A molecular mechanism for the topographic alignment of convergent neural maps

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Abstract Sensory processing requires proper alignment of neural maps throughout the brain. In the superficial layers of the superior colliculus of the midbrain, converging projections from retinal ganglion cells and neurons in visual cortex must be aligned to form a visuotopic map, but the basic mechanisms mediating this alignment remain elusive. In a new mouse model, ectopic expression of ephrin-A3 (EfnA3) in a subset of retinal ganglion cells, quantitatively altering the retinal EFNA3 gradient, disrupts cortico-collicular map alignment onto the retino-collicular map, creating a visuotopic mismatch. Genetic inactivation of ectopic EFNA3 restores a wild-type cortico-collicular map. Theoretical analyses using a new mapping algorithm model both map formation and alignment, and recapitulate our experimental observations. The algorithm is based on an initial sensory map, the retino-collicular map, which carries intrinsic topographic information, the retinal EFNAs, to the superior colliculus. These EFNAs subsequently topographically align ingrowing visual cortical axons to the retino-collicular map.

DOI: 10.7554/eLife.20470.001

Introduction

Brain function relies on the efficient processing of sensory information, which in turn requires the proper formation and interaction of multiple sensory maps of the world. The superior colliculus (SC) of the midbrain is a major hub for sensory processing, as it receives organized inputs from visual, auditory, and somatosensory modalities (May, 2006). The SC is a laminated structure controlling visuo-spatial orientation and attention (May, 2006; Krauzlis et al., 2013). As such, defective visual processing in the SC has been associated with psychiatric conditions (Mathis et al., 2015). Visual information reaches the superficial layers of the SC, which are innervated both by retinal ganglion cells (RGCs - the retino-collicular projection) and by layer V neurons of the primary visual cortex V1 (the cortico-collicular projection). During development in the mouse, the retino-collicular map forms during the first post-natal week, and is followed by the cortico-collicular map, which develops between P6 and P12 (Triplet et al., 2009). These visuotopic maps must be aligned to ensure efficient modulation of the SC’s retinal response by V1 inputs (Zhao et al., 2014; Liang et al., 2015). It has been suggested that the formation of the visuotopy is a stochastic process instructed by a balanced contribution of molecular cues and correlated neuronal activity (Chandrasekaran et al., 2005; Tsigankov and Koulakov, 2006; 2010; Triplet et al., 2009; Ackman and Crair, 2014;
Owens et al., 2015). However the basic principles and underlying molecular mechanisms governing the alignment of converging maps have not been fully described.

Potential candidates are gradients of Eph receptor tyrosine kinases (EPHs) and their membrane-bound ligands, the ephrins (EFNs), already known to control retino-collicular map formation. In the mouse, EPHA4/A5/A6 receptors are present on projecting RGCs in a low-nasal to high-temporal gradient. RGC axons are repelled when their EPHAs contact and are activated by collicular EFNA2/A5, expressed in a low-rostral to high-caudal gradient in the SC (Lemke and Reber, 2005; Cang and Feldheim, 2013). Counter-gradients of ligands (EFNA2/A5/A6) and receptors (EPHA3/A4/A7) are also present in the RGCs and the SC respectively, but their role remains controversial (Feldheim et al., 2010; Triplett and Feldheim, 2012; Sutterlin et al., 2012; Weth et al., 2014). In V1, gradients of EPHA4/A7, running from high-lateral to low-medial have also been observed, and evidence from genetic analyses suggest their involvement in the development of cortico-collicular projections (Cang et al., 2005; Wilks et al., 2010). Moreover, the formation of the cortico-collicular map requires retinal input (Khachab and Bruce, 1999; Triplett et al., 2009), but again, the underlying molecular mechanisms remain elusive.

The complex expression patterns of Epha/Ena gradients in both projecting and target structures require the development of incisive in vivo approaches to address, in a selective and quantitative manner, the function of these gradients in specific neuronal projections. Here, we analyzed the role of retinal Efna gradients in visuotopic map formation in the SC. We generated a unique mouse model in which EFNA3 is ectopically expressed only in Isl2(+) RGCs, therefore quantitatively disrupting the EFNA gradients exclusively in the retina without affecting Ephas/Efnas expression in other visually-related structures. Surprisingly, Isl2-ires-Efna3 knock-in mice (referred as Efna3 KI) exhibited normal retino-collicular/geniculate maps and normal ipsi/contralateral projections. In marked contrast, the formation of the cortico-collicular map was severely disrupted, leading to a mix of single and duplicated projections and generating a mismatch with the retino-collicular map. The causal role of EFNA3 ectopic expression was further confirmed when in vivo inactivation by co-expressed EPHA3 receptor in the same Isl2(+) RGCs restored a wild-type map. Theoretical modelling recapitulated our experimental observations in normal and aberrant conditions induced by EFNA3 ectopic expression, therefore validating the basic principle and mechanism of map alignment. This mechanism suggests that a leading sensory map carries positional information cues for further sensory projections, within the same modality, to align and adjust.

Results

Knock-in mice for Efna3 ectopic expression in Isl2(+) RGCs show normal retino-collicular and retina-geniculate projections

To test the role of retinal EFNA ligands in visuotopic map formation, we generated knock-in mice in which a full length Efna3 cDNA was inserted, flanked by an internal ribosome entry site (ires), into the 3’-UTR region of the Isl2 (Isl2) gene (Figure 1—figure supplement 1), similar to a previous approach (Brown et al., 2000). Immunohistochemical staining confirmed ectopic expression of EFNA3 in somata and proximal axons of Isl2(+) RGCs in postnatal day 1 (P1) and P8 Efna3 homozygous knock-in (Efna3KI/KI) mice compared to wild-type (WT) littermates (Figure 1A–F) without affecting Isl2 expression at P1 (Figure 1G,H). Efna3KI/KI mice present two intermixed sub-populations of RGCs: Isl2(−) cells, expressing wild-type levels of Efna3, and Isl2(+) cells expressing additional Efna3 (Figure 1E–F,I–N). EFNA3 is observed on RGC axons in vitro (Figure 1O,Q’). Ectopic expression of EFNA in Isl2(+) RGCs did not induce perceptible changes in synaptic layers or retinal organization (Figure 2A,B). Quantitative transcript (mRNA) measurement on RGC cell bodies, performed and standardized as described previously (Reber et al., 2004; Claudepierre et al., 2008) confirmed a two-fold increase of Efna3 mRNA and normal levels of Efna2/a5 mRNA in nasal, central and temporal Efna3KI/KI mutant retinas when compared to WT littermates (Figure 2C). Together, these data confirm a two-fold ectopic expression of Efna3 in Isl2(+) Efna3KI/KI RGCs, which generates an oscillating high-nasal to low-temporal gradient in Efna3 expression in the Efna3 KI mouse, very similar to the oscillating Ephas gradient we previously described for Isl2-Epha3 knock-in mice (Reber et al., 2004).
### Figure 1. Validation of the Efna3 KI model 1.

