Title: Dopaminergic modulation of locomotor network activity in the neonatal mouse spinal cord

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Abstract:

Dopamine is now well established as a modulator of locomotor rhythms in a variety of developing and adult vertebrates. However, in mice while all five dopamine receptor subtypes are present in the spinal cord it is unclear which receptor-subtypes modulate the rhythm. Dopamine receptors can be grouped into two families – the D_{1/5} receptor group and the D_{2/3/4} group that have excitatory and inhibitory effects respectively. Our data suggest that dopamine exerts contrasting dose-dependent modulatory effects via the two-receptor families. Our data show that administration of dopamine at concentrations greater than 35 µM slowed and increased the regularity of a locomotor rhythm evoked by bath application of 5-HT and NMA. This effect was independent of the baseline frequency of the rhythm that was manipulated by altering the NMA concentration. We next examined the contribution of the D_{1} and D_{2} receptor-like families on the rhythm. Our data suggest that the D_{1}-like receptor contributes to enhancing the stability of the rhythm. Overall, the D_{2}-like family had a pronounced slowing effect on the rhythm however, quinpirole, the D_{2}-like agonist, also enhanced rhythm stability. These data indicate a receptor-dependent delegation of the modulatory effects of dopamine on the spinal locomotor pattern generator.
Introduction

Neural circuits that produce basic rhythmic motor patterns of locomotion that, in vertebrates, reside primarily in the spinal cord and are subject to neuromodulation from a wide range of sources both intrinsic and extrinsic to the spinal cord (Jankowska et al., 1967a; 1967b; Kiehn and Kjaerulff, 1996; Kiehn et al., 1999; Schmidt and Jordan, 2000; Madriaga et al., 2004; Gordon and Whelan, 2008; Dunbar et al., 2010; Sharples et al., 2014). Monoamines are an important group of neuromodulators that are released onto spinal cord circuits and are critical for the expression of locomotion (Sharples et al., 2014). Additionally, monoamines also endow these circuits with the necessary flexibility to control the precision, timing, and constancy of locomotor behaviors in order to adapt to different internal and external demands, essential for survival (Grillner, 2003; Miles and Sillar, 2011).

In contrast with the other monoamines, the role of dopamine in controlling spinal locomotor circuits has been largely neglected in the mammal. What we do know is that dopamine is released within the spinal cord during stepping activity (Gerin and Privat, 1998; Jordan and Schmidt, 2002) to alter motor output (Barbeau and Rossignol, 1991; McCrea et al., 1997; Madriaga et al., 2004; Han et al., 2007; Han and Whelan, 2009; Lapointe et al., 2009; Clemens et al., 2012) and modulate sensory transmission in a variety of species. Dopaminergic fibers project from the diencephalon (A11 area) and all five dopamine receptors are present in the ventral horn of the adult mouse spinal cord (Yoshida and Tanaka, 1988; Ridet et al., 1992; Weil-Fugazza and Godefroy, 1993; Holstege et al., 1996; Qu et al., 2006; Zhu et al., 2007), where motor circuits are located. The five dopamine receptor types are divided into two dopamine receptor families that have been distinguished based on biochemical studies. \(D_1\) and \(D_5\) receptors belonging to the \(D_1\) – like family elicit excitatory post-synaptic effects, and the \(D_2, D_3\) and \(D_4\) receptors belonging to the \(D_2\) – like family elicit inhibitory effects on both pre-synaptic and post-synaptic terminals (Missale et al., 1998; Neve and Seamans, 2004).
The key role for dopaminergic transmission in locomotion is highlighted by the fact that L-DOPA-elicited air stepping in intact neonatal rats is blocked by D_1-like and D_2-like receptor antagonists (McCrea et al., 1997) and that D_1 agonists can promote stepping in adult mice (Lapointe et al., 2009). Additionally, previous work has emphasized that dopamine can modulate ongoing drug-evoked locomotor rhythms and in rats can even evoke locomotor-like activity (Schotland et al., 1995; Jiang et al., 1999; Whelan et al., 2000; Barrière et al., 2004; Madriaga et al., 2004; Humphreys and Whelan, 2012; Milan et al., 2014). Collectively, these studies emphasize an important role for dopamine in the modulation of locomotor function in the developing and adult spinal cord.

An important step in understanding how dopamine exerts sustained neuromodulatory actions on central pattern generating (CPG) networks is to identify the receptors activated by dopamine. Previous reports in the neonatal mouse have investigated the underlying dopamine receptors that contribute to ongoing locomotor rhythms evoked by either pharmacological application of 5-HT (Madriaga et al., 2004), or electrical stimulation of the afferents that reside in the cauda-equina (Gordon and Whelan, 2006a). These studies, as well as others in the rat, provide evidence that dopamine’s excitatory effects are mainly mediated via a D_1-like receptor based pathway (Seth et al., 1993; Barrière et al., 2004). Similar roles for the D_1-like receptor systems are also found to modulate locomotor activity in Xenopus tadpoles (Clemens et al., 2012) and lamprey (Schotland et al., 1995). In contrast, the role of D_2-like receptor subfamily in motor control at the level of the spinal cord of mammals is less understood.

