Dopamine exerts activation dependent modulation of spinal locomotor circuits in the neonatal mouse

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Running Head: Dopaminergic modulation of locomotor networks is activity dependent

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Author Contributions
Dr. Patrick Whelan conceived and designed the experiments with assistance from Jennifer Humphreys. Jennifer Humphreys collected, and analyzed the data. Both Dr. Patrick Whelan and Jennifer Humphreys contributed to the writing and editing of the manuscript.
ABSTRACT

Monoamines can modulate the output of a variety of invertebrate and vertebrate networks including the spinal cord networks that control walking. Here we examine the multiple changes in the output of locomotor networks induced by dopamine (DA). We found that DA can depress the activation of locomotor networks in the neonatal mouse spinal cord following ventral root stimulation. By examining disinhibited rhythms, where the Renshaw cell pathway was blocked, it was found that DA depresses a putative recurrent excitatory pathway that projects onto rhythm generating circuitry of the spinal cord. This depression was D\textsubscript{2} but not D\textsubscript{1} receptor dependent and was not due exclusively to depression of excitatory drive to motoneurons. Furthermore, the depression in excitation was not dependent on network activity. We next compared the modulatory effects of DA on network function by focusing on a serotonin and a N-methyl-DL-aspartate evoked rhythm. In contrast to the depressive effects on a ventral root evoked rhythm, it was found that DA stabilized a drug evoked rhythm, reduced the frequency of bursting and increased amplitude. Overall these data demonstrate that DA can potentiate network activity while at the same time reducing the gain of recurrent excitatory feedback loops from motoneurons onto the network.

Keywords: Neuromodulators, CPG, Motor Systems
INTRODUCTION

Monoamines released onto spinal cord networks are critical for the expression and control of locomotion (Miles and Sillar, 2011). In contrast with the other monoamines, the role of DA in controlling spinal locomotor circuits has been largely neglected in the mammal. What we do know is that DA is released within the spinal cord during stepping activity (Gerin and Privat, 1998) to increase motor output and modulate sensory transmission in a variety of species (Barbeau and Rossignol, 1991; Schotland et al., 1995; McCrea et al., 1997; Millan, 2002; Madriaga et al., 2004; Han et al., 2007; Lapointe et al., 2009; Clemens et al., 2012). DA receptors and putative DA fibers projecting from the diencephalon (A11 area) are present in the ventral horn of the adult spinal cord (Yoshida and Tanaka, 1988; Ridet et al., 1992; Weil-Fugazza and Godefroy, 1993; Holstege et al., 1996; Qu et al., 2006), an area where motor circuits are located. Accordingly, mice with lesions of the A11 area show deficits in motor control (Clemens et al., 2006). Microdialysis measurements in both neonatal and adult rodents show release of DA and its metabolites in the ventral horn of neonatal rats during fictive locomotion (Gerin and Privat, 1998; Jordan and Schmidt, 2002). The key role for DA transmission in locomotion is highlighted by the fact that L-DOPA-elicited air stepping in intact neonatal rats is blocked by DA receptor antagonists (McCrea et al., 1997) and that D₁ agonists can promote stepping in adult mice (Lapointe et al., 2009). Previous work has emphasized that DA can modulate ongoing drug evoked locomotor rhythms (Schotland et al., 1995; Jiang et al., 1999; Whelan et al., 2000; Barrière et al., 2004). However, drug-evoked rhythmicity represents a rather defined state and we wished to contrast this modulation with a distinct mode of central pattern generator (CPG) activation.
Evidence over the last decade suggests that stimulation of recurrent collaterals from motoneurons activates spinal CPGs, (Mentis et al., 2005; Machacek and Hochman, 2006; O'Donovan et al., 2010) likely independently of the Renshaw circuit (Bonnot et al., 2009) (Delpy et al., 2008). While the function of this pathway is not fully understood, one possibility is that it could act to reinforce ongoing network activity. The gain of recurrent positive feedback circuits needs to be carefully controlled (Douglas et al., 1995; Staley et al., 1998). While the gain of the Renshaw pathway is controlled by postsynaptic GABAergic and glycinergic mechanisms (Windhorst, 1996; Gonzalez-Forero and Alvarez, 2005), less is known about the recurrent excitatory pathway. Machacek et al. (2006), showed that noradrenaline could promote excitability in a recurrent excitatory pathway. Interestingly 5-HT appeared to inhibit activity of this excitatory pathway.

Our previous work demonstrated an excitatory effect of DA on motoneuronal spike rates that were due to changes in intrinsic neuronal properties and increased glutamatergic synaptic transmission (Han et al., 2007; Han and Whelan, 2009). Overall, our data, and that of others, establish that DA has a net excitatory neuromodulatory effect on neurons within the ventral spinal cord. In our current work we show that DA exerts more complex neuromodulatory effects on rhythmic activity that are dependent on the mode of evoking CPG activity. We first examine the effects of DA on the modulation of ventral root evoked rhythmic activity, and establish that DA attenuates this activity. Our data suggest that DA inhibits an excitatory recurrent circuit via a D2 mediated signaling pathway. We then contrast the effects of DA on modulating an established NMDA and 5-HT locomotor rhythm. Contrary to the ventral root evoked rhythms, we establish that DA potentiates locomotor activity by boosting the amplitude of the evoked bursts and decreasing frequency. These data suggest that DA exerts complex activation dependent
modulation of rhythm networks and acts to decrease the gain of the recurrent excitatory pathway. A portion of these results have been published in abstract form (Humphreys and Whelan, 2011)).