(A–F) Immunohistochemical staining using anti-EFNA3 (LS-C6547, LS-Bio) in P1 (A, B) and P8 (C) WT (n = 3 animals) and P1 (D, E) and P8 (F) Efna3KI/KI (n = 5 animals) RGCs. In (E, F) arrows indicate EFNA3 WT expression level in RGCs, arrowheads indicate putative ectopic (knock-in) EFNA3 expression in RGCs. The asterisk highlights strong EFNA3 labelling in the RGC fiber layer. Scale bars represent 200 μm (A, D), 20 μm (B–F). (G–H) Immunostaining of ISL2 in P1 WT (n = 3 animals) (G) and Efna3KI/KI (n = 5 animals) (H) RGCs in the ganglion cell layer. Arrowheads indicate ISL2(+) RGCs. Scale bars represent 20 μm. (I–N) Immunostaining of ISL2 (I, L), EFNA3 (LS-C6547, LS-Bio) (J, M) and merged (K, N) in P1 and P7 Efna3KI/KI (n = 5 animals) RGCs. Arrowheads indicate ISL2(+) RGCs with high EFNA3. Arrows indicate ISL2(-) RGCs with wild-type EFNA3 level. Scale bars represent 10 μm. (O, Q') EFNA3 (LS-C6547, LS-Bio) (O, Q, Q') and axonal marker TAU (P, Q, Q') co-immunostaining on P6 RGC in culture (DIV 5) (n = 3 animals). (Q') shows an EFNA3/TAU co-labelled RGC axon at higher magnification. Scale bars represent 10 μm. GCL, ganglion cell layer; RGC, retinal ganglion cell; DIV, days in vitro; WT, wild-type.

DOI: 10.7554/eLife.20470.002

The following figure supplement is available for figure 1:

Figure 1 continued on next page
To study retino-collicular map formation, we performed focal anterograde DiI labelling in the retina of P7 mice and analyzed the termination zones (TZs) in the SC at P8, as described previously (Brown et al., 2000; Reber et al., 2004; Bevins et al., 2011). For quantitative analysis, we measured the locations of the termination zones (TZs) along the rostral-caudal axis of the SC and the location of the focal DiI injections along the nasal-temporal axis of the retina (Figure 3—figure supplement 1).

![Figure 2](image-url)

**Figure 2.** Validation of the Efna3 KI model II. (A, B) Synaptophysin (red) and DAPI (blue) staining in P34 WT (n = 4 animals) (A) and Efna3KI/KI (n = 4 animals) (B) retinas. Scale bars represent 50 μm. (C) Efna mRNAs relative to nasal WT quantified in acutely isolated RGCs from nasal (N), central (C) and temporal (T) retinas and normalized against Gapdh and Hprt in P3 WT and Efna3KI/KI (WT, n = 6 animals/12 retinas; Efna3KI/KI, n = 8 animals/16 retinas, variables are normally distributed, two-way ANOVA: Efna2 genotype F_{11,3} = 1.79, p = 0.11; Efna5 genotype F_{11,3} = 2.08, p = 0.07; Efna3 genotype F_{11,3} = 8.11, ***p<0.001). WT, wild-type; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; OS, outer and inner segments; RGCs, retinal ganglion cells.

DOI: 10.7554/eLife.20470.004
Cortico-collicular maps are duplicated in Isl2-Efna3KI mutants

To test whether retinal EFNA guidance cues influence the formation of the V1 cortico-collicular map, we traced cortico-collicular projections from V1 cortex by focal DiI injection in P14 mice and analyzed the location of the TZs in the SC at P15. Quantitative analyses using ImageJ revealed a remarkable duplication of the cortico-collicular map for single injections along the lateral-medial axis of the V1 in 47% of Efna3KI/KI (n = 9/19, Figure 4A, Figure 4—figure supplement 1A, Figure 4—figure supplement 2A) and 43% of Efna3KI/+ (n = 7/16, Figure 4B, Figure 4—figure supplement 1B) animals when compared to WT littermates (n = 9, Figure 4C, Figure 4—figure supplement 1C, Figure 4—figure supplement 2B) demonstrating heterogeneity of the phenotype in Efna3 KI mutants.

If this heterogeneity is caused by genetic variations between animals, the same type of projections (either single or duplicated) should be observed in both colliculi of a given animal. This was not observed in 60% of Efna3KI/KI (n = 3/5) and 57% of Efna3KI/+ (n = 4/7) animals when we traced the cortico-collicular projections in both left and right colliculi (Figure 4D,D'). Therefore, genetic variation is unlikely to contribute to the map heterogeneity between animals of the same genotype. This heterogeneity is most likely the consequence of a stochastic process of map formation, as suggested previously (Owens et al., 2015). In Efna3KI/KI animals showing the same type of projections between colliculi, (40% of the mice) all of these projections were duplicated whereas in the Efna3KI/+ animals, the remaining 43% presented only single projections in both left and right colliculi, suggesting an effect of the level of Efna3 ectopic expression onto cortico-collicular map duplication. As expected, all WT animals tested (n = 4) showed single projections in both colliculi (Figure 4D).

Next, we calculated the average distance of separation (ΔS_{exp}) between the duplicated maps in Efna3 KI mutants as a percentage of the rostral-caudal axis of the SC (Figure 4A,B). This revealed a significant two-fold difference in map separation between Efna3KI/KI (ΔS_{exp} median = 13%, n = 9) and Efna3KI/+ (ΔS_{exp} median = 7%, n = 7) animals, which correlates with the presence of one or two copies of the Isl2-ires-Efna3 allele (Figure 4E). Since Isl2 is not expressed in the cortex (see Figure 5B), these results suggest that Efna3 ectopic expression in Isl2(+) RGC axons/TZs destabilizes the stochastic process of cortico-collicular mapping, leading to map duplication in Efna3 KI animals. This in turn implies that V1 EphA-positive cortical axons sense varying EFNA3 levels on RGC terminals by direct contact with these terminals in the SC (Cang et al., 2005) and anterograde labelling from retina and V1 cortex showed that both cortical and retinal axons terminals overlap in the superficial layers of the SC (Figure 4F) (Phillips et al., 2011; Owens et al., 2015). To further analyze the presence of EFNA3 of retinal origin in the SC, we performed EFNA3 immunohistochemistry coupled to retinal Dil injections on collicular sections. Dil labelled RGC axons were observed in the SC, as well as a strong collicular EFNA3 staining (Figure 4—figure supplement 3A–F, Figure 5—figure supplement 1A–D'). However, no EFNA3 staining on single Dil-labelled RGC axons could be detected in neither Efna3KI/KI nor WT littermates (Figure 4—figure supplement 3A–F). EFNA3 immunohistochemistry on P3 Efna3KI/KI and WT optic nerve (ON) longitudinal sections (proximal, middle and distal to the optic cup) showed the presence of EFNA3 on fibers within the proximal part of the ON in Efna3KI/KI and WT littermates (Figure 4—figure supplements 4A,A', D,D') but not on the proximal part of Efna3KI/KI ON (Figure 4—figure supplement 4H). EFNA3 signal was also detected in the middle part of the ON in Efna3KI/KI but not in WT (Figure 4—figure supplements 4B,B', E,E') and was nearly impossible to discern in the distal part of Efna3KI/KI ON (Figure 4—figure supplements 4C,C', F,F'), indicating that EFNA3 is present on RGC axons. This was
Figure 3. Retinal anterograde mapping and retrograde Dil labelling in P8 animals. (A) Retino-collicular maps generated by the Leave-One-Out method at P8 in WT (n = 38 animals), Efna3KI/+ (n = 15 animals) and Efna3KI/KI (n = 43 animals) mutants. Grey lines represent each map for each leave-one-out run. Each dot represents an individual mouse. (B–G) P8 Dil retrograde labelling (red) coupled to immuno-staining (green) of EFNA3 (B–D) and ISL2 (E–G) in Efna3KI/KI (n = 4 animals) mutant retinas. Arrowheads indicate ISL2(+) / high EFNA3 Dil labelled RGCs. Scale bars represent 10 μm. (H–K)
Figure 3 continued

Anterograde labelling after focal DiI injections in the retina showing retino-geniculate projections from the temporal-ventral (T–V) (H, I) and nasal-ventral (N–V) (J, K) retinal quadrants between Efna3KI/KI (H, J), (n = 4 animals) and WT (I), (K), (n = 4 animals) P8 littermates. Scale bars represent 400 μm. (L–Q) Anterograde labelling after full-eye injections showing eye-segregation in P8 WT (n = 3 animals) SCs (whole-mount top view: L), coronal sections: M, (M’) and dLGN (N) and inEfna3KI/KI (n = 4 animals) SCs (whole-mount top view: O); coronal sections: P, (P’) and dLGN (Q). Scale bars represent 250 μm (L–P’), 100 μm (N, Q). WT, wild-type; GCL, ganglion cell layer; T, temporal; N, nasal; R, rostral; C, caudal; dLGN, dorsal lateral geniculate nuclei; SC, superior colliculus.

