June 4, 2008

Endogenous dopamine suppresses initiation of swimming in pre-feeding zebrafish larvae.

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Keywords: spinal cord, central pattern generator, neuromodulation, motor neuron, motor control, MPTP.

Abbreviated title: Dopaminergic suppression of swimming in larval zebrafish

Number of figures and tables: 11 Figures; 0 Tables

Number of pages: 37

Abstract: 245 words; Introduction: 555 words; Discussion: 1421 words.

Acknowledgements: Supported by the NIH Director's Pioneer Award (HTC). The authors thank Dr. Mark Masino for help with the extracellular recording technique, Dr. James Demas for assistance with the behavior and members of the Cline lab for helpful discussions.
Abstract

Dopamine is a key neuromodulator of locomotory circuits, yet, the role that dopamine plays during development of these circuits is less well understood. Here, we describe a suppressive effect of dopamine on swim circuits in larval zebrafish. Zebrafish larvae exhibit marked changes in swimming behavior between 3 days post fertilization (dpf) and 5dpf. We found that swim episodes were fewer and of longer durations at 3dpf than at 5dpf. At 3dpf, application of dopamine as well as bupropion, a dopamine reuptake blocker, abolished spontaneous fictive swim episodes. Blocking D2 receptors increased frequency of occurrence of episodes and activation of adenylyl cyclase, a downstream target inhibited by D2-receptor signaling, blocked the inhibitory effect of dopamine. Dopamine had no effect on motor neuron firing properties, input impedance, resting membrane potential or the amplitude of spike after-hyperpolarization. Application of dopamine either to the isolated spinal cord or locally within the cord does not decrease episode frequency, whereas dopamine application to the brain silences episodes suggesting a supraspinal locus of dopaminergic action. Treating larvae with 10μM 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine hydrochloride (MPTP) reduced catecholaminergic innervation in the brain and increased episode frequency. These data indicate that dopamine inhibits the initiation of fictive swimming episodes at 3dpf. We found that at 5dpf, exogenously applied dopamine inhibits swim episodes yet the dopamine reuptake blocker or the D2 receptor antagonist have no effect on episode frequency. These results lead us to propose that endogenous dopamine release transiently suppresses swim circuits in developing zebrafish.
Introduction

Locomotion is achieved by the rhythmic activity of motor-pattern generating circuits (Grillner 2003; Kiehn 2006). Descending projections to these pattern generating circuits regulate their activation through the release of fast-acting neurotransmitters and slower acting neuromodulators (Barriere et al. 2005; El Manira et al. 1997; Li et al. 2006; Marder and Bucher 2001; McLean and Sillar 2003; Nishimaru et al. 2000; Roberts et al. 1998). As an animal develops, its locomotory behavior becomes more flexible and mature (Clarac et al. 2004; Saint-Amant and Drapeau 1998; Sillar et al. 1991) and in some cases, even undergoes dramatic changes (Combes et al. 2004). Proper maturation of locomotory behavior requires maturational changes in the neural circuits generating motor commands. Neuromodulators have been implicated in triggering the developmental maturation of pattern generating circuits (Branchereau et al. 2002; Brustein et al. 2003a; Fenelon et al. 2003; Sillar et al. 1995; Straus et al. 2000) and they may achieve this by affecting neurogenesis (Marsh-Armstrong et al. 2004), synaptogenesis (Niitsu et al. 1995), synaptic strength (McDearmid et al. 1997), intrinsic membrane properties of individual neurons within the network (Han et al. 2007; Sillar et al. 1995) or by changing the influence of other neuromodulators on target networks (McLean and Sillar 2004).

Dopamine is a key neuromodulator involved in the control of motor systems in both invertebrates and vertebrates (Crisp and Mesce 2004; Kiehn and Kjaerulff 1996; Marder and Eisen 1984; Schotland et al. 1995). Loss of brain stem dopaminergic neurons leads to movement disorders in humans as well as non-human primates, rodents, and fish (Bretaud et al. 2004; Dauer and Przedborski 2003; Lam et al. 2005;
McKinley et al. 2005). Furthermore, dopamine receptor blocking agents prescribed as anti-psychotics induce movement disorders (Dauer and Przedborski 2003). The effect of dopamine on the initiation (Kiehn and Kjaerulff 1996; Madriaga et al. 2004; Whelan et al. 2000) and frequency of motor patterns (Schotland et al. 1995; Svensson et al. 2003b) has been well-studied. Given the importance of dopamine in the initiation and control of locomotory behavior in established neural circuits, we tested whether dopamine controls the initiation of swimming in a developing vertebrate, i.e., the larval zebrafish.

Locomotion in larval zebrafish evolves from slow tail flips at 18 hours post-fertilization (hpf), to escape swimming at 28 hpf to robust spontaneous swimming at 5 days post fertilization (dpf) (Brustein et al. 2003b; Buss and Drapeau 2001). As late as 3dpf, larvae show very little spontaneous swimming but by 5dpf, larvae swim actively for foraging. In zebrafish, dopaminergic neurons are seen as early as 24hpf (McLean and Fetcho 2004a). By 3dpf, dopaminergic neurons are seen in the ventral diencephalon, the hypothalamus, the preoptic region and the pretectum (McLean and Fetcho 2004a; Rink and Wullimann 2002). Also, putative dopaminergic fibers densely innervate the mesencephalon, rhombencephalic reticulospinal neurons and the spinal cord (McLean and Fetcho 2004a; b).

Here, we show that motor patterns generated by larval zebrafish at 3dpf are vastly different from those at 5dpf. The spinal cord in 3dpf zebrafish larvae is capable of initiating a high frequency of spontaneous fictive swimming episodes, but dopamine, acting via D2 receptors, selectively suppresses the initiation of spontaneous fictive swimming episodes. However, at 5dpf, endogenous release of dopamine does not
suppress spontaneous swimming episodes, suggesting differential dopamine modulation of circuits involved in the initiation of spontaneous swimming at these two stages.
Methods

Adult wildtype zebrafish were obtained from a commercial supplier (Scientific Hatcheries, Huntington Beach, CA) and maintained in aquarium tanks at 28°C. Embryos were collected in a trap every morning and maintained in clean fish water in a water bath at 28°C.