The aim of the current investigation was to build on and extend previous observations of the dopaminergic modulation of rhythmic activity in the neonatal mouse spinal cord, by investigating the effects of dopamine, and the D_1 and D_2-like agonists on a pre-existing locomotor-like rhythm evoked by pharmacological application of 5-HT and NMA. In the rat spinal cord, application of 5-HT and NMA is sufficient to induce stable, rhythmic activity (Cazalets et al., 1992; Cowley and Schmidt, 1995; Kjaerulff and Kiehn, 1996; Cowley and Schmidt, 1997; Kremer and Lev-Tov, 1997). However in the mouse, application of 5-HT and
NMA can at certain concentrations generate less coordinated rhythmic activity between the left and right sides of the spinal cord (Jiang et al., 1999; Whelan et al., 2000). Therefore, we specifically used this relatively unstable locomotor-like rhythm to highlight which aspects of rhythmic activity were altered by dopamine application. Our results demonstrate that dopamine application as low as 35 µM stabilizes a pre-existing 5-HT, NMA-evoked locomotor-like rhythm and slowed the rhythm frequency. The underlying boost in excitation leading to a stabilization of rhythmic output was found to be both D1 and D2 dependent, however, in other studies a D1 effect was noted although D2 effects were not ruled out (Barrière et al., 2004). Our results using a combination of pharmacology and transgenic D3-receptor knockouts points to dopamine’s ability to slow down the frequency of the rhythm to be mediated by D2-dependent signaling mechanisms. Our data highlights an important contribution for dopamine receptor activation in the maintenance and stability of ongoing CPG network activity. A portion of our data has been published in abstract form (Humphreys and Whelan, 2011).

Methods

Ethical approval & animals

Experiments were performed on neonatal Swiss Webster mice of 0-3 days old (P0-P2) (N=142). Experiments were also conducted on D3 receptor knockout mice (D3KO; strain B6.129S4-

Drd3tm1dac/J; stock # 002958, Jackson Laboratory, Bar Harbor, ME, n=10) and their appropriate associated wild-type (WT) controls (C57BL/6, n=6). All procedures used were approved by the University of Calgary Health Sciences Animal Care Committee and the East Carolina University Institutional Care and Use Committee, respectively.

Tissue preparation: Spinal cord isolation

Animals were anaesthetized by cooling or i.p. injection of ketamine (90 mg/mL), xylazine (10 mg/mL), decapitated and eviscerated to expose the vertebral column. The remaining tissue was placed ventral side up in a dissection chamber filled with room-temperature oxygenated (95% O2, 5% CO2) artificial cerebrospinal fluid (aCSF) (128 mM NaCl, 4 mM KCl, 1.5 mM CaCl2, 1 mM
MgSO$_4$, 0.5 mM Na$_2$HPO$_4$, 21 mM NaHCO$_3$, 30 mM D-glucose), and spinal cords were exposed via a ventral laminectomy and dorsal and ventral roots cut. The spinal cord was removed and left to stabilize for 15-20 minutes before transferring to a recording chamber, ventral side up, with oxygenated aCSF and gradually heated from room temperature to 27° C. The spinal cord was allowed another 20 minutes to further stabilize before attaching the ventral roots of the second and fifth lumbar segments (L2 and L5) with tight-fitting suction electrodes. D$_3$KO experiments followed the same dissection procedure; however, ventral root recordings were made at room temperature (approximately 21-22° C) from D$_3$KO and WT cords. In these experiments, a WT and a D$_3$KO preparation were tested in parallel in the same recording chamber to ensure identical experimental conditions.

**Pharmacology**

Fictive locomotion was elicited in isolated spinal cord and intact hind-limb preparations by bath-application of N-methyl-D(L)-aspartic acid (NMA, 5 µM; Sigma-Aldrich) and 5-hydroxytryptamine (5-HT, 10 µM; Sigma-Aldrich). To measure the modulatory effects of dopamine on the rhythm, most experiments involved bath application of 50 µM dopamine; however, a subset of experiments also involved bath application of dopamine at 1 µM, 10 µM, 30 µM, 35 µM and 40 µM of dopamine (Sigma-Aldrich). In a subset of experiments we targeted the D$_1$–like (D$_1$ /D$_5$) and D$_2$–like subfamilies (D$_2$/D$_3$/D$_4$). Receptor-selective dopamine agonists used were the D$_1$-like agonist SKF-81297 (20 µM; Tocris) and the D$_2$-like agonist quinpirole (20 µM; Sigma-Aldrich). Dopamine receptor-selective antagonists used were the D$_1$-like antagonist LE 300 (1-4 µM; Sigma-Aldrich), and the D$_2$-like receptor antagonists L-741, 626 (6-12 µM; Sigma-Aldrich) and sulpiride (20 µM; Sigma-Aldrich) and the D$_3$-preferring antagonist SB-277,011-A (1 µM; Tocris). In all experiments a wash of 500-800 mL of aCSF containing the baseline drugs was performed to restore baseline conditions after drug testing.

