Inverse modulation of motor neuron cellular and synaptic properties can maintain the same motor output

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Abstract

Although often examined in isolation, a single neuromodulator typically has multiple cellular and synaptic effects. Here, we have examined the interaction of the cellular and synaptic effects of 5-HT in the lamprey spinal cord.

5-HT reduces the amplitude of glutamatergic synaptic inputs and the slow post-spike afterhyperpolarisation (sAHP) in motor neurons. We examined the interaction between these effects using ventral root activity evoked by stimulation of the spinal cord. Whilst 5-HT reduced excitatory glutamatergic synaptic inputs in motor neurons to approximately 60% of control, ventral root activity was not significantly affected. The reduction of the sAHP by 5-HT increased motor neuron excitability by reducing spike frequency adaptation, an effect that could in principle have opposed the reduction of the excitatory synaptic input. Support for this was sought by reducing the amplitude of the sAHP by applying the toxin apamin before 5-HT application. In these experiments, 5-HT reduced the ventral root response, presumably because the reduction of the synaptic input now dominated. This was supported by computer simulations that showed that the motor output could be maintained over a wide range of synaptic input values if they were matched by changes in postsynaptic excitability. The effects of 5-HT on ventral root responses were altered by spinal cord lesions: 5-HT significantly increased ventral root responses in animals that recovered good locomotor function, consistent with a lesion-induced reduction in the synaptic effects of 5-HT, which thus biases its effects to the increase in motor neuron excitability.

Keywords: Spinal cord, neuromodulation, 5-HT, lamprey, spinal cord injury

Glossary

sAHP – slow afterhyperpolarisation

SiIN – small ipsilateral inhibitory interneuron

EIN – excitatory interneuron

Introduction

Neuromodulators confer behavioural flexibility by modifying the functional properties of hard-wired circuits. A single neuromodulator typically affects several cellular and synaptic properties (Buonomano and Merzenich, 1998; Harris-Warrick and Johnson, 2010). These effects can be synergistic (e.g. an increase in excitatory and decrease in inhibitory inputs) or antagonistic (e.g. increased presynaptic transmitter release combined with reduced postsynaptic sensitivity; see Harris-Warrick and Johnson, 2010; Lilvis and Katz 2013). The interactive effects of different modulators (Brezina, 2010) and multiple effects of a single modulator, together with the potential for concentration, time, and state-dependent influences (Power and Sah, 2008; Levitan and Levitan, 1988; Parker, 2015), provide the potential for considerable flexibility of modulatory effects.

5-HT is among the best studied neuromodulators. Its effects have been studied in detail in the spinal cord of several animals where both sensory inputs and motor outputs are affected (Schmidt and Jordan, 2000; Jordan et al. 2008). In the lamprey, 5-HT slows the frequency of fictive locomotor activity (Harris-Warrick and Cohen, 1985) as it does in most other systems (Jordan et al 2008; but see Sillar et al. 1998), although its effects differ somewhat in intact animals (Kemnitz et al. 1995; Becker and Parker, 2015). This network effect has been linked to a 5-HT-mediated reduction of a calcium-dependent potassium conductance underlying the slow afterhyperpolarisation (sAHP) following action potentials, which can influence spike frequency adaptation and increase neuronal excitability (Wallen et al. 1989). While this effect has been claimed to account for the changes in fictive and simulated network activity (e.g. Grillner et al. 1995; Grillner et al. 2005; Hellgren et al.1992), this conclusion is complicated by the wide range of cellular and synaptic effects of 5-HT. These include a hyperpolarisation of the resting membrane potential, a reduction of glutamatergic synaptic transmission, and an increase or decrease in inhibitory inputs (Biro et al. 2006; Buchanan and Grillner, 1991; Harris-Warrick and Cohen, 1985; Parker and Grillner, 1999, 2000; Parker, 2006; Svensson et al. 2001).

We have shown that the cellular and synaptic effects of 5-HT differ after spinal cord lesions (Becker and Parker, 2015). We aimed to determine the mechanisms underlying this change in 5-HT modulation by examining 5-HT effects on ventral root activity evoked by spinal cord stimulation in lesioned and unlesioned animals. This approach was chosen as it offers a simpler assay for 5-HT effects than fictive locomotion, where the marked variability

of the fictive output complicates analyses (McClellan, 1990; Wallen and Williams, 1984; Parker and Srivastava 2013). However, we found that 5-HT did not reduce the ventral root response in unlesioned animals as expected, despite a significant reduction of glutamatergic synaptic inputs. Here, we provide evidence that inverse effects of 5-HT on neuronal excitability and synaptic inputs can maintain the same motor neuron output, providing an example of interactive cellular and synaptic effects by a single modulator (Harris-Warrick and Johnson 2010). We also show that this interaction is altered after spinal cord lesions, consistent with a change in neuromodulatory effects after spinal injury.

Experimental procedures

Juvenile adult lampreys (Pertomyzon marinus) were obtained from commercial suppliers (Acme Lamprey Company, Maine, USA). All experiments were conducted under license of the UK Home Office (Animals Scientific Procedures Act 1986) and the approval of the local ethical committee. All attempts were made to minimise the number of animals used and any suffering.

