

Dopamine from the Brain Promotes Spinal Motor Neuron Generation during Development and Adult Regeneration

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SUMMARY

Coordinated development of brain stem and spinal target neurons is pivotal for the emergence of a precisely functioning locomotor system. Signals that match the development of these far-apart regions of the central nervous system may be redeployed during spinal cord regeneration. Here we show that descending dopaminergic projections from the brain promote motor neuron generation at the expense of V2 interneurons in the developing zebrafish spinal cord by activating the D4a receptor, which acts on the hedgehog pathway. Inhibiting this essential signal during early neurogenesis leads to a long-lasting reduction of motor neuron numbers and impaired motor responses of free-swimming larvae. Importantly, during successful spinal cord regeneration in adult zebrafish, endogenous dopamine promotes generation of spinal motor neurons, and dopamine agonists augment this process. Hence, we describe a supraspinal control mechanism for the development and regeneration of specific spinal cell types that uses dopamine as a signal.

INTRODUCTION

Several local signals are known to influence neurogenesis in the developing ventral spinal cord of zebrafish and other vertebrates (Fuccillo et al., 2006; Maden, 2006). The most prominent

example is the floor-plate-derived hedgehog signal that promotes the setup of ventral progenitor domains and generation of different cell types from these domains (Fuccillo et al., 2006). However, it has been shown in other central nervous system (CNS) regions that projection axons also influence cell proliferation in target areas. For example, thalamocortical axons increase mitotic activity in the cortex (Dehay et al., 2001), olfactory axons increase cell cycle length in the olfactory bulb primordium (Gong and Shipley, 1995), and enucleation reduces mitotic rates in the tectum of frogs (Kollros and Thiesse, 1988). Such mechanisms may be important to match maturation of the long-range projection axons with that of their distant targets. In the spinal cord, different pools of cells are generated in a highly ordered spatiotemporal fashion (Grillner and Jessell, 2009; Levine et al., 2012). In the zebrafish model, great strides are being made in our understanding of the maturation of the brain stem command neurons and spinal targets. In both CNS regions, recruitment of neurons for different locomotor outputs is determined by the time and place of their birth (Kinkhabwala et al., 2011; McLean et al., 2007). Such a system should be exquisitely dependent on the exact coordination of development. Thus, signals from axons descending from the brain to the spinal cord could act on spinal progenitor cell proliferation and differentiation to match the developmental states of brain stem and spinal cord.

The dopaminergic projection from the brain to the spinal cord is an excellent candidate for such a coordinating signal, because axons from diencephalic dopaminergic neurons invade the spinal cord during neuronal differentiation and provide the only source of dopamine in the spinal cord of zebrafish (McLean and Fetcho, 2004a, 2004b). Furthermore, dopamine has been identified as a factor influencing neurogenesis in the developing

brain (Ohtani et al., 2003; Popolo et al., 2004) and adult brain (Höglinger et al., 2004; Yang et al., 2008).

Embryonic signals are often reactivated in regeneration. In contrast to that of mammals, the adult spinal cord of zebrafish (Meletis et al., 2008; Shihabuddin et al., 2000) regenerates spinal cell types such as motor neurons (Reimer et al., 2008) after a lesion, and these processes are influenced by a number of “developmental” signals (Dias et al., 2012; Goldshmit et al., 2012; Reimer et al., 2009). Hence, the adult zebrafish is one of the best characterized adult vertebrate models for successful spinal cord regeneration, in which the influence of descending projections on cellular regeneration can be tested.

Here we show that dopamine from the brain promotes generation of motor neurons in the developing spinal cord at the expense of V2 interneurons, which is essential for later motor performance of free-swimming larvae. We identify activation of the D4a receptor interacting with the hedgehog pathway as the signal transduction mechanism. Moreover, dopamine promotes generation of spinal motor neurons in the lesioned spinal cord of adult zebrafish. Thus, our results demonstrate that descending axons regulate the generation of spinal neurons during development and adult regeneration and identify dopamine signaling as a powerful regulator of spinal neurogenesis.

RESULTS

We hypothesized that dopamine is a brain-derived signal that influences generation of spinal cell types during development and regeneration.

Endogenous Brain-Derived Dopamine Promotes Motor Neuron Development

We analyzed the endogenous origin of dopamine by detecting Tyrosine hydroxylase (TH) enzymes, which are rate limiting for dopamine synthesis, by in situ hybridization. *th1* and *th2* (Chen et al., 2009) messenger RNAs (mRNAs) were only detectable in the brain, with *th1* showing most pronounced expression in the diencephalon at 24 and 48 hr postfertilization (hpf) (Figure S1B available online). Caudally growing TH1 immunoreactive axons from the brain had not reached the spinal cord at 24 hpf ($n = 7$ embryos), reached somite 7 ± 0.2 ($n = 8$ embryos) by 33 hpf (Figures 1A–1C), and reached somite 18 ± 2.4 ($n = 10$ embryos) by 56 hpf (McLean and Fetcho, 2004a). This is in agreement with direct measurements of dopamine in the trunk and tail region by high-pressure liquid chromatography (HPLC), which showed detectable levels at 48, but not at 24, hpf (Figure 1D). TH1⁺ axons grew close to ventral pMN progenitor cells in the ventral spinal cord. pMN progenitors were identified by single fluorescence in embryos double transgenic for Olig2:dsRed, labeling pMN progenitors and motor neurons, and HB9:GFP, labeling motor neurons (Figure 1C) (Shin et al., 2003). A colabeling study of HB9 immunoreactive motor neurons with a proliferation marker in the spinal cord indicated that almost half of all motor neurons were still born between 24 and 51 hpf (Figure S1A). Hence, TH1⁺ axons from the diencephalon are the only detectable source of dopaminergic innervation of the embryonic spinal cord, and their spatiotemporal distribution is consistent with a role in the regulation of motor neuron generation.

To determine whether dopamine was indeed important for motor neuron formation, we reduced dopaminergic input from the brain to the spinal cord using three independent manipulations, morpholino knockdown of *th1*, genetic reduction of dopaminergic cells in *orthopedia* (*otp*) mutants (Kastenhuber et al., 2010; Ryu et al., 2007), and toxic ablation using 6-OHDA.

A morpholino to *th1* abolished TH1 immunoreactivity in embryos (Figure 1E) and reduced the number of spinal HB9 immunoreactive motor neurons by 35% at 33 hpf (Figure 1F). A similar result was obtained in HB9:GFP transgenic embryos (Figure S1D) (Flanagan-Steet et al., 2005). Importantly, this phenotype was fully rescued by replacing the endogenous dopamine signal through the application of pergolide, an agonist for the D2 class of dopamine receptors (D2-like: D2, D3, D4) (Goldstein et al., 1980), from 24 to 33 hpf (Figure 1F). Similarly, development of a late-developing (mostly after 24 hpf), dorsally projecting sub-type of motor neurons, transgenically labeled by a promoter fragment for islet-1 (islet-1:GFP) (Higashijima et al., 2000), was also significantly inhibited by morpholino treatment. This was fully rescued by another, chemically distinct D2-like agonist R(–)-propylnorapomorphine (NPA) (Figure S1E). Thus, endogenous dopamine is important for the development of different classes of spinal motor neurons and can be substituted by dopaminergic drugs.

In the *otp* mutant, dopaminergic input to the spinal cord is reduced due to a mutation in the *orthopedia1a* homeodomain transcription factor, which is essential for the development of many dopaminergic neurons projecting to the spinal cord (Kastenhuber et al., 2010; Ryu et al., 2007). We found that the number of HB9⁺ motor neurons in these mutants was reduced by 25% compared to nonhomozygous siblings at 33 hpf (Figures 1G–1I). This supports that endogenous dopamine promotes generation of motor neurons.

