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Altered GABA and somatostatin modulation of proprioceptive feedback after spinal cord injury in lamprey

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GABA: Gamma-Aminobutyric acid

Abstract

While various changes occur after spinal cord lesions, their influence on functional recovery is generally unclear. We have shown changes in proprioceptor and locomotor network properties below lesion sites in the lamprey spinal cord. The proprioceptive system offers a particularly tractable model for analyzing these changes. Here, we have sought evidence for changes in neuromodulatory effects below lesion sites by comparing the effects of GABA and somatostatin, both of which are located around the edge cells, on proprioceptive responses in lesioned and unlesioned spinal cords.

Exogenously applied GABA significantly reduced or abolished bending-evoked responses in unlesioned animals. In lesioned animals bending-evoked responses were stronger and certain of the effects of exogenously applied GABA were reduced. However, blocking endogenous GABA with bicuculline significantly potentiated responses in lesioned but not unlesioned animals. This suggested that the potentiated responses in lesioned animals were nevertheless associated with stronger tonic GABAergic inhibition. There were significant differences in these effects when lesioned animals were separated on the basis of their degree of recovery: notably, bicuculline only potentiated responses in animals that recovered good locomotor function, suggesting a need for raised endogenous GABA levels. Somatostatin alone did not affect edge cell responses in lesioned or unlesioned animals, but in lesioned animals it reduced and thus further weakened the inhibitory effects of GABA.

There are thus multiple changes in sensory modulation in the lesioned spinal cord, and differences in these effects may influence the degree of recovery.

Keywords: lamprey spinal cord, functional recovery, mechanosensory neurons, neuromodulation, GABA, somatostatin.

Various anatomical and functional changes occur after spinal cord lesions (Muir and Steeves, 1997; De Leon et al., 1998, 1999; Edgerton et al., 2001; Pearson, 2001; Rossignol et al., 2001; Wolpaw and Tennissen, 2001; Dietz et al., 2002; Fawcett, 2002; Li and Bennett, 2003; Harkema, 2008; Lavrov et al., 2008; Marsh et al., 2011; Rossignol and Frigon, 2011; Roy and Edgerton, 2012). Approaches to restoring function ultimately require insight into these changes, but in many cases their underlying mechanisms and influence on recovery is unknown.

Neuromodulation has been studied as a potential mechanism for promoting functional recovery after spinal cord injury (see Rossignol, 2001; Parker, 2005). Neuromodulation offers the potential of evoking diverse but integrated changes in system components (e.g. Harris-Warrick et al., 1998). However, while neuromodulators have a range of effects in lesioned animals, the analysis of these effects is complicated by variability that can be introduced by the type of injury, time after lesioning, cell specific effects of neuromodulators, and potential state-dependent effects caused by interactions between modulators. Developing rational pharmacological strategies to restore locomotor function after injury requires an understanding of modulatory systems in the intact spinal cord (to identify candidate target systems) and also in the lesioned spinal cord (to identify any changes that will alter their effects).

The larval lamprey is a model system for examining regeneration and functional recovery after spinal cord lesions (Rovainen, 1979; Cohen et al., 1988; McClellan, 1994).

The larval transformation to the adult state begins after several years, and thus larvae offer a

developmentally stable system in which to investigate spinal cord function and plasticity (see Cohen et al 1988). We have used this system to examine changes below lesion sites, and have shown an increase in spinal cord excitability associated with changes in cellular and synaptic properties and synaptic ultrastructure (Cooke and Parker, 2009), and an increase in the strength of proprioceptive feedback (Hoffman and Parker, 2011). The proprioceptive system offers a particularly tractable model system for analyzing functional changes after lesioning, as the proprioceptive edge cells that are depolarized by stretch of the body during swimming are located on the lateral margin of the spinal cord and can be examined in response to imposed movements of the isolated spinal cord (Rovainen, 1979; Grillner et al., 1982, 1984; McClellan and Sigvardt, 1988). The edge cells function in the same way as mammalian muscle spindles (Vinay et al., 1996) and could contribute to the sensory entrainment of locomotion (McClellan and Sigvardt, 1988). In addition to receiving phasic locomotor-related inhibitory and excitatory inputs from the locomotor network (Vinay et al., 1996), the edge cells receive somatostatin and GABAergic inputs from neurons located around the central canal (Christenson et al., 1991). GABA and somatostatin are co-localized and both can hyperpolarize the edge cells (Christenson et al., 1991).