**Electrophysiological recordings**

Neurograms were obtained from isolated spinal cord preparations by drawing segmental ventral roots from both the right and left ventral roots in the second lumbar (L2) segment and one or both, left and right ventral roots in fifth lumbar (L5) segment into tight-fitting suction
electrodes filled with bath aCSF. Neurograms were amplified (100-10,000 times), filtered (low
pass 1kHz), digitized (Digidata 1440, Molecular Devices, Sunnyvale, CA), acquired using Clampex
10 software (Molecular Devices, Sunnyvale, CA) and saved on a laboratory computer for offline
analysis. All data obtained during isolated spinal cord experiments were analyzed using custom-
written programs (Matlab, Mathworks, Natick, MA, USA; Spinalcore, A. Lev-Tov) and
commercially available programs (Spike2, CED, Cambridge, UK).

Data analyses

Continuous recordings of drug-evoked locomotor activity during isolated spinal cord
experiments were high-pass filtered (100 Hz) and rectified before performing a Morlet cross-
wavelet spectrogram analysis of ventral root bursting pairs in Spinal Core (Mor and Lev-Tov,
2007) producing coherent power spectrograms. For convenience non-significant regions are
omitted from the spectrogram, colours indicate the logarithmic power of the signal, which
shows the power of the signal at a given frequency. High-power regions are assigned warm
colours (i.e. red and orange) and low-power regions are assigned “cool” colours (i.e. green and
blue). The high-power band representing the locomotor activity was then identified and
selected as a region of interest for analysis. Regions of interest were segmented into twenty
bins over the course of a ten-minute trial from rhythms evoked under different drug conditions,
and analyzed to extract three main features including rhythm frequency, coherent power and
phase of the pattern. Data from each of the twenty bins of the respective ten-minute trial were
normalized to the average of the twenty bins from the control or baseline condition.
Normalized bins were then averaged across ten-minute trials of the respective experimental
conditions.

Separate dose response experiments were conducted for the D$_1$-like agonist, SKF-81297 and
the D$_2$-like agonist, quinpirole (1, 3, 10, 30, 100 µM) by recording spontaneous ventral root
activity from single L2 roots. This permitted us to record from up to four separate isolated
spinal cords simultaneously to ensure equivalent experimental conditions between
preparations. Data over a ten-minute period of time were high pass filtered (100 Hz) and the
root mean square (RMS) of bursting activity measured. A response ratio was calculated
between the RMS of bursting activity between 10 and 20 minutes after addition of SKF- 81297 to the bath relative to a ten-minute baseline recording.

Statistics

All statistical tests were conducted on the raw, unnormalized data. Paired t-tests were conducted on experiments involving repeated measures assessment at two points in time and repeated measures analysis of variance (ANOVA) were conducted on experiments involving assessment at three or more points in time. Tukey post hoc analysis was conducted when significant effects were detected using ANOVAs with a significance level of p<0.05. Unpaired t-tests were conducted for between group comparisons of two groups and a one-way ANOVA for comparisons of more than two groups. Circular statistics were conducted on phase analyses of rhythmic locomotor activity to test for directionality (Rayleigh’s test, p < 0.05).

Results

Dopamine promotes stability and reduces frequency of ongoing locomotor activity

Bath application of monoamines such as 5-HT (rat/mouse) or dopamine (rat) to neonatal isolated spinal cord preparations is capable of eliciting a robust locomotor-like pattern of activity (Nishimaru et al., 2000; Barrière et al., 2004; Madriaga et al., 2004). In contrast, our work demonstrates that in the mouse, bath application of dopamine alone did not elicit a strong locomotor pattern of activity, but rather low frequency rhythmic bursting activity that does not show the left-right alternation of a basic locomotor pattern (Figure 1 Bi &Bii). Our work and that of others demonstrates that bath-applied dopamine does play an important role in rodents in the stabilization of fictive locomotor activity. What is not known are the receptor mechanisms that mediate these effects in mice. To study the mechanisms of dopaminergic modulation of locomotion, we made use of an unstable pre-existing locomotor rhythm evoked solely by bath application of 5-HT and NMA in the neonatal mouse spinal cord isolation preparation. This provides the advantage of being able to ascertain the stabilizing effects of dopamine on rhythms coupled with an analysis of effects on frequency.
The baseline rhythm evoked by 5-HT and NMA consisted of bursting activity that alternated between left and right ventral root neurograms within the same segment and alternating bursts between L2 and L5 neurograms on the ipsilateral side of the cord (Figure 1Ci). In the spectrogram this was apparent as a locomotor frequency band around 0.4 Hz (Figure 1D). Addition of 50 µM of dopamine to this rhythm resulted in an initial degradation of stability of the rhythm during the first three minutes. After approximately three minutes a more stable (power: n=8, F(2,14)=22.6, P<0.001; Figure 1 D & Eii) and lower frequency (n=8, F(2,14)=17.8, p<0.001; Figure 1D & Ei) alternating rhythm between left and right L2 ventral root bursts emerged (Figure 1Cii) comparable to the one previously reported (Humphreys and Whelan 2012). Similar effects were observed when comparing left-right alternation of L5 neurograms (n=7, frequency: F(2,12)=9.2, p<0.01, Figure 1Fi; power: F(2,12)=19.9, p<0.001, Figure 1, Fii) and ipsilateral alternating activity between L2 and L5 ventral roots (n=8, frequency: F(2,14)=12.6, p<0.001, Figure 1Fi; power: F(2,14)=20.0, p<0.0001; Figure 1Fii). There was no change in the phase or vector length of the alternating pattern with the addition of dopamine to the pre-existing locomotor rhythm (data not shown). In a separate set of experiments we determined that 35 µM of dopamine was the lowest concentration required to significantly stabilize and reduce the frequency of an ongoing 5HT, NMA-evoked locomotor rhythm (n=35, Figure 2).