Next, we reduced the number of dopaminergic cells in the brain with the specific toxin 6-hydroxydopamine (6-OHDA) (Ding et al., 2004) (dimethyl sulfoxide [DMSO] treated: 16.7 ± 0.88 neurons per embryo, $n = 18$; 6-OHDA treated: 4.6 ± 0.79 neurons, $n = 18$; $p < 0.0001$; Figure S1C). This inhibited development of islet-1:GFP⁺ motor neurons in the spinal cord and was fully rescued by NPA application. Hence, 6-OHDA did not act as a general neurotoxin but rather acted on motor neuron numbers by reducing the dopamine signal in the spinal cord. Combined, these manipulations show that dopamine from descending axons controls the generation of spinal motor neurons (schematically depicted in Figure 1J).

To determine whether dopamine specifically promotes motor neuron development, we applied the dopamine agonist pergolide (24–33 hpf) to *vsx1*:GFP embryos in which prospective V2 interneurons are labeled. These cells originate from the p2 progenitor zone just dorsal to the pMN progenitor zone for motor neurons (Kimura et al., 2008). Remarkably, pergolide treatment led to a reduction in the number of *vsx1*:GFP⁺ interneurons by 29% (confirmed by Chx10 labeling; data not shown), whereas in the same embryos, the number of HB9⁺ motor neurons was increased by 48% (Figure 2A). Interestingly, there was a small *vsx1*:GFP⁺/HB9⁺ hybrid population of cells, which was strongly reduced after pergolide treatment, indicating that dopamine signaling may act on neuroblast differentiation or sharpening of progenitor domain boundaries.

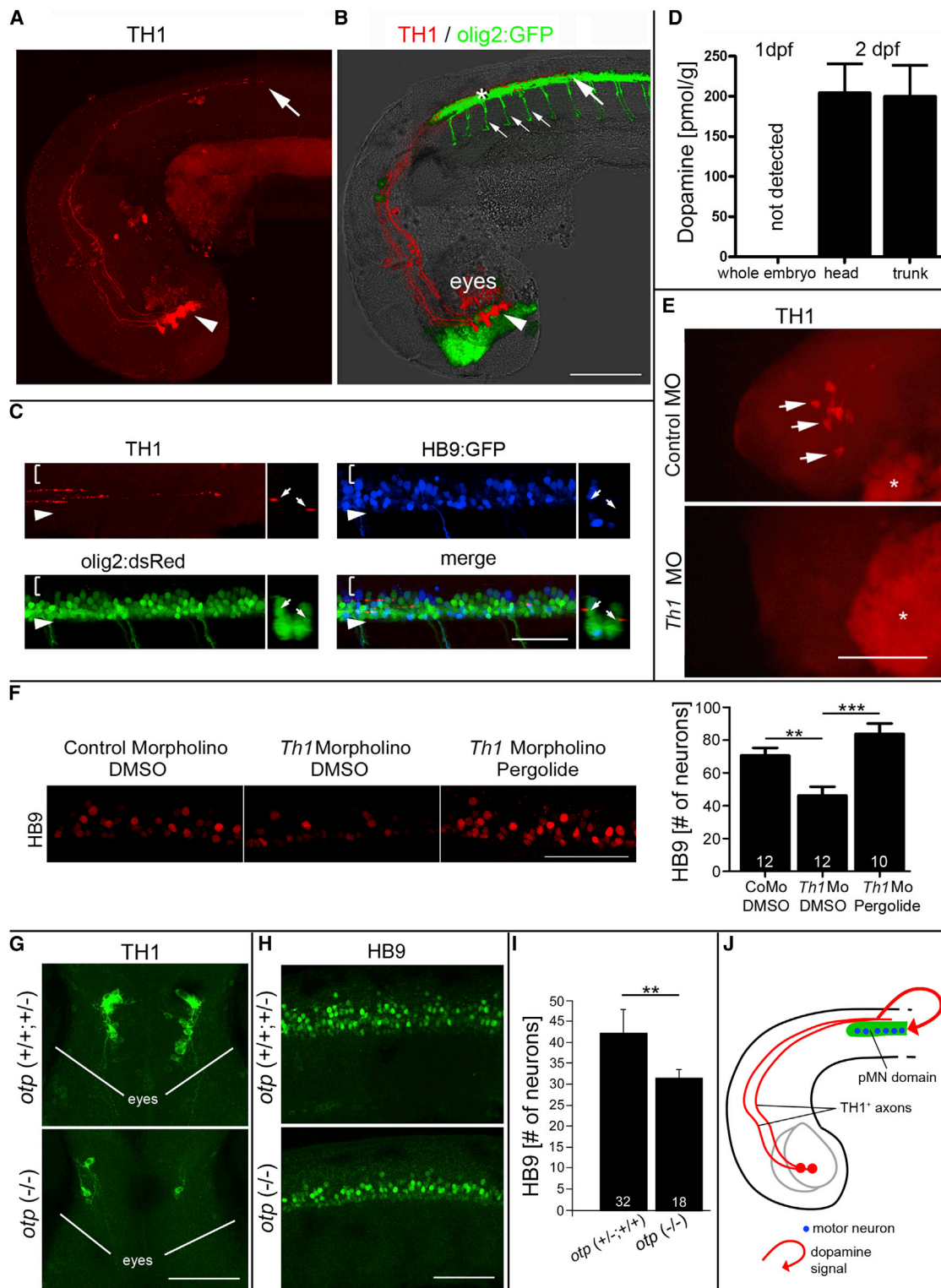


Figure 1. Dopamine from the Brain Promotes Motor Neuron Development

(A and B) In a lateral view of (A) TH1 immunohistochemistry only and (B) combined with olig2:GFP fluorescence and light microscopy, diencephalic TH1⁺ neurons (arrowheads; 33 hpf) and axonal growth cones of their spinal projections (large arrows) are labeled as they grow in close proximity to olig2:GFP⁺ cells in the ventral spinal cord. Small arrows in (B) indicate motor axons.

(C) TH1 labeling reveals close contact of axons (small arrows in coronal views) with pMN progenitors (olig2:dsRed⁺/HB9:GFP⁻). Brackets indicate dorsal spinal cord; arrowheads indicate ventral border of spinal cord.