Here, we have used edge cell responses to imposed bending of the isolated larval spinal cord and their modulation by GABA and somatostatin to investigate if modulatory effects are altered by spinal cord lesions.

Experimental Procedures

Larval lampreys (*Petromyzon marinus*) between 100-130 mm were obtained from commercial suppliers (Acme Lamprey Company, Maine, USA). To perform spinal cord transections, animals were anaesthetized by immersion in MS-222 (300 mg/ml, pH adjusted

1 to 7.4), and a ~5 mm dorsal incision was made approximately 1 cm below the last gill to
2 expose the spinal cord. The spinal cord was then transected completely with iridectomy
3 scissors. Following transection, animals were kept at 23° C for 8-10 weeks (unlesioned
4 animals were also kept at this temperature). At this time most animals have recovered
5 locomotor function (McClellan, 1994), and the injury at the lesion site had healed completely.
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13 Locomotor function in unlesioned and lesioned animals was assessed from video and
14 electromyogram recordings and scored according to a six-point scale (see Cooke and Parker,
15 2009; Hoffman and Parker, 2011). EMG recordings were made by inserting bipolar teflon
16 insulated electrodes (0.075 mm in diameter) into the muscle under MS-222 anesthesia.
17 Electrodes were inserted approximately 0.5 cm rostral and caudal to the lesion site. After
18 recovery from anesthesia muscle activity was recorded as the animals swam in a plastic
19 aquarium (29 x 23.5 x 5.5 cm). Swimming activity was initiated by lightly pinching the tail or
20 head of the animal using serrated forceps. Swimming performance was scored independently
21 by two experimenters. For the extremes of swimming performance used in this analysis (i.e.
22 stage 1/2 and 5/6) there was total agreement on the rating.
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38 To examine movement-related feedback in the isolated spinal cord, lesioned and
39 unlesioned animals were anaesthetized with MS-222 and the spinal cord and notochord from
40 the trunk region (i.e. between the last gill and the start of the dorsal fin) were removed in
41 oxygenated lamprey Ringer at 4°C (Ringer contents: 138 mM NaCl, 2.1 mM KCl; 1.8 mM
42 CaCl₂; 2.6 mM MgCl₂; 4.0 mM D-(+)-glucose; 2.0 mM HEPES; 0.5 mM L-glutamine,
43 bubbled with O₂ and adjusted to pH 7.4 with 1 M NaOH). The isolated spinal cord and
44 notochord was pinned to a Sylgard lined chamber and bathed in lamprey Ringer at 9° C in all
45 experiments. This is a different temperature to that at which the animals were maintained.
46 This was due to competing temperature requirements: recovery is optimal at warmer
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temperatures (Cohen et al., 1999), whereas the isolated spinal cord survives better at lower temperatures (experiments are routinely performed at the temperature we used). As the lamprey is cold-blooded and lives in streams at temperatures from just above freezing to approximately 20° C, the differences in temperature should not introduce any significant **issues**. The spinal cord was fixed so that only approximately 1.5 cm of the rostral end could move (Fig. 1). The bending point was thus approximately 0.5 cm below the lesion site. A syringe tip (25-gauge) was attached to a vertical arm connected to a custom made motor that moved the vertical arm sinusoidally 1 cm to the left and right of center (this displacement was comparable to previously used bending parameters; Kiemel and Cohen, 2001; Tytell and Cohen, 2008; McClellan and Sigvardt, 1988; Hoffman and Parker, 2011). We have only used 1 Hz stimulation here as a previous study showed that 0.1-1 Hz stimulation revealed little frequency-dependent effect on bending responses in control and lesioned animals (Hoffman and Parker, 2011). 1 Hz stimulation also provided a physiologically-relevant stimulus within the range used previously (Kiemel and Cohen, 2001; Tytell and Cohen, 2008; McClellan and Sigvardt, 1988), and it allowed us to assess changes over repetitive trials in control and in the presence of modulators, while reducing the risk of introducing accumulative long-term changes that could occur if multiple stimulation frequencies were examined (Kiemel and Cohen, 2001). The syringe tip was bent to create a hook that was inserted through the ventral side of the notochord approximately 2 mm from the rostral end (i.e. the end free to move). Imposed sinusoidal movements of the isolated spinal cord/notochord that mimicked bending during swimming were programmed in MatLab. Bending trials lasted for 60 s, and consisted of the initial 20 s of no bending, 20 s of bending, and the final 20 s of no bending. These trials were repeated every 2 min, to give three trials in control, drug, or wash. We saw no cumulative change in bending responses over the repetitive trials.