Animals were anesthetized with MS-222 (300mg/mL, pH adjusted to 7.4) and the spinal cord and notochord were removed from the trunk region (i.e., between the last gill and the start of the dorsal fin) in oxygenated lamprey Ringer at 4°C (Ringer contents: 138 mM NaCl, 2.1 mM KCl; 1.8 mM CaCl2; 2.6 mM MgCl2; 4.0 mM D-(+)-glucose; 2.0 mM HEPES; 0.5 mM L-glutamine, bubbled with O2 and adjusted to pH 7.4 with 1 M NaOH). The spinal cord and notochord were pinned to a Sylgard lined chamber at 10°C and superfused with lamprey Ringer at 10°C.

The spinal cord was left attached to the notochord to prevent potential damage to the ventral roots upon isolation of the cord. Ventral root activity was evoked using an extracellular stimulating electrode placed on the dorsal surface of the spinal cord to cover the lateral tract on one side. 5-HT consistently reduces the amplitude of reticulospinal inputs from descending axons in this tract (Buchanan and Grillner,1991). Extracellular activity was recorded from an electrode placed on a ventral root 3-5 segments caudally to the stimulation electrode. A single stimulation of the dorsolateral tract to evoke a single or unpatterned burst of ventral root activity was given at 1.5-2 times the threshold needed to evoke a single ventral root spike: this stimulation was delivered ten times at a frequency of 0.1Hz. The stimulation strength and frequency of delivery was not altered once the experiment started. The cord-evoked ventral root activity was quantified from the peak of the averaged rectified and integrated activity over a period of either 50ms or 150ms after the stimulation (Ullström et al 1999; Cooke and Parker 2009).

Single or paired intracellular recordings were made from motor neurons and spinal cord interneurons using thin walled micropipettes filled with 3 M potassium acetate and 0.1 M potassium chloride. Motor neurons were identified by recording orthodromic spikes in a ventral root following current injection into their somata. Excitability was examined by injecting 100ms depolarising current pulses (0.5-2.5 nA) into the cells using discontinuous current clamp (DCC; sampling frequency between 2-3 KHz). The sAHP was assessed from

single action potentials evoked in motor neurons by 1ms depolarising current pulses using DCC. The sAHP amplitude was measured as the peak hyperpolarisation that occurred >10ms following the action potential. Synaptic inputs were evoked in motor neurons by stimulation of the dorsolateral column in the same way as used to evoke ventral root activity above. Unless stated otherwise, all cellular and synaptic properties in control and after 5-HT application were examined from a membrane potential of -65mV maintained using current injection in DCC (5-HT could hyperpolarise cells by 1-2mV). In some cases when the response of individual motor neurons was examined to cord stimulation, depolarising current injection was needed so that the synaptic input caused the cell to spike. In these cases the effects of 5-HT were examined at the same membrane potential before and after 5-HT application.

To examine the effects of 5-HT after spinal cord lesions, animals received a complete transection of the spinal cord approximately 1cm behind the last gill (see Cooke and Parker, 2009). Animals were then left to recover for 8-10 weeks. By the end of this period the incision site had healed and most animals had recovered full locomotor function (McClellan, 1994). Animals were scored behaviourally on a six point scale that ranged from stages 1-2 (no recovery of locomotor function) to stages 5 or 6 (almost complete or complete recovery; see Cooke and Parker 2009 for details). Once the swimming behaviour had been scored the animal was anaesthetised and the spinal cord removed for experiments as above.

Drugs were purchased from Sigma-Aldrich and applied to the isolated spinal cord by superfusion using a peristaltic pump. 5-HT (1-10uM) was superfused for ten minutes after it had replaced the Ringer superfusing the cord (the time needed for the 5-HT solution to replace the normal Ringer solution was determined from the time needed for a dye to fill the bath). Because the initial response to stimulation could vary markedly in different experiments, all values were normalised to the mean of the control values. Statistical analyses were performed using t-tests or a one-way ANOVA with a Tukey post-hoc test.

Model

A simple model was built in MATLAB using Simulink (Version 6.3 R14SP3). The simulation used a generic neuron to represent the motor neuron and a pre-synaptic neuron to represent the inputs stimulated extracellularly in this study.