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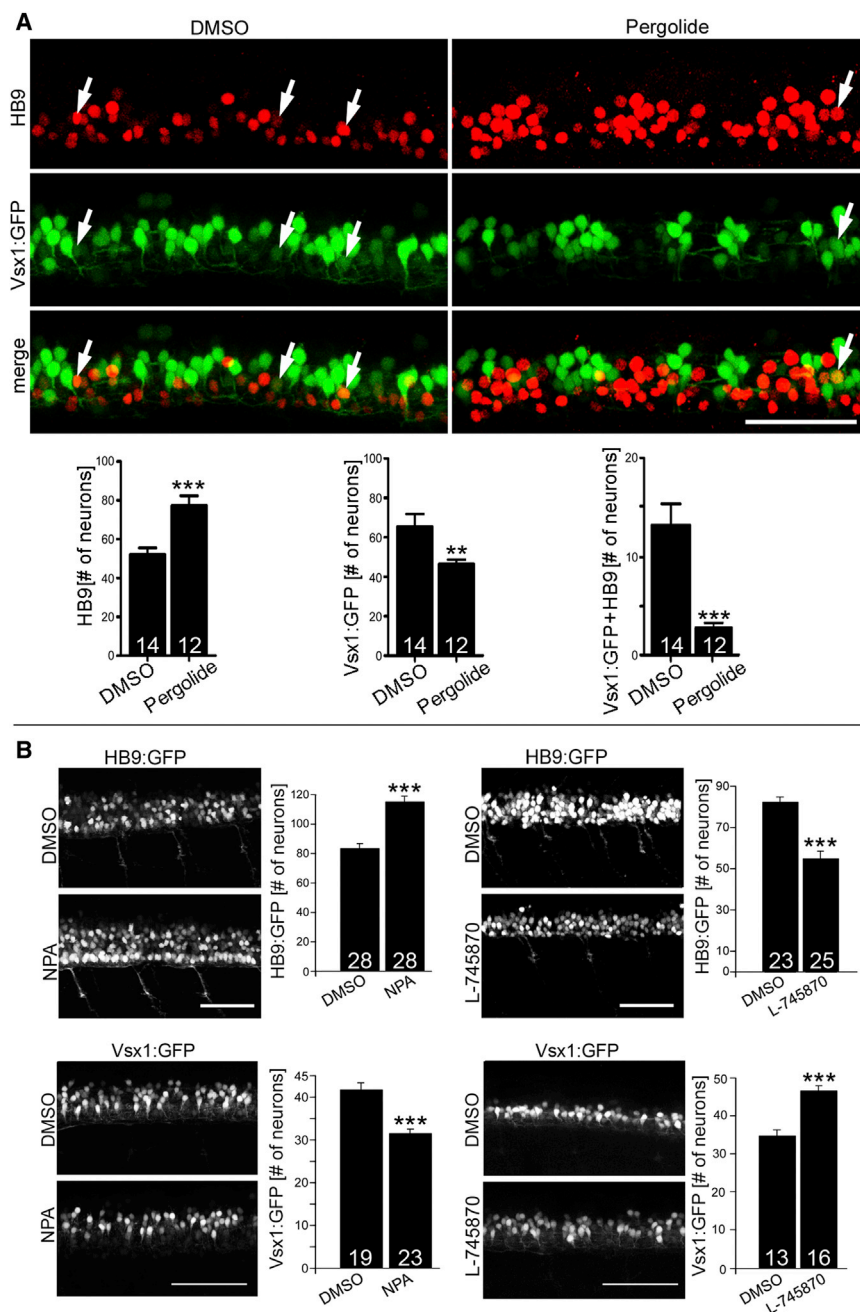


Figure 2. Dopamine Signaling Inversely Affects Motor Neuron and V2 Interneuron Numbers

Lateral trunk views at 33 hpf are shown (drug exposure: 24–33 hpf).

(A) Treatment with the dopamine agonist pergolide leads to an increase of HB9 immunoreactive motor neurons and a reduction of transgenically labeled presumptive V2 interneurons (vsx1:GFP) in the same embryos. A small subpopulation of double-labeled cells is also strongly reduced (Mann-Whitney U test; *** $p < 0.0001$, ** $p < 0.001$).

(B) NPA, another dopamine agonist, increases the number of HB9:GFP⁺ motor neurons and decreases the number of vsx1:GFP⁺ cells (left column). The D4 antagonist L-745870 has the inverse effect on numbers of these cell types (right column). Error bars represent SEM. Scale bars, 50 μ m. See also Figure S2.

et al., 2002) interneurons were unaffected by treatment with the agonist NPA (Figure S2D). This suggests that dopamine signaling specifically promotes motor neuron generation at the expense of V2 but does not affect some other interneuron cell types.

The D4a Receptor Is Required to Generate Appropriate Numbers of Spinal Motor Neurons and V2 Interneurons

To identify the receptor for dopamine in the spinal cord, we surveyed dopaminergic drugs in islet-1:GFP embryos. This indicated that those drugs with a positive action on the D4 receptor (agonists, reuptake inhibitor) promoted, and antagonists inhibited, the development of islet-1:GFP⁺ motor neurons in a dose-dependent manner (Figures S2A and S2C; see Table S1 for all drugs). Importantly, the action of the potent D2-like agonist NPA was abolished by the highly D4-specific antagonist L-745870, indicating exclusive action through D4 receptors (Figure S2B).

Conversely, using the dopamine D4 antagonist L-745870 reduced the number of HB9:GFP⁺ motor neurons and increased the number of vsx1:GFP⁺ interneurons (Figure 2B). Glyt2:GFP⁺ (McLean et al., 2007) and pax2:GFP⁺ (Picker

PCR on fluorescence-activated flow-sorted (purity > 87%) pMN progenitor cells and motor neurons at 26 hpf (before olig2⁺ oligodendrocytes are present) indicated strong expression of *d4a* in olig2:dsRed⁺/HB9:GFP[−] pMN progenitor cells

(D) Consistent with TH1⁺ axon growth, HPLC detects dopamine in 48 hpf embryos but not in 24 hpf embryos.

(E and F) Injection of a morpholino to *th1* leads to (E) loss of TH1 immunoreactivity (indicated by arrows and lateral views of heads; asterisks indicate autofluorescence of the yolk sac) and (F) a significant reduction in the number of HB9⁺ motor neurons (lateral spinal cord views; 33 hpf), which is fully rescued by pergolide application (drug exposure 24–33 hpf; one-way ANOVA, $p = 0.0001$, with Bonferroni's multiple comparison, ** $p < 0.001$, *** $p < 0.0001$).

(G–I) In the *otp* mutant, fewer dopaminergic neurons are present in the brain, as indicated (G) in dorsal views of heads by TH1 immunohistochemistry, and motor neuron numbers in the spinal cord are reduced, as indicated in (H) lateral views of HB9 immunohistochemistry and (I) quantification thereof. ** $p < 0.01$. (J) Model of the spatial relationship of descending dopaminergic axons with the spinal pMN domain.

Error bars represent SEM. Scale bars, 200 μ m (A and B), 50 μ m (C, F, and H), and 100 μ m (E and G). See also Figure S1.

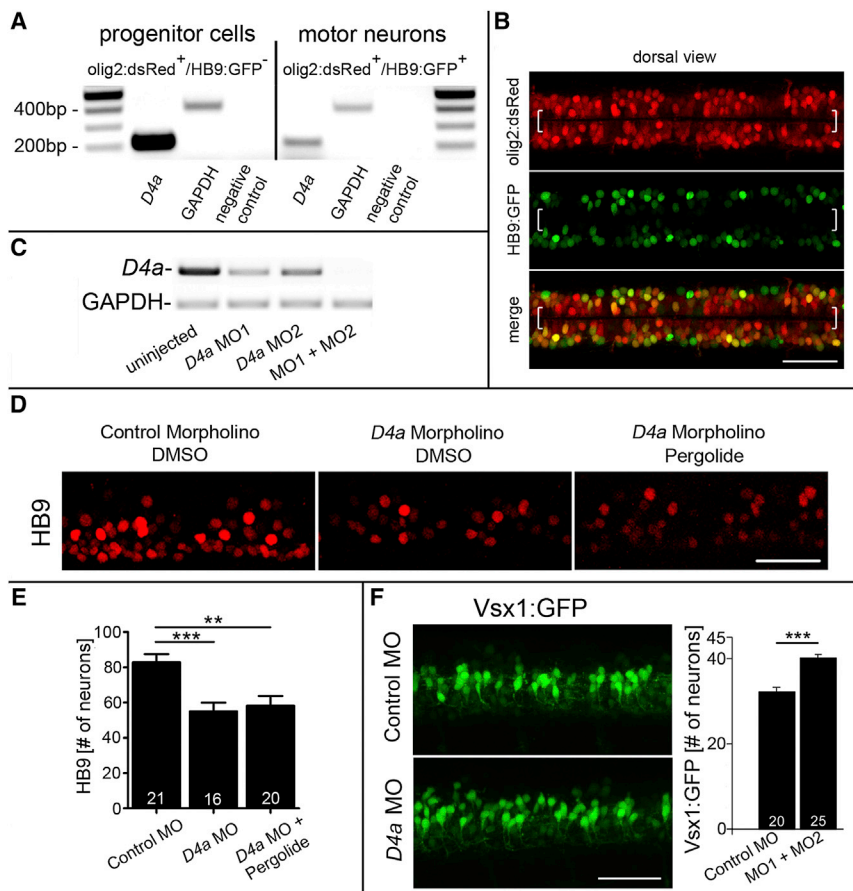


Figure 3. The D4a Receptor Is Necessary to Mediate the Action of Dopamine on Neurogenesis

(A) PCR analysis on fluorescence-activated cell-sorted cells from olig2:dsRed/HB9:GFP double-transgenic embryos shows that *d4a* receptor mRNA is enriched in pMN progenitors, compared to motor neurons.