DOI: 10.7554/eLife.20470.005

The following figure supplements are available for figure 3:

Figure supplement 1. Anterograde DiI labelling showing the focal injection in the retina (white star in A) and the corresponding termination zones in the SC (B).

DOI: 10.7554/eLife.20470.006

Figure supplement 2. Representative examples of collicular parasagittal sections showing labelled anterograde retinal termination zones in P8 Efna3KI/KI (A) and WT (B) littermates.

DOI: 10.7554/eLife.20470.007

further confirmed by co-localization of EFNA3 with NF200 on fibers (Figure 4—figure supplement 4I–J’ and Figure 1B–F, O–Q’). Retrograde labelling confirmed that the cortico-collicular projections originate from layer V neurons in V1 cortex in P14 Efna3KI/KI animals (Figure 5A). No bi-cistronic expression of Isl2-ires-Efna3 in either SC or V1 cortex of WT and Efna3KI/KI animals could be detected (Figure 5B), ruling out any indirect effects of Efna3 ectopic expression. Transcript analyses in colliculi and V1 cortices revealed similar levels of Efna2/a3/a5 and EphA4/a7 receptors in Efna3KI/KI compared to WT littermates at P7 (Figure 5C) excluding indirect effects due to local changes of Ephas/Efna genes expression. Similar level of EFNA3 between Efna3KI/KI and WT littermates was further confirmed by immunostaining of EFNA3 (Figure 5—figure supplement 1A–E).

Genetic cis-inactivation of ectopic EFNA3 expression in RGCs restores a wild-type cortico-collicular map

If the defective cortico-collicular maps in the Efna3 KI animals were solely due to Efna3 ectopic expression in Isl2(+) RGCs, then inactivation of this ectopic expression should rescue the phenotype and restore a wild-type map. Previous work showed that co-expression of EFNA3 ligand and EPHA receptor in the same cell leads to their mutual inactivation through cis-masking (Falivelli et al., 2013). To accomplish this in vivo, we generated double heterozygous mice carrying Epha3 on one allele of the Isl2 gene and Efna3 on the second allele (Epha3KI/Efna3KI). Immunohistochemical staining confirmed co-expression of EFNA3 and EPHA3 in acutely isolated double heterozygous RGCs (Figure 6A). Importantly, previous studies demonstrated that ectopic expression of the EPHA3 receptor in Isl2(+) RGCs in heterozygous Epha3KI/+ animals results in a duplicated retino-collicular map (Figure 6—figure supplement 1, Brown et al., 2000). Remarkably, co-expression of Epha3 and Efna3 in Epha3KI/Efna3KI double-mutant mice reverted the duplications, leading to normal retino- and cortico-collicular maps (Figure 6B,C). This double rescue of both maps indicates that cortico-collicular defects in Efna3 KI animals were caused by ectopic expression of EFNA3 in Isl2(+) RGCs and were restored by concomitant expression of EPHA3. The cis-inactivation mechanism was cross-validated by the presence of a normal retino-collicular map in the Efna3KI/Epha3KI double mutant mice, indicating EPHA3 inactivation (Figure 6C). To further evaluate any residual EFNA3 or EPHA3 signaling activity, we generated Epha3KI/Efna3KIxEpha4 knock-in/out compound mutants. In these mice, decreasing the overall level of retinal EPHA receptors by eliminating EPHA4 expression would reveal subtle changes in retinal EPHA signaling strength (Reber et al., 2004; Bevins et al., 2011). According to the Relative Signaling model in the Epha3KI/+;Epha4+/— and Epha3KI/+;Epha4−/— compound mutant animals analyzed previously (Reber et al., 2004) any residual EPHA3 signaling on map formation would generate duplicated retinal Tzs, particularly in the caudal part of the SC where nasal RGCs axons, expressing low levels of EPHA receptors, project. Retinal DiI anterograde tracing revealed no retino-collicular duplications, even in the caudal-most pole of the SC, in Epha3KI/Efna3KIxEpha4+/— and Epha3KI/Efna3KIxEpha4−/— compound mutants, confirming inactivation of EPHA3 and EFNA3 in Isl2(+) RGCs (Figure 6D,E). Altogether, these results suggest that EPHA3 and EFNA3 ectopic expression in the same RGCs lead to their mutual inactivation and
Figure 4. Anterograde cortico-collicular mapping in P14 animals. (A–C) Cortico-collicular maps generated by the Leave-One-Out method at P15 in Efna3Kl/Kl (n = 19 animals) (A), Efna3Kl/+ (n = 16 animals) (B) and WT (n = 9 animals) (C) mutant littermates. Grey lines represent each map for each leave-one-out run for duplicated and single maps. Each dot represent an individual mouse. (A’–C’) show examples of single (A’, B’, C’) and duplicated (A’’, B’’)) cortical TZs in collicular sagittal sections from different genotype together with the corresponding injections in V1 (lower panels in A’–C’). The corresponding coordinates are labelled by red arrows (duplicated) or a black arrow (single). Scale bars represent 400 μm (A’–C’), 1 mm (insets in A’–C’). (D–D’) Percentage of heterogeneous duplicated/single (D/S) or homogeneous single/single (S/S) or duplicated/duplicated (D/D) projections in both colliculi of the same animal (D) (Efna3Kl/Kl, n = 5 animals; Efna3Kl/+ , n = 7 animals; WT, n = 4 animals). (D’) shows an example of cortico-collicular TZs from left and right colliculi and corresponding V1 injections in a P15 Efna3Kl/+ animal. Scale bars represent 400 μm (for colliculi), 1 mm (for V1). (E) Mean separation between duplicated maps measured experimentally (ΔSexp) in Efna3Kl/Kl (median = 13%, 1st quartile = 10%, third quartile = 13%, n = 9 animals) and Efna3Kl/+ (median = 7%, 1st quartile = 6%, third quartile = 9.5 % n = 7 animals) (non-parametric Mann and Whitney test, **p=0.020). (F) Sagittal section of P15 Efna3Kl/Kl (n = 4 animals) Dil labelled cortical projection (left) and merged image of CTB-488 full-eye fill and cortical Dil projection (right). Scale bars represent 200 μm. L, lateral; M, medial; R, rostral; C, caudal; S, single; D, duplicated; TZ, termination zone; WT, wild-type.

DOI: 10.7554/eLife.20470.008

The following figure supplements are available for figure 4:

Figure supplement 1. Representative examples of collicular parasagittal sections showing cortical terminations zones and the corresponding injections in V1 in Efna3Kl/Kl (A), Efna3Kl/+ (B) and WT (C) P15 littermates.