MATERIALS AND METHODS:

Ethical Approval

Experiments were performed on Swiss Webster mice (Charles River Laboratories, Wilmington, MA), postnatal day 0 (P0) to P3 [weight 1.5 - 2.9 g; n = 37]. The animals were chilled by hypothermia, decapitated and eviscerated using procedures approved by the University of Calgary Animal Care Committee which following the guidelines published by the Canadian Council on Animal Care.

Tissue preparation

*Isolated spinal cord preparations:* The remaining tissue was placed in a dissection chamber filled with carbogenated (95% O₂, 5% CO₂) artificial CSF (aCSF) (in mM: 128 NaCl, 4 KCl, 1.5 CaCl₂, 1 MgSO₄, 0.5 Na₂HPO₄, 21 NaHCO₃, and 30 D-glucose). A ventral laminectomy exposed the cord, and the ventral and dorsal roots were cut. The spinal cord was transversely transected at T5 and was gently removed from the vertebral column to the level of the cauda equina. Once dissections were complete, the aCSF containing the isolated spinal cord was allowed to warm up to room temperature (~24 – 25°C) in the dissection chamber (~30 min). Then, the preparation was transferred to the recording chamber and superfused with carbogenated aCSF (concentrations similar to dissecting solution). The bath solution was then heated gradually from
room temperature to 27°C (Whelan et al., 2000). The spinal cords were then allowed another 30 min to recover in the recording chamber.

Isolated spinal cord with attached sciatic nerve: In some preparations, the sciatic nerve was carefully dissected out and exposed several millimeters away from the spinal cord. Caution was taken to ensure that the sciatic nerve was dissected in continuity with the parent ventral roots (L3-L6). All dorsal roots were cut.

Pharmacology

We investigated the effects of several dopaminergic (DAergic) agonists and antagonists on drug evoked and ventral root/sciatic nerve evoked rhythmic activity. In this study we targeted the D1-like (D1/D5) and D2-like subfamilies (D2/3/4) which are present within the mouse spinal cord (Zhu et al., 2007). Specific DA agonists used were: D1-receptor: SKF-81297 (20 µM; Tocris). D2 receptors quinpirole, (20 µM; Sigma-Aldrich). Additionally, we also investigated the effects of DA during entrainment of disinhibited rhythms that were generated by bath application of the glycine receptor antagonist strychnine (2 µM; Sigma-Aldrich), the GABA<sub>A</sub> antagonist picrotoxin (50 µM; Sigma Aldrich) and the GABA<sub>B</sub> antagonist CGP-35348 (CGP, 50 µM; Sigma-Aldrich) (Mandadi et al., 2009). Disinhibited rhythms are slow synchronous rhythms that occur after GABA and glycinergic receptors are blocked.

Electrophysiological recordings

Tight fitting suction electrodes were used to record neurograms from the right and left lumbar ventral roots two (L2) and from a single lumbar five ventral root (L5). A locomotor-like rhythm
exhibits a classic neurogram signature, evident by an alternating left/right L2 neurogram pattern, as well as an ipsilateral alternation between the L2 and L5 neurograms. When this pattern was observed we classified the evoked rhythm as locomotor-like. Neurograms were amplified (100 – 10,000 times), filtered (DC - 1 kHz), and digitized (Digidata 1322A, Molecular Devices, Sunnyvale, CA) for future analysis. All data collected were analyzed using custom written programs (MatLab, Math Works, Natick, MA; SpinalCore, A. Lev-Tov) and commercially available programs (Clampfit, Molecular Devices & Spike2, CED, Cambridge, UK). Rhythmic activity was elicited by bath application of drugs or by stimulation of the ventral roots or sciatic nerve (Whelan et al., 2000; Bonnot et al., 2009).

**Drug evoked locomotion:** In certain experiments, pharmacological agents were used to evoke rhythmicity (Jiang et al., 1999; Whelan et al., 2000). These drugs included N-methyl-D(L)-aspartic acid (NMA, 5 μM), and 5-HT (10 μM). To measure the modulatory effects of DA, 50 μM DA was added to the bath. Note this concentration of DA remained the same for all drug evoked and ventral root/sciatic nerve stimulation experiments and was selected based on concentrations used by several labs to elicit robust bouts of rhythmicity (Jiang et al., 1999; Whelan et al., 2000).

**Ventral root stimulation-evoked locomotion:** Square-wave stimulation trains were delivered to the ventral roots (L5 or L6) to antidromically activate spinal motoneurons (stimulus duration: 500 μs, trains: 4 Hz, stimulus intensity: 2 - 10 μA, train duration: 10 s). In some preparations, we antidromically stimulated the sciatic nerve (stimulus duration: 500 μs, trains: 4 Hz, stimulus intensity: 50-100 μA, train duration: 10 s). To determine the stimulus evoked response threshold (T), pulse trains were delivered at increasing intensities until a depolarization response could be detected (2 - 10 μA) in the ipsilateral L2 ventral root. Pulse trains were delivered once every
three minutes at a constant intensity throughout an experiment (Whelan et al., 2000). Control
rhythms were recorded for at least 15 min before pharmacological agents were added. For some
experiments, stimuli were delivered to the L5 ventral root (5 pulses, 50 - 100 µA, 200 µs
duration, at 4 Hz) and neurogram recordings were made from the L6 ventral root.