Larval swimming behavior

A single larva was placed in a shallow translucent plastic dish filled with fish water. Larvae swam in a 5cm wide circular arena. Swimming behavior was recorded for fifteen minutes using a Hamamatsu ORCA ER CCD camera fitted with a Nikon 50mm zoom lens at 20 frames per second. The position of the larva in each frame was detected by background subtraction and the displacement was calculated from previous frame. The total displacement for fifteen minutes was calculated by adding the displacements in each frame.

Extracellular Suction Recordings

Recordings were performed as described in Masino and Fetcho (Masino and Fetcho 2005) with minor modifications (see Fig. 2A). Briefly, larvae were anaesthetized in 0.1% Tricaine (MS222) and pinned laterally through their notochord onto Sylgard using fine tungsten wire (California Fine Wire, Grover Beach, CA). We then paralyzed the larvae by replacing the MS222 with Danio external saline containing curare (in mM: 134 NaCl; 2.9 KCl; 1.2 MgCl₂; 10 HEPES; 10 Glucose; 0.01 d-Tubocurarine; 2.1 CaCl₂; pH 7.8; 290 mmol/Kg). Using fine tungsten wire, we peeled the skin to expose the musculature and the brain. Using thin-walled borosilicate capillaries with no filament (Sutter Instruments, Novato CA), we pulled large-tipped pipettes and filled them with
Danio external saline. We positioned these close to the muscles and aspirated the fibers one by one to expose the spinal cord in two or three segments so that bath-applied drugs would permeate easily into the spinal cord. A micropipette filled with Danio saline (0.7 to 1.5 MΩ) was positioned very close to the myotomal boundary of one of the anterior segments and mild suction was applied. This resulted in the muscles, as well as the axons innervating them to be drawn up into the micropipette and the action potentials traveling down these axons could be recorded. We recorded mostly from muscle segments in the rostral one-third of the animal except during the local dopamine application experiments (see below). Multi-unit spiking activity was recorded using Multiclamp 700A amplifier and digitized using Digidata 1320 and pClamp 9.0 suite of software.

For local application of dopamine, patch pipettes were filled with 10mM dopamine containing sulforhodamineB (Invitrogen, Carlsbad, CA). The third or fourth segment from the caudal end of the spinal cord was exposed by aspirating muscle fibers as explained above and the patch pipette containing dopamine was gently inserted into the cord. Mild positive pressure was applied while monitoring the extent of fluorescence. Pressure was applied until the solution traveled at least six segments rostrally. Recordings were made from segments within the extent of dopamine injection.

Whole-cell Patch Clamp Recording

The larva was pinned out and the spinal cord exposed as described above. Patch pipettes were pulled from borosilicate glass (Sutter Instruments, Novata, CA) and filled with internal solution (in mM: K Gluconate 115; KCl 15; MgCl2 2; HEPES 10; EGTA 10; Mg-ATP 3.94; pH 7.2; 290 mOsm; Pipette resistance 10-14 MΩ). The patch
pipette was placed in the bath and in current clamp mode, the pipette offset and capacitance were calculated. Then the amplifier was switched to voltage-clamp mode and a giga-seal was formed with a ventrally located cell body. After adjusting for pipette capacitance, the seal was broken to achieve whole-cell configuration. The amplifier was switched to current clamp mode and DC current was injected to keep the cell membrane potential near –65mV. The bridge resistance and pipette capacitance were compensated for. Current pulses of varying amplitudes and about 1s duration were injected and the resulting membrane potential was recorded. The cell was filled with fluorescent dye included in the internal solution and at the end of the recording motor neuronal identity was confirmed from the morphology of the cell.

**Drugs**

Saline containing drugs at stated concentrations were bath-applied using a switching manifold and drugs tended to have an effect within 10 to 15 minutes of bath application. Some drugs were dissolved in 0.1% Dimethyl sulfoxide (DMSO) prior to further dilution in saline. 0.1% DMSO by itself did not have an effect on motor pattern activity (data not shown). Dosages for all drugs used were determined in preliminary experiments. Drugs were obtained from Sigma Chemical Company, St. Louis, MO (dopamine, bupropion hydrochloride, N-methyl d-aspartic acid, L741,626) and Tocris Inc, Ballwin, MO (S(-) sulpiride).

**Data Analysis**

Spikes were extracted offline using Spike2 software (Cambridge Electronic Design) and spike times were sorted into bursts, episodes and bouts using custom scripts written in Neuroexplorer (Nex Technologies, Littleton, MA) and Matlab (The
Mathworks Inc, Natick, MA). Bouts were defined as intervals during which three or more spikes occurred and after which there was an inter-spike interval of at least 10s. Episodes were defined as time intervals during which three or more spikes occurred and after which there was an inter-spike interval (ISI) of at least 100ms. Bursts were defined as time intervals during which one or more spikes occurred and after which there was an ISI of at least 10ms. Burst period and episode period were calculated as the time between successive burst and episode start times respectively. Episode duration was calculated as the time between the start and end of an episode. Parameters such as episode period, duration and burst period were calculated using custom scripts written in Matlab. Swim episodes were counted in a 10-minute window and plotted.

For the firing rate vs. current injected plots, the instantaneous firing rate was calculated as the inverse of the first ISI evoked by current of certain amplitude. The slope of the linear part of the firing rate vs. current curve was calculated to obtain gain. The input impedance and resting membrane potential were calculated as the slope and the y-intercept respectively of the voltage vs. current plot. Data were plotted using Microsoft Excel and SigmaPlot. Statistical testing was performed with Statview. In general the Mann-Whitney U test was used to test for significant differences in episode periods and durations because these data were non-normally distributed. The Student’s T-test was used for burst period data because these passed the test for normality. Where present, error bars indicate standard error of the mean (SEM).

*MPTP treatment*
Embryos were reared in normal fish water for 24 hours at 28°C. 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine hydrochloride (MPTP-HCl) was purchased from Sigma in 10mg rubber-stoppered bottles. 50mM stock MPTP solution was made by injecting 1mL fish water containing 0.01% Tween-80 into the bottle. The stock solution was further diluted to 10µM and 1 day old embryos were placed in it. Control embryos were reared in 0.01% Tween-80 solution. At 3dpf embryos were rinsed several times in fish water and the spontaneous swim episodes were recorded as described above.