Extracellular recordings were made from the surface of the lateral tract using glass suction electrodes to monitor movement-related sensory feedback (see Grillner et al., 1982; McClellan and Sigvardt, 1988). Movement-related responses in this region reflect the activity of edge cells, intraspinal mechanoreceptors with cell bodies on the lateral edge of the spinal cord (Rovainen, 1979). The lateral tract also contains the axons of descending brainstem neurons (Rovainen, 1979). While we cannot unequivocally identify edge cells from extracellular recordings, as the imposed movements were behaviorally relevant (1 cm deflections at 1 Hz) it would seem unlikely that non-proprioceptive axons running in the lateral tracts would have been activated as they would then also be activated by normal swimming movements. As the edge cells are stretch-sensitive the activation of edge cells with contralateral or ipsilateral projecting axons (Vianan di Prisco et al., 1990) can be determined from the response to stretch (ipsilateral axon) or compression (contralateral axon) relative to the side that the recording is made from. Recordings were made approximately 7 segments caudal to the bending point (we have seen no difference in effects at 3, 7, and 12 segments caudal to the bending point; Hoffman and Parker, 2011). The edge cells also have ascending axons (see Tang and Selzer, 1979). We have only examined descending effects here to match our previous analysis (Hoffman and Parker, 2011), but rostral or caudal bending that activates descending or ascending axons, respectively, show similar effects (McClellan and Sigvardt, 1988), although there is some controversy over whether there is a difference in the entrainment of locomotor activity at the rostral and caudal ends (McClellan and Sigvardt, 1988, Tytell and Cohen, 2008). In all recordings, the peak of the wavelength represented compression (i.e. bending of the spinal cord to the same side as the recording electrode), while the trough corresponded to stretch (i.e. bending to the contralateral side; see Fig. 1).

Activity evoked during bending was quantified by detecting spikes offline using pClamp9 software (Clampfit 9.0, Axon Instruments Inc.). The time of each spike was logged

1 along with the time of bending onset and offset. Analyses were then performed in Matlab (see
2 Fig. 2) to calculate the frequency of spikes, the peak phase activity (PPA; the phase of the
3 oscillation at which the highest instantaneous probability of a spike occurs relative to the
4 bending cycle), and the grouping of activity around the PPA (fPPA; this was calculated as the
5 number of spikes occurring within ± 150 ms of the PPA divided by the total number of spikes
6 in the cycle).

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Drugs were purchased from Sigma-Aldrich. They were dissolved in the Ringer's solution and bath applied for 10 min unless stated otherwise. The data presented here are taken from two batches of animals examined over a two year period, and does not include data from previous bending analyses (Hoffman and Parker, 2011). There is variability of effects generally in unlesioned (Parker, 2003; Parker and Bevan, 2007) and lesioned animals (Cooke and Parker, 2009; Hoffman and Parker, 2011). As a result in examining pharmacological effects we have not relied on assumptions that previous effects should have occurred but have repeated where necessary analyses that serve as a precursor for subsequent analyses (e.g. GABA with somatostatin or bicuculline with apamin): the control group for each analysis can thus differ. All graphs and statistical analyses were performed using GraphPad Prism. Statistical analyses were performed using Wilcoxon matched pairs test for paired comparisons or a Kruskal-Wallis test with a post-hoc Dunns test for multiple comparisons. The graphs show mean responses $\pm$ SEM.

50 Results

53 *GABA reduces bending responses in both lesion and unlesioned spinal cords*

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In unlesioned animals responses to bending showed adaptation over repeated cycles (ratio of 10th response to the 1st was 0.4 ± 0.08 for contralateral bending and 0.79 ± 0.33 for