Data Analyses

Analysis of frequency and spectral power using non-stationary analysis techniques:

Spinalcore is a wavelet-based data analysis software developed by the Lev-Tov group (Mor and
Lev-Tov, 2007) that we have applied to neurogram signals recorded during fictive locomotion
induced by pharmacology and by electrical stimulation. Briefly, the pairs of neurogram signals
(Right L2 (RL2) - Left L2 (LL2)), were rectified, integrated, and low-pass filtered. The program
utilizes Wavelet Transformation (WT) and WT coherence approaches to analyze sections of the
processed neurogram. Spectrograms were constructed to graphically represent the frequency
components of the neurogram signals with respect to time. Illustrated in the spectrogram
representations, the x-axis represents the time course of the recording, in seconds; the y-axis
represents the range of frequencies where locomotor rhythms occur, from approximately 0.1 to 5
Hz. The colors that are plotted on the graph represent logarithmic power, which shows the
amplitude of a given frequency component (y-axis) at that time (x-axis) in the recording. High-
power regions are arbitrarily assigned “warm” colors (i.e., red and orange), and low-power
regions are assigned “cool” colors (i.e., green and blue). The spectrograms show which
frequencies are more strongly represented in the recording and how those frequency components
may change over the course of the recording. Once the spectrograms were produced, a high
power band representing the cross WT of the segmental L2 neurograms became apparent. This
band was then selected in its entirety as a region of interest for further analysis. Regions of
interest were segmented into 6 bins across the time course of the experiment, and analyzed to produce the following parameters: mean frequency, mean coherence, and mean power, for each bin. Bins were then averaged together, and resulting values were normalized to control conditions. Normalized values were calculated by dividing each sweep value by the maximum control value obtained in each experiment, leading to the generation of a mean control value hovering around 1 with an accompanying SD. Phase values were obtained for each experiment and plotted as a circular plot with length of the vector and phase (degrees) as variables (Oriana Software, Kovach Computing Services, UK). A Rayleigh test was performed to examine whether the distribution was random or clustered (Zar, 2009). Significant values were in the range of P < 0.001 to P < 0.05 (GraphPad Software Inc, La Jolla, CA).

Analysis of burst amplitude:

Burst amplitudes were calculated using Spike 2 software (CED, Cambridge, UK). Two ten minute segments of data were identified for analysis. One segment was taken immediately prior to drug administration while the other 10 minute segment was centered around the point where the DA drugs had the greatest effect. Neurograms were rectified, integrated and the peak amplitudes of the bursts were measured and binned (10 bins per 10 minute time segment). Data amplitudes were normalized to average control values for each experiment. A two way analysis of variance was performed to measure the DAergic effect across time (GraphPad).

RESULTS

DA modulation of ventral root evoked rhythmic activity.
In the neonatal rodent, antidromic stimulation of motoneuron axons can trigger alternating rhythmic activity, via non-Renshaw cell pathways (Mentis et al., 2005). We made use of this approach here to test the modulatory role of DA on recurrent pathway elicited rhythmic activity. We were able to evoke rhythmic activity recorded from segmental L2 and contralateral L5 ventral roots of the isolated neonatal mouse spinal cord by applying a brief train of stimuli to either the L5 or L6 ventral root (Mentis et al., 2005; Bonnot et al., 2009). First we established a regular rhythm (Fig 1Bi-ii; \( n = 16 \)). Stimulation of the L5/L6 ventral root evoked a slow tonic depolarization of electrotonic potentials that was potentiated with each successive stimuli of the stimulus train (Figure 1Bi). Similar to other reports (Bonnot et al., 2009) we found that evoking a rhythm using VR stimulation was much less reliable than using other approaches such as dorsal root stimulation or pharmacological activation (Whelan et al., 2000). In our hands, VR stimulation produced rhythmic activity in around 25% of preparations. The mean frequency of the rhythm was \( 1.51 \text{Hz} \pm 0.17 \text{SD} \) with a phase of \( 162^\circ \) and a vector length of \( 0.80 \) (\( n=16 \); Rayleigh’s test \( p<0.001 \); Fig 1Biii). Of the preparations that produced bouts of rhythmicity, 7 out of 16 produced coordinated (segmental L2, and L2-L5 locomotor activity; Fig 1Bi-ii), while the remaining 9 preparations produced segmental L2 alternation with little or no L5 bursting. Because of this we focused on analysis of segmental L2 alternating activity in this paper.

*Addition of DA abolishes a ventral root evoked locomotor rhythm.*

In our first set of experiments, we bath applied 50 µM DA, and found that the ventral root evoked rhythmic activity was abolished in all preparations (\( n=5 \); Fig 2A). In Figure 2C we produced a representative set of spectrograms illustrating ventral root evoked rhythmic activity following the addition of DA. The inhibition of the ventral root evoked response is not likely
due to hyperpolarization of the motoneurons since DA increased the tonic discharge of the L2 neurograms (Fig 2Aii). Also previous work has shown that 50 µM DA has a net depolarizing effect on motoneurons (Han et al., 2007). Washing with 500-700ml of regular aCSF restored the power and frequency of ventral root evoked rhythmic activity and slow depolarization of the electrotonic potentials to control levels (Fig 2Aiii; Fig 2D).