Given that different neuronal populations are recruited at different frequencies of locomotor activity (Zhong et al., 2011) this could alter the modulatory effects of dopamine. To address this we tested the ability of dopamine to modulate ongoing locomotor activity at different locomotor frequencies by varying the concentration of NMA (5 – 12 µM) (Talpalar and Kiehn, 2010) in the bath (n=17, frequency range = 0.29-0.79 Hz; Figure 3A). As expected, increasing the concentration of NMA in the bath significantly increased the frequency of the rhythm (One-Way ANOVA: F(3,13)=7.9, p=0.003; Figure 3A). However, the ability of dopamine to modulate the rhythm frequency and power remained true independent of the induced locomotor frequency (Figure 3B & C).

D1-like and D2-like receptor mechanisms of modulation
To test the different dopamine receptor family contribution to the modulation of an ongoing 5-HT and NMA-evoked rhythm, we focused specifically on the left-right alternating pattern of the L2 neurograms. Application of the D₁-like agonist SKF-81297 (20 µM) increased the stability of the ongoing 5-HT and NMA-evoked rhythm (Figure 4Ci) evident by a significant increase in normalized power (n=11; F(2,20)=6.59, p=0.006; Figure 4Cii & Eii) but had no effect on frequency (n=11, F(2,20)=1.5, p=0.25; Figure 4Ei). Followed by a 500 mL wash of aCSF containing 5-HT and NMA restored the rhythm to control conditions. 20 µM of the D₁-like agonist was utilized based on a dose-response experiment using spontaneous ventral root bursting as a probe of motor circuit excitability (N=20). These experiments determined the EC₅₀ to be 10 µM (Figure 4 Bi & Bii).

To further explore the role of dopamine during ongoing locomotor activity evoked by combined application of 5-HT, NMA and dopamine (50 µM), D₁-like antagonists were bath applied in separate experiments to perturb the rhythm. In 4/5 preparations, bath application of the D₁-preferring antagonist LE300 (1-4 µM) resulted in degradation of the rhythm within 5-10 minutes (n=5, F(2,8)=6.14, p=0.02, Figure 4 Dii & Eii) and was restored to original conditions following a wash with 500-700mL of aCSF containing 5HT, NMA and dopamine.

In a separate set of experiments, addition of the D₂-like agonist, quinpirole (20 µM), to the 5-HT and NMA-evoked rhythm (Figure 5Ci) increased the stability indicated by a significant increase in power (n=7, F(2,12)=5.8, p=0.02; Figure 5Cii & Eii) and reduction in frequency (n=7, F(2,12)=8.9, p<0.004; Figure 5Cii & Ei). The D₂-like agonist activity persisted after a wash with 500-700mL of aCSF containing 5-HT and NMA, indicating the possibility for long lasting effects of D₂-like receptor activation on rhythm stability. Dose response experiments (N=18) determined the IC₅₀ of quinpirole’s ability to reduce spontaneous motor activity to be 12 µM (Figure 5Bi & Bii). We then tested the ability of the D₂ antagonist L741,626 to reduce the quinpirole mediated effects. Similar to previous experiments, bath application of the D₂-like agonist, quinpirole (20 µM) to a locomotor rhythm evoked by 5-HT and NMA resulted in a significant reduction in frequency (p<0.01) and increase in stability (p<0.05) of the rhythm. Subsequent bath application of the D₂ receptor antagonist, L-741,626 (12 µM), offset the
quinpirole-induced effects, increasing the frequency ($F(3,12)=7.03$, $p=0.005$) and reducing the stability ($F(3,12)=4.23$, $p=0.029$) back to that of the baseline 5-HT and NMA-evoked rhythm (data not shown).

Surprisingly, bath application of the D$_2$-like antagonist L-741,626 (6 µM) had no effect on the 5HT, NMA and dopamine-evoked locomotor rhythm ($n=5$, frequency: $F(2,8)=1.36$, $p=0.31$; power: $F(2,8)=4.5$, $p=0.049$; Tukey post hoc $p>0.05$). To test for the possibility of competitive binding of L-741,626 and dopamine on D$_2$ receptors the concentration of the antagonist was doubled and bath applied to a rhythm evoked by 5-HT, NMA and a lower concentration of dopamine (35 µM); however still no effect was observed ($n=5$, frequency: $F(2,8)=1.2$, $p=0.36$; power: $F(2,8)=1.5$, $p=0.29$; data not shown).