The neuron block had a Na^+, delayed rectified K^+, leak K^+ current, and a calcium-dependent potassium channel (KCa). The resting potential for model neurons was -70mV. The peak value of the action potential was +50mV. Voltage dependent Na^+ and K^+ channels were modelled using Hodgkin-Huxley equations:

$$I_{Na} = \overline{G_{Na}} m^3 h (V - E_{Na})$$

$$I_K = \overline{G_K} n^4 (V - E_K)$$

$$I_{leak} = G_{leak} (V - V_{rest})$$

in which m, h and n were defined as:

$$\frac{dm}{dt} = \alpha_m(V)(1 - m) - \beta_m(V)m$$

$$\frac{dh}{dt} = \alpha_h(V)(1 - h) - \beta_h(V)h$$

$$\frac{dn}{dt} = \alpha_n(V)(1 - n) - \beta_n(V)n$$

These Rate Functions were defined as:

$$\alpha_m(V) = \frac{A_{\alpha_m}\left(V - B_{\alpha_m}\right)}{\left(1 - e^{\left(B_{\alpha_m} - V\right)/C_{\alpha_m}}\right)}$$

$$\beta_m(V) = \frac{A_{\beta_m}(B_{\beta_m} - V\ )}{\left(1 - e^{\left(V - B_{\beta_m}\right)/C_{\beta_m}}\right)}$$

$$\alpha_h(V) = \frac{A_{\alpha_h}\left(B_{\alpha_h} - V\right)}{\left(1 - e^{\left(V - B_{\alpha_h}\right)/C_{\alpha_h}}\right)}$$

$$\beta_h(V) = \frac{A_{\beta_h}}{\left(1 + e^{\left(B_{\beta_h} - V\right)/C_{\beta_h}}\right)}$$

$$\alpha_n(V) = \frac{A_{\alpha_n}\left(V - B_{\alpha_n}\right)}{\left(1 - e^{\left(B_{\alpha_n} - V\right)/C_{\alpha_n}}\right)}$$

$$\beta_n(V) = \frac{A_{\beta_n}(B_{\beta_n} - V)}{\left(1 - e^{\left(V - B_{\beta_n}\right)/C_{\beta_n}}\right)}$$

The Rate Constants were:

$$A_{\alpha_m} = 0.2 \times 10^6, B_{\alpha_m} = -45 \times 10^{-3}, C_{\alpha_m} = 1 \times 10^{-3}$$

$$A_{\beta_m} = 0.06 \times 10^6, B_{\beta_m} = -54 \times 10^{-3}, C_{\beta_m} = 20 \times 10^{-3}$$

$$A_{\alpha_h} = 0.08 \times 10^6, B_{\alpha_h} = -45 \times 10^{-3}, C_{\alpha_h} = 1 \times 10^{-3}$$

$$A_{\beta_h} = 0.4 \times 10^3, B_{\beta_h} = -41 \times 10^{-3}, C_{\beta_h} = 2 \times 10^{-3}$$

$$A_{\alpha_n} = 0.02 \times 10^6, B_{\alpha_n} = -45 \times 10^{-3}, C_{\alpha_n} = 0.8 \times 10^{-3}$$

$$A_{\beta_n} = 0.005 \times 10^6, B_{\beta_n} = -35 \times 10^{-3}, C_{\beta_n} = 0.4 \times 10^{-3}$$

Voltage dependent Ca^{2+} channels provide the main source of intracellular calcium for activating the KCa channels underlying the sAHP. These calcium channels were modeled to give action potentials with an early fast voltage dependent K^+ channel AHP, and a sAHP. Equations and parameters used to describe the N-type calcium channel and calcium-dependent potassium channel were based on previous studies (Booth et al. 1997; Huss et al. 2007):

$$I_{CaN} = \overline{G_{CaN}} m^2 h (E_{CaN} - V)$$

where the activation variable *m* and inactivation variable *h* are defined as:

$$\frac{dm}{dt} = \frac{m_\infty - m}{\tau_m}$$

$$\frac{dh}{dt} = \frac{h_\infty - h}{\tau_h}$$

m_∞ and h_∞stand for the steady state of *m* and *h*. They are determined by the following equations:

$$m_\infty = \frac{1}{1 + e^{(V - B_{m_\infty})/C_{m_\infty}}}$$

$$h_\infty = \frac{1}{1 + e^{(V - B_{h_\infty})/C_{h_\infty}}}$$

The K_{Ca} channel activation level was determined by the intracellular calcium level. The $[Ca^{2+}]_i$ increased proportionally to the size of inward calcium current, and decayed according to the time constant of removal:

$$\frac{d[Ca^{2+}]_i}{dt} = A_{Ca} I_{Ca} - \frac{[Ca^{2+}]_i}{B_{Ca}}$$

Where A_{Ca} is the intracellular influx rate of calcium, and B_{Ca} is the time constant of removal. The activation of calcium-dependent potassium channel was determined by:

$$z = \frac{[Ca^{2+}]_i}{[Ca^{2+}]_i + B_{KCa}}$$

B_{KCa} describes the half-activation concentration of the intracellular calcium pool.

The amplitude of sAHP was controlled by changing the influx rate of calcium and the time constant of removal of the intracellular calcium pool. The Ca^{2+}-dependent K^+ current

peaks and decays with a time course dependent on the Ca^{2+} influx; as Ca^{2+} load increases, peak amplitude increases and the current decay is slowed (Sah 1996). To increase the sAHP, the influx of calcium was increased by 50% and the decay rate of intracellular calcium pool was decreased by 50%: to decrease the sAHP the influx rate was decrease by 50% and the decay rate was increased by 50%.