(B) In a dorsal view of double-transgenic embryos, olig2:dsRed⁺/HB9:GFP⁻ pMN progenitors (brackets) and double-labeled motor neurons are visible.

(C) *d4a* morpholino knockdown is efficient, as shown by PCR.

(D) *d4a* knockdown (MO1) reduces the number of HB9⁺ motor neurons, which cannot be rescued by pergolide (exposure 24–33 hpf).

(E) Quantification of motor neurons in (D) (ANOVA, $p = 0.0003$ with Bonferroni's posttest, ** $p < 0.01$, *** $p < 0.0001$).

(F) *d4a* knockdown increases the number of vsx1:GFP⁺ interneurons (*** $p < 0.0001$).

Error bars represent SEM. Scale bars, 50 μ m (B, D, and F). See also Figure S3 and Table S1.

but not in olig2:dsRed⁺/HB9:GFP⁺ motor neurons (Figures 3A and 3B). Two independent splice-site-directed morpholino antisense oligonucleotides strongly reduced detectability of the wild-type transcript in PCR and almost completely abolished *d4a* expression when given in combination (Figure 3C). Morpholinos reduced numbers of HB9⁺ motor neurons by 34% at 33 hpf (1 mM MO1). This could not be rescued by application of pergolide (Figures 3D and 3E; drug exposure 24–33 hpf), as opposed to the effect of knockdown of *th1*, which was rescued by the drug (cf. Figure 1F). This shows that the D4a receptor is crucial for mediating the effect of dopamine. Similarly, HB9:GFP⁺ motor neuron numbers were reduced by 49% (1 mM MO1), 47% (1 mM MO2), and 40% (0.5 mM MO1 + 0.5 mM MO2) by *d4a* morpholino treatment at 33 hpf (Figure S3B). Development of islet-1:GFP⁺ motor neurons was also strongly inhibited by *d4a* morpholino application and could not be rescued by the agonist NPA (Figure S3A). The observations that the magnitude of the effect of *d4a* knockdown is comparable to that of *th1* knockdown and that *d4a* knockdown could not be rescued by dopamine agonists indicate that the contribution of D4b and D4rs (expressed in olig2:GFP⁺ cells; data not shown) is marginal and that these receptors cannot compensate for a lack of D4a. Interestingly, combining morpholinos did not lead to a stronger effect on motor neuron numbers than single morpholino (Figures S3A and 3B), suggesting that the gene expression level of *d4a* is limiting for the action of dopamine on motor neuron generation.

indicate that the D4a receptor is essential to mediate the action of dopamine on spinal neuron generation.

Dopaminergic axons and dopamine are present in the spinal cord too late (later than 24 hpf) to influence early motor neuron generation, which starts at 9 hpf in zebrafish (Myers et al., 1986). Accordingly, neither *d4a* morpholino nor D4 antagonist (L-745870, 16–24 hpf) treatment affected motor neuron numbers at 24 hpf (data not shown). Moreover, at 33 hpf, when *d4a* MO reduces the number of motor neurons at trunk segments 6 and 7, where TH1⁺ axons are present, no significant reduction was observed at segments 16 and 17 where dopaminergic axons are not present in the same embryos (Figure S3C; cf. Figures 3D and 3E). This shows that the endogenous dopamine signaling is restricted in space and time to the spinal level reached by the growing TH1⁺ axons.

Dopamine Signaling Promotes Motor Neuron Progenitor Cell Development

Dopamine does not increase motor neuron numbers by preventing developmental cell death of motor neurons, since this does not naturally occur in zebrafish (Lewis and Eisen, 2003). Moreover, treatment with 6-OHDA or L-745870, which reduces motor neuron numbers, did also not lead to detectable cell death in the ventral spinal cord, as determined by TUNEL labeling (data not shown).

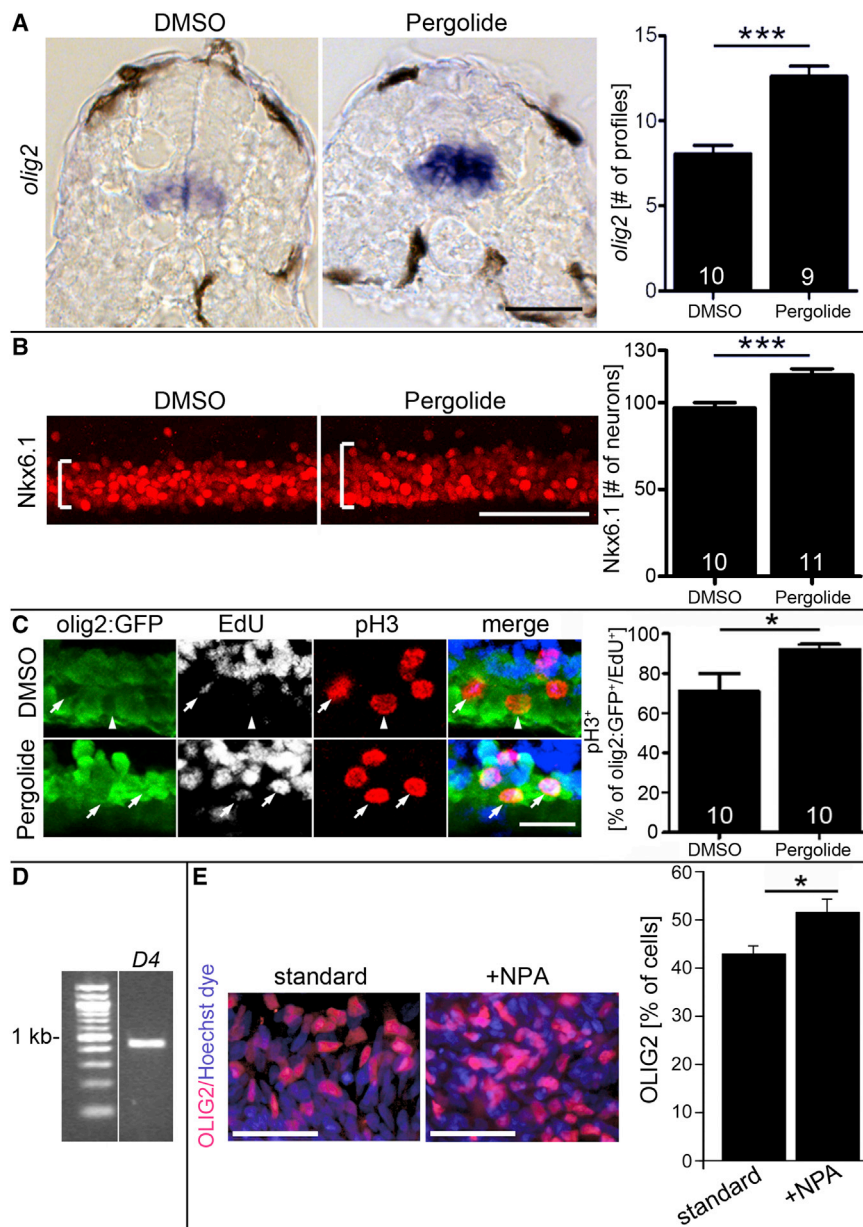


Figure 4. Dopamine Agonists Affect Ventral Progenitor Cells

(A) The *olig2* mRNA expressing pMN progenitors are increased in number and labeling intensity after pergolide treatment (24–33 hpf; cross-sections; ****p* < 0.0001).