In an additional set of experiments we bath-applied the D$_2$-preferring antagonist, sulpiride (20 µM), to an ongoing locomotor rhythm evoked by 5-HT, NMA and 35 µM dopamine which resulted in a significant increase in rhythm frequency ($n=4$, $F(2,6)=17.0$, $p<0.001$; Figure 5Cii & Eii) with no change in stability ($F(2,6)=1.53$, $p=0.29$; Figure 5Cii & Ei). Based on the comparative binding affinities of L-741,626 for D$_2$-receptors and sulpiride being a more general D$_2$-like antagonist (D$_2$,D$_3$,D$_4$) and that the D$_3$ receptor has the highest affinity to dopamine, we considered the possibility that the frequency modulation of dopamine may be in part mediated by D$_3$-receptors.

With this in mind, experiments were conducted on D$_3$ receptor knockout mice. We found that the baseline locomotor rhythm (evoked by only 5-HT and NMA) frequency was significantly higher in the D$_3$KO isolated spinal cords compared to the wild type ($n=12$: WT; $n=6$, frequency=0.23±0.03 Hz; D$_3$KO; $n=6$, frequency=0.39 ± 0.08 Hz: un-paired t-test: $t(10)=-3.07$, $p=0.017$; Figure 6B). Note that in these experiments, the wild-type and knockout animals were always side-by-side in the same chamber – subject to the same conditions (Figure 6A, cf. Methods). Dopamine (50 µM) elicited a similar robust reduction in frequency and increase in stability of the 5-HT and NMA-evoked locomotor rhythm in both wild-type and knock-outs ($N=12$: WT; $n=6$, D$_3$KO; $n=6$: frequency: $F(2,10)=5.97$, $p=0.02$; Power: $F(2,10)=15.06$, $p=0.0003$; Figure 6C & D). As these data were obtained at 21-22º C, we specifically tested the possible role of temperature.
While we did observe a temperature effect on initial rhythm frequency we found that the respective WT data obtained at 21 and 27°C could be normalized to each other (data not shown). Therefore, the direct comparison between WT and D3KO in the same dish and at the same temperature suggests that temperature did not contribute to the impact of dopamine’s modulatory actions.

Discussion

The ability for spinal dopamine to modulate ongoing locomotor activity is of growing interest in the field of motor control. Over the past decade, work in vivo has implicated an important role for dopamine to generate and maintain stepping behavior (McCrea et al., 1997; Gerin and Privat, 1998; Jordan and Schmidt, 2002; Lapointe et al., 2009). Additionally, in vitro work has highlighted the ability of dopamine to modulate ongoing locomotor rhythms in several vertebrate species (Schotland et al., 1995; Jiang et al., 1999; Whelan et al., 2000; Barrière et al., 2004; Madriaga et al., 2004; Gordon and Whelan, 2006b; Clemens et al., 2012). However, experimental protocols in the mouse have often relied upon stable control rhythms induced either pharmacologically or by electrical stimulation, to test the modulatory effectiveness of dopamine. This has raised some questions regarding whether the effects of dopamine are masked by the presence of a stable control rhythm. Therefore in the current study, we sought to expand upon an existing body of research (Kjaerulff and Kiehn, 1996; Whelan et al., 2000; Barrière et al., 2004; Madriaga et al., 2004; Gordon and Whelan, 2006a) and test what aspects of a less coordinated, pharmacologically induced rhythm were modulated by the presence of dopamine. The results indicate that the presence of dopamine induced a more stable, slower rhythm. Indeed, our work suggests that D1 – like and D2 – like receptors have distinct roles in controlling rhythm stability with the D2 receptors playing an important role in the modulation of rhythm frequency.

The potential for dopamine to modulate ongoing locomotor rhythms in vitro was first recognized in the lamprey (Schotland et al., 1995) and later in other vertebrate systems (Smith et al., 1988; Kiehn and Kjaerulff, 1996; Barrière et al., 2004; Madriaga et al., 2004; Gordon and
Whelan, 2006a). Initial experiments described dopamine’s ability to dose-dependently slow down the cycle frequency of swimming behaviour (Schotland et al., 1995) with lower concentrations increasing rhythm frequency. Similar dose-dependent modulatory effects on spontaneous locomotor activity have been reported in the pre-metamorphic Xenopus tadpole such that low concentrations of dopamine activate D$_2$-like receptors and reduce spontaneous locomotor activity whereas higher concentrations activate the D$_1$-like receptors and facilitate spontaneous locomotor activity (Clemens et al., 2012). More recent work in the isolated mouse has reported an excitatory role for dopamine on network output (Whelan et al., 2000; Madriaga et al., 2004; Gordon and Whelan, 2006a). Our current work adds to this growing body of research in the following important ways: First, the number of studies investigating the dopaminergic modulation of ongoing locomotor rhythms in the neonatal mouse is limited. Although a report in the neonatal rat documenting dopamine’s role during ongoing locomotor activity exists, we cannot assume the underlying receptor subtypes activated during fictive locomotion are the same. Second, of the limited studies that do exist in the mouse, two of them have relied upon evoking robust control rhythms prior to investigating dopamine’s modulatory effects (Madriaga et al., 2004; Gordon and Whelan, 2006a). Therefore, it is arguable that the magnitude of dopamine’s effect on a pre-existing stable rhythm was not fully revealed. The remaining study that showed dopamine’s modulatory effects on an uncoordinated control rhythm (Whelan et al., 2000) did not ascribe distinct roles for the underlying dopamine receptor families responsible for mediating dopamine’s effects. We present data demonstrating that D$_1$-like receptor pathways may act in parallel with D$_2$-like receptor pathways to increase the stability of a pre-existing 5-HT, NMA induced rhythm. Evidence in the brain and spinal cord support the finding that a synergy between D$_1$ and D$_2$ receptor based signalling pathways exists, and functions to produce appropriate behavioral output in the brain and spinal cord (Braun and Chase, 1986; Missale et al., 1998; LaHoste et al., 2000; Barrière et al., 2004). However, it is important to note that application of D$_1$-like antagonist LE 300 resulted in degradation of the stable 5-HT, NMA, dopamine-evoked locomotor-like rhythm, while D$_2$-like antagonist L-741, 626 with preferential binding affinity for D$_2$ only degraded rhythm stability in the presence of the D$_2$-like agonist, quinpirole, and not
when dopamine was present. This points to the possibility that at the D\textsubscript{2}-mediated effects may be masked by stronger D\textsubscript{1}-mediated effects at the higher concentrations (50 \(\mu\)M and 35 \(\mu\)M) of dopamine used in these experiments.