**Whole-mount Immunohistochemistry**

3dpf larvae were fixed in 4% paraformaldehyde at 4°C overnight and then rinsed in phosphate-buffered saline (PBS). Larvae were pinned on Sylgard and the skin covering the brain was carefully peeled. The yolk and the eyes were removed. The jaw was removed to expose the ventral surface of the brain because many of the TH-positive cell groups are located on the ventral side. All incubations were performed at 4°C. After blocking overnight in 10% goat serum in PBS-Triton-X100 (PBST), larvae were washed several times in PBST and then incubated in 1:400 rabbit anti-mouse tyrosine hydroxylase antibody (MAB318, Chemicon, Temecula, CA) for 2 days. The larvae were washed several times in PBST and then incubated in chicken anti-rabbit IgG coupled to Alexa 594 overnight (Invitrogen, Carlsbad, CA). Larvae were washed several times in PBS and the brains were mounted ventral side up on glass slides with Prolong mounting medium (Invitrogen, Carlsbad, CA) and coverslipped. Brains were imaged on a Zeiss LSM 510 confocal microscope with 543nm excitation and a 560nm long-pass filter. Images were analysed offline using LSM Image Browser and Adobe Photoshop software. Control and MPTP-treated larvae were processed for
immunohistochemistry in parallel and imaged under the same conditions. Images were analyzed offline for number of TH-positive cell bodies using ImageJ software.
Results

Newly-hatched zebrafish larvae (~2.5dpf) have intact touch-evoked swimming responses but show very little spontaneous swimming. However, by 5dpf, larvae swim robustly in all 3 dimensions (Brustein et al. 2003b; Buss and Drapeau 2001). To quantify this change in swimming behavior, we recorded movies of larval swimming at 3dpf and 5dpf and calculated the total displacement of larvae over 15 minutes (Figure 1; supplementary movies 1 and 2). The average total displacement of larvae in 15 minutes is significantly greater at 5dpf than that at 3dpf (Fig. 1B, P<0.01, n = 7).

To understand the neural basis for such a dramatic change in swimming behavior, we recorded fictive motor patterns using suction electrodes at the nerve muscle junction in paralyzed preparations (Fig. 2A). In the 3dpf larvae, such recordings revealed motor patterns organized into spontaneous episodes of fictive swimming (referred to as “episodes” henceforth). These episodes were clustered together into bouts of activity and bouts of episodes lasted several seconds with periods on the order of several tens of seconds (Fig. 2B, top trace). Each bout consisted of many episodes of swimming, with the episode periods ranging from hundreds of milliseconds to several seconds (Fig. 2B, second trace). Within each episode of swimming, bursts of spikes occurred with intervals ranging from 20 to 50ms. Bursts consisted of one to two spikes and the spiking interval within a burst was less than 10ms (Fig. 2B bottom two traces). Figure 2C shows the frequency distribution of the inter-spike intervals (ISIs) for the data in Figure 2B and represents typical ISI distributions seen at 3dpf. ISIs separating spikes in successive bursts (inter-burst intervals) are centered at approximately 30ms and there is a broad distribution of longer ISIs with no detectable peaks. The peak seen at
ISI<10ms corresponds to spike intervals within bursts (intra-burst intervals). These data demonstrate that spontaneous motor patterns are organized into rhythms at multiple time scales.

**Fictive swim motor patterns show significant differences between 3dpf and 5dpf**

We then compared fictive swim motor patterns recorded at 3dpf with those recorded at 5dpf. At 3dpf, episodes occurred at relatively low frequency (Fig. 3A, top trace). When these episodes occurred, they were clustered into bouts (Fig. 3A, bottom trace and Fig 2B). However, at 5dpf, episodes occurred at a much higher frequency (Fig. 3B, top trace) and a bout structure was absent (Fig. 3B, bottom trace). The number of episodes in a ten minute recording window increased from 22.3 ± 4.9 at 3dpf (n =14) to 222 ± 23 at 5dpf (n=11; P<0.001, Fig. 3C). The cumulative probability distribution of episode periods at 3dpf and at 5dpf (Fig. 3D) shows that at 5dpf, there was no episode period greater than ~50s. The distributions of episode periods at 3dpf and 5dpf were similar for short episode periods but diverged at the long end of the distribution (P<0.001, n = 14 at 3dpf and 11 at 5dpf). At 3dpf, short episode periods were those occurring inside of a bout of episodes and these were similar to those seen at 5dpf (compare periods for bottom trace in Figs. 3A and 3B). However, at 3dpf, bouts were separated by long intervals and these were responsible for the long tail of the period distribution. Episode duration also changed significantly between 3dpf and 5dpf. At 3dpf, episodes had an average duration of 1.78 ± 0.32s (n=14) while at 5dpf episode duration was 0.45 ± 0.09s (n=11). The distribution of episode durations at 5dpf was shifted towards shorter durations compared to 3dpf (Fig. 3E) and the two distributions
were significantly different from each other (P<0.0001). Concomitant with the developmental decrease in episode durations, there was also a decrease in the number of bursts per episode from $42.3 \pm 7.5$ at 3dpf to $14.2 \pm 2$ bursts per episode at 5dpf (Fig. 3I, n = 26, P<0.001). When the motor pattern was observed at faster time scales, we noticed an increase in the number of spikes per burst from 3dpf to 5dpf (Fig. 3F and 3G; P<0.001, n =26). However, there was no significant change in the average burst period between 3dpf and 5dpf (3dpf: 35.1 ± 1 msec; 5dpf: 34.7 ± 0.001, n=26, P=0.934). Thus, maturation of the spinal cord swim circuit from 3dpf to 5dpf was marked by a decrease in the period and duration of episodes, a decrease in the number of bursts per episode and an increase in the number of spikes per burst.