DOI: 10.7554/eLife.20470.009

Figure supplement 2. Collicular termination zones on parasagittal sections revealed after double Dil – DiD V1 anterograde injections in P15 Efna3Kl/Kl (A) and WT (B).

DOI: 10.7554/eLife.20470.010

Figure 4 continued on next page
confirm that cortico-collicular mapping duplications observed in Efna3 KI animals are the consequence of retinal EFNA3 ectopic expression.

**In silico modelling and theoretical analysis of cortico-collicular map alignment**

Our results indicate that the level of EFNA3 on RGC projections innervating the SC influences the mapping and alignment of cortico-collicular projections. To simulate and explore the mechanism of mapping and alignment of cortico-collicular projections. To simulate and explore the mechanism of mapping and alignment of cortico-collicular projections, we created a 3-step map alignment model based on an algorithm originally developed to model retino-collicular mapping (Tsigankov and Koulakov, 2006; 2010; Owens et al., 2015). Our version generates first the retino-collicular map based on retinal Eph receptor and collicular Eph ligands graded expression. The second step transposes the retinal Epha gradients onto the rostral-caudal axis of the SC according to the layout of the retino-collicular map generated in step one. In the third step, the cortico-collicular map is generated based on cortical Eph receptor expression and the transposed retinal Efna gradients in the SC. Each map is generated by a stochastic process based on balanced forces between repellent EphA forward signaling and associating correlated neuronal activity (Tsigankov and Koulakov, 2006; 2010; Owens et al., 2015) (see Experimental Procedures). To improve the validity of our model, we replaced the theoretical values of the retinal EphA and EFNA gradients previously used (Tsigankov and Koulakov, 2006; 2010; Pfeifenberger et al., 2006; Owens et al., 2015) by our experimental quantification of retinal Epha mRNAs (Reber et al., 2004) (R_A(x)^Ephas) and Efna mRNAs (L_A(x)^Efna) (Figure 7A,B) reasonably assuming, as made previously (Reber et al., 2004), that the measurement of mRNA differences translates linearly into differences in protein expression. L_A(x)^Efna best fit equations are:

\[
L_{A3}(x)^{\text{retina}} = (1/0.56)^{2} \exp(-0.014x) \quad R^2 = 0.97
\]

\[
L_{A2}(x)^{\text{retina}} = (1/0.54)^{2} \exp(-0.008x) \quad R^2 = 0.93
\]

\[
L_{A3}(x)^{\text{retina}} = 0.44 \quad R^2 = 0.97
\]

The total Efna gradients in the WT retina is:

\[
L_A(x)^{\text{retina} \ WT} = L_{A3}(x)^{\text{retina} \ WT} + L_{A2}(x)^{\text{retina} \ WT} + L_{A3}(x)^{\text{retina} \ WT}
\]

\[
L_A(x)^{\text{retina} \ WT} = (1/0.56)^{2} \exp(-0.014x) + (1/0.54)^{2} \exp(-0.008x) + 0.44
\]

The total Efna gradients in the mutant retinas are:

\[
L_A(x)^{\text{retina} \ KI} = L_A(x)^{\text{retina} \ WT} + \Delta L_{A3} \quad \text{with } \Delta L_{A3} = 0.22 \text{ in Efna3KI/+}
\]

\[
L_A(x)^{\text{retina} \ KI} = (1/0.56)^{2} \exp(-0.014x) + (1/0.54)^{2} \exp(-0.008x) + 0.44 + 0.22
\]

\[
L_A(x)^{\text{retina} \ KI/KI} = (1/0.56)^{2} \exp(-0.014x) + (1/0.54)^{2} \exp(-0.008x) + 0.44 + 0.44
\]

Equations were derived from semi-quantitative in situ hybridization as performed previously (Reber et al., 2004) (Figure 7A,B) and from our transcripts analyses measuring the relative expression levels of Efna in acutely isolated RGCs (Figure 2C). We observed graded expression of Efna2 and Efna5 along the nasal-temporal axis of the retina, whereas Efna3 is homogeneously expressed in WT animals (Figure 7A,B). Curve fitting analysis using MATLAB revealed the equation L_A(x)^{\text{retina} \ WT}
Figure 5. V1 retrograde labelling and Ephas/Efnas quantitative expression in V1 and SC. (A) Retrograde labelling by collicular Dil injection in P14 Efna3KI/KI (n = 4 animals) and WT (n = 3 animals) showing the localization of the projecting V1 cortical neurons in layer V (Dil, red; DAPI, blue). Scale bars represent 100 μm. (B) PCR amplification products from P7 RNA (35 cycles) in Efna3KI/KI (n = 3 animals per genotype). A single amplicon of 90 bp, corresponding to an amplified sequence located in the ires cassette of the targeted construct, is observed only in Efna3KI/KI retina. (C) Box-plot of Efna2/a3/a5 and Epha4/a7 mRNA fold-expression levels relative to wild-type (WT) at P7 in the superior colliculus and V1 cortex of Efna3KI/KI mutants (n = 4 animals for each genotype; two colliculi/V1 per animal, variables are normally distributed, one sample t-test: superior colliculus: Efna2, p = 0.17; Efna3, p = 0.48; Efna5, p = 0.50; Epha4, p = 0.09; Epha7, p = 0.26 – V1 cortex: Efna2, p = 0.09; Efna3, p = 0.61; Efna5, p = 0.84; Epha4, p = 0.07; Epha7, p = 0.58).

DOI: 10.7554/eLife.20470.013

The following figure supplement is available for figure 5:

Figure supplement 1. EFNA3/DAPI immunohistochemical staining in P3 (A – A', C, C') and P8 (B – B', D, D') Efna3KI/KI (A – B'), WT (C – D') and Efna3-/- (E) SCs sagittal sections.

DOI: 10.7554/eLife.20470.014
Figure 6. Retino- and cortico-collicular maps in Epha3KI/Efna3KI double heterozygous and compound Epha3KI/Efna3KI::Epha4 knock-in/out mutants. (A) Co-immunostaining of EFNA3 (LS-C6547, LS-Bio; green) and EPHA3 (red) in P4 acutely isolated Epha3KI/Efna3KI (n = 8 animals/15 retinas) RGCs. Arrowheads indicate EPHA3/EFNA3 co-expression in the same RGCs. Scale bars represent 10 μm. (B, C) Cortico-collicular (n = 11 animals) (B) and retino-collicular (n = 11 animals) (C) projections. (D) Epha3KI/Efna3KI::Epha4+/−. (E) Epha3KI/Efna3KI::Epha4−/−. Nasal injections and Temporal injection.
modelling WT retinal Eph ligands expression along the nasal-temporal axis (Equations 1-4). The two-fold increase of Eph3 in Eph3KI/KI RGCs compared to WT (Figure 2C) was included into the model by adding a constant $D_{\text{LA3}}$ to $L_{\text{A}(x)}^{\text{retina WT}}$ thus generating the $L_{\text{A}(x)}^{\text{retina KI}}$ alternating ectopic expression in the Eph3 KI retinas (Equation 5, 6). The 3-step map alignment model simulates the

Figure 6 continued

collicular (n = 18 animals) (C) maps generated by the Leave-One-Out method at P15 and P8 respectively in Eph3KI/Ephna3KI double mutants. (D, E) Examples of retino-collicular projections for nasal and temporal retinal injections in P8 Eph3KI/Ephna3KI::Epha4+/− (n = 6 animals) (D) and Eph3KI/Ephna3KI::Epha4+/− (n = 6 animals) (E) mutants. Scale bars represent 200 μm. T, temporal; N, nasal; R, rostral; C, caudal; L, lateral; M, medial; RGCs, retinal ganglion cells.