1 ipsilateral bending; only contralateral adaptation was significant; Wilcoxon matched pairs
2 test, $p < 0.05$, $n = 9$; Fig. 3A). The peak phase activity (PPA) in unlesioned animals varied
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4 between experiments (418 ± 116 ms; Fig. 3Bi), which could reflect the recording of cells
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6 with different response patterns in different experiments. The fraction of spikes within 150
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8 ms of the peak phase activity (fPPA) was 0.56 ± 0.13 (Fig. 3Bii), which suggested some
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10 degree of phase locking around the peak activity in individual experiments. GABA (1 mM;
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12 Christenson et al., 1991) significantly reduced the response to ipsilateral and contralateral
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14 bending on both the first and tenth responses ($n = 9$, $p < 0.05$, Kruskal-Wallis; Fig. 2, 3A), an
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16 effect consistent with the inhibitory effect of GABA on the edge cells (Christenson et al.,
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18 1991). In GABA, the bending-evoked responses were abolished in 6 of the 9 experiments and
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20 thus its effects on adaptation, PPA, and fPPA could not be examined statistically due to the
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22 low sample size (see Fig. 3Bi, Bii for a summary of the effects where activity was evoked).
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24 The effects of GABA had recovered on wash-off for 30 min-1 h.
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32 In lesioned animals ipsilateral and contralateral bending-evoked responses were
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34 significantly potentiated compared to unlesioned animals ($n = 46$; $p < 0.05$, Kruskal-Wallis;
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36 compare Fig. 3A, C; see also Hoffman and Parker, 2011) and adaptation was not significant
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38 for either ipsilateral or contralateral bending (Fig. 3C). As in unlesioned animals, the 1st and
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40 10th contralateral bending-evoked responses were significantly reduced in 1 mM GABA ($n =$
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42 24, $p < 0.05$, Kruskal-Wallis; Fig. 3C), and recovered on wash-off for 30 min-1 h. The
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44 magnitude of the reduction of the contralateral bending-evoked responses by GABA did not
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46 differ significantly in lesioned or unlesioned animals ($p > 0.05$, Kruskal-Wallis). However, in
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48 contrast to contralateral bending-evoked effects, responses to ipsilateral bending were not
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50 significantly reduced by GABA in lesioned animals ($n = 24$, $p > 0.05$, Kruskal-Wallis; Fig.
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52 3C), suggesting a reduced GABAergic inhibitory effect. There was no significant effect of
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54 GABA on the rate of adaptation for either ipsilateral or contralateral bending (data not
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shown). The PPA was significantly reduced by GABA (from 459 ± 76 ms to 230 ± 84 ms, $p < 0.05$, Wilcoxon matched pairs test; Fig. 3Di), but the fPPA was not significantly affected (0.5 ± 0.08 compared to 0.49 ± 0.09 in GABA, $p > 0.05$, Wilcoxon matched pairs test; Fig. 3Dii).

The GABA_A receptor antagonist bicuculline potentiates bending responses in lesioned spinal cords

The larger bending-evoked responses in lesioned animals could reflect reduced endogenous GABAergic inhibition. This was examined using the GABA_A receptor antagonist bicuculline (10 μ M; Tegnér et al., 1993). In unlesioned animals bicuculline had no significant effect on bending responses ($p > 0.05$, Kruskal-Wallis, $n = 5$; Fig. 4A,Ci), suggesting against the antagonism of a significant basal GABAergic inhibition of the edge cells (bicuculline also failed to affect the centrally-generated phasic modulation of the edge cells in unlesioned adult animals; see Vinay et al., 1996). However, in lesioned animals bicuculline increased the ipsilateral and contralateral bending-evoked responses, with significant effects occurring at the 10th response ($p < 0.05$, $n = 14$, Kruskal-Wallis; Fig. 4B). This increase over repeated bending cycles resulted in facilitation of the response rather than the adaptation seen in control (Fig. 4B, Cii). Bicuculline did not affect the PPA or fPPA in unlesioned animals (data not shown). In lesioned animals it significantly reduced the PPA (from 486 ± 77 ms to 168 ± 69 ms, $n = 6$, $p < 0.05$, Wilcoxon matched pairs test; Fig. 4Di) but did not significantly affect the fPPA (from 0.35 ± 0.02 to 0.39 ± 0.03 , $n = 6$, $p > 0.05$, Wilcoxon matched pairs test; Fig. 4Dii).