**Dopamine is a modulator of swim initiating circuits in zebrafish larvae**

The output of the swim circuit differed between 3 and 5dpf in zebrafish larvae suggesting that the swim circuit undergoes developmental modifications during this time. Because dopaminergic innervation of the CNS develops relatively early, it is an ideal candidate for regulating the maturation of the swim motor circuit. Therefore, first we tested whether dopamine altered swim circuit output in larval zebrafish at 3dpf. At 3dpf, bath application of saline containing 10µM dopamine abolished all episodes in 4 out of 4 larvae (Fig. 4A), however, episodes could still be evoked by a flash of light in the presence of dopamine in 4 of 4 larvae (Fig.4A, middle trace, arrow). The number of bursts per episode (49.8 ± 24.7), the number of spikes per burst (1.46 ± 0.3) and the burst cycle periods (0.041 ± 0.007s) in such light-evoked episodes were not significantly different from those seen during spontaneous episodes in control saline
Episodes returned after dopamine was rinsed out of the bath (Fig. 4A).

To test if dopamine is released endogenously and if the endogenously released dopamine can suppress fictive swim episodes, we bath applied 200µM bupropion hydrochloride, a dopamine reuptake blocker, to 3dpf larvae. Bath application of 200µM bupropion hydrochloride silenced episodes reversibly in 4 out of 4 larvae at 3dpf (Fig. 4B). These data indicated that both exogenously applied dopamine and endogenously released dopamine could silence episodes in the 3dpf larval locomotor network.

In contrast to the effect of dopamine and bupropion, application of L741,626, a D2 receptor-specific antagonist, increased the frequency of swim episodes compared to that in control saline (Fig. 4C). In 5 of 5 larvae, the number of episodes in 10 minutes increased after application of L741, 626 (Fig. 4D, P<0.02). When we plotted the cumulative probability distribution of episode periods in control saline and in L741, 626 (Fig. 4E), we found that the distributions were significantly different (P<0.05, n = 175 episodes from 5 larvae). The distributions were overlapping for episode periods less than 1s, however, they diverged for longer episode periods. The distribution of episode durations was not significantly different between control and L741, 626 (Fig. 4F, P =0.65, n = 180 episode durations from 5 larvae). Similarly, application of sulpiride, another D2-specific antagonist, also increased the number of episodes significantly (Fig. 4G and H, Control: 39 ± 23; Sulpiride: 194 ± 55, P<0.01). However, there was no change in the number of spikes per burst, the burst cycle period or the number of bursts per episode.
Activation of the D2-like family of receptors (consisting of D2, D3 and D4 receptors) suppresses adenylyl cyclase activity (Missale et al. 1998) and leads to a reduction in cellular cAMP levels (Fig. 5A). We asked if the effect of dopamine on fictive swimming can be occluded by interfering with the downstream signaling of D2 receptors. To this end, we first silenced the spontaneous activity with dopamine and then bath applied forskolin, a cell-permeable activator of adenylyl cyclase (Fig. 5A). In preparations that were silenced with 100µM dopamine (Fig. 5B, middle trace), adding 10µM forskolin to the saline resulted in a high frequency of episodes (Fig. 5B, bottom trace), indicating that forskolin was able to over-ride the suppressive effects of dopamine even at relatively high concentrations of dopamine. In 5 of 5 larvae, application of dopamine along with forskolin increased the number of episodes in ten minutes (Fig. 5C, control: 39 ± 13; Forskolin + Dopamine: 397 ± 94; P<0.02). Again the cumulative distribution of episode periods in control and in forskolin plus dopamine was significantly different (Fig. 5D, 522 episode periods from 5 larvae, P<0.001). Forskolin, even in the presence of dopamine caused a shift of episode periods towards shorter period values and an absence of episode period values above 50s. Importantly, activation of adenylyl cyclase blocked the suppressive effects of dopamine on episodes. Application of forskolin along with dopamine also significantly decreased episode durations – the cumulative distribution of episode durations shifted to the left (Fig. 5E, 539 episode durations from 5 larvae, P<0.001), and modestly increased the average burst period (Figs. 5F and G, control: 0.033 ± 0.003 s, Forskolin+Dopamine: 0.04 ± 0.0018 s, P<0.05). Forskolin did not affect the number of spikes per burst or the number of bursts per episode.
Effect of dopamine on motor neuronal firing properties

By 3dpf, the spinal cord is innervated by tyrosine hydroxylase immunoreactive fibers (TH-fibers) (McLean and Fetcho 2004a), some of which originate from the dopaminergic neurons of the posterior tuberculum (McLean and Fetcho 2004b). These spinal cord TH-fibers are closely apposed to motor neuronal cell bodies and proximal dendrites (McLean and Fetcho 2004b), suggesting a direct effect of dopamine on motor neurons. To investigate if dopamine affects motor neuronal firing properties, we performed whole-cell recordings in current clamp mode from motor neurons in control saline and in 10µM dopamine. In control saline, motor neurons showed episodes of spiking (Fig.6A, left), which correspond to the episodes recorded extracellually. The same motor neuron showed no spontaneous spiking activity in the presence of 10µM dopamine consistent with the results obtained with extracellular recording (Fig. 6A, right). To assay if dopamine modifies the neuronal input-output relationship, we recorded spikes generated by increasing amounts of injected current in control saline or in 10µM dopamine (Fig. 6B). The instantaneous firing rate, calculated as the inverse of the first inter-spike interval evoked by current of given amplitude was not affected by dopamine (Figs. 6B and 6C). Dopamine did not significantly alter the neuronal gain (the slope of the firing rate vs. current plot; Fig. 6D, P = 0.838), input impedance (Fig. 6E, P = 0.713), resting membrane potential (Fig. 6F, P = 0.969) or after-hyperpolarization amplitude (Fig. 6G, P = 0.232, n=35 cells in control and 24 cells in dopamine).

Effect of dopamine on spinal circuits
We investigated the effect of dopamine on local spinal cord circuits by employing two methods: one was to assay the effect of dopamine on the isolated spinal cord and two was to apply dopamine locally within the cord in the intact animal. We spinalized 3dpf zebrafish by severing the tail at the level of the rostral 4th–5th segments. After isolation of the tail, no spontaneous activity could be detected (data not shown). NMDA application induced episodic activity as previously reported (McDearmid and Drapeau 2006); Fig. 7A top trace). Addition of 100µM dopamine to the saline containing NMDA did not suppress episodes (Fig. 7A, bottom trace). There was no reduction in the number of NMDA-evoked episodes in ten minutes when dopamine was added to the saline (Fig. 7E, P=0.2). Dopamine had no effect on the number of spikes per burst or bursts per episode but it increased the average burst period (NMDA: 0.047± 0.002 s, NMDA+DA: 0.057± 0.003 s, P=0.032, n=4).