The following figure supplement is available for figure 6:

Figure supplement 1. Retino-collicular map in P7 Eph3KI/+ mutants (n = 11 animals).

DOI: 10.7554/eLife.20470.016

Figure 7. Retinal Ephas quantification. (A) Section of P2 mouse retina (n = 6 retinas from three animals from different litters) hybridized with Epha5, Epha2 and Epha3 probes. Quantification was performed for 10 segments of the RGC layer along the nasal-temporal axis. Scale bar represent 100 μm. (B) Epha2/3/5 expression profiles (dots) fitted by the equations (lines) corresponding to equations (1), (2) and (3) respectively.

DOI: 10.7554/eLife.20470.017
sequential mapping of 100 RGCs onto a 1D array of 100 SC neurons along the rostral-caudal axis, followed by 100 V1 cortical neurons innervating the SC. We made two assumptions: (1) endogenous collicular EFNAs are no longer active for incoming V1 axons as they have been engaged previously in RGCs axon guidance by binding to retinal EPHAs (forward signaling) which leads to the cleavage of their extracellular domains (Janes et al., 2005), (2) retinal EFNA3 alone cannot provide positional information in the SC as its expression is not graded in the RGCs. Consequently, a proportion of graded retinal EFNA2/A5 acts together with retinal EFNA3 to provide positional information in the SC. After 10^7 iterations per run (n = 20 runs) for each genotype, stable and organized retino-collicular maps were formed (Figure 8A,D,G). Thereafter, a proportion of retinal Efn3 gradients were transposed in the SC (Figure 8B,E,H) following to the retinal projections layout and then the cortico-collicular maps are generated in a similar fashion (Figure 8C,F,I). To account for map heterogeneity observed experimentally, we further analyzed the maps generated using a linear regression (Figure 8C,F,I, red lines) and an exclusion parameter (EP) (Figure 8F,I, dashed grey lines), which corresponds to the variability of the WT single map (Figure 8C, dashed grey lines, σ_WT = 2.18 %) added to the genotype-specific average map separation (ΔS_exp) calculated in Efna3 KI animals (Figure 4E, EFna3KI/KI = 15.18%; EFna3KI/+ = 9.18 %; Figure 8F,I). The points, simulating the position of the cortico-collicular TZs along the rostral-caudal axis of the SC, located outside EP correspond to duplicated projections whereas the points located within EP correspond to single projections (Figure 8F,I). The percentages of duplicated projections generated by the model were similar to the percentages of experimentally measured duplications (Figure 4A,B) for both Efna3KI/KI and Efna3KI/+ animals (one sample t-test, EFna3KI/KI, p = 0.17; Efna3KI/+, p = 0.22; Figure 8J).

These results indicated that the 3-step map alignment model simulates both the retino- and cortico-collicular mapping and accurately recapitulates the normal and defective visual maps. It predicts the stochastic nature of the mapping abnormalities in Efna3KI/+ and Efna3KI/KI animals due to the Efna3 ectopic expression in a subset of RGCs. Hence the model provides further evidence that retinal EFNA3 contributes to the alignment of the cortico-collicular map by providing positional information in the SC for ingrowing V1 axons carrying EPHAs.

Discussion

EPHAs and EFNAs are present in opposed gradients within visual areas and in corresponding gradients between connected areas. These features preclude the identification of basic molecular mechanisms using full knock-out approaches. We therefore generated a unique and powerful mouse model in which gradients of EFNAs are quantitatively perturbed in the RGCs only. Together with theoretical modeling, we describe a molecular mechanism and associated principles governing the alignment of converging topographic neural maps in the brain.

Retinal EFNA3 in cortico-collricular mapping

We showed that modestly elevated expression of EFNA3 exclusively in a subset of RGCs disturbed cortico-collricular map alignment in the SC, pointing to a mechanism where retinal EFNA3 provides positional information to ingrowing V1 cortico-collicular axons. Further confirmation came from the genetic inactivation of ectopic EFNA3 using co-expressed EPHA3 receptor. EPHA3/EFNA3 coexpression in the same cell, including RGCs, abolishes their trans-binding to EFNAs and EPHAs (Connor et al., 1998; Hornberger et al., 1999; Menzel et al., 2001; Carvalho et al., 2006; Falivelli et al., 2013; Klein and Kania, 2014). We therefore generated double heterozygous mutants, Efna3KI/Epha3KI. The power of this approach resides in the fact that each individual Efna3KI/+ and Epha3KI/+ mutant shows robust visuotopic map abnormalities (Brown et al., 2000; Triplet et al., 2009). The presence of WT retino- and cortico-collricular maps in the Efn3KI/Epha3KI double heterozygous mutants provides compelling evidence that both EFNA3 and EPHA3 were inactivated in Isi2(+) RGCs. Further evidence of EFNA3/EPHA3 inactivation came from the presence of normal retino-collricular maps in compound mutants Efna3KI/Epha3KI::Epha4 knock-in/out mutants. Although we cannot rule out the occurrence of an altered expression/function of other yet unidentified molecules involved in mapping (e.g., semaphorins, L1) by ectopic EFNA3 expression, our results confirm the causal role of retinal EFNA3 ectopic expression on cortico-collicular alignment defects. They suggest that retinal projections play an instructive role in cortico-collricular map formation.
Figure 8. Theoretical analysis and modelling. (A–I) Retino- (A, D, G) and cortico- (C, F, I) collicular maps generated by the 3-step Map alignment model in WT (A, C), Efna3KI/KI (D, F) and Efna3KI/+ (G, I) after n = 20 runs and $10^7$ iteration/run. The parameters used for the retino-collicular modelling in the three genotypes are identical. Translated retinal Efn-As gradients (% of retinal expression: Efna2 = 100%, Efna3 = 100% and Efna5 = 80%) into the SC in WT (B), Efna3KI/KI (E) and Efna3KI/+ (H). Red lines in (C, F, I) represent the linear regression. Variability of the WT map in (C) is calculated by $\sigma_{WT} = \ldots$

Figure 8 continued on next page
The heterogeneity of the cortico-collicular phenotype in the Efna3 KI mutants, revealed by a mix of single and duplicated projections, is in accordance with previous data showing a variable penetrance of the retinotopic mapping abnormalities in constitutive or conditional Efna knock-outs (Feldheim et al., 2000; Cang et al., 2008; Suetterlin and Drescher, 2014; Owens et al., 2015). Such variable penetrance of the mutant phenotype can be explained by the stochastic nature of map formation driven by opposing forces resulting from EPHA signaling, which tends to separate neighboring RGCs through repulsion, and correlated neuronal activity, which tends to reinforce neighboring RGCs projections on adjacent target cells (Cang et al., 2008; Triplett et al., 2009; Owens et al., 2015). The general pattern of V1 collicular projections is consistent with the involvement of cortical EPHA receptor gradients (high-lateral to low-medial) (Cang et al., 2005) repelled by low-rostral to high-caudal EFNA gradients of retinal origin in the SC (forward signaling). In the Efna3 KI animals, retinal EFNA3 oscillation in the SC organizes neighbor-neighbor relationships of V1 and RGC Tzs locally through repulsion inducing a small distance of map duplication. Our model is consistent with a retinal-matching model, suggesting that retinal inputs are required for proper cortico-collicular mapping (Khachab and Bruce, 1999; Triplett et al., 2009; Cang and Feldheim, 2013).