The synapse block

An action potential in the Neuron Block triggered the Synapse Block to generate an excitatory postsynaptic potential (EPSP). The EPSP shape was determined by the PSP buffer. The initial EPSP Amplitude was set by a standard impulse response function $f(t)$:

$$EPSP = maxamplitude \times f(t)$$

Where $f(t)\ is$:

$$f(t) = \begin{cases} e^{-\tau_1 t} - e^{-\tau_2 t}, & t - t_1 < T \\ 0, & otherwise \end{cases}$$

This gave an exponential PSP decay. A synapse was defined by four parameters: the time when a spike was detected t_1, duration T, rise time constant τ_1, and decay time constant τ_2. The parameters of the impulse response function were:

$$Max\ amplitude = 0.004, \tau_1 = 0.1, \tau_2 = 0.3$$

Results

5-HT consistently reduces the amplitude of glutamatergic inputs from reticulospinal and excitatory interneurons (EIN) to motor neurons in the lamprey spinal cord (Buchanan and Grillner, 1991; Parker and Grillner, 1999), and thus seems to have a depressive effect on excitatory synaptic inputs. 5-HT also reduces the slow calcium-dependent potassium channel-evoked afterhyperpolarisation (sAHP) following an action potential, which leads to an increase in cellular excitability (Wallen et al. 1989). These effects have been studied in mature adults (Buchanan and Grillner, 1991; Parker and Grillner, 1999; Wallen et al. 1989). As the analysis here used juvenile animals, and effects in these animals can differ to those in adults (see Parker and Gilbey, 2007), we had to examine the cellular and synaptic effects of 5-HT to determine if there were any developmental differences in its effects. The synaptic input evoked by stimulating the dorsolateral column of the spinal cord was reduced by 1μM 5-HT to 62% of control (from 4.2 ±1.2 to 2.6 ±1mV, n=7, $p<0.05$, paired t-test; Fig. 1Ai,Aii). This is comparable, although slightly reduced, to the reduction in adults (reduction to ~40% for reticulospinal and EIN inputs to adult motor neurons (Buchanan and Grillner, 1991; Parker and Grillner, 1999)). 5-HT also reduced the sAHP to 75% of control (from 3.1 ±0.46 to 0.76 ±0.41mV, n=8, $p<0.05$, paired t-test; Fig. 1Bi,Bii). This is within the range reported in adults (48-85%; Hill et al 1992). As in adults, the reduction of the sAHP increased the excitability of motor neurons in response to 100ms depolarising current pulses (Fig. 1Ci,Cii).

The reduction of excitatory inputs to motor neurons by 5-HT was expected to reduce the ventral root response. However, 5-HT (1μM or 10 μM) did not significantly affect the peak integrated ventral root response evoked by stimulating the dorsolateral column of the spinal cord (Fig. 2Ai-Aiii; n=25, $p>0.05$, one-way ANOVA; n=3 increased, n=19 unchanged, n=3 reduced). Measuring the peak integrated response represents the summed activity over the measurement period and does not reflect differences in the distribution of the activity over this time period. In nine cases there seemed to be no obvious difference in the distribution of the rectified but not integrated ventral root activity, but in approximately half of the experiments in which there was no significant change in the peak integrated response, ventral root activity was altered by 5-HT to a smaller initial value that was longer lasting than the control response (n=10; Fig. 2Aii). We thus also measured the integrated response over the first 50ms after the stimulation to examine the effect of 5-HT on the peak response. There was again no significant reduction in the integrated response by 5-HT over this time (Fig. 2Ai-Aiii; n=25, $p>0.05$, one-way ANOVA). In contrast to the effects of 5-HT, the integrated

ventral root response was significantly reduced to 75% of control by bath application of glycine (1mM, n=10 of 12. P<0.05, one-way ANOVA; Fig. 2Ai), thus showing that ventral root responses evoked in this way could be reduced by decreasing motor neuron excitatability.

In addition to modulating excitatory glutamatergic inputs, 5-HT could also act on inhibitory inputs from small ipsilateral inhibitory interneurons (SiIN; Buchanan and Grillner 1988). A 5-HT mediated reduction of inhibitory inputs from these cells could reduce the effects of the reduction in excitatory inputs. The effect of 5-HT on SiIN-evoked synaptic transmission was thus examined by making paired recordings from SiINs and motor neurons. 5-HT (1μM) failed to affect the amplitude of the monosynaptic IPSP (n=17, p>0.05, paired t-test). However, its effects were more varied than on glutamatergic synapses, where 5-HT consistently reduces the input (Buchanan and Grillner 1991; Parker and Grillner 1999). In 40% of paired recordings from SiINs onto motor neurons, 5-HT increased the IPSP amplitude (n=7), in 40% it reduced it (n=7,), and it had no effect in the remaining three connections (Fig. 2B). When the connections were separated into these groups, there was a significant effect of 5-HT (p<0.05, one-way ANOVA), which the post-hoc test showed was due to a significant effect on the reduction of the IPSP amplitude.