These results suggested that despite the potentiated responses to bending in lesioned animals, the edge cells were subject to greater endogenous tonic GABAergic inhibition. This

seems paradoxical since bending-evoked responses are increased and GABA is less effective in lesioned animals, but endogenous GABAergic inhibition is higher. However, this could simply be explained by non GABA-related effects of bicuculline on calcium-dependent potassium (K_{Ca}) channels underlying spike frequency adaptation (Debarbieux et al., 1998). This effect could increase cellular excitability independently of any GABAergic antagonism. That bicuculline had no significant effect in unlesioned animals argued against a general blocking effect of bicuculline on edge cell K_{Ca} channels, but given that many changes occur after lesioning (Cooke and Parker, 2009) we cannot be sure that the sensitivity to this effect did not differ in lesioned animals. The potential effect of bicuculline on K_{Ca} channels was thus studied directly using apamin (2 μ M; Hill et al., 1992), which reduces the K_{Ca} -mediated sAHP (Hill et al., 1992). Apamin did not significantly affect bending-evoked responses in control or lesioned animals ($p > 0.05$, $n = 5$, Kruskal-Wallis; Fig. 4E), suggesting against a significant influence of the K_{Ca} channels on spiking. In the presence of apamin bicuculline was still able to significantly increase the response ($n = 5$, $p < 0.05$, Kruskal-Wallis; Fig. 4E), suggesting that the effects of bicuculline were not mediated through K_{Ca} channels.

GABAergic effects are influenced by the degree of recovery

These results suggest that there are changes in the GABAergic regulation of proprioceptive activity in lesioned animals. Animals can show varying degrees of recovery and most animals normally recover good locomotor function (assessed as stage 5 or 6 on a six-point functional scale; Cooke and Parker, 2009), but some animals can also show various dysfunctions (stage 1 or 2; see Hoffman and Parker, 2011). To examine whether the degree of recovery was associated with differences in edge cell modulation we examined the effects of

1 GABA in animals that recovered good (stage 5/6) or poor locomotor function (stage 1/2;
2 Cooke and Parker, 2009).
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5 The contralateral and ipsilateral bending responses in stage 1/2 animals (n = 16) and
6 stage 5/6 animals (n = 12) did not differ statistically ($p > 0.05$, Kruskal-Wallis; Fig. 5A),
7 suggesting that the potentiation of sensory responses does not simply correlate with the
8 degree of recovery (see Hoffman and Parker, 2011). However, responses in both stage 1/2
9 and stage 5/6 animals were significantly greater than those in unlesioned animals ($p < 0.05$,
10 Kruskal-Wallis; see Fig. 3A, Fig. 5A). In response to GABA, there was a significant
11 reduction of the 1st contralateral bending response in stage 1/2 (n = 8) and stage 5/6 animals
12 (n = 11, $p < 0.05$, Kruskal-Wallis; Fig. 5B, C), but while reduced the 1st or 10th ipsilateral
13 effects were again not significantly affected ($p > 0.05$, Kruskal-Wallis; data not shown).
14 However, while the 10th contralateral response was significantly reduced by GABA in stage
15 5/6 animals it was not significantly affected in stage 1/2 animals (compare Fig. 5B, C),
16 suggesting reduced GABAergic effects in poorly recovered animals. In addition, significant
17 effects of bicuculline were also only seen in animals that recovered to stage 5/6 (n = 8), not in
18 those that recovered poorly (n = 6; $p < 0.05$, Kruskal-Wallis; Fig. 5D, E), which suggested
19 that tonic GABA levels were only increased in the animals that recovered well.
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46 *Somatostatin inhibits GABA effects in lesioned spinal cords*

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50 The lateral edge of the spinal cord receives both somatostatin and GABAergic inputs
51 (Christenson et al., 1991). GABA and, less reliably, somatostatin can hyperpolarize the edge
52 cells (Christenson et al., 1991). The effects of somatostatin and GABA were thus also
53 examined sequentially here. In unlesioned animals GABA (1 mM) again significantly
54 reduced or abolished ipsilateral and contralateral bending-evoked activity ($p < 0.05$, Kruskal
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Wallis test, $n = 5$; Fig. 6A). Somatostatin ($1 \mu\text{M}$) applied after GABA effects had recovered on wash-off had no significant effect on the bending response ($p > 0.05$, $n = 5$, Kruskal Wallis test; Fig. 6A), and GABA applied a second time in the presence of somatostatin was able to significantly reduce activity to a comparable extent to that in the absence of somatostatin ($p < 0.05$, $n = 5$, Kruskal Wallis test; Fig. 6A). There was thus no indication of a significant effect of somatostatin on bending-evoked responses, and no indication of any interaction of somatostatin with GABA in unlesioned animals.