In our model, retinal inputs carry molecular cues, EFNA3 and likely other retinal EFNAs present at the level of the Tzs to provide positional information for ingrowing V1 axons (Figure 9). EFNA3 could be detected on RGC axons by immunohistochemical staining on proximal and middle parts of the optic nerve but fell below detection limit in the distal part and in the SC. We cannot exclude that a proportion of collicular EFNAs also contributes to the cortico-collicular mapping process. Altogether, our data suggest that transposed retinal EFNAs in the SC instruct cortico-collicular map alignment and act together with correlated neuronal activity pattern shared between RGCs and V1 axons (Triplett et al., 2009; Cang and Feldheim, 2013). Triplett and collaborators (Triplett et al., 2012) suggested a gradient-matching model which posits that collicular EFNAs are required for the mapping of somatosensory inputs to the SC. These inputs behave similarly to the retino-collicular projections as they also require collicular EFNAs, although in different layers. In contrast, the requirement of SC-produced EFNAs for cortico-collicular mapping is unlikely. As mentioned by Triplett and colleagues, in this particular scenario, cortico-collicular projections in previously characterized Epha3KI/KI mutants would have led to a single TZ in the SC, leading to a mismatch between cortico- and retino-collicular maps. However, this was not observed (Triplett et al., 2009).

**Retinal EFNA3 in retino-collicular mapping**

The presence of non-duplicated retino-collicular maps in both Efna3KI/+ and Efna3KI/KI mutants, as revealed by DiI tracing, suggests that retinal EFNA3 does not play a significant role in the formation of this map, consistent with previous work on Efna3-null mutants (Pfeiffenberger et al., 2006). Several hypotheses have been raised as to how retinal EFNAs may participate in retino-collicular map formation using in vitro, ex vivo and in vivo approaches in mouse and chick. For example, EFNAs on RGC axons are activated by collicular EPHAs (reverse signaling), leading to axon repulsion (Rashid et al., 2005; Lim et al., 2008; Yoo et al., 2011) or branch inhibition in the SC (Yates et al., 2001). In our mouse model, this mechanism would generate a segregation between Isl2(−) and Isl2 (+) RGC axons in the SC, the latter being more repelled by collicular EPHAs. However, such a segregation did not occur, suggesting that retinal EFNA3 is not involved in such a repulsive mechanism by collicular EPHAs. In vitro and in vivo transfection in chick neurons suggested that retinal EFNAs bind to co-expressed retinal EPHAs in the same RGCs leading to inactivation/masking of the EPHA receptors rendering those axons less sensitive to EFNA3 binding in the target tissue (Connor et al., 1998; Hornberger et al., 1999; McLaughlin and O’Leary, 1999; Menzel et al., 2001; Yin et al., 2004;
In our model, this mechanism would lead to a stronger inactivation of the EPHA4/A5/A6 receptors in the \( \text{IsI2}(+) \) RGCs expressing high levels of EFNA3, compared to RGCs with normal EFNA3 expression level. This would create two population of RGCs with different EPHA signaling strength, similarly to the EphA3KI mutants (Brown et al., 2000; Reber et al., 2004; Triplett et al., 2009; Bevins et al., 2011). In this context, according to the Relative Signaling model (Brown et al., 2000; Reber et al., 2004; Bevins et al., 2011), duplicated retino-collicular maps (partial or full) should be observed. Our results rather indicate that EFNA3 ectopic expression does not inactivate endogenous co-expressed EPHA4/A5/A6 receptors in RGCs. Nevertheless, we showed specific inactivation of EPHA3 by ectopic EFNA3, suggesting distinctive interactions in cis between EFNA/EPHA pairs as previously observed (Yin et al., 2004; Falivelli et al., 2013; Klein and Kania, 2014). Conditional ablation revealed that high retinal EFNA5 on nasal RGCs axons prevents temporal RGC axons from targeting the caudal SC through fiber-fiber interaction (Suetterlin and Carvalho et al., 2006).

Figure 9. Schematic representation of the map alignment mechanism. RGCs axons in WT and Efna3 KI animals project to the SC during the first postnatal week and form the retino-collicular map through forward signaling activated by collicular EFNA3s and fiber-fiber interactions (left side). In both WT and Efna3 KI animals, the retino-collicular map is single and coherent. In WT and Efna3 KI, each RGCs axon is loaded with a different concentration of retinal EFNA3s (high-nasal, low-temporal) carried to the SC. Due to the coherence of the retino-collicular map, a smooth retinal EFNA3 gradient is formed in the SC in WT, whereas in Efna3 KI animals, \( \text{IsI2}(+) \) RGCs axons carry extra EFNA3 (in red) which creates an oscillatory retinal EFNA3 gradient once transposed in the SC (right side). During cortico-collicular mapping, V1 axons carrying EPHA receptors are facing a smooth gradient of retinal EFNA3s in the SC, leading to a single coherent map in WT. In Efna3 KI animals, V1 axons carrying EPHA receptors are facing an oscillatory gradient of retinal EFNA3s (due to ectopic expression of EFNA3 in \( \text{IsI2}(+) \) RGCs) which leads to the duplication of the cortico-collicular map (43% in Efna3KI/+ and 47% in Efna3KI/KI) through local neighbor–neighbor relationships of V1/RGCs TZs via repulsion. C, caudal; R, rostral; WT, wild-type; TZ, termination zone; RGCs, retinal ganglion cells.

DOI: 10.7554/eLife.20470.019
Drescher, 2014). In our mice, such a mechanism would have generated a local duplication between Isl2(+) and Isl2(-) RGCs or an extension of the TZs in the SC which was not observed, suggesting that retinal EFNA3 is not involved in fiber-fiber interaction. However, our results do not exclude the contribution of the fiber-fiber interaction mechanism to retino-collicular map development. Together with recent results (Suetterlin and Drescher, 2014), our data further suggest that retinal EFNA3 and EFNA5 present distinct effects on visuotopic mapping and imply a member-specific role of retinal EFNAs in map formation. This is in contrast with retinal EPHA receptors which are considered to be functionally interchangeable (Reber et al., 2004; Bevins et al., 2011).

Theoretical modelling further confirms mapping mechanism

Previous work modelled the stochastic nature of retino-collicular map formation based on the Koulakov model (Tsiganov and Koulakov, 2006; 2010; Owens et al., 2015). Here, we have substantially modified and expanded this algorithm using our measured Efna expression data instead of theoretical values and simulated the retina- and cortico-collicular mapping process sequentially. We assumed that retinal Efna3 expression alone cannot provide positional information, due to its homogeneous expression profile in WT RGCs. Therefore other EFNAs or other guidance molecules, either retinal or collicular, must participate in the regulation of map alignment. Retinal EFNA5 has been recently shown to participate in RGCs fiber-fiber interactions (Suetterlin and Drescher, 2014) but a given proportion may contribute to map alignment. We chose to retain 80% of the retinal EFNA5 level and 100% of retinal EFNA2/A3 levels in the algorithm. Moreover, contribution of endogenous collicular EFNAs to cortico-collicular map alignment seems unlikely as these mediated previous RGCs axons guidance (Janes et al., 2005), therefore collicular EFNA levels were not included in the V1 projection step of the model. The 3-step map alignment model replicates experimental features of both retina- and cortico-collicular maps observed in WT and Efna3 KI animals. It validates a stochastic mechanism of retinal-dependent molecular cues, involving EFNA3, providing positional information in the SC for V1 axons which then coordinate with correlated neuronal activity (Triplet et al., 2009) to align visuotopic maps.

These data raise important new questions as to the function of the different endogenous EFNAs in the formation of topographic maps, requiring additional development of cell-specific gene targeting approaches. From a functional standpoint, this new principle may serve as a general framework for sensory map alignment, where positional information, carried by a leading map, acts together with correlated activity, enabling precise adjustments of subsequent projection alignment.