The potential parallel reduction of feedforward inhibition from the SiINs by 5-HT could have offset the effects of the reduction in excitatory inputs to potentially leave the motor neuron excitation:inhibition ratio, and thus ventral root response, unchanged. This possibility was examined using strychnine (1μM) to block inhibition and thus allow the effects of 5-HT to be examined on excitatory inputs in isolation. Strychnine alone resulted in a significant increase in the ventral root response, suggesting that feedforward inhibition was evoked by cord stimulation (n=7, p<0.05; Fig. 2Ci-Ciii). However, when 5-HT was applied in the presence of strychnine there was again no significant change in the ventral root response when it was integrated over 50ms or 150ms after the stimulation (n=7, p>0.05, one-way ANOVA; Fig. 2Ci-Ciii). This suggests that a parallel reduction in inhibitory transmission did not account for the failure of 5-HT to affect the ventral root response.

As synaptic inputs were necessarily suprathreshold to evoke ventral root activity, a reduction of the sAHP after an action potential could also influence the motor neuron response. A large sAHP would act as a non-synaptic inhibitory influence by reducing excitability after the first spike. As a result, a reduction of the sAHP by 5-HT would be

equivalent to a disinhibition that could overcome the reduction of the excitatory synaptic input. The two best characterised effects of 5-HT, a reduction of excitatory synaptic inputs and of the sAHP, could thus have opposite effects on motor neuron responses, with the net effect potentially causing no net change in the ventral root output. To examine this we recorded intracellularly from motor neurons. Dorsolateral cord stimulation that was large enough to evoke at least two spikes in the motor neuron was used (in some experiments depolarising current injection was needed to allow the input to evoke spiking), and 5-HT was applied for 10 minutes. In 4 of 9 experiments spiking was abolished by the reduction of synaptic inputs, which removed the possibility of examining the influence of the sAHP, but in the other five experiments despite a reduction of the excitatory synaptic input by 5-HT (measured by alternating between supra and subthreshold stimulation; Fig. 3Aii) motor neuron spiking was maintained at the control level in three and increased in two experiments. Examining motor neuron activity throughout the stimulation period suggested that the influence of the cellular and synaptic effects of 5-HT differed over time: spiking was initially reduced or abolished in 3 of 5 experiments (Fig. 3Bi-Bii), but recovered to the control level or above towards the end of the 10 minute period (Fig. 3Biii).

While this experiment shows that the same level of spiking can be maintained in single cells despite the reduction in the synaptic input, in approximately half of the intracellular experiments spiking was reduced or abolished. This was a four-fold higher proportion than with ventral root recordings, where only 12% showed a reduction of the ventral root response in 5-HT. The difference in proportions of effects could relate to the differences introduced by monitoring summed ventral root activity or the activity in a single motor neuron. The influence of the inverse changes in the synaptic input and the sAHP was thus tested further on ventral root responses in the intact spinal cord by attempting to disconnect the two effects. The removal of the synaptic effect was attempted by reducing the synaptic input to motor neurons using CNQX (2-5μM). This reduced the synaptic input by approximately 40% (data not shown), an effect that should have allowed less scope for a reduction of the synaptic input and thus bias the effect of 5-HT to the increased excitability caused by the reduction of the sAHP. However, CNQX alone could reduce (n=4) or increase (n=5) the ventral root response, and 5-HT in CNQX also had variable effects (reduction in 2, increase in 3, no effect in 2; data not shown). The increase in CNQX alone was unexpected, but it could have related to the reduced feedforward activation of inhibitory interneurons, resulting in a relative increase in motor neuron excitability (see Fig. 2Ci-Ciii). Instead of

using the variable effects of CNQX, the influence of the sAHP was removed using apamin (Hill et al 1992). Apamin (1μM) reduced the sAHP from 2.7±0.5 to 0.9±0.3mV, n=3). While the sAHP was not abolished, its reduction should have biased the summed effect of 5-HT towards the influence of the reduction of the synaptic input. In the presence of 1μM apamin the ventral root response was reliably and significantly increased (n=8 of 8, $p<0.05$, one-way ANOVA; Fig. 4A, Bi-Bii), consistent with the excitatory effect of a reduced sAHP in motor neurons (Wallen et al 1989). In 6 of 8 experiments when 5-HT (10μM) was applied in the presence of apamin the ventral root response was significantly reduced ($p<0.05$; Fig. 4A, Bi,Bii). This result was thus consistent with the reduction of the sAHP opposing the effects of a reduction of the excitatory synaptic input.

Model of the interactive cellular and synaptic effects

We used a computer model to examine the relevant contributions of the synaptic input and sAHP reduction to the motor output by regulating both parameters separately (Fig. 5A-C). As expected, a reduction of the synaptic input without a change in the sAHP reduced the activity of the output motor neuron, whereas a reduction of the sAHP without a change in the synaptic input increased motor neuron activity (Fig. 5B). Combining varying reductions of the synaptic input with reductions of the sAHP resulted in effects that altered the motor neuron output relative to control. When the synaptic input was small and the sAHP large the motor neuron output was reduced, and when the synaptic input was large and the sAHP was small the motor neuron output was increased. A certain spiking level in the motor neuron could occur when the synaptic input was reduced by varying amounts providing the sAHP was also reduced (Fig. 5A,C). For example, a fixed output of three spikes in the motor neuron could occur with a synaptic input that ranged from 1.5-0.8mV, matching the ~50% reduction of synaptic inputs seen experimentally, provided the sAHP was reduced from its nominal value of 2 down to 0.4, matching the experimental range of the sAHP reduction by 5-HT (Fig. 5A).