In the current investigation we show that dopamine does not demonstrate a concentration-dependent bidirectional influence on locomotor rhythm frequency in the mouse spinal cord as reported in the lamprey (Schotland et al., 1995) and *Xenopus* (Clemens et al., 2012). Additionally, L-741, 626 exerted no effects on rhythm frequency in the presence of dopamine. This was unexpected given that application of D\textsubscript{2} – like receptor agonist quinpirole decreased rhythm frequency (Figure 5) and the broader D\textsubscript{2}/D\textsubscript{3} antagonist, sulpiride selectively increased the rhythm frequency with dopamine on board. Based on this, we considered the possibility that the frequency modulation may be mediated by the D\textsubscript{3} receptor subtype. To overcome the challenges of selectively targeting the D\textsubscript{3} receptor population with traditional pharmacological approaches we utilized a transgenic D\textsubscript{3} receptor knock out mouse to test this hypothesis. In contrast to our hypothesis, dopamine still reduced rhythm frequency of both the wild type and D\textsubscript{3} KO. Interestingly, when testing D\textsubscript{3} KO and wild type (WT) animal spinal cord preparations in parallel and at the same ambient temperature, we found that the baseline frequency of locomotor rhythms evoked by 5-HT and NMA was significantly higher in D\textsubscript{3} KO than in WT. These data suggest that D\textsubscript{3} receptors exert an inhibitory influence on the locomotor-generating circuitry, and that the lack of this receptor in the D\textsubscript{3} KO releases the CPG in the transgenic animals from the D\textsubscript{3} receptor-mediated inhibition. While the mechanism underlying this phenotypical switch remains speculative, it is conceivable that the dysfunction of the D\textsubscript{3} receptor gives rise to an increase in spinal D\textsubscript{1} receptor protein levels that in turn might additionally fasten the locomotor rhythm observed in the D\textsubscript{3} KO, similar to recent findings that suggested a D\textsubscript{1} receptor upregulation when testing pain-related sensorimotor pathways in D\textsubscript{3} KO animals (Brewer et al, 2014).

We made use of pharmacological and transgenic approaches to examine the role for the D\textsubscript{2} receptors in the modulation of rhythm frequency. Given that dopamine was capable of
reducing rhythm frequency in the D₃KO spinal cords and the more selective D₂ antagonist (L-741, 626) increasing rhythm frequency in the absence of D₁ receptor activity in wild type cords, it is likely that D₂ receptors mediate this effect. These findings point to the fact that if we wish to fully understand the role that dopamine plays in the modulation of spinal motor circuits, we must not consider just the receptor families (i.e. D₁-like and D₂-like), but the individual receptor types (i.e. D₁-D₅), which are all expressed non-uniformly across the lumbar spinal cord, with all five receptor subtypes expressed in motor neurons (Zhu et al., 2007). It should be noted that this receptor characterization was conducted in P14-age mice, and therefore, consideration must be given toward the potential developmental receptor expression profile in the first 5 postnatal days. Furthermore, consideration must also be given toward the possibility of cross-family heterodimers that form for example, between D₁-D₂ (George and O'Dowd, 2007) or D₁-D₃ (Surmeier et al., 1996; Cruz-Trujillo et al., 2013).