Next, we injected dopamine either into the caudal spinal cord or into the rhombencephalic ventricle while recording episodes extracellularly. When 10mM dopamine (1000X bath-applied levels) was pressure-injected into the caudal cord, such that four to six segments were exposed to dopamine, episodes were not affected (Fig. 7C top and middle trace) and the number of episodes in ten minutes remained unchanged (Fig. 7D, P=1, n=6, paired T-test). The distribution of episode periods was also not significantly altered (316 episode periods from 6 larvae, P=0.254). By contrast, in the same animal, when 10mM dopamine was injected into the rhombencephalic ventricle, episodes were abolished (Fig. 7C, bottom trace) and the number of episodes fell to zero (Fig. 7D). This suggests that the target circuits of dopamine that mediate the suppression of episodes are more likely to lie within the brain and not in the spinal cord.
**Effect of dopamine on swimming in 5dpf larvae**

We investigated the effect of dopamine in 5dpf larvae, first on swimming behavior and then on fictive swimming in paralyzed preparations. We collected movies of larval swimming behavior in normal fish water and in fish water containing 10µM dopamine for 15 minutes each. 5dpf larvae showed robust spontaneous swimming (supplementary movie 2 and Fig. 8A, left) whereas the same larvae placed in saline containing 10µM dopamine showed reduced swimming (supplementary movie 3 and Fig. 8A, right). The average total displacement of larvae in 15 minutes in dopamine was significantly smaller than in normal saline (Fig. 8B; Control: 264.5 ± 46.9 cm; DA: 83.5 ± 24.4 cm, P<0.05, n=7).

When we applied dopamine to paralyzed 5dpf larvae, we found a significant decrease in the number of episodes (Fig. 8C and D; n=7, P<0.001). In the presence of dopamine, the cumulative distribution of episode periods was significantly different from that seen in control and had a long tail like that seen at 3dpf (Fig. 8E; 529 episode periods from 7 larvae, P<0.001). The two distributions were similar for short episode periods (<10s) but diverged for longer episode periods. In particular, at 5dpf, in control saline, period values greater than 50s were absent, however, periods as long as 500s were seen in the presence of dopamine. Similarly, dopamine shifted the distribution of episode durations towards longer durations (Fig. 8F; 536 episode durations from 7 larvae, P<0.001). There was no effect on the number of spikes per burst or the average burst period in the presence of dopamine.
**Endogenously released dopamine does not suppress spontaneous motor episodes at 5dpf**

We recorded fictive swim episodes from 5dpf larvae in the presence of the dopamine reuptake blocker, bupropion and observed that bupropion did not affect episode number (Fig. 9A and B, P=0.438, n =6). The distribution of episode periods in control and in bupropion were similar (Fig. 9C; 1209 episode periods from 6 larvae at 5dpf, P = 0.385), however, episodes were of significantly shorter duration in bupropion than in control saline (Fig. 9D; 1215 episode durations from 6 larvae at 5dpf, P<0.001). The number of spikes per burst or the average burst periods were not affected. Thus, in contrast to the suppressive effect of bupropion on spontaneous motor episodes at 3dpf, the accumulation of extracellular endogenous dopamine with bupropion treatment was not sufficient to decrease spontaneous activity at 5dpf.

**Blocking D2 receptors does not affect episode activity at 5dpf**

We asked if blocking D2 receptors at 5dpf would yield the same effects as it did in the 3dpf larva. We found that bath application of the D2 receptor antagonist, Sulpiride did not affect the number of swim episodes (Fig. 10A and B, n=6, P = 0.86). Sulpiride did not affect the distributions of episode periods and durations (Fig.10C and D; P = 0.766 for episode periods and P = 0.705 for durations) nor did it change the number of spikes per burst, the burst cycle period or the number of bursts per episode.

**Larvae with compromised dopaminergic circuitry have a higher frequency of occurrence of swim episodes**
All of our data thus far suggest that in the 3dpf zebrafish larva, the motor circuitry for generating episodes is functional but suppressed by dopaminergic input and that by 5dpf dopaminergic signaling may not be as effective at suppressing the motor output. If this is the case, lesioning the dopaminergic neurons specifically should relieve this suppression and lead to a higher frequency of fictive swim activity at 3dpf. To test this hypothesis, we used 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine hydrochloride (MPTP), a neurotoxin that has been shown to kill dopaminergic neurons in zebrafish larvae at 50µM (Bretaud et al. 2004). When we treated larvae with 50µM MPTP, larvae exhibited a curved body and were completely still. They did not show any escape responses and the musculature appeared rough and irregular. At a lower dose (10µM), larvae had normal appearance. To test if 10µM MPTP affects dopaminergic neurons, we maintained 1dpf larvae in normal fish water or 10µM MPTP for 48 hours and immunostained them for tyrosine hydroxylase (TH), a marker for catecholaminergic neurons. Dopaminergic cells are found in the olfactory bulb, the subpallium, the pretectum, the pre-optic region, the ventral diencephalon and in the caudal hindbrain. The largest group of TH-positive cells is found in the ventral diencephalon (Fig. 11Ai and Aii, (McLean and Fetcho 2004a; Rink and Wullimann 2002)). Although MPTP treatment did not appear to affect the number of TH-positive cell bodies in any of the above regions (Fig. 11B), it severely reduced the TH-positive fiber systems in the brain (Fig. 11Aiii-viii). Specifically, TH-immunoreactivity (IR) in the post-optic commissure (POC) was reduced (Fig. 11Aiii and iv) and TH-IR in the neuropil of the optic tectum was not detectable in the MPTP-treated larvae (Fig. 11Av and vi). TH-IR fibers in the rhombencephalon that have been shown to innervate reticulospinal circuitry (McLean
and Fetcho 2004b) were greatly reduced (Fig. 11Avii and viii). Overall, 10µM MPTP
treatment reduced innervation of the brain by TH-positive axonal fibers suggesting that
the toxin treatment reduced dopaminergic inputs to target circuits.