To confirm that these DAergic effects could be replicated following stimulation of a peripheral nerve versus a ventral root we applied a stimulus to a portion of exposed sciatic nerve where the ventral roots were left intact but the dorsal roots were cut (Fig 3A; n = 3). Stimulation of the sciatic nerve (5 pulses, 4Hz, 50-100 µA) resulted in rhythmic bursting from the recorded neurograms (3/3 preparations; Fig 3B). In a separate set of control experiments we cut the ventral roots following eliciting depolarizing burst and as expected the depolarizing response was suppressed (2 preparations; data not shown). Following bath application of DA, sciatic nerve stimulation could not produce any detectable rhythmic bursting (3/3 preparations; Fig. 3Bii).

Consistent with DA’s effect on ventral root evoked bursting, we observed an increase in tonic discharge in ventral root neurograms (compare Fig 3Bi with 3Bii).

It was found that a train of pulses applied to the ventral root could elicit long-latency depolarizing events with an average latency to peak of 143 ms (Bonnot et al., 2009). The emergence of these slow long-latency responses was found to be highly correlated with the ability of ventral root stimulation to elicit locomotion (Bonnot et al., 2009). Taking this into consideration, we placed a stimulating electrode at the tip of the L5 ventral root, and recorded long-latency depolarizing responses from the adjacent L6 ventral root which were superimposed on a slow depolarization (Fig 3Ci, 104 ms ± 26.74 SD, range: 71-175 ms, n = 3). In the presence
of DA these slow long-latency responses were completely blocked (Fig 3Cii), consistent with our
observation that DA abolished ventral root evoked rhythmicity.

Taken together, the most parsimonious conclusion is that the inhibitory actions of DA during ventral root evoked locomotion are on the recurrent collateral pathways which have access to the CPG network. We next designed a set of experiments to test whether DA’s actions were due to an inhibition of the excitatory recurrent collateral pathway.

*DA application disrupts ventral root evoked entrainment of burst activity in disinhibited cords.*

Stimulation of lumbar ventral roots with trains of pulses can entrain a purely excitatory rhythm, otherwise known as a disinhibited rhythm (Machacek and Hochman, 2006; Bonnot et al., 2009). These reports provide evidence for the existence of an excitatory recurrent collateral pathway having access to the CPG network. Consequently, we tested whether DA’s effects during ventral root stimulation are mediated, at least in part, by inhibiting transmission of an excitatory recurrent collateral pathway onto the CPG network. In the following experiments we tested whether DA can prevent rhythm entrainment of disinhibited rhythms.

After establishing a disinhibited rhythm by adding the glycine receptor antagonist strychnine (2 μM), GABA_A antagonist picrotoxin (50 μM), and GABA_B antagonist CGP (50 μM) to the isolated spinal cord (Mandadi et al., 2009), we measured the natural frequency of rhythmic bursts to determine the optimal frequency for the stimulus trains. Once the entrainment frequency was determined, we then used this frequency to apply a 5 pulse, 4 Hz stimulus (50-100 μA) to the L5 or L6 ventral root (Fig 4A). As illustrated in Fig 4, under control conditions, nearly all of the stimulus trains were able to entrain disinhibited bursting. However, when DA was added to the bath, the probability that each stimulus train successfully entrained the
disinhibited rhythm and evoked a burst was significantly reduced (Fig 4Bii, C, Eii; \( n = 6; P < 0.0001 \)). These data suggest that during DA application, stimulation of excitatory recurrent collateral pathways and subsequent transmission onto CPG networks is depressed.

Contribution of D₁/D₂ agonists to ventral root evoked locomotion

We next examined which DA-receptor families contribute to the inhibition of the presumed excitatory recurrent excitatory pathway. Given that Zhu and colleagues (2007) found a predominate expression of D₂-like receptors within the ventral horn and motoneurons of the mouse lumbar spinal cord, we hypothesized that activation of the D₂-like receptor family was involved in the DAergic inhibition of ventral root evoked rhythmic activity. After establishing a consistent ventral root evoked rhythmic rhythm, we bath applied either a D₁ or D₂ agonist in separate experiments to isolate which DA receptor family contributed to the DAergic inhibition of ventral root evoked rhythmic activity (Fig 5). Following bath application of the D₁ agonist SKF-81297 (20 \( \mu \)M), no observable changes in the normalized power or frequency of the ventral root evoked rhythm were observed (Fig 5A – D; \( n = 5 \)). However, upon application of the D₂ agonist quinpirole (20 \( \mu \)M) the ventral root evoked rhythm was significantly depressed in all experiments (Fig 5E – H; \( n = 5 \)). Specifically, we observed a 69% decrease in normalized power, suggesting that activation of the D₂-like family most likely underlies the effects seen during DA application. Wash with 800 ml of regular aCSF was unable to completely restore rhythmic activity to control levels.

DA modulation of rhythmic activity is state dependent
Given the unexpected depressive effects of DA on ventral root evoked locomotor activity it was important to compare DA’s modulatory effect on a rhythm evoked following bath application of 5-HT and NMA.

Addition of DA increases the stability of a 5-HT, NMA evoked locomotor rhythm.