In lesioned animals ($n = 8$) the initial response to bending was greater and the effects of GABA were reduced (they were again only significant for contralateral, not ipsilateral bending responses (Fig. 6B). Somatostatin applied after wash-off of GABA again had no significant effect on edge cell response ($p > 0.05$, $n = 8$; Fig. 6B). However, when GABA was subsequently applied in the presence of somatostatin its significant effects on contralateral bending-evoked responses were now abolished ($p > 0.05$, $n = 8$, Kruskal Wallis test; Fig. 6B). An occluding effect of somatostatin on GABA-evoked inhibition thus occurred in lesioned animals.

Discussion

This study has examined the modulation of proprioceptive responses after spinal cord lesions in the lamprey. The results suggest that there are differences in neuromodulation in lesioned animals, and that some of these effects were associated with the degree of functional recovery.

The edge cells underlying proprioceptive responses have excitatory ipsilateral or contralateral inhibitory projections (see Fig. 7). As the edge cells are stretch-sensitive,

1 ipsilateral bending (i.e. bending toward the same side as the recording electrode) will
2 compress rather than stretch edge cells on that side (see Fig. 1). The evoked response to this
3 bending is thus assumed to reflect the activity of edge cells with crossing inhibitory axons
4 activated by stretch on the opposite side of the body (Viana Di Prisco et al., 1990). Responses
5 to contralateral bending will reflect the activation of excitatory edge cells ipsilateral to the
6 recording electrode (see Viana Di Prisco et al., 1990; see Fig. 7). GABA markedly reduced
7 all proprioceptive responses in unlesioned animals, but it had reduced effects in lesioned
8 animals as it did not significantly reduce ipsilateral bending-evoked responses. Conversely,
9 blocking endogenous GABA_A with bicuculline had significantly stronger effects in lesioned
10 animals suggesting greater endogenous GABA effects. Somatostatin had no direct effect in
11 lesioned or unlesioned animals, but in lesioned animals it blocked the effects of GABA,
12 suggesting the potential for regulation of GABA inhibition by the release of co-localized
13 somatostatin (Christenson et al., 1991).

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32 The reduced inhibitory effects of GABA in lesioned animals could reflect several
33 mechanisms. The effects of inhibitory transmitters coupled to chloride channels can change
34 as the chloride equilibrium potential is depolarized due to changes in potassium-chloride co-
35 transport (Boulenguez et al., 2010). However, if this effect was occurring here bicuculline
36 should have reduced and GABA should have increased excitability, opposite effects to those
37 seen here. Higher endogenous GABA levels after lesioning could have reduced the effects of
38 exogenously applied GABA, although there would need to be specific regional differences in
39 GABA levels to account for the differences in ipsilateral and contralateral bending-evoked
40 responses. There may be some difference in the effects of GABA_A and GABA_B-mediated
41 responses, but these remains to be determined. Finally, the reduced effects of GABA on
42 ipsilateral bending responses in lesioned animals could reflect a change in the properties or
43 numbers of GABA receptors on contralaterally projecting cells. Whatever the mechanism, the

1 differences in ipsilateral and contralateral bending-evoked responses suggest some functional
2 re-organization of the proprioceptive system after lesioning.
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5 In any integrated system changes in one property must be balanced by changes in
6 other properties. Disturbances in the interaction between spinal cord locomotor networks and
7 their inputs could lead to various motor dysfunctions (see Bennett, 2008). Potentiated
8 proprioceptive responses may reflect the increase in edge cell excitability (see Hoffman and
9 Parker, 2011). The stronger endogenous GABA inhibition suggested by the effects of
10 bicuculline may reflect a necessary compensation in response to this effect that regulates the
11 potentiated sensory inputs. The responses when endogenous GABA effects were blocked by
12 bicuculline could be very strong and lead to facilitating high frequency bursts of edge cell
13 activity, suggesting that the lesion-induced changes in the edge cells lead to the potential for
14 runaway proprioceptive excitation in lesioned (but not unlesioned) animals when GABA-
15 mediated inhibition is compromised. While a causal link has not been determined, the
16 reduced effects of bicuculline in animals that recovered poorly suggest that an increase in
17 sensory input (which occurred in animals that recovered well or poorly) may contribute to
18 poor recovery if it is not associated with increased endogenous GABA inhibition.
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40 The lack of effect of somatostatin was surprising, as it is reported to hyperpolarize
41 edge cells (Christenson et al., 1991). However, somatostatin inhibition was less reliable than
42 that of GABA, and unlike GABA its effects were revealed when the edge cells were activated
43 by the cord being stretched (see Christenson et al., 1991). These features all suggest a more
44 subtle and potential state-dependent role for somatostatin than for GABA. In our experiments
45 somatostatin alone had no significant effect on edge cell responses in lesioned or unlesioned
46 animals. However, in lesioned animals somatostatin occluded the effects of exogenously
47 applied GABA. This suggests that rather than a direct effect on the edge cells, somatostatin
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1 may instead modulate GABAergic inhibition. This will result in disinhibition, and thus
2 promote stronger proprioceptive inputs.
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8 *Functional effects*