When we collected extracellular recordings of spontaneous episodes from control
and MPTP-treated larvae, we found that the episodes occurred much more frequently in
the latter group (Fig. 11C). Larvae treated with 10µM MPTP from 1-3dpf had
significantly more episodes in 10 minutes at 3dpf compared to control larvae (Fig. 11C
and D, P<0.001, n=31 for control, n = 20 for MPTP). The episodes appeared normal
and the burst periods in MPTP-treated larvae were similar to control larvae (Fig. 11C,
bottom trace, compare to Fig. 2B, P=0.942). The number of spikes per burst and the
number of bursts per episode were not significantly different in control and MPTP-
treated larvae.
Discussion

Zebrafish larvae show marked changes in swim output between 3dpf and 5dpf. First, episodes of swimming were much more frequent and shorter in duration at 5dpf compared to 3dpf. Next, the swim bursts consisted of more spikes at 5dpf than at 3dpf, similar to the ontogeny of swim motor patterns in *Xenopus laevis* tadpoles (Sillar et al. 1991). At 3dpf, when we applied dopamine exogenously, or when we increased endogenously released dopamine with a dopamine uptake inhibitor, swim episodes were abolished. Conversely, when we blocked dopaminergic inputs with a D2 receptor antagonist or when we compromised the dopaminergic circuit with the neurotoxin MPTP, larvae exhibited a higher frequency of swim episodes. These data indicate that zebrafish larvae at 3dpf are capable of generating fictive swimming episodes at a high frequency but that the initiation of episodes is suppressed by endogenously released dopamine. Subsequently, at later larval stages, endogenously released dopamine is unable to suppress initiation of swimming and the larva exhibits more frequent episodes of swimming. We propose that dopaminergic neuromodulation helps to keep the functional but immature swimming circuit of a 3dpf larva largely silent.

_transient nature of dopaminergic suppression of swim circuits_

We found that exogenous dopamine suppressed swim episodes at 3dpf and 5dpf. To test if endogenously released dopamine affected fictive swim episodes at both stages, we used bupropion, which blocks dopamine reuptake. We found that while bupropion silenced episodes at 3dpf, it had no effect on episode frequency at 5dpf even though dopaminergic neurons and projections to the spinal cord are still present at 5dpf.
This suggests that the endogenous release of dopamine is unable to exert a suppressive effect on swim circuits at 5dpf. One caveat is that bupropion also blocks noradrenaline reuptake, and therefore, some of the effects of bupropion that we see might be mediated by noradrenaline instead of dopamine. However, bupropion has been shown to be twice as effective at the dopamine transporter as it is at the noradrenaline transporter (Horst and Preskorn 1998). Further, bupropion has been used effectively in earlier studies in lamprey to establish a role for endogenous dopamine in the modulation of locomotory patterns (Schotland et al. 1995; Svensson et al. 2003b), spinal neuron intrinsic properties (Schotland et al. 1995) and the strength of reticulospinal synaptic inputs (Svensson et al. 2003a). It was also shown by HPLC analysis that when spinal tissue was incubated with bupropion, the concentration of extracellular dopamine significantly increased (Schotland et al. 1995). Lastly, consistent with the lack of effect of bupropion, we found that application of D2 receptor antagonists did not affect episode frequency at 5dpf. Taken together, these results lead us to propose that endogenously released dopamine has a transient suppressive effect on swim circuits in zebrafish larvae. The inability of endogenously released dopamine to suppress the output of the swim circuit at 5dpf may be driven by other ontogenic events such as the maturation of other modulatory inputs (Brustein et al. 2003a) or the increase in excitatory synaptic drive within the cord (Buss and Drapeau 2001).

Locus of dopaminergic action

(McLean and Fetcho 2004a; Rink and Wullimann 2002).
We found that dopamine had no effect on motor neuronal intrinsic properties and that it did not suppress NMDA-evoked episodes in the isolated cord. In the intact animal, when we injected dopamine locally within the cord so as to expose 4-6 segments to dopamine, swim episodes were not affected. The most parsimonious explanation for these results is that the locus of dopaminergic suppression of swim episodes is supraspinal, however, it is possible that the local injection of dopamine into the spinal cord did not reach enough number of spinal segments to inhibit episode initiation. There are about 30 spinal segments in zebrafish and suppression of 20% of the segmental oscillators may be insufficient to prevent the triggering of episodes. Nevertheless, it is not technically feasible to expose all of the spinal cord without also exposing supraspinal centers to dopamine in this small animal. It should be possible in the future to confirm the locus of dopamine's action through the use of calcium imaging of descending projection neurons.

*Dopaminergic terminal loss in response to MPTP treatment*

We used a dose of MPTP lower than those used in previous studies in zebrafish (Bretaud et al. 2004; Lam et al. 2005; McKinley et al. 2005). This low dose of MPTP reduced the number of TH-labeled fibers in many areas of the brain. This observation is particularly interesting because in Parkinson's disease, dopaminergic neurons seem to undergo a “dying back” process by which the dopaminergic axonal process progressively dies culminating in the death of the cell body itself. This view is substantiated by MPTP toxicity studies in the monkey where loss of striatal dopaminergic terminals precedes loss of substantia nigra cells and in the rat where
protection of striatal terminals prevents the loss of substantia nigra cells (Dauer and Przedborski 2003). These studies suggest that many of the early clinical symptoms of Parkinson’s disease could be the effect of loss in terminals and reduction in dopamine release to postsynaptic targets even before cell death has begun. Consistent with this, cDNA microarray analysis of rat substantia nigra has revealed that MPTP treatment results in reduced expression of messages coding for proteins involved in axonal transport, vesicle docking and transmitter release (Miller et al. 2004). Taken together with previous studies, our results suggest that a low dose of MPTP is an effective tool to compromise dopaminergic cell function without inducing cell death.

Developmental modulation of locomotory circuits

During development, animals face changing behavioral needs. For example, with increasing body lengths, tadpoles and fish larvae encounter increasing Reynolds numbers. Therefore, the force required for propulsion needs to increase. Another example is a change in mode of locomotion, such as in amphibians, where the locomotory networks switch from a pre-metamorphic tail-driven axial swimming output to a post-metamorphic limb-driven hopping/swimming output (Combes et al. 2004). The maturation of locomotory circuits in line with the animal’s behavioral needs can be triggered by neuromodulators. For instance, in zebrafish, serotonin increases swim episode frequency (Brustein et al. 2003a) and it has been suggested that serotonin produces its effects by modulating chloride homeostasis (Brustein and Drapeau 2005). Although serotonergic neurons and fibers are present quite early in development, serotonin fails to affect episode frequency earlier than 4dpf (Brustein et al. 2003a).
fact, the maturation of sertonergic system by 5dpf may partially underlie the relief from
dopaminergic suppression of swim episodes that we see.