Materials and methods

Generation of the Isl2-Efna3KI mice, animals and housing

The targeting construct containing a ribosomal entry site (IRES) followed by the mouse Efna3 ORF-SV40polyA and the selection cassette PGK-Neo was inserted by homologous recombination into the 3′ untranslated region of the Islet-2 gene locus as previously performed (Brown et al., 2000). The mouse line was generated by the Mouse Clinic Institute, project IR3483 (Ilkirch-France) in a C57/Bl6J background. Mice were hosted in a 12 hr/12 hr light-dark condition, fed ad lib. at the CNRS UMS3415 Chronobiotron (Strasbourg – France). All procedures were in accordance with national (council directive 87/848, October 1987) and European community (2010/63/EU) guidelines. Official agreement number for animal experimentation is A67-395, protocol number 01831.01 (M.R). Male and female C57/Bl6J Efna3KI, Epha3KI and Epha4KO mice and pups were genotyped by PCR from genomic DNA from tail biopsies as described previously (Dottori et al., 1998; Reber et al., 2004). Primers are available in Supplementary Files. Immunohistochemistry and mapping analyses were made blind to genotype.

Projections analysis/mapping

Anterograde and retrograde Dil (1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate), DiD (1,1′-dioctadecyl-3,3,3′,3′- tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate) and/or retrograde CTB-488 (Cholera Toxin B subunit-Alexa 488) labelling were performed as described (Reber et al., 2004; Triplet et al., 2009). Whole-mount SC were processed as described and TZs were plotted along the rostral-caudal axis on Cartesian coordinates (y axis) (Reber et al., 2004). For
Retino-geniculate and binocular tracings were performed as described in Claudepierre et al., 2008 and plotted on Cartesian coordinates (x axis). V1 cortices were photographed as whole-mount and focal injections plotted along the V1 lateral-medial axis (x axis) (Triplett et al., 2009). Retino-collicular and cortico-collicular maps were generated using non-parametric smoothing technique, termed LOESS smoothing (Efron and Tibshirani, 1991), to estimate the profile of the one-dimensional mapping either from retina to SC, or from V1 to SC. To estimate the variability in a mapping containing N data points, we repeated the procedure N times with N-1 datapoints, each time dropping a different datapoint. This is termed a ‘leave-one-out’ method and was used in the R Project for Statistical Computing (RRID:SCR_001905). The script is available in Source code 1 file. Retino-geniculate and binocular tracings were performed as described (Pfeifferberger et al., 2006) on Efn3Kt/Kt (n = 4) and WT (n = 3) P7 animals. Analyses of the cortico-collicular projections were performed using the thresholding function in ImageJ (RRID:SCR_003070).

**Immunohistochemistry**

Retinas, ONs, SCs and V1 cortices were dissected after animal perfusion with PFA4% in PBS1X, post-fixed O/N in PFA4%/PBS1X, cryoprotected in PFA4%/sucrose 30%/PBS1X for several hours at 4°C. Retinas and ONs were cryostat-sectioned (14–18 μm), SCs and V1 cortices were vibratome-sectioned (40 μm) and processed for immunohistochemistry. Experiments were performed at least three times. Briefly, sections were incubated in blocking solution (PBS 1X/BSA 1%/normal goat serum 10%) for 1 hr at RT then incubated with primary antibody O/N at 4°C in PBS 1X/BSA 1%/normal goat serum 1%. The following day, sections were washed (3 × 5’ in PBS 1X at RT) and incubated for 1 hr at RT with secondary Alexa-labelled antibodies in PBS1X/BSA 1%/normal goat serum 1%. After three washes at RT (3 × 5’ PBS 1X), slides were mounted in Aqua-Polymount (Polysciences Europe GmbH, Eppelheim, Germany) and visualized under a confocal microscope (Leica SP5 II, Leica Microsystems, Wetzlar, Germany). Sections were imaged using Leica LASAF software. Antibodies: anti-Is12 (1/400, ref. LS-C165303, LifeSpan Biosciences Inc., RRID:AB_2126601), anti-Efna3 (1/300, ref. LS-C6547, LifeSpan Biosciences Inc., RRID:AB_797145), anti-EphA3 (1/100, ref. LS-C150188, LifeSpan Biosciences Inc., RRID:AB_11187174), anti-Efna3 (ref. 36–7500, 1/200, Invitrogen, Invitrogen Co., RRID:AB_2533278), anti-synaptophysin (1/200, ref. S5768, Sigma-Aldrich), anti-Tau (1/200, MAB3420, Merck Millipore), anti-NF200 (1/200, ref. N5389, Sigma-Aldrich), anti-rabbit Alexa 488 (RRID:AB_10373124), anti-goat Alexa 594 (RRID:AB_10562717), anti-mouse Alexa 555 (RRID:AB_10561552) (1/500, Invitrogen, Invitrogen Co.).

**Quantitative RT-PCR**

V1 cortices, superficial layers of the SC and retinas were freshly dissected. Retinas were cut in three equal pieces along the NT axis (Nasal, Central, Temporal RGCs) and RGCs acutely isolated (Steinmetz et al., 2006; Claudepierre et al., 2008). Total RNA was extracted and quantified as previously described (Mathis et al., 2015). Briefly, relative quantification was performed using the comparative Delta Ct method. Triplicates were run for each sample and concentration for the target gene and for two housekeeping genes (hypoxanthine-guanine phosphoribosyl transferase – Hprt and glyceraldehyde 3-phosphate dehydrogenase – Gapdh) were computed. Primers are listed in Supplementary File.

**Retinal ganglion cell isolation**

P3/P4 retinas were freshly dissected and RGCs were isolated and purified (>99%). For details see Steinmetz et al. (2006); Claudepierre et al., 2008). Briefly, cells were cultured in Neurobasal medium (Gibco/Invitrogen) supplemented with (all from Sigma, except where indicated) pyruvate (1 mM), glutamine (2 mM; Gibco/Invitrogen), N-acetyl-l-cysteine (60 μg ml⁻¹), putrescine (16 μg ml⁻¹), selenite (40 ng ml⁻¹), bovine serum albumin (100 μg ml⁻¹); fraction V, crystalline grade), streptomycin (100 μg ml⁻¹), penicillin (100 U ml⁻¹), triiodothyronine (40 ng ml⁻¹), holotransferrin (100 μg ml⁻¹), insulin (5 μg ml⁻¹) and progesterone (62 ng ml⁻¹), B27 (1:50, Gibco/Invitrogen), brain-derived...
neurotrophic factor (BDNF; 25 ng ml\(^{-1}\); PeproTech, London, UK), ciliary neurotrophic factor (CNTF; 10 ng ml\(^{-1}\); PeproTech) and forskolin (10 μm; Sigma). After isolation, RGCs were either treated for RNA extraction or fixed with PFA4% 15′ at RT and processed for immunohistochemistry. Stainings were performed and cells were visualized as described above.

**Semi-quantitative in situ hybridization and gradient fitting of retinal Efna expression**

Analysis of the expression of the Efna was performed as previously described (Reber et al., 2004) on nasal-temporal 20 μm thick sections of P1/P2 WT retinas. Probes used were: mouse Efna2 (NM007909.3, 879 bp, pos. 387–1266), mouse Efna3 (NM010108, 791 bp, pos. 208–999) and Efna5 (NM207654, 696 bp, pos. 189–885). Experimental values (mean +SD, Efna2/a3/a5, for each Efna, n = 18 sections total, 3 sections/retina, from 6 retinas –2 left, 2 right- from 3 animals) were plotted along the nasal-temporal axis and fitted using MATLAB (RRID:SCR_001622).