(B) Nkx6.1 labeling is expanded (brackets) after pergolide treatment (24–33 hpf; lateral views; ****p* < 0.0001).

(C) A higher proportion of pMN progenitors in M phase (*olig2:GFP*⁺/pH3⁺) is labeled with EdU 2 hr after exposure, indicating shortened G2 phase, after pergolide treatment (24–33 hpf; lateral views; **p* < 0.05; arrows indicate triple-labeled cells, and arrowhead indicates a cell that is *olig2:GFP*⁺/pH3⁺/EdU⁺).

(D and E) In human embryonic stem cell cultures, driven toward motor neuron differentiation, PCR indicates expression of the *d4* receptor at day 16 (D) and the proportion of OLIG2⁺ cells is significantly (*p* = 0.0109) increased by NPA at day 16 (E). Error bars represent SEM. Scale bars, 50 μ m (A, B, and E) and 20 μ m (C). See also Figure S4.

were labeled by EdU, suggesting that exposure time was short enough to only label pMN progenitors. There was an increase of 37% in the number of *olig2:GFP*⁺/EdU⁺ cells, which was comparable to the increase in the number of *olig2* mRNA-expressing profiles, indicating that the number of *olig2:GFP*⁺/EdU⁺ cells reflected the expansion of the pMN domain and that there was no change in the rate of pMN progenitors that can be labeled by EdU. However, the rate of pH3-labeled *olig2:GFP*⁺ progenitors that were also labeled by EdU was increased by 30% (Figure 4C), indicating a shortened G2 cell cycle phase in pMN progenitors (Peco et al., 2012).

Specific expression of *d4a* by pMN progenitors and altered proliferative activity suggest direct action of dopamine on pMN cells. To determine whether

Next, we analyzed the size of the pMN progenitor pool by in situ hybridization of *olig2*, showing a larger and more intensely labeled pMN zone with an increase of 57% in the number of *olig2* mRNA-expressing profiles after pergolide treatment (Figure 4A). The number of cells immunopositive for the ventral progenitor domain transcription factor Nkx6.1 was also increased by 20% (Figure 4B). Neither factor showed ectopic dorsal expression. This suggests that progenitor pool expansion may explain the increase in motor neurons after increased dopamine signaling.

To determine whether the proliferative activity of the expanded pMN pool was affected by dopamine agonist treatment, we incubated *olig2:GFP* embryos with the S-phase marker EdU and combined this with M-phase labeling using anti-phospho-histone 3 (pH3) antibodies 2 hr later. Only medial *olig2:GFP*⁺ cells

agonist actions on motor neuron generation can occur in the absence of an intact spinal network, we used human embryonic stem-cell-derived neural stem cells driven toward motor neuron differentiation (Li et al., 2005b). We found that *d4* receptor mRNA was expressed in neurospheres (Figure 4D). Importantly, application of the dopamine agonist NPA led to a significant 20% increase in the number of OLIG2⁺ progenitor cells at early differentiation stages (Figure 4E). Subsequently, at later differentiation stages, a 21% increase in the number of HB9⁺ motor neurons was observed (Figures S4A and S4B). Conversely, the number of FOXN4⁺ V2 interneurons (Li et al., 2005a) was decreased by 29% (Figures S4C and S4D). All of these observations resemble the in vivo action of dopamine agonists, suggesting direct action of dopamine on motor neuron progenitor cells in the absence of a spinal network.

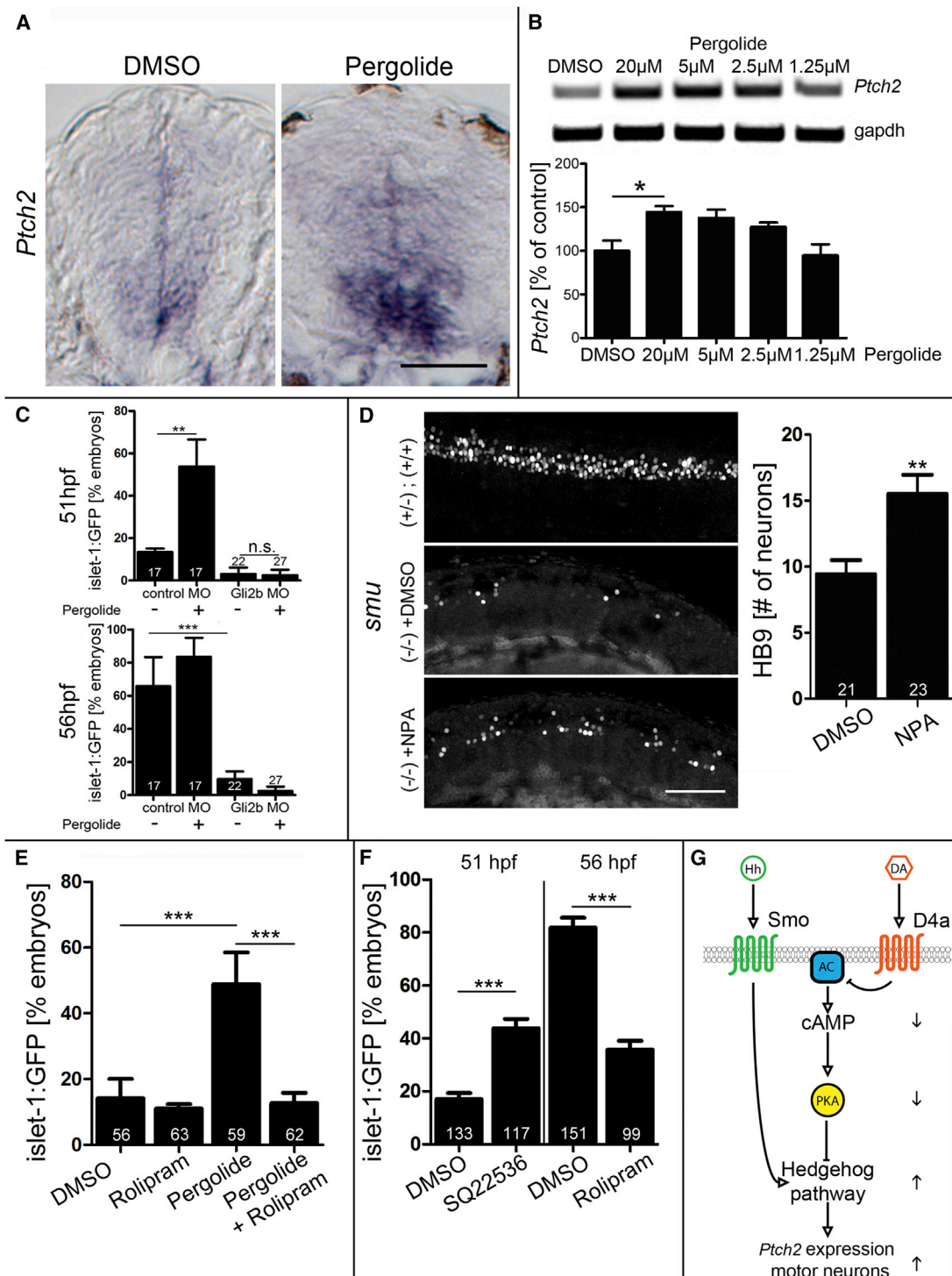


Figure 5. Dopamine Signaling Stimulates the Hedgehog Pathway by Attenuating cAMP/PKA Activity

(A) Pergolide increases *patched2* (*ptch2*) expression in in situ hybridization on spinal cross-sections, retaining the ventrodorsal expression gradient (exposure 24–33 hpf).

(B) PCR indicates a dose-dependent increase in *patched2* expression after pergolide exposure (Kruskal-Wallis, $p = 0.0102$ with Dunn's multiple comparison, * $p < 0.05$).