Similar to previous reports, we observed that DA alters several aspects of rhythmic activity evoked by bath application of 5-HT and NMA (Whelan et al., 2000; Barrière et al., 2004). Figure 6B shows the bursting pattern recorded from the segmental L2 ventral root neurograms following bath application of 5-HT and NMA. Generally, the 5-HT, NMA-evoked activity was comprised of bouts of alternating bursts, with intermittent short pauses of tonic activity. Following application of DA for 10 minutes, the relatively unstable 5-HT, NMA-evoked rhythm began to convert into a more stable, alternating rhythm in 6/6 preparations. Normalized power scores increased following addition of DA \((n = 6; \, P < 0.05; \, \text{Fig 6Di})\). Burst amplitude significantly increased five minutes after the addition of DA and reached a peak 3 minutes later \((n = 6; \, P < 0.001 \text{ to } 0.05; \, \text{Fig 6E})\). As the rhythmic bursting pattern became more stable in the presence of DA, the frequency of the rhythm slowed considerably from an average of ~0.4 Hz to ~0.3 Hz \((n = 6; \, P < 0.001; \, \text{Fig 6Dii})\). To summarize power and frequency changes over time, we produced a representative spectrogram illustrating a 5-HT, NMA evoked rhythm following application of DA at two different time points (Fig 6C). The advantage of this approach is that it allows one to examine an experiment in it’s entirety. Overall, bath application of DA facilitated locomotion by increasing the burst amplitude, stabilizing the rhythm as indicated by an increase in power, and also decreasing the rhythm frequency, during bath application of 5-HT and NMA.
DISCUSSION

The main result of this paper is that DA exerts activation specific modulation of spinal cord CPG output. Our data suggest that DA, via a D\textsubscript{2} based signaling system, blocks a recurrent excitatory pathway that likely projects onto the CPG (Fig. 7). On the other hand, when the CPG is already active, DA promotes a more regular rhythm and increases burst amplitude.

Control of recurrent excitatory feedback by dopamine

Several studies illustrate that stimulation of recurrent collaterals from motoneurons can elicit network activity (Wenner and O’donovan, 1999; 2001; Hanson and Landmesser, 2003; Mentis et al., 2005; Bonnot et al., 2009). Our work shows that DA inhibits rhythmic activity evoked from recurrent collaterals. Several lines of evidence support this conclusion. First we found that DA reversibly abolished long-latency potentials evoked following ventral root stimulation. Bonnot and colleagues found that ventral root stimulation evoked similar slow long-latency potentials, likely through a polysynaptic pathway, and that the presence of the potentials correlated highly with the ability to elicit locomotion (Bonnot et al., 2009). DAergic inhibition of these long-latency potentials are not dependent on a rhythm being evoked, suggesting that the pathway may lie outside the CPG itself. Second, when fast inhibitory transmission was blocked, entrainment of the excitatory rhythm by ventral root stimulation was reduced by DA. This suggests that DA is acting on an excitatory pathway that can access the CPG and rules out a role for Renshaw cells under these conditions. Our data does not exclude a role for Renshaw cells when inhibitory blockers are absent. DA could disinhibit GABA/glycinergic projections onto Renshaw cells allowing them to inhibit motoneuronal drive. It has been demonstrated that Renshaw cells normally receive inhibitory drive during the active portion of L2 rhythmic bursting in the
neonatal mouse (Nishimaru et al., 2006). However, there is no data suggesting selective
depression of DA on inhibitory pathways within the spinal cord, and evidence suggests that
Renshaw cells receive both excitatory and inhibitory inputs during locomotor activity
(Nishimaru et al., 2006). While we do not know the mechanism for the DA evoked depression a
possible candidate is depression of EPSCs from recurrent collaterals onto postsynaptic cells.
Depression of evoked EPSCs from dorsal root afferents onto motoneurons by DA has been
observed in neonatal rats accompanied by an attenuation of short-term depression suggesting a
presynaptic locus (Lev-Tov and Pinco, 1992; Barrière et al., 2008).
A possible need for the regulation of the excitatory pathway may be because DA and other
monoamines tend to increase the input-output ratio of motoneurons (Rekling et al., 2000). For
example DA boosts the spike frequency of motoneurons by increasing excitatory glutamatergic
transmission, decreasing $I_A$, and decreasing the $SK_{ca}$ based conductances in neonatal mice (Han
et al., 2007; Han and Whelan, 2009). The net increase in motoneuron spike frequency following
DA administration could explain the need to decrease the gain of the recurrent excitatory
pathway. In other areas of the brain where recurrent positive feedback exists, such as the CA3
region of the hippocampus (Bains et al., 1999) or neurons in the cortex (Douglas et al., 1995),
gain is regulated to ensure that the dynamic firing range of neurons is maintained.
Our work shows that the DAergic inhibition of the recurrent excitatory pathway is at least partly
mediated by D$_2$ - like receptor mechanisms. This concurs with evidence showing expression of
D$_2$ receptors within the ventral and dorsal horns of the mouse spinal cord (Zhu et al., 2007). Our
work has not identified the downstream D$_2$ mediated signaling mechanism but one possibility are
the metabotropic glutamate (mGluR) 1 class of receptors which have been shown to contribute to
the excitatory ventral root activation of locomotor networks (O'Donovan et al., 2010). Evidence
suggesting this as a possibility comes from the basal ganglia where mGluR activity is closely
regulated by D₁ and D₂ receptors (Conn et al., 2005). The depressive effects on recurrent
excitatory pathways using D₂ agonists are consistent with the DA and D₂ agonist effects on
afferent transmission within the spinal cord (Millan, 2002; Gordon and Whelan, 2006). Recent
work has found a concentration dependent effect of DA on spinal circuits in Xenopus embryos.
Specifically they demonstrated that low doses of DA preferentially activated D₂-like receptors
due to the higher binding affinity of DA to the D₂ receptors. Higher doses then activated the D₁-
like system producing an excitatory effect on CPG function and overcoming the D₂ inhibitory
effects (Clemens et al., 2012). However, in the excitatory recurrent pathway, it appears that it is a
primarily a D₂ based signaling pathway and the D₁ system has a minor role to play in modulating
the rhythm.
Ventral root afferents in mouse have been found to contain C fibers (Biscoe et al., 1982), but
their functional role and connectivity within the spinal cord remains ambiguous (Hildebrand et
al., 1997). While C-fibre afferents appear to be present in the ventral roots, it is not clear where
they enter the cord, and evidence suggests that few if any fibers are observed at the PNS/CNS
border (Hildebrand et al., 1997). Also latency measurements following antidromic activation of
ventral roots in Renshaw Cells did not show evidence for long latency EPSPs which would
suggest C fibre activation (Mentis et al., 2005). Another possibility for ventral root bursting
discussed by O’Donovan and colleagues is that the ventral root stimulation may elevate
extracellular $K^+$ concentrations in the ventral horn (Marchetti et al., 2001; Bonnot et al., 2009).
In this scenario, D₂ agonists would inhibit network interneurons countering the effects of
increases in extracellular $K^+$ in promoting bursting. However the kainate/AMPA glutamate
receptor blocker NBQX could block ventral root evoked activation of the network, while
permitting dorsal root evoked rhythmic activity, suggesting that the network itself was not in a
dormant state (Bonnot et al., 2009). Similarly, in the same report it was found that mGluR1
antagonists could depress the rhythm. Thus, the most parsimonious hypothesis is that the
observed excitatory responses are caused by recurrent collaterals projecting via interposed
interneurons onto the CPG.