Neuromodulators can suppress the expression of a motor output until it is
needed. The network for generating lung breathing is mature in pre-metamorphic *Rana*
tadpoles but it is inhibited by GABA<sub>B</sub> receptor activation (Straus et al. 2000). Similarly,
the stomatogastric ganglion in embryonic lobsters is capable of generating adult-like
motor patterns but is suppressed from doing so by central modulatory inputs (Fenelon et
al. 2003).

Motor patterns in newly hatched *Xenopus* tadpoles consist of single-spike bursts
but in 24 hours, this nascent motor pattern evolves to one in which there are several
impulses per burst cycle (Sillar et al. 1991). Serotonin acts as a maturation factor for
this process (Sillar et al. 1995) and it was shown that presence of exogenous serotonin
increases burst durations in the rostral cord around the time that raphe inputs arrive at
the cord. In subsequent stages, more caudal segments of the cord were affected by
serotonin (Sillar et al. 1992). Likewise, removal of serotonin or blockade of 5HT1a
receptors prevented the occurrence of multi-spike bursts in post-hatchling tadpoles
(Sillar et al. 1995). Our results in 3dpf and 5dpf larvae also show that in larval zebrafish
the motor pattern evolves from single-spike bursts to multi-spike bursts, similar to the
ontogeny of the *Xenopus* motor pattern.

Dopamine is known to activate motor patterns in the rat (Kiehn and Kjaerulff
1996). In mouse, dopamine increases the excitability of motor neurons and the
excitatory synaptic transmission impinging on them (Han et al. 2007). In lamprey,
dopamine decreased burst frequency when present at high concentrations and had the
opposite effect at lower concentrations (Svensson et al. 2003b). Dopamine also reduced the amplitude of the slow after hyperpolarization in spinal neurons in lamprey (Schotland et al. 1995). Here, we propose a developmental role for dopamine in regulating swim circuit activity in larval zebrafish. We suggest that endogenously released dopamine acts transiently to regulate swim episode frequency in larval zebrafish. Such differential neuromodulation during development might be fundamental for the maturation of network function, not only in locomotory circuits but also for neural circuits in general.
**Figure Legends**

Figure 1: Larvae show increased swimming at 5dpf compared to 3dpf. A. Swimming trajectories of 3dpf and 5dpf larvae over 15 minutes of video recording. Scalebar: 5cm. B. Total displacement of 3dpf and 5dpf larvae in 15 minutes. N = 7 larvae each at 3dpf and 5dpf.

Figure 2: Motor patterns in larval zebrafish. A. Schematic of the experimental preparation illustrating extracellular and whole-cell recording configurations. B. Spontaneous motor patterns recorded extracellularly at 3dpf, shown from top to bottom at increasing time resolution to reveal their temporal organization. Top: Spontaneous episodes of fictive swimming (episodes) are clustered together into bouts. Second trace: A single bout is expanded to show individual episodes of fictive swimming. Third trace: Every episode consists of rhythmic bursting. Bursts are separated by about 30ms. Last trace: Bursts consist of one to three spikes at 3dpf. C. Distribution of the inter-spike intervals (ISIs) for the data shown in B. Peaks correspond to spike intervals within a burst (0<ISI<10ms) and spike intervals between bursts (20-40 ms). Longer ISIs correspond to intervals between episodes and intervals between bouts of episodes.

Figure 3: Fictive swim motor patterns are different in 3dpf and 5dpf larvae when examined at multiple time scales. A. Episodes recorded from a 3dpf larva show bouts of episodes (top trace). One of these bouts indicated by the thick grey bar is expanded in the bottom trace to show individual episodes. B. Extracellular recordings from a 5dpf larva show increased number of spontaneous episodes (top trace), which are not tightly
clustered into bouts. The region indicated by the thick grey bar is expanded in the bottom trace. C. The number of episodes recorded in a 10 minute window is significantly higher at 5dpf compared to 3dpf (n = 14 for 3dpf larvae and 11 for 5dpf larvae) D. Cumulative probability distribution of episode periods at 3dpf (black dots) and 5dpf (grey dots) shows that distributions differ at longer episode periods. E. Cumulative probability distribution of episode durations at 3dpf (black dots) and 5dpf (grey dots) indicate significantly longer episode durations at 3dpf compared to 5dpf. F. At faster time scales, motor patterns at 3dpf (top trace) and 5dpf (bottom trace) are different in the number of spikes per burst, although burst cycle periods are not different. G. The average number of spikes per burst increases from 3dpf to 5dpf. H. The average burst cycle period remains the same between 3dpf and 5dpf. I. The number of bursts per episode significantly decreases at 5dpf in agreement with the decrease in episode duration.

Figure 4: Dopamine suppresses swim episode initiation at 3dpf. A. Recordings from a 3dpf larva in control saline (top trace), in 10μM dopamine (middle trace) and in control saline after rinsing off dopamine (bottom trace). Arrow indicates episode evoked by a light stimulus. B. Episodes in a 3dpf larva in control saline (top trace), in 200μM Bupropion (middle trace) and after rinsing off Bupropion (bottom trace). C. Extracellular recording showing episodes in a 3dpf larva in control saline (top trace), and in a D2 receptor specific antagonist (L741,626, bottom trace). D. Application of D2 receptor antagonist L741,626 increases the frequency of occurrence of episodes at 3dpf significantly. E. Cumulative probability distribution of episode periods in control saline
(black dots) and in saline containing L741,626 (grey dots). F. Cumulative probability
distribution of episode durations in control saline (black dots) and in saline containing
the D2 antagonist L741,626 (grey dots). G. Extracellular recording of fictive swim
episodes in a 3dpf larva in control saline (top trace) and in saline containing another D2
specific blocker, sulpiride (bottom trace). H. Application of sulpiride significantly
increased episode frequency.