What is clear is that dopamine is a potent modulator of locomotor network activity; however, if we wish to more fully understand the role of spinal dopamine in motor control we must further examine how dopamine shapes different components of the network. This includes the modulation of intrinsic properties and synaptic inputs to motor neurons and other populations of neurons that make up the pattern formation elements and rhythm generating components of locomotor CPGs. Indeed dopamine does increase the excitability of motor neurons by reducing potassium conductances (Iₐ, SKCa) (Han et al., 2007) and increasing excitatory glutamatergic transmission onto motor neurons (Han and Whelan, 2009) that could contribute to dopamine’s excitatory increase on burst amplitude. But further work is required to probe how dopamine modulates other components of the locomotor network. For example, in the lamprey, the frequency modulation of the rhythm by dopamine has been attributed to modulation of inhibitory commissural interneurons that participate in the generation of the left-right alternating pattern of fictive swimming (Hill et al., 2003a; Wang et al., 2011). Furthermore, dopamine’s reduction in rhythm frequency has also been suggested to be in part due to a decrease in descending excitatory reticulospinal input to motor neurons (Svensson et
al., 2003) and also increasing activity of inhibitory commissural interneurons that participate in the generation of the left right alternating pattern of fictive swimming (McPherson and Kemnitz, 1994; Kemnitz, 1997; Hill et al., 2003b; Wang et al., 2011). Until recently, targeting similar circuitry in the mammalian spinal cord was highly inaccessible compared to those in the lamprey. Recent advances in optogenetic methods will serve as a valuable tool to interrogate circuit function in the control of locomotion and provide further insight into the mechanisms that dopamine act to shape locomotor activity.

Conclusion

We have demonstrated that dopamine modulates ongoing spinally-generated locomotor activity but is insufficient to evoke coordinated patterns of locomotor-like activity when applied on its own. Specifically, dopamine stabilizes ongoing locomotor activity through both D₁-like and D₂-like receptor mechanisms. We provide evidence that activation of the D₂ receptors play a role in dopamine’s ability to reduce the frequency of the rhythm. Our experiments with the D₃ knockout mice suggest that the D₃ receptors may be constitutively involved in the regulation of rhythm frequency in the absence of dopamine; however our data do not exclude a role for D₄ receptors that are also highly expressed within the ventral horn of the spinal cord (Zhu et al., 2007). Collectively these data provide a receptor-based understanding of dopamine’s modulation of spinal cord CPGs.
References


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**Figure Captions**

**Figure 1:** Dopamine reduces locomotor rhythm frequency and increases rhythm stability. Ventral root neurogram recordings from an isolated spinal cord of a neonatal mouse (A). Bath application of dopamine alone (50 μM) evokes low frequency rhythmic bursting activity in ventral root pairs that does not resemble a locomotor pattern (Bi & Bii). When dopamine is applied during a pre-existing fictive locomotor rhythm evoked by 5-HT (10 μM) and NMA (5 μM) (Ci) it reduces the frequency of the rhythm (Cii, Ei, Fi) and increases the stability of the rhythm indicated by an increase in power (Cii, Eii, Fii). The spectrogram (D) depicts a cross-wavelet analysis of a locomotor rhythm recorded from left and right L2 ventral root neurograms evoked by 5-HT and NMA in the first 600 seconds with subsequent addition of dopamine for up to 20 minutes after addition of dopamine. Rhythm frequency is displayed on the y-axis and rhythm power displayed as warm or cool colours with warmer colours representing higher power. Line and bar graphs depict the average normalized frequency and power of the locomotor rhythm (±SEM) from neurograms recorded in the left and right L2, L5 and ipsilateral L2-L5 bursting activity. Bar graphs depict normalized data averaged over ten minute time intervals during respective drug conditions.

**Figure 2:** Dose dependent modulatory effects of dopamine on the locomotor rhythm. Concentrations of dopamine as low as 35 μM are sufficient to reduce the frequency (A) and increase the power (B) of pre-existing 5-HT and NMA-evoked locomotor activity. Data are presented as mean ± SEM frequency (A) and power (B) normalized to baseline rhythm evoked by 5-HT and NMA alone. Asterisks denote significant differences from baseline 5-HT, NMA evoked rhythm (not shown) from a ten-minute time window between 10 and 20 minutes after dopamine application (repeated measures ANOVA, Tukey post hoc, p<0.05)

**Figure 3:** Locomotor rhythm frequency evoked by 5-HT and NMA was manipulated by modifying the concentration of NMA in the bath. Higher concentrations of NMA combined with 10 μM 5-HT increase rhythm frequency (A). Data are presented as mean ± SEM rhythm frequency with the number of preparations in parentheses above each point. Asterisks denote significant differences (Tukey post hoc * p<0.05, ** p<0.01) (A). The degree of modulation by dopamine on locomotor rhythm frequency (B) and power (C) is not dependent on the pre-existing rhythm frequency. Control rhythm frequency is displayed on the x axis with the degree of modulation on the y axis for each experiment (black dots) normalized to baseline control rhythm frequency (B) and power (C).

**Figure 4:** D₁-like receptor activation increases rhythm stability with no effect on frequency. Spontaneous activity from single ventral root neurograms of the isolated spinal cord (A) were used to determine the EC₅₀ for D₁-mediated excitation of motor output (B). The EC₅₀ for SKF-81297 was calculated as 10 μM by generating a response ratio between the root mean square
of activity in a 10-20 minute window after drug application compared to a ten-minute baseline (B). Data are presented as mean ± SEM response ratio as a function of SKF-81297 concentration (B). Bath application of the D₁-like receptor agonist, SKF-81297 during a 5-HT, NMA-evoked rhythm (Ci) increased stability (Cii & Eii) but had no effect on frequency (Cii & Ei). Similarly, the D₁-like antagonist, LE300 (1-4 μM) destabilized the locomotor rhythm (Dii & Eii) but had no effect on frequency (Dii & Ei) of a rhythm evoked by 5-HT, NMA and dopamine (Di).