**Dopaminergic modulation of 5-HT/NMA locomotor rhythms.**

Given that the inhibitory actions of DA on the ventral root evoked rhythm were mediated by the
D₂ family of receptors, we reexamined whether similarities would be observed in the DA
modulation of drug evoked activity. The DA effects on the drug evoked rhythm were
qualitatively different. We observed that DA decreased the frequency, and increased the
amplitude of the rhythm. These data are similar to what was described for the mouse (Whelan et
al., 2000; Madriaga et al., 2004). The modulatory effects of DA on locomotion are qualitatively
similar to that observed in other species (Grillner, 2003; Barrière et al., 2004). Less is known
about the excitatory mechanism in the mouse. However, in motoneurons intracellular recordings
show that DA can increase the input resistance, lower the AHP (via SKCa dependent
mechanisms) and decrease IA conductances (Han et al., 2007). Collectively, these mechanisms
substantially accelerate spike firing in motoneurons and increase the slope of the F-I relationship.

**Relation to actions of other monoamines**

It is interesting that a closely related catecholamine, noradrenaline (NA), has different effects
compared to DA (Machacek and Hochman, 2006). In contrast to the effects of NA (Machacek &
Hochman, 2006), DA appears to decrease the gain of the recurrent excitatory pathway. In this
regard, DA appears to share an inhibitory regulatory role with 5-HT (Machacek and Hochman, 2006). These data also suggest that the actions of DA are not likely due to conversion to NA and are also supported by the replication of DA’s effects by D₁ and D₂ agonists. Differential effects of monoamines have been observed in many areas of the brain and spinal cord as well as invertebrate networks (Miles and Sillar, 2011). For example, NA and 5-HT were observed to mutually enhance group II afferent transmission but were also observed to act in an antagonistic fashion (Jankowska et al., 2000). When one considers commissural interneurons, monoamines have been shown to have contrasting effects depending on the pathway being activated (Hammar et al., 2004). For example 5-HT facilitated group II input onto these cells, but NA inhibited transmission. On the other hand both 5-HT and NA facilitated reticulospinal input onto commissural interneurons. 5-HT has been shown to have heterogeneous effects on mouse commissural interneurons depending on whether they descended or bifurcated after crossing the midline (Zhong et al., 2006).