**In silico replication of the duplication of the cortico-collicular map**

The Koulakov model in MATLAB (RRID:SCR_001622) (Tsigankov and Koulakov, 2006; 2010) was used to simulate the formation of both the retino- and cortico-collicular maps in the presence of an oscillatory Efna gradients in the target structure. Each brain structure (retina, SC, V1) is modelled as a 1-d array of 100 neurons (N) in each network. Two maps are generated: first, the map from retina to SC; second, the map from V1 to SC. Each map is modelled sequentially in the same way. This model consists in the minimization of affinity potential (E) which is computed as follows:

\[
E = E_{\text{act}} + E_{\text{chem}}
\]

At each step, this potential is minimized by switching two randomly chosen axons probabilistically according to the degree such a switch reduces the energy in the system by Delta E (ΔE). The probability of switching, p, is given by: \(p=1/(1 + \exp(4\Delta E))\).

\(E_{\text{chem}}\) is expressed as follows:

\[
E_{\text{chem}} = \sum_{i \in \text{TZS}} \alpha \left( [R_A(i) - R_A(j)][L_A(i') - L_A(j')] \right)
\]

where \(\alpha\) is the strength (\(\alpha = 200\)), \(R_A(i)\) and \(R_A(j)\) the receptor concentration in the retina and V1 at location \((i)\) and \((j)\), and \(L_A(i')\) and \(L_A(j')\) the ligand concentration at the corresponding position \((i')\) and \((j')\) in the SC. The contribution of activity-dependent process is modelled as:

\[
E_{\text{act}} = -\gamma/2 \sum_{i,j|\text{synapses}} C_{ij} U(r)
\]

where \(\gamma = 1\) is the strength parameter, \(C_{ij}\) is the cross-correlation of neuronal activity between two RGCs (or V1) neurons during spontaneous activity located in \((i)\) and \((j)\), and \(U\) simulates the overlap between two SC cells. Here, we use \(C_{ij} = \exp(-r/R)\), where \(r\) is the retinal distance between RGC \((i)\) and \((j)\), \(R = 0.11 \times N\), and \(U(r') = \exp(-r'^2/2d^2)\), where \(r'\) is the distance between two SC points \((i')\) and \((j')\), \(d = 3\) and \(N = 1\) to 100 neurons.

Receptor and ligand gradients were modelled as follows:

**Retinal EphA gradients** (Reber et al., 2004):

\[
R_A(x)_{\text{retina}} = 0.26 \exp(0.023x) + 1.05
\]

**Cortical V1 EphA gradients** (Tsigankov and Koulakov, 2006; 2010):

\[
R_A(x)^{\text{V1}} = \exp\left(-x/N\right) - \exp\left((x - 2N/(N)) + 1\right)
\]

**Collicular Efna gradients** (Tsigankov and Koulakov, 2006; 2010):

\[
L_A(x)^{\text{SC}} = \exp\left((x - N)/N\right) - \exp\left((-x - N)/N\right)
\]

\(L_A(x)_{\text{retina}}\) is the Efna gradient which was modelled by an exponential fitting of the in situ hybridization data. These retinal Efna gradients (80% of Efna5 expression and 100% of Efna2 and Efna3) are transposed to the SC for the simulation of the cortico-collicular map.

Retinal Efna gradients equation along the nasal (0) – temporal (100) axis (see Results):
L_A(x)^{retina WT} = L_{A5}(x)_{retina} + L_{A2}(x)_{retina} + L_{A3}^{retina}

L_A(x)^{retina WT} = (1/0.56) \times \exp(-0.014x) + (1/0.54) \times \exp(-0.008x) + 0.44

(Figure 7) where x = 1 to N and N = 100 representing the neurons along each axes (L-M in V1, N-T in retina). When transposed along the rostral-caudal axis in the SC (retina → SC), retinal Efna gradients are flipped along the x axis and become:

L_A(x)^{retina→SC} = (1/0.56)^*\exp(-0.014(100 - x)) + (1/0.54)^*\exp(-0.008(100 - x)) + 0.44

= 0.56^*\exp(0.014x) + 0.54^*\exp(0.008x) + 0.44

The oscillatory gradient was generated by randomly attributing to 50% of collicular TZs an over-expression of Efna3 (ΔL_{A3}) with ΔL_{A3} = 0.44 for homozygotes and ΔL_{A3} = 0.22 for heterozygotes. Iterations were ran for 10^7 epochs. 3-step map alignment model scripts can be found in Source Code two file.

Quantitative analysis of theoretical maps
To determine the amount of duplication that could be found in heterozygotes and homozygotes mutants a linear regression was calculated using implemented functions in Matlab. The residuals were then used to calculate the percentage of duplication. Duplications were considered when values were outside EP = 9.18% for Efna3KI/+ and EP = 15.18% for Efna3KI/KI, which corresponds to the averaged experimental distances measured between duplicated termination zones (ΔS_exp) to which was added the wild-type map variability (average of residuals, \(s_{WT} = 2.18 \%\), n = 20 runs). Twenty runs were performed and averaged to find the proportion of duplicated termination zones for Efna3 KI.

Data/codes availability

Acknowledgements
We are grateful to Dr. Feldheim for providing biological material from Efna3KO mice. We thank Dr. Sophie Reibel-Foisset, Laurence Huck and Noémie Charley-Adele (UMS 3415 CNRS – Chronobiotron, Strasbourg, France) for mouse colony management, Dr. Nick Bevins and Dr. Alexandre Charlet for technical help, Dr. Melinda Owens for providing the Kouakov/Triplett scripts in MATLAB and Dr. Alexandra Rebsam for providing a batch of DiD. ES, MP, AB and MR performed the experiments. SE generated the Leave-One-Out algorithm. GL and MR analyzed the Efna ISH quantitation. ES, SE and MR generated the theoretical modelling. ES, SE FWP and MR analyzed the results. MR wrote the manuscript with the active participation of all the authors. Material requests and correspondence should be addressed to MR email: michael.reber@inserm.fr. This work was supported by CNRS and University of Strasbourg – Institute for Advanced Study (MR).

Additional information

Funding

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<th>Funder</th>
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<td>Université de Strasbourg</td>
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The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Author contributions
ES, Data curation, Software, Formal analysis, Validation, Methodology; SJE, Conceptualization, Software, Validation, Methodology, Writing—review and editing; AB, MP, Data curation, Methodology; FWP, Conceptualization, Resources, Methodology, Writing—review and editing; GL, Resources, Methodology, Writing—review and editing; MR, Conceptualization, Resources, Data curation, Software, Formal analysis, Supervision, Funding acquisition, Validation, Investigation, Visualization, Methodology, Writing—original draft, Project administration, Writing—review and editing

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Ethics
Animal experimentation: All animal procedures were in accordance with national (council directive 87/848, October 1987) and European community (2010/63/EU) guidelines. Official agreement number for animal experimentation is A67-395, protocol number 01831.01 (M.R).

Additional files

Supplementary files
- Source code 1. Leave-one-out ‘Leave-one-out’ script in R.
  DOI: 10.7554/eLife.20470.020
- Source code 2. 3-step map alignment model 3-step map alignment script in MATLAB
  DOI: 10.7554/eLife.20470.021
- Supplementary file 1. Sequences of the primers used for genotyping and expression analyses.
  DOI: 10.7554/eLife.20470.022

References