10 While spinal cord excitability and sensory responses are potentiated below lesion sites
11 (Cooke and Parker, 2009; Hoffman and Parker, 2011), we ultimately need to know how these
12 effects influence recovery. In this respect we have so far been unsuccessful in that we have
13 been able to make few correlations between the functional changes and the degree of
14 recovery (Cooke and Parker, 2009; Hoffman and Parker, 2011). The lack of correlation may
15 reflect the marked variability of functional effects which may need larger sample sizes or
16 alternative analyses (see West, 2010; Aradi and Soltesz, 2002). We also have to consider that
17 the qualitative scoring of recovery is relatively subjective. The extremes of the scale that we
18 have used (stage 1/2 and 5/6) can be unequivocally defined, but there are almost certainly
19 going to be differences in mechanisms between and within these stages. The scoring scale
20 that we have used thus only allows us to relate the effects studied to differences in behavioral
21 performance. Relating effects to their underlying mechanisms will only be possible with
22 greater insight into the changes that occur below lesion sites (Cooke and Parker, 2009;
23 Hoffman and Parker, 2011).
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45 Our results show that even when performing a simple analysis in what is considered
46 to be a relatively simple system there are complex and wide-ranging changes. In unlesioned
47 animals GABA essentially abolished all edge cell responses. In lesioned animals it only
48 significantly reduced responses to contralateral bending. Why should responses to ipsilateral
49 bending be less affected by GABA in lesioned animals? As outlined above, the edge cells are
50 stretch-sensitive and have axons that project ipsilaterally and contralaterally (Rovainen,
51 1974). Bending ipsilateral to the recording site (i.e. compression; see Fig. 7) will reflect the
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activity of inhibitory edge cells with crossing axons, while responses to contralateral bending (stretch) will reflect the activation of edge cells with excitatory ipsilaterally-projecting axons (Viana Di Prisco et al., 1990). The edge cells also receive glutamatergic and glycinergic inputs generated centrally by ipsilateral and contralateral locomotor networks, respectively (Vinay et al., 1996; see Fig. 7). These effects will lead to increasing stretch on the inactive side of the body, which will eventually activate edge cells on that side. These edge cells will then provide ipsilateral excitation to promote activity in the previously silent half-center in that segmental network and contralateral inhibition to suppress activity in the active half-center (Viana Di Prisco et al., 1990). The inhibition of contralateral bending-evoked responses (i.e. to stretch) by GABA will suppress ipsilateral excitation on the stretched side, while the reduced effects of GABA on ipsilateral bending responses means that the inhibitory effect on the contralateral side will be less affected and thus relatively stronger. In lesioned animals the release of GABA will thus functionally introduce a bias to sensory-evoked inhibition over excitation. This may be a necessary adaptation to the increased excitability of spinal locomotor networks (Cooke and Parker, 2009; Hoffman and Parker, 2011) where added sensory-evoked excitation may be maladaptive. This is supported by the difference in bicuculline effects in stage 1/2 and stage 5/6 animals. The interactive effect of somatostatin offers a way of regulating this if needed, reducing the effects of GABA on contralaterally-evoked responses to increase ipsilateral excitation. The changes in modulatory effects of GABA thus offer the potential to regulate the relative degree of inhibitory and excitatory feedback to locomotor networks. Co-release of somatostatin (Christenson et al., 1991), possibly as a result of relatively intense activity of the GABA/somatostatin-containing cells located around the central canal (Verhage et al., 1991), thus offers a further regulatory level of proprioceptive activity.