**Functional Considerations**

The modulatory effects of DA on motoneurons are biased towards increasing the input-output spike rates. Overall this acts to robustly promote rhythmic motor output. A possible consequence is that the gain of the recurrent excitatory pathway from motoneurons onto rhythm generating populations of interneurons could increase to a point where the drive could lead to destabilization of the rhythmic pattern. However, it is important to emphasize that the functional effects of DA will likely differ depending on several variables, concentration at the synapse, intrinsic properties of the neurons, specific pathways, receptor distribution, and the state of the second messenger system (Clemens et al., 2012). Data from adult animals suggests that DA can potentiate stepping
in adult animals (Lapointe et al., 2009) which suggests conserved modulatory mechanisms
during development. On the other hand the VR evoked depolarizing responses in mice may
represent a functionally immature excitatory circuit. Certainly we see evidence for differences in
circuitry during development (Hanson and Landmesser, 2003; Mentis et al., 2006). These
caveats, which are interesting in their own right, remain to be tested. Further it is not known
whether ventral root evoked rhythmicity is equivalent to neurochemical evoked fictive
locomotion (Whelan et al., 2000). The flexor-extensor recruitment of individual muscles has
been demonstrated to differ depending on the mode of activation (Klein et al., 2010), suggesting
that ventral root evoked rhythms could evoke different patterns.
The current work provides evidence that DA can exert complex changes in spinal network
function that are dependent on the mode of activation. A hypothesis for further investigation is
whether DA can functionally depress excitatory recurrent collateral transmission onto ventral
horn interneurons.
ACKNOWLEDGEMENTS

Dr. Patrick Whelan is a Senior Scholar of the Alberta Heritage Foundation for Medical Research (Alberta Innovates – Health Solutions). The research was supported by the Canadian Institutes of Health Research. Ms. Humphreys was supported by a Dr. Fong studentship from the Hotchkiss Brain Institute. We would like to acknowledge technical assistance from Dr. Aleksandra Krajacic.

GRANTS

This work was supported by a CIHR operating grant and grants from the University of Calgary.

AUTHOR CONTRIBUTIONS

Dr. Patrick Whelan conceived and designed the experiments with assistance from Jennifer Humphreys. Jennifer Humphreys collected, and analyzed the data. Both Dr Patrick Whelan and Jennifer Humphreys contributed to the writing and editing of the manuscript.
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FIGURE CAPTIONS

Figure 1: A, schematic diagram of the isolated spinal cord preparation showing recording (black electrodes (segmental L2 and one L5 ventral roots) and stimulation sites (red electrode). Stimulation trains were applied to the L5 ventral root (4 Hz, 5-10 μA, 10 s; n = 5) once every 3 minutes. B. Neurograms illustrating a locomotor-like sequence following stimulation of the L5 ventral root. (Bi). Neurogram recordings filtered using a low-pass filter (DC - 1kHz) to illustrate slow depolarization (arrow represents the start of stimulation), and expanded in (Bii) (100Hz - 1kHz) to focus on rhythmic bursting. (Biii). Circular plot summarizing results from several experiments (n = 15). Each dot represents a single experiment with associated phase and vector length. The arrow represents the mean phase and vector length. Phase is represented in degrees (0 - 360°).

Figure 2: Activation of rhythmic activity by stimulation of motor axon recurrent collaterals is abolished by application of DA. A, DC recordings from the right and left L2 ventral roots under control conditions (Ai), addition of DA (Aii), and wash with 500 ml of regular aCSF (Aiii) following stimulation of the L5 ventral root. B, alternating activity between the left and right sides is highlighted by the rectangle. Traces were band-pass filtered (100 Hz - 1 kHz). C, cross-wavelet spectrograms of segmental L2 neurogram recordings during ventral root evoked rhythmic activity under control (Ci) and DA (50 μM) conditions (Cii) The power in the cross-wavelet spectra is shown in a color-coded logarithmic scale on the right (see Materials and Methods). Note: spectrogram time scales vary depending on the length of a rhythmic bout. High power regions were abolished with the
addition of DA compared to control. \textbf{D}, graphs illustrating normalized power and normalized frequency. Error bars represent standard deviation. Note: there are no bars associated with DA application because no rhythmic activity was evoked.

\textbf{Figure 3: DAergic modulation extends to peripheral nerve stimulation and inhibits long-latency depolarizations.}

\textbf{A}, schematic of the isolated spinal cord preparation with the cut sciatic nerve dissected free. \textbf{B}, Stimulation of the sciatic nerve (10 s trains, 4 Hz, dorsal roots cut) evoked rhythmic bursts of activity under control conditions (Bi) but not when DA was bath applied (Bii). \textbf{C}, average of 5 DC recordings of the right L6 ventral root during a 4 Hz stimulus train (5 stim, 50-100 µA; n = 3) applied to the right L5 ventral root under control and DA conditions and following wash. Under control conditions, each stimulus of the stimulus train evoked slow long-latency depolarizing potentials (indicated by the arrows) that were superimposed on top of a tonic depolarization (Ci). In the presence of DA, the ability of each stimulus to evoke slow long-latency potentials was abolished (Cii). Washout restored the long-latency potentials (Ciii).

\textbf{Figure 4: The probability of ventral root evoked entrainment of disinhibited cords decreases in the presence of DA.}