(C) Gli2b morpholino inhibits development of islet1:GFP⁺ motor neurons. This is not rescued by pergolide (56 hpf), capable of promoting motor neuron development in siblings (51 hpf) (** $p < 0.01$, *** $p < 0.0001$).

(D) NPA partially rescues impaired development of HB9 immunolabeled motor neurons in the smoothened (*smu*) mutant at 33 hpf (*** $p < 0.0001$; lateral trunk views are shown).

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Evidence that D4a Acts through cAMP/PKA on the Hedgehog Pathway

We hypothesized that D4a activity could act by reducing cyclic AMP (cAMP) levels, because the D4 receptor is a known negative regulator of the cAMP/protein kinase A (PKA) pathway (Asaumi et al., 2006; Wang et al., 2002). To mimic the action of D4 receptor activity, we used SQ22536, an inhibitor of adenylate cyclase that reduces cAMP levels (Fabbri et al., 1991). Indeed, the drug significantly promoted development of islet-1:GFP⁺ motor neurons, similar to the D2-like agonist pergolide. Conversely, rolipram, an inhibitor of phosphodiesterase IV that increases cAMP levels (Lelkes et al., 1998), strongly inhibited the appearance of islet-1:GFP⁺ motor neurons (Figure 5F). Importantly, when coapplied with the dopamine agonist pergolide, rolipram abolished the promoting effect of pergolide on motor neuron generation (Figure 5E). These results support the hypothesis that dopamine promotes motor neuron development by lowering cAMP levels.

Since cAMP directly controls activity of PKA (Beaulieu et al., 2007), a known negative regulator of hedgehog signaling (Hammerschmidt et al., 1996), dopamine signaling could augment hedgehog signaling, which is of pivotal importance in the regulation of neurogenesis in the ventral spinal cord. Indeed, we found that pergolide application (incubation from 24 to 33 hpf) significantly increased the expression levels of the direct hedgehog target gene *patched2* (formerly *patched1*) in a dose-dependent manner, as detected by PCR (Figure 5B). In situ hybridization for *patched2* also indicated increased labeling intensity (Figure 5A). However, the ventral^{high} to dorsal^{low} gradient was retained, suggesting augmentation of hedgehog signaling by dopamine.

PKA is thought to act on *gli* transcription factors, including *gli2* (Pan et al., 2009; Tuson et al., 2011), which are the effectors of the hedgehog pathway. Generation of islet1:GFP⁺ motor neurons depends on *gli2b* (Ke et al., 2008). We confirm that knockdown of *gli2b* inhibits the appearance of islet-1:GFP⁺ motor neurons. Crucially, this is not rescued by application of the dopamine agonist pergolide (Figure 5C). This suggests that dopamine signaling converges on the hedgehog pathway at the level of *gli* transcription factors.

Next, we aimed to rescue hedgehog signaling in a mutant of the hedgehog receptor smoothened, *slow muscle omitted* (*smu*), with a dopamine agonist. We used a weak allele of *smu*, which leads to reduced hedgehog activity (Varga et al., 2001). Very few HB9⁺ motor neurons differentiated in homozygous *smu* mutants. NPA treatment (16–33 hpf) increased the number of motor neurons by 65%, indicating a significant rescue compared to nonhomozygous embryos from the same clutches (Figure 5D). However, motor neuron numbers were not restored to those in control embryos, in part due to the inability of dopamine to influence early generation of motor neurons (discussed earlier), which still depends on hedgehog signaling. Thus, dopa-

mine is likely to signal through cAMP/PKA on the hedgehog pathway to influence spinal neurogenesis (summarized in Figure 5G).

Lack of Early Dopamine Signaling Leads to a Long-Term Reduction in Motor Neuron Numbers and Impairs Motor Behavior in Older Larvae

We asked whether dopamine-dependent generation of motor neurons during early development is important for later motor neuron numbers and motor behaviors. Larvae hatch between 2 and 3 days postfertilization (dpf), as of when they have to escape predation and become active predators themselves at 5 dpf. Up to 4 dpf, embryos are mostly immobile and swim in short bursts, whereas they show constant swimming behavior at later free-feeding stages (Brustein et al., 2003). We reduced D4 signaling with the drug L-745870 starting at 24 hpf and washed out the drug at 72 hpf to avoid potential direct actions of the drug on neurotransmission or network maturation (Lambert et al., 2012). After this treatment, reduced numbers of HB9:GFP⁺ motor neurons were still observed at 9 dpf (Figure 6A). Similarly, morpholino knockdown of *th1* led to reduced motor neuron numbers compared to controls at 14 dpf (data not shown). Hence, the dopamine signal during the late phase motor neuron development (24–51 hpf; Figure S1A) is indispensable for having an appropriate number of motor neurons at later developmental stages, when the larval motor system has to be fully operational.

Next, we tested the escape response of larvae (4 dpf) that had previously been treated with L-745870 (incubation from 24 to 72 hpf) with a tail touch response test (McLean and Fetcho, 2011). Drug-treated embryos reacted to being touched 27% less often by swimming away from the stimulus than control-treated animals (Figure 6B). Targeted ablation of roughly a quarter of the motor neurons using a nitroreductase ablation system also led to a significantly reduced frequency of tail touch responses, indicating that a reduced number of motor neurons is sufficient to produce such a phenotype (Figure S5). Moreover, at 9 dpf, drug-treated larvae covered 14% less distance in a given time period than controls and, despite maximal velocities not being significantly different, their high mobility frequency was reduced by 37% during spontaneous swimming (Figure 6C). This shows that reducing dopamine signaling during early development impairs vital motor behaviors at later stages.

Dopamine Promotes Regeneration of Motor Neurons after Adult Spinal Cord Lesion

Given that developmental signals can be recruited again during adult regeneration, as is the case for hedgehog signaling during motor neuron regeneration in adult zebrafish (Reimer et al., 2009), we tested whether dopamine signaling could also promote adult motor neuron regeneration. Complete transection

(E) The phosphodiesterase inhibitor rolipram abolishes the promoting effect of pergolide on development of islet-1:GFP⁺ motor neurons.

(F) The adenylate cyclase inhibitor SQ22536 mimics the action of pergolide by promoting islet-1:GFP⁺ motor neuron development, and rolipram inhibits development of islet-1:GFP⁺ motor neurons (***p < 0.0001).

(G) Model for dopamine action. By inhibiting adenylate cyclase (AC), dopamine (DA), acting through the D4a receptor, reduces the inhibitory influence of cAMP-dependent PKA on the hedgehog pathway, which is normally activated by hedgehog (Hh) acting through the Smoothened receptor (Smo). Upward and downward arrows indicate positive or negative influences, respectively, of D4a activity.

Error bars represent SEM. Scale bars, 50 μ m (A and D).