Figure 5: Activation of adenylyl cyclase over-rides dopaminergic inhibition. A: Schematic
diagram showing the known signaling cascades downstream of D2 receptor activation
by dopamine (DA). B. Low frequency episodes are present in control saline (top trace)
but are abolished in 100μM dopamine (middle trace). 10μM Forskolin increased
episodic activity even in the presence of 100μM dopamine (bottom trace). C. Episode
frequency was significantly increased when Forskolin and Dopamine were bath-applied
together compared to control saline. D. Pooled data from 5 larvae at 3dpf shows that in
the presence of forskolin and dopamine, episode period distribution is significantly
shifted towards shorter episode periods (control: black; forskolin plus dopamine: grey).
E. Cumulative probability distribution of episode durations in control saline (black dots)
and in saline containing forskolin and dopamine (grey dots). F. Traces in B expanded to
show bursts in control saline (top trace) or in forskolin plus dopamine (bottom trace). G.
Average burst period ± SEM in control saline ('Ctrl') and in Forskolin plus dopamine
('Frsk+DA').
Figure 6: Dopamine does not affect the intrinsic membrane properties of motor neurons.
A. Whole-cell current clamp recording from a motor neuron in control saline (left) and the same neuron in dopamine (right). B. Spiking induced in a motor neuron (top traces) in response to injection of current (bottom traces) in control saline (left) and in dopamine (right). C. Current injected vs. the instantaneous rate of spiking is plotted for the traces shown in B. Control: black circles; Dopamine: open triangles. D. Summary data from 33 cells in control saline and 24 cells in 10μM dopamine showing mean ± SEM of neuronal gain, input resistance, resting membrane potential and amplitude of the after-hyperpolarization of motor neurons.

Figure 7: Dopaminergic suppression of episodes likely occurs in the brain. A. Episodes of fictive swimming induced in an isolated tail by the application of NMDA (top trace) were not suppressed by the addition of dopamine to the bath saline (bottom trace). B. Episode frequency in isolated tail exposed to NMDA first and then to a mixture of NMDA and dopamine (‘+DA’). C. Local injection of 10mM dopamine into the posterior spinal cord (schematic diagram shown on the left) does not alter the frequency of episodes (right, top trace – control; middle trace – dopamine injected within spinal cord), however injection into the brain immediately suppressed episodes (bottom trace). D. Pressure injection of 10mM dopamine into the spinal cord (‘SC’) did not decrease the frequency of episodes but injecting into the brain (‘Brn’) abolishes all episodes in 6 of 6 larvae.

Figure 8: Exogenous dopamine decreases initiation of swimming at 5dpf. A. Swimming trajectories of a 5dpf larva in control fish water (left) and in dopamine (right) over 15
minutes. Scalebar: 5 cm. B. Total displacement in 15 minutes of 5dpf larvae in control fish water and in dopamine. C. Fictive swim episodes in control saline (top trace), in saline containing 10μM dopamine (middle trace) and after rinsing out dopamine (bottom trace). D. Exogenous dopamine reversibly decreases episode frequency at 5dpf. E. Cumulative probability distribution of episode periods in 5dpf larvae in control saline (black dots) and in dopamine (grey dots). F. Cumulative probability distribution of episode durations in 5dpf larvae in control saline (black dots) and in dopamine (grey dots).

Figure 9: Effect of the dopamine reuptake blocker bupropion on fictive swimming at 5dpf. A. Episodes in a 5dpf larva in control saline (top trace), in 200μM bupropion (middle trace) and after rinsing off bupropion (bottom trace). B. Bupropion did not significantly alter the frequency of episodes in 5 dpf larvae. C. Cumulative probability distribution of episode periods at 5dpf in control saline (black dots) and in 200μM bupropion (grey dots). D. Cumulative probability distribution of episode durations at 5dpf in control saline (black dots) and in 200μM bupropion (grey dots).

Figure 10: The D2 receptor specific antagonist sulpiride did not affect fictive swim episodes at 5dpf. A. Extracellular recording of fictive swim episodes at 5dpf in control saline (top trace) and in sulpiride (bottom trace). B. Episode frequency of 5dpf larvae did not change when sulpiride was applied. C. Cumulative probability distribution of episode periods at 5dpf in control saline (black dots) and in sulpiride (grey dots). D. Cumulative
probability distribution of episode durations at 5dpf in control saline (black dots) and in sulpiride (grey dots).

Figure 11: Ablating catecholaminergic circuitry derepresses the 3dpf larval swim circuit. A. MPTP treatment causes an overall reduction in TH-immunoreactive fibers in the CNS. i, iii, v, vii: Control; ii, iv, vi, viii: MPTP-treated. i, ii: TH-immunoreactive cell bodies in the ventral diencephalon. iii, iv: TH-immunoreactive cell bodies in the pre-optic region and labeled fibers in the post-optic commissure (white arrowhead). v, vi: Labeled cell bodies in the pre-tectal region and labeled fibers within the tectal neuropil (white arrowheads). vii, viii: Labeled fiber tracts in the rhombencephalon. TH-positive cell bodies in the caudal hypothalamus are visible at the top. All images were taken from a ventral perspective. Rostral is up in all images. Scale bars are 20µm. B. Number of TH-immunoreactive somata in in the CNS of control and MPTP-treated larvae. NA indicates the purely noradrenergic population of neurons in the locus coeruelus. C. Episodes recorded from a sham-treated larva (top trace) and from a larva treated for 48 hours with 10µM MPTP (middle trace). The episodes have been expanded in the bottom trace to show that the bursts appear normal in MPTP-treated larvae. D. The frequency of episodes in MPTP-treated larvae is significantly higher than in sham-treated larvae. The horizontal bars indicate the medians for each set of data.
References


Figure 1.
Figure 2.
Figure 3.
Figure 4.

A. Control

10μM Dopamine

Rinse

B. Control

200μM Bupropion

Rinse

C. Control

5μM L741,626

1min

D. Episodes in 10 min.

E. Cumulative Probability

Episode period, s

F. Cumulative Probability

Episode duration, s

G. Control

1mM Sulpiride

1min

H. Episodes in 10 min.

Ctrl
Sulpiride
Figure 5.
Figure 6.
Figure 7.
Figure 8.
Figure 9.
Figure 10.
Figure 11.