Effects of 5-HT in lesioned animals

5-HT can have different modulatory effects on animals that have recovered from spinal cord lesions, in particular the reduction of glutamatergic synaptic inputs which is a very consistent effect in the unlesioned spinal cord is absent after recovery from spinal cord lesions (Becker and Parker 2015). We thus also examined the effects of 5-HT on ventral root responses in lesioned animals. In poorly recovered animals (stage 1 or 2; see Experimental Procedures) the effects of 5-HT above the lesion site matched that in unlesioned animals, no significant effect of 5-HT on the ventral root response (n=6; Fig. 6A). However, below the lesion site 5-HT significantly reduced the ventral root response (n=6, $p<0.05$, one-way ANOVA; Fig. 6A). In animals that recovered well (stage 5 or 6), 5-HT significantly increased the ventral root response both above and below the lesion site (n=9, $p<0.05$, one-way ANOVA; Fig. 6B). This may reflect the combination of the lack of a reduction of the synaptic input by 5-HT in lesioned animals (Becker and Parker 2015) with the significant reduction of the sAHP that occurred in animals that recovered well (Becker and Parker, 2015), changes that together will shift the effect of 5-HT towards the cellular modulation and thus increase motor neuron excitability. The relationship between the degree of recovery and the change in the ventral root response by 5-HT was shown by the significant correlations between recovery and the change in the ventral root response by 5-HT above ($r^2=0.3$, $p<0.05$) and below the lesion site ($r^2=0.70$, $p<0.05$; Fig. 6C).

Discussion

This study used a simple experimental approach to examine the effects of 5-HT on ventral root activity evoked by spinal cord stimulation. We expected that the significant reduction in excitatory synaptic input to motor neurons by 5-HT (Buchanan and Grillner, 1991; Parker and Grillner, 1999) would reduce the ventral root response. However, the ventral root response was usually unchanged by 5-HT, despite the significant reduction of the synaptic input. This seemed to reflect an increase in motor neuron excitability due to the reduction of the motor neuron sAHP by 5-HT (Wallen et al. 1989), which reduced the influence of the reduction in excitatory synaptic drive. A balance between these two effects could thus maintain the motor neuron and ventral root output at the same level despite the significant changes in cellular and synaptic properties.

We have only used stimulation of the dorsolateral column, and it is possible that this effect does not occur for stimulation in other regions. An obvious question is why multiple

cellular and synaptic modulatory mechanism would interact to have no overall effect on motor output. The issue with any exogenous application of a neuromodulator is that it removes the normal context of modulation. Under normal conditions, the two effects could be recruited separately as a result of the controlled spatial release of 5-HT. In rat basolateral amygdala, two calcium-activated potassium currents (I_{SK} and sI_{AHP}) influence the slow afterhyperpolarisation (sAHP; Power and Sah, 2008). Acetylcholine modulates the sAHP and thus spike frequency adaptation through a muscarinic inhibition of the sI_{AHP} and muscarinic potentiation of I_{SK}. The inhibitory effect on sI_{AHP} dominates during tonic bath application of ACh but the excitatory effect on I_{SK} dominates during short focal application onto the soma and proximal dendrites, suggesting a temporal and spatial dependence to the cholinergic modulation. Effects could also be concentration-dependent, possibly through differential receptor sensitivity. The sAHP modulation and the network effect of 5-HT may be mediated by 5-HT1A-like receptor (Wikström et al. 1995), while the reduction of glutamatergic transmission from reticulospinal axons is suggested to be mediated by a 5-HT1B/D receptor (Schwartz et al 2005). In the *Aplysia* bursting neuron R15, 5-HT has concentration-dependent effects on currents with opposite effects: low levels of 5-HT potentiate an inward-rectifying potassium current that slows the burst frequency, but higher levels of 5-HT activate a subthreshold calcium current that can override the potassium current to convert R15 from a bursting to a tonic spiking neuron (Levitan and Levitan, 1988). We have used 5-HT concentrations of 1 or 10µM, with 10µM being the higher range used in examining 5-HT effects (see Buchanan and Grillner 1990; Wikstrom et al 1995). We used this higher level to ensure that the lack of effect of 5-HT on ventral root responses was not due to reduced sensitivity to 5-HT in this developmental stage. Effects were the same with 1 or 10µM, suggesting no concentration-dependent differences over the range we have used. The different effects of 5-HT could also be selected in a state-dependent manner (Parker, 2015), where the current functional state of the cell or network could lead to a bias towards one or other effect. Finally, opposing effects like the sAHP and synaptic modulation may provide a mechanism that constrains single effects to prevent "over-modulation" by ensuring that the system does not go too far in one direction (Harris-Warrick and Johnson, 2010). In this case the sAHP-mediated changes in excitability could ensure that the reduction of the synaptic input does not cause the motor output to fail.