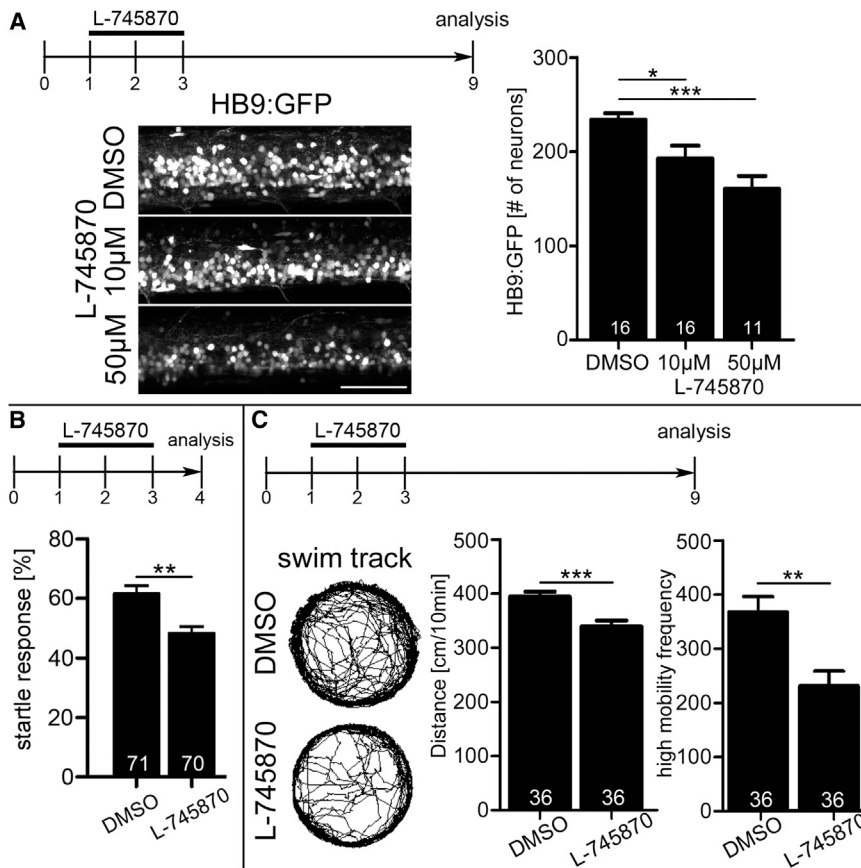


Figure 6. Early Disruption of Dopamine Signaling Has Long-Lasting Consequences for Motor Neuron Numbers and Swimming Behavior

(A) Motor neuron numbers (lateral trunk views) are significantly reduced at 9 dpf by exposure to 10 μ M L-745870 from 24 to 72 hpf (Kruskal-Wallis $p = 0.0102$ with Dunn's multiple comparison, $*p < 0.05$, $***p < 0.0001$). Scale bar, 25 μ m.

(B and C) Incubation of embryos from 1 to 3 dpf decreases (B) the frequency of a startle response after touch at 4 dpf and (C) the total distance moved and high mobility frequency at 9 dpf ($**p < 0.01$; $***p < 0.0001$).

Error bars represent SEM. See also Figure S5.

Consistent with these rostrocaudal asymmetries in dopamine signal and receptor expression, almost twice as many newly generated motor neurons, identified by intense HB9 immunoreactivity or HB9:GFP transgene expression (Reimer et al., 2008), were found rostral than caudal to the lesion site in untreated animals at 14 dpf (Figure S6B). To directly show that dopaminergic axons promoted motor neuron regeneration rostral to the lesion site, we effectively and specifically ablated these axons with 6-OHDA (Figure S6C). This treatment reduced the number of newly generated motor neurons rostral to the lesion site by 43% at 14 dpf.

of the spinal cord elicits generation of new motor neurons rostral and caudal close to the lesion site, which peaks at 14 days post-lesion (dpi) (Reimer et al., 2008). At that time point, mostly dopaminergic TH1⁺ axons, which are exclusively derived from the brain, show sprouting rostral to the lesion site, but regrowth to the caudal spinal cord is only observed at later time points after the lesion (Figure 7A) (Kuscha et al., 2012). Rostral to the lesion site, some TH1⁺ axons were located in close proximity to the olig2:GFP⁺ ependymoradial glial motor neuron progenitor cells (Reimer et al., 2008) at the ventricle, indicating that dopamine from TH1⁺ axons can reach adult motor neuron progenitor cells (Figure 7B). The adult spinal cord, like the embryonic spinal cord, is devoid of *th2* expression (Kuscha et al., 2012). Hence, also in the adult spinal cord, dopaminergic innervation is exclusively derived from the brain and, after spinal cord transection, a significant difference exists in the availability of dopamine between the rostral and caudal spinal cord.

In situ hybridization for the *d4a* receptor indicated lesion-induced upregulation in the ependymal progenitor zone of the rostral, but not caudal, spinal cord (Figure 7A; Figure S6A). *d4a* mRNA was increased in the entire ependymal zone, including the ventrolateral pMN-like motor neuron progenitor domain. PCR analysis confirmed this result (Figure 7E). Other *d2*-like receptors were not conspicuously expressed in the ependymal zone (Figure S6A). This demonstrates that dopamine signal (TH1⁺ axons) and receptor (*d4a* mRNA) are present to promote motor neuron regeneration only rostral to the lesion site.

As expected from the very low number of TH1⁺ axons caudal to the lesion site, 6-OHDA ablation of TH1⁺ axons had no significant effect there (Figure 7C). This supports that endogenous dopamine from descending axons promotes regeneration of spinal motor neurons.

Next, we asked whether the lack of dopamine caudal to the lesion site can be substituted for by dopamine agonist application. We repeatedly injected the agonist NPA to mimic dopaminergic innervation (injections at 3, 5, 6, 7, 8, and 9 dpf; analysis at 14 dpf). First we asked whether this treatment was able to induce *d4a* expression caudal to the lesion site and whether it led to increased expression of *patched2*. Indeed, PCR indicated that expression of both genes was significantly increased compared to untreated controls, demonstrating that the agonist treatment leads to upregulation of the *d4a* receptor and activation of the hedgehog pathway (Figure 7E). Rostral to the lesion site, NPA treatment did not lead to a further increase in lesion-induced *patched2* expression or *d4a* expression, indicating that dopamine agonist treatment in addition to the endogenous signal was not effective. *d4a* and *patched2* expression even appeared reduced, suggesting negative feedback regulation; however, this was not statistically significant.

As expected from the PCR results, we found that, caudal to the lesion site, the number of HB9⁺ motor neurons was strongly increased by NPA treatment by 79% (Figure 7D), whereas rostral

