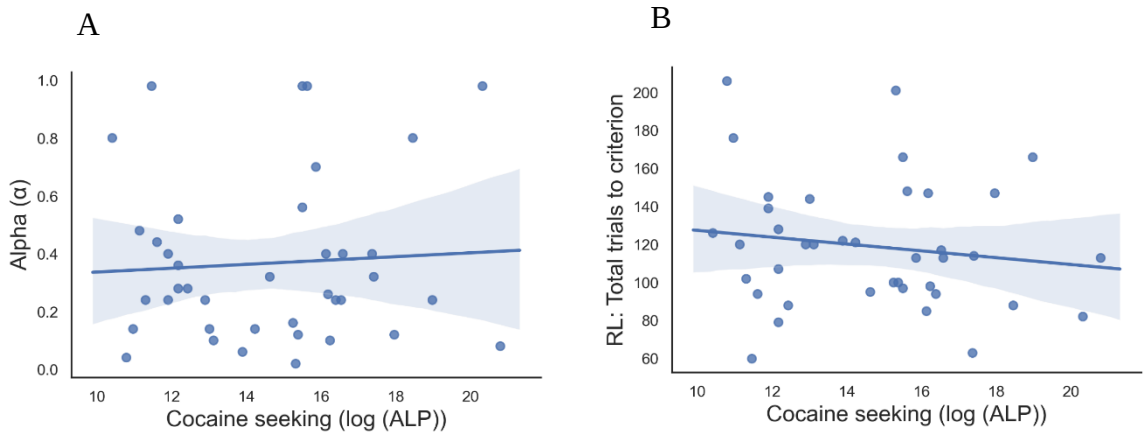


Fig7. Compulsive cocaine seeking is not related to **(A)** alpha, the learning rate derived from computational trial-by-trial analysis of reversal learning performance or **(B)** total trials to criterion, an overall, and more conventional analysis of learning on a reversal learning paradigm. *Abbreviations*: RL, reversal learning.

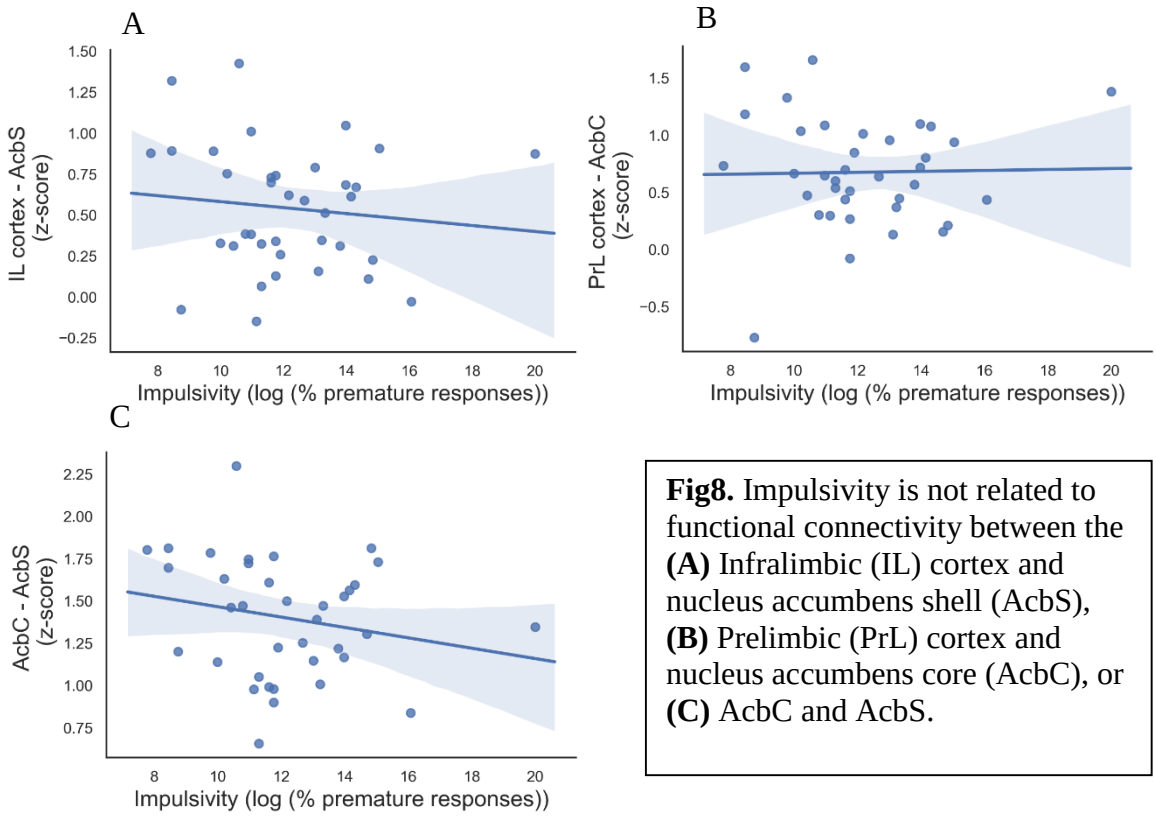


Fig8. Impulsivity is not related to functional connectivity between the **(A)** Infralimbic (IL) cortex and nucleus accumbens shell (AcbS), **(B)** Prelimbic (PrL) cortex and nucleus accumbens core (AcbC), or **(C)** AcbC and AcbS.

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