In addition to the separate selection of the two effects under different conditions, it is possible that they are designed to happen simultaneously, an effect that will shift motor

neuron excitability from a synaptic to a cellular driven mechanism. Maximization of information transmission per energy used is suggested to be an important functional principle in nervous systems. In the cerebral cortex and cerebellum the highest percentage of ATP use is associated with synaptic transmission (Howarth et al. 2012). Rather than being fixed, energy use may be modifiable. The combination of a reduction of synaptic transmission with the sAHP reduction shown here for 5-HT could provide an energetically favourable shift from synaptic to cellular-driven motor neuron activation that allows the same motor output to be generated from an approximately 50% smaller postsynaptic input.

An obvious question is how these effects would relate to the 5-HT-mediated network modulation. 5-HT reduces the frequency of fictive locomotion (Harris-Warrick and Cohen, 1985). If the motor neuron output remains constant in the presence of 5-HT, as suggested here, various changes in the network output, including the slowing of fictive activity by 5-HT, could still occur through premotor changes (motor neurons have been considered to be pure output elements of the network and thus to have no effect on patterning locomotor activity (Wallen and Lansner 1984), an assumption that probably needs revising (see Buchanan 1999)). The reduction of the magnitude of the initial response but longer-lasting spiking (Fig. 2Aii) may underlie longer-lasting bursts of motor neuron activity associated with slower swimming. Amongst other effects, 5-HT blocks calcium-dependent potassium channels (KCa) to reduce the sAHP and prolong NMDA-induced depolarisations in various cell types (Wallén et al.1989). Simulations of KCa channel effects can mimic the network modulation by 5-HT (e.g. Grillner et al. 1995; Hellgren et al. 1992), but the simulation only examined the sAHP modulation, and to simulate the experimental effect of 5-HT certain assumptions had to be made (e.g. removal of ipsilateral inhibition; Hellgren et al 1992 see also Meer and Buchanan 1992 for limitations of the KCa effect on the network). A later simulation (Kozlov et al. 2001) examined the effects of 5-HT on synaptic transmission from inhibitory and excitatory premotor interneurons (Parker and Grillner, 1999), and showed that these effects, with extrapolation of effects to connections between network interneurons, could also account for the network modulation. In combination the varied cellular and synaptic effects of 5-HT could increase or decrease the network output (Kozlov et al. 2001). The network effects of 5-HT are thus complex, and involves more than just modulation of KCa channels. These, and other, effects of 5-HT (Parker, 2006), could all influence the net change in the locomotor network output.

5-HT can have different effects after spinal cord lesions (Becker and Parker 2015). The 5-HT effects shown here also differed in the lesioned spinal cord. In animals that failed to recover locomotor function, effects of 5-HT above the lesion site effects matched those in unlesioned animals, but below the lesion site 5-HT now reduced the ventral root response. We do not have enough cellular data in poorly recovered animals to know if this reflects changes in the sAHP or synaptic modulation (over 80% of animals recover good locomotor function, and a larger sample size is needed to examine poor recovery). However, 5-HT can evoke a significant hyperpolarisation of the membrane potential below the lesion site in poorly recovered animals, an effect that could have contributed to the reduced ventral root response (Becker and Parker 2015). In contrast, in animals that recovered good locomotor function 5-HT significantly increased the ventral root response both above and below the lesion site. This may reflect the failure of 5-HT to reduce glutamatergic synaptic inputs while significantly reducing the sAHP amplitude in animals that show good recovery (see Becker and Parker, 2015), effects that will shift the effects of 5-HT towards the cellular modulation that increases motor neuron activity. This effect, as with other changes after lesioning (Cooke and Parker, 2009; Hoffman and Parker 2011), may be an adaptation that helps to increase the motor output in the face of the reduction in descending synaptic excitation.

While the ventral root response to dorso-lateral column stimulation was used as a simpler experimental system to reduce the number of factors that had to be considered to understand the 5-HT modulation, several aspects still complicate the analysis. Stimulation will evoke direct reticulospinal inputs to motor neurons, and potentially also excitatory and inhibitory inputs from reticulospinal feedforward activation of EINs and SiINs (this is suggested by the significant increase in the ventral root response in strychnine). 5-HT can affect transmission at all of these synapses. The sAHP will be reduced in motor neurons to increase their excitability, and if we assume that the sAHP is reduced in inhibitory and excitatory interneurons (Biro et al. 2006; but see Parker, 2006) interneuron excitability will be increased which could lead to activity-dependent synaptic changes that can be modulated by 5-HT (5-HT can convert the depression of EIN inputs to motor neurons into facilitation, but as thus occurs from a markedly reduced initial EPSP amplitude the excitatory input is still reduced; Parker and Grillner 1999). This could also influence the effect of the apamin experiment by altering the activity of any network interneurons activated by stimulation of descending axons, and thus change the input to motor neurons and the motor output (Jia and Parker, 2016; Parker, 2006). 5-HT could thus affect several properties, some that seem to

occur so consistently as to seem obligatory (reduction of glutamatergic inputs, reduction of the sAHP in motor neurons), and others that are variable (effects on resting membrane potential, inhibitory transmission, and on interneuron sAHP amplitudes; Parker, 2006). The more variable effects could allow for modification of the response under different conditions. This seemingly simple response thus seems open to a potentially wide range of modulation.