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39 Figure 1: (A) Diagram of the experimental set-up. The isolated spinal cord is fixed to a
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41 Sylgard-lined chamber. The rostral region of the spinal cord (to left) is free to move and is
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43 attached to a motor driven in MatLab to generate sinusoidal movements of the spinal cord at
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45 1 Hz (compression corresponds to bending ipsilateral to the recording site, stretching to
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47 bending to the contralateral side relative to the recording site).
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56 Figure 2: Example of the Matlab bending analysis. (A) A 20 s bending frame in an
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58 unlesioned animal, and (B) after 10 min application of 1 mM GABA. (Ai, Bi) The time (x-
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axis) and phase as a fraction of 1 bending cycle (y-axis) of spikes occurring through the whole experiment. The bar shows when bending occurred and each black dot represents a spike. (Aii, Bii) Raster plot showing when spikes occurred relative to the bending cycle (shown in Biv). (Aiii, Biii) A normalized probability density function (PDF) (relative to the bending cycle) that models each spike as a Gaussian with a standard deviation of 10 ms and truncated at 50 ms. The solid line shows the mean value of the PDF and the dotted line shows the mean + 2 standard deviations. Note the difference in scale in Aiii and Biii.

Figure 3: The effects of GABA on bending-evoked responses in control and lesioned animals.

(A) The effects of 1 mM GABA on the 1st and 10th responses to bending contralateral and ipsilateral to the recording electrode in an unlesioned animal. The asterisk on this and other graphs represents significant effects at $p < 0.05$. Graphs showing the peak phase activity (PPA; Bi) and fraction of activity around the peak phase (fPPA; Bii) in unlesioned animals in control and 1 mM GABA. (C) The effects of 1 mM GABA on bending responses in animals recorded 8-10 weeks after a complete spinal cord lesion. Note the difference in the y-axis scale in 3A and C. Note also that in lesioned animals GABA did not significantly affect ipsilateral bending responses. Graphs showing the PPA (Di) and fPPA (Dii) in lesioned animals in control and in GABA. (Ei) Traces showing the effects of GABA in an unlesioned animal. (Eii) Traces showing the effects of GABA in a lesioned animal.

Figure 4: Blocking endogenous GABA with bicuculline potentiates bending-evoked responses in lesioned animals. (A) Graph showing the effects of the GABA_A receptor antagonist bicuculline (10 μ M) in spinal cords from unlesioned animals. (B) Graph showing the potentiating effects of bicuculline on bending-evoked responses in lesioned animals.

Traces showing the effects of bicuculline in an unlesioned (Ci) and lesioned animal (Cii).
Graphs showing the effects of bicuculline on the PPA (Di) and fPPA (Dii) in control and
bicuculline. (E) The effects of bicuculline applied after application of the calcium-dependent
potassium channel antagonist apamin (2 μ M). Note that bicuculline was still able to
potentiate bending-evoked responses after apamin application.

Figure 5: The influence of the degree of recovery on the modulation of bending-evoked
responses. (A) Graph showing bending response in animals that recovered poorly (defined as
stage 1/2) or well (defined as stage 5/6 animals; see Experimental Procedures). The
differences between these stages were not statistically significant. Graphs showing the effects
of GABA (1 mM) in animals that recovered poorly (B) or well (C). Note that the contralateral
bending-evoked response was not significant in stage 1/2 animals. Graphs showing the effects
of bicuculline (10 μ M) in animals that recovered poorly (D) or well (E). Note also that
bicuculline potentiation of the responses was absent in stage 1/2 animals.

Figure 6: The interactive effects of GABA and somatostatin on bending-evoked responses.
(A) Graph showing the effects of GABA (1 mM) and somatostatin (1 μ M) on bending-
evoked responses in unlesioned animals. Note that GABA significantly reduced responses on
its own, and in the presence of somatostatin, but that alone somatostatin had no significant
effect. (B) The effects of GABA, somatostatin, and somatostatin and GABA in lesioned
animals. Note that the effects of GABA were reduced in the presence of somatostatin as the
effects of GABA on contralateral bending-evoked effects were no longer significant in the
presence of somatostatin. (C) Traces showing the effects of GABA and somatostatin in a
lesioned animal.

Figure 7: Diagram showing the connectivity between the edge cells and the locomotor network. The large square boxes show half-center networks on the left and right side of the body. The active half-center causes contraction of that side (right) and stretches the contralateral side (left). The active networks send excitatory inputs (white circles) to ipsilateral edge cells, and inhibitory inputs (black circles) to contralateral edge cells. Edge cells on the inactive (stretched) side of the body send excitatory inputs ipsilaterally (i.e. to the inactive half-center) and inhibitory inputs contralaterally (to the active half-center).

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