Spectrograms depict cross wavelet analysis of neurogram activity from left and right L2 ventral roots over time with the rhythm frequency on the y axis and rhythm stability indicated by the power as the colour bands. A more stable rhythm is displayed as warmer colours (C & D). Data are presented as mean ± SEM normalized rhythm frequency (Ei) and power (Eii) over a ten-minute window between 10 and 20 minutes after dopamine application and are normalized to a ten minute window of the control rhythm evoked by 5-HT and NMA. Asterisks denote significant differences from baseline control rhythm (repeated measures ANOVA, Tukey post hoc) with p<0.05.

Figure 5: D₂-like receptor activation increases rhythm stability and decreases rhythm frequency. Spontaneous activity from single ventral root neurograms of the isolated spinal cord (A) were used to determine the IC₅₀ for D₂-mediated inhibition of motor output (B). The IC₅₀ for quinpirole was calculated as 12 μM by generating a response ratio between the root mean square of activity in a 10-20 minute window after drug application compared to a ten-minute baseline (B). Bath application of the D₂-like agonist quinpirole (20 μM) decreased the frequency (Cii & Ei) and increased stability (Cii & Eii) of a pre-existing locomotor rhythm evoked by 5-HT and NMA (Ci). Similarly, bath application of the D₂-like antagonist, sulpiride (20 μM) increased the frequency (Dii & Ei) but had no effect on stability (Cii & Eii) of a rhythm evoked by 5-HT, NMA and dopamine (Di). Bar graphs represent mean ± SEM rhythm frequency (Ei) and Power (Eii) normalized to baseline control rhythm conditions. Spectrograms depict cross wavelet analysis of neurogram activity from left and right L2 ventral roots over time with the rhythm frequency on the y axis and rhythm stability indicated by the power as the colour bands. A more stable rhythm is displayed as warmer colours (C & D). Data are presented as mean ± SEM normalized rhythm frequency (Ei) and power (Eii) over a ten minute window between 10 and 20 minutes after dopamine application and are normalized to the control rhythm evoked by 5-HT and NMA. Asterisks denote significant differences from baseline control rhythm (repeated measures ANOVA, Tukey post hoc) with p<0.05.

Figure 6: 5-HT and NMA-evoked locomotor activity was recorded from left and right L2 ventral root of wild type and D₃KO in parallel and in the same recording chamber to ensure identical experimental conditions (A). Baseline rhythm frequency was higher in D₃KO (blue) compared to the wild type (red) (B). Data are presented as mean ± SEM rhythm frequency with asterisks denoting significant difference between groups (p<0.05). Subsequent application of dopamine
resulted in a significant reduction in locomotor rhythm frequency (C) and increase in stability (D). Data are presented as mean ± SEM normalized rhythm frequency (C) and power (D) over a ten minute window between 10 and 20 minutes after dopamine application and are normalized to a ten minute window of the control rhythm evoked by 5-HT and NMA. Asterisks denote significant differences from baseline control rhythm (repeated measures ANOVA, Tukey post hoc) with p<0.05.
A. Ventral roots
   Rec. electrode

B. Spontaneous Activity (No Drugs)
   LL2
   RL2

C. 5-HT (10 μM) & NMA (5μM)
   LL2
   RL2
   RL5
   LL5

D. RL2-LL2 Spectrogram

E. Normalized Frequency
   Time (minutes)

F. Normalized Power
   Time (minutes)
A. Bi.

Control- 5-HT & NMA

RL2

LL2

5-HT, NMA & DA

RL2

LL2

Bii.

Response Ratio

EC_{50} = 10\mu M

Ci.

Control- 5-HT & NMA + D_1 Agonist (SKF-81297)

Cii.

5-HT, NMA + D_1 Antagonist (Le300)

Di.

Control- 5-HT, NMA & DA

Dii.

5-HT, NMA, DA + D_1 Antagonist (Le300)

Ei.

Normalized Frequency

Eii.

Normalized Power

[SKF-81297]
A. Bi. Quinpirole (100 μM) 5-HT, NMA

B. Bii. 5-HT, NMA

Ci. Control- 5-HT & NMA

C. Cii. + D₂ Agonist (Quinpirole)

Di. Control- 5-HT, NMA & DA

D. Dii. + D₂ Antagonist (Sulpiride)

E. Ei. Quinpirole (20 μM)

E. Eii. Sulpiride (20 μM)

** IC₅₀ = 12 μM

Normalized Frequency

Normalized Power
Parallel WT and D3KO setup

A. Parallel WT and D3KO setup

Wild Type D3KO

B. Frequency (Hz)

Wild Type D3KO

C. Normalized Frequency

Wild Type D3KO

D. Normalized Power

Wild Type D3KO

- Control- 5-HT, NMA
- 5-HT, NMA, DA (10-20 mins)
- Post-wash, 5-HT, NMA