Finally, the balance between multiple effects of modulators and the variability of these effects may be needed to match the intrinsic variability of cellular and synaptic parameters (Buchanan 1993; Parker 2003). Just as a circuit can function with the relative balance between two or more variable parameters (Prinz et al.2004), normal modulation could occur through a relative balance of modulatory effects on two or more properties. For example, consider cellular property *x* that can have two variable values *x1* (high) and *x2* (low) that generate network output z as a result of *x1* and *x2* being balanced by property y that varies as *y1* (high) and *y2* (low): *z* can occur with a combination of either *x1+y2* or *x2+y1*. If modulation of the network (*z'*) occurred by acting only by reducing *x*, when *x* was in the low value state *x2*, there would be a reduction in the modulator effect that would prevent the network output from being altered to *z'*. If the modulator instead acts to reduce both *x* and *y*, in the case of *x2+y1* network modulation *z'* can occur through the effect on the high value parameter *y1*. We know that cellular and synaptic parameters can vary across a wide range. The multiple effects of modulators may reflect the need for a modulator to be as variable as the cellular properties that it acts on.

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Figure legends

Figure 1: (Ai) Graph showing the effects of 5-HT (1µM) on glutamatergic synaptic transmission onto motor neurons evoked by stimulation of the lateral tract. (Aii) Traces showing the effects of 5-HT (1µM) on synaptic transmission. (Bi) Graph showing the effects of 5-HT (1µM) on the slow afterhyperpolarisation (sAHP) in motor neurons. (Bii) Traces showing the reduction of the sAHP by 5-HT. (Ci, Cii) Effects of 5-HT (10µM) on motor neuron excitability in response to depolarising current pulses. On this and other graphs, * represents a statistically significant effect.

Figure 2: The effects of 5-HT on dorsolateral column stimulation-evoked ventral root responses. (Ai) Graph showing the lack of effect of 5-HT (1 and 10µM for 10 minutes) on the magnitude of rectified and integrated ventral root responses measured either over 50ms or 150ms after the stimulation, and the significant reduction by 1mM glycine. (Aii) Rectified traces of ventral root activity in control and 1 and 10µM 5-HT. (Aiii) Rectified and integrated traces in control and 1 and 10µM 5-HT. Unless stated otherwise the thicker line represents the control response. (B) The effects of 5-HT on inhibitory synaptic transmission from small ipsilateral inhibitory interneurons (SiIN) to motor neurons. The effects of 5-HT were variable: it could increase, decrease, or have no effect on synaptic transmission. These three effects are shown separately. (Ci) Graph showing the lack of effect of 5-HT on the integrated ventral root activity when inhibition was blocked by strychnine (1µM). (Cii) Rectified traces of ventral root activity in control, strychnine, and 1 and 10µM 5-HT. (Ciii) Rectified and integrated traces in control, strychnine, and 1 and 10µM 5-HT.

Figure 3: Effects of 5-HT on motor neuron excitability. (Ai) Traces showing the effects of 5-HT on synaptically-evoked spiking in motor neurons. Note that the spiking was not reduced despite the reduction in the summed synaptic input (Aii; gray trace shows the effect in 5-HT).

(Bi-Biii) Changing effects of 5-HT during bath application. (Bi) Control, (Bii) 5min after 5-HT had replaced the Ringer superfusing the cord, and (Biii) 8min after 5-HT.

Figure 4: Graph showing the effects of apamin (1μM) on ventral root responses and on 5-HT modulation. Apamin alone increased the ventral root response, while subsequent application of 5-HT (10μM) reduced it. (Bi) Rectified traces showing ventral root activity in control, apamin, and 5-HT. (Bii) Integrated traces of rectified ventral root activity in control, apamin, and 5-HT.

Figure 5: Model of the effects of the effects of 5-HT on the reduction of glutamatergic synaptic inputs and the sAHP. (A) Graph showing the range of motor neuron spike outputs for various combinations of changes in the amplitude of the summed synaptic input and the sAHP. (B) Traces showing the effects of individual reductions of the sAHP and synaptic input (given as percentage of control). (C) Traces showing that the same motor neuron output can be evoked from different combinations of a synaptic and sAHP reduction. As the synaptic input is decreased the output can be maintained by a reduction of the sAHP.

Figure 6: Graphs showing the effects of the effects of 5-HT (1 and 10μM) on ventral root responses in lesioned animals that recovered poorly or well. (A) The effects in poorly recovered animals above and below the lesion site, and (B) effects in animals that recovered well. Note the differences in 5-HT effects depending on the degree of recovery, especially for below lesion changes. (C) Graph showing the relationship between the degree of recovery and the change in the ventral root response by 1μM 5-HT relative to control above and below the lesion site.