\textbf{A}, schematic diagram of an isolated spinal cord preparation illustrating stimulation (L6 ventral root) and recording sites (adjacent L5 ventral root) \textbf{B}, comparison in which stimulation of the L6 ventral root (5 pulses, 4 Hz, 50-100 µA) was able to entrain disinhibited rhythmic bursting under control (Bi) but not DA (Bii) conditions. \textbf{C}, graph illustrating the percentage of ventral root
stimulation evoked bursts in the presence of strychnine, picrotoxin, and CGP, vs. the percentage of evoked bursts following the addition of DA (one-way repeated measures ANOVA, $P < 0.0001$; $n = 6$). Error bars represent standard deviation. **D,** Illustration depicting parameters measured to obtain phase value: (Di) $a$ – cycle period of entrainment stimulus train delivered to the L6 ventral root; (Dii) $b$ – time to burst onset of adjacent L5 ventral root relative to the beginning of the stimulus train. **E,** graph illustrates a disinhibited rhythm entrained by ventral root stimulation. Illustrated are plots of phase $(b/a)$ vs. time (one representative experiment) depicting two sequences of rhythmic activity taken prior to and following application of DA. The endogenous frequency of the disinhibited rhythm was strongly entrained by delivering a stimulus train once ever 45 sec, indicated by the nearly constant phase value through the sequence (Ei). The endogenous frequency of the disinhibited rhythm increased in the presence of DA, such that a stimulus train needed to be delivered once every 25 sec (Eii). The progressive increase in phase indicates that in the presence of DA, the disinhibited rhythm was no longer entrained by ventral root stimulation.

**Figure 5:** Application of D$_2$ – like receptor agonist depresses ventral root evoked rhythmic activity while D$_1$ receptor agonist does not.

**A,** cross-wavelet spectrograms of segmental L2 neurogram recordings under control conditions (Ai) and with the addition to D$_1$ receptor agonist SKF-81297 (20 μM; Aii). **B,** raw traces of L2 neurogram recordings under control conditions (Bi), addition of SKF-81297 (Bii) (same experiment as A). **C – D,** graphs show normalized power and normalized frequency under control and SKF-81297 conditions ($P < 0.05$; paired $t$ test; $n = 9$). Error bars represent standard deviation. **E,** cross-wavelet spectrograms of segmental L2 neurogram data under control
conditions (Ei) and with the addition to D2-like receptor agonist quinpirole (Eii; 20 μM). F, raw traces of L2 neurogram recordings under control conditions (Fi), and following bath application of quinpirole (Fii) (same experiment as E). G – H, graphs show normalized power and normalized frequency under control and quinpirole conditions (P < 0.05; one-way repeated measures ANOVA; n = 5). Error bars represent standard deviation. Note: in 3/5 experiments, rhythmic activity persisted in the presence of quinpirole, however activity was depressed from control levels. Therefore, we report the normalized power and normalized frequency scores for these experiments in G and H. All raw traces depicted illustrate the first 5 s of a 10 s stimulus train.

Figure 6: DA stabilizes a 5-HT, NMA evoked locomotor rhythm:
A, schematic diagram of the isolated spinal cord preparation showing recording sites. B, raw traces from the segmental L2 neurogram with 5-HT and NMA in the bath (Bi), 2 minutes after DA (50 μM) was added to the bath containing 5-HT and NMA (Bii), and 6 minutes after DA was added to the bath. C, cross-wavelet spectrogram showing DA’s effects over a 10-minute period. Epoch 1 illustrates the lower power region representing the 5-HT-NMA evoked locomotor rhythm. Epoch 2 illustrates the increase in power associated with 2 min of DA application to the 5-HT, NMA evoked rhythm. Epoch 3 illustrates the high power region associated with 6 min application of DA (Epoch 1 – 3 are the same experiment as Bi – Biii). D, graphs showing normalized power (Di) and normalized frequency (Dii) under 5-HT, NMA and 5-HT, NMA, DA conditions (P < 0.05; paired t test; n = 6). Error bars represent the standard deviation. Ei, graph displaying increase in burst amplitude over 10 minute period, taken immediately following the addition of DA to a pre-existing 5-HT-NMA evoked rhythm. Each time point represents burst
amplitude, normalized to 5-HT-NMA values, per bin ($n = 6$; Two-way repeated measures ANOVA; P < 0.001 to P < 0.05). **Eii**, graph showing the averaged L2 neurograms for one representative experiment. Graph was constructed by using a peak detection algorithm [see Materials and Methods]. Black line represents the averaged 5-HT, NMA burst amplitude across a ten minute time; Red line represents the 5-HT, NMA, DA burst amplitude across a 10 minute period.

**Figure 7: DA exerts activation dependent modulation over locomotor CPG networks.**

The present results suggest that pharmacological activation of CPG networks is facilitated by increasing the amplitude of the bursts and the stability of the rhythm. Additionally, our work suggests that activation of CPG networks by excitatory recurrent collateral pathways is inhibited by a D$_2$-like mediated pathway. Excitatory and inhibitory synaptic connections are shown by vertical lines and small circles, respectively. Dashed lines represent putative sites of DA modulation. The question mark represents an unidentified excitatory interneuron that receives excitatory drive from recurrent motor axon collaterals. MN, motoneuron; RC, Renshaw cell.
Figure 6

A. Schematic diagram of spinal cord with labeled structures: ventral roots and sci. electrode.

B. Waveforms recorded during different conditions:
   - Bi: 5-HT, NMA
   - Bii: 5-HT, NMA + DA (2 min)
   - Biii: 5-HT, NMA + DA (6 min)

C. Spectrogram showing frequency and time bins for conditions 1, 2, and 3.

D. Graphs showing normalized power and normalized frequency for:
   - Di: SHF, NMA, SHF, NMA + DA
   - Dii: SHF, NMA, SHF, NMA + DA

E. Graph showing normalized burst amplitude over time bins (1 min each) with a scale of 10 μV and 1 ms.
DA based modulation

CPG - CPG

D1- Mediated Excitation

MN

RC

D2- Mediated Inhibition

To Muscle