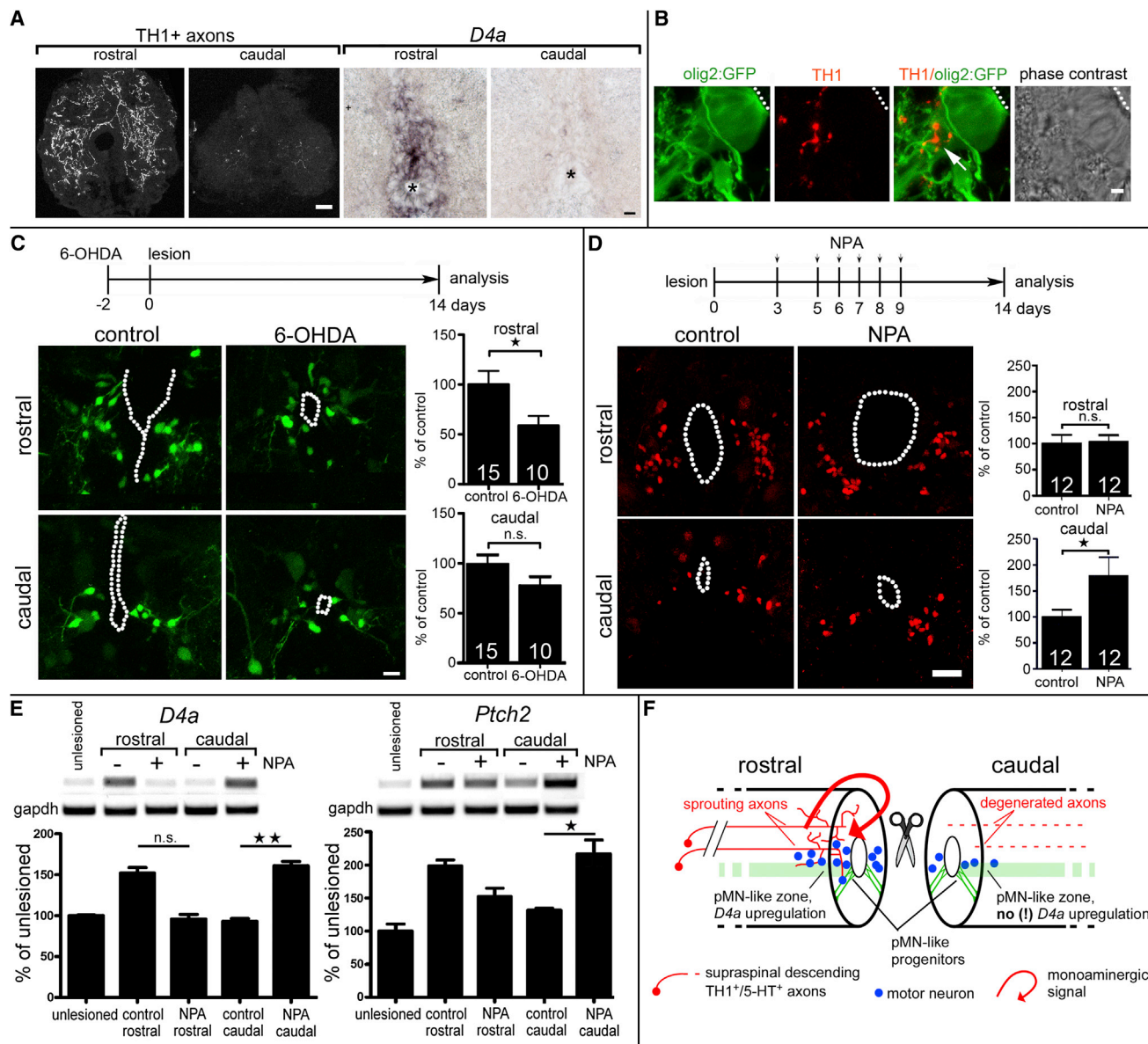


Figure 7. Dopamine Promotes Motor Neuron Regeneration in the Adult Spinal Cord

Spinal cross-sections are shown at 14 dpl; central canal is indicated by dots or asterisks.

(A) TH1+ axons and *d4a* mRNA expression are detectable mainly rostral to a spinal lesion site.

(B) Higher magnification shows apposition (arrow) of TH1+ axons with radial processes of olig2:GFP+ motor neuron progenitor cells.

(C) Ablation of dopaminergic axons with 6-OHDA reduces (p < 0.05) numbers of newly generated HB9:GFP+ motor neurons only rostral to a spinal lesion site.

(D) NPA injections significantly (p < 0.05, one sided) increase the number of HB9+ motor neurons only caudal to a spinal lesion site.

(E) NPA injections increase *d4a* (left) and *patched2* (right) expression only caudal to the lesion site. Example gels and quantitative densitometric analyses are shown (*p < 0.05, **p < 0.01; Kruskal-Wallis with Dunn's posttest).

(F) Schematic summary of dopamine signaling in relation to motor neuron regeneration at 14 dpl.

Error bars represent SEM. Scale bars, 40 μ m (A, left), 20 μ m (A, right), 5 μ m (B), and 15 μ m (C and D). See also Figure S6.

to the lesion site, no further augmenting effect on lesion-induced motor neuron generation was observed. In related experiments, BrdU labeling of the adult olig2:GFP+ pMN like zone showed an increased number of olig2:GFP+/BrdU+ cells where increased numbers of motor neurons were observed, indicating a reaction of progenitor cells to the dopamine agonist, similar to development (Figure S6D).

Together, these data indicate that brain-derived dopamine promotes motor neuron regeneration in the spinal cord in adult zebrafish, at least in part by augmenting hedgehog signaling, which is critical for motor neuron regeneration (Reimer et al., 2009) (summarized in Figure 7F). Importantly, the absence of dopaminergic input can be substituted by injections of a dopamine agonist to increase motor neuron regeneration.

DISCUSSION

Our results show that dopamine is a signal by which descending axons regulate neurogenesis in the ventral spinal cord. The augmenting effect of dopamine signaling on motor neuron generation is recapitulated during adult spinal cord regeneration. We demonstrate a conserved signaling pathway that is essential for correct neurogenesis and function in the motor system. This pathway can be pharmacologically stimulated to promote regeneration of motor neurons in an adult vertebrate.

Developmental Roles of Descending Dopaminergic Projections

Several observations, including knockdown of *th1*, genetic or toxic reduction of dopaminergic axons, and receptor knockdown demonstrated that dopamine, acting through the D4a receptor, increased motor neuron numbers at the expense of V2 interneurons during development. Interestingly, glycinergic signaling affects numbers of Pax2 immunoreactive interneurons but not motor neurons in developing zebrafish embryos, indicating that different neurotransmitters may control different subsets of neurons and their progenitors (McDermid et al., 2006). Of note, the only detectable source of dopamine comes from the descending dopaminergic axons from the diencephalon, which are among the first descending axons to grow into the spinal cord during development (McLean and Fetcho, 2004a, 2004b). Hence, dopamine from descending axons presents a long-range signal that influences generation of different cell types in the spinal cord, the target area for descending axons. The dopamine signal is important because an early reduction in motor neuron numbers by compromising dopamine receptor function is not compensated for at later stages. Moreover, larvae are impaired in their escape response as early as 4 dpf, and even at 9 dpf larvae show reduced and weaker spontaneous swimming. These changes are not trivial, since a reduced touch response directly increases the risk of being eaten on multiple encounters with a predator. Even a slightly altered pattern of swimming at a later stage will let an individual stand out from a shoaling group to a predator. Reduced motor performance after earlier blocking of the dopamine signal could be a direct consequence of fewer motor neurons being generated, as ablation of motor neurons leads to similar deficits in the escape response. However, increased numbers of V2 interneurons and alterations in the connectivity of the motor system, observed by dopamine manipulations at later stages (Lambert et al., 2012), may contribute.

The recruitment of interneurons and motor neurons to different swimming behaviors in response to signals from the brain stem critically depends on the order in which the neurons are born (McLean et al., 2007). The same holds true for the command neurons in the brain stem (Kinkhabwala et al., 2011). The descending dopaminergic projection is in an ideal spatiotemporal position to coordinate the maturation of these distant CNS locations. We show here that manipulating this signal leads to an over- or underproduction of cell populations in the spinal cord at critical time points, which has detrimental effects for the functioning of the locomotor system.

Dopamine Promotes Adult Motor Neuron Regeneration

We demonstrate by loss- and gain-of-function experiments that dopamine from the brain profoundly influences the properties of spinal progenitor cells in the lesioned adult spinal cord. Similar to other developmental signals, such as hedgehog (Reimer et al., 2009) and notch (Dias et al., 2012), dopamine signaling is recapitulated during adult regeneration. Our study demonstrates that the analysis of developmental signals can lead the way to discovering pathways with the potential to augment adult regeneration of neuronal cell types. Remarkably, dopamine signaling not only increases regeneration of motor neurons but also changes the receptor status of spinal progenitors. This is indicated by upregulation of *d4a* expression in progenitor cells only rostral to the spinal lesion site (where dopaminergic axons are present) during nonmanipulated regeneration and the experimental induction of expression caudal to the lesion site by an agonist. Taken together, these data show an influence of descending projections on progenitor cells in the lesioned adult spinal cord.

Dopamine Likely Acts Directly on Spinal Progenitor Cells

The *d4a* receptor is strongly expressed by embryonic pMN progenitor cells and only very weakly by their motor neuron progeny, as shown by PCR of fluorescence-activated cell-sorted cell populations. During adult regeneration, *d4a* is specifically upregulated in the ventricular progenitor zone, and progenitor cells are directly contacted by dopaminergic axons during development and after an adult lesion, such that it is likely that dopamine acts directly on progenitor cells. This is supported by the observation that a dopamine agonist increased numbers of motor neuron progenitors and motor neurons in human embryonic-stem-cell-derived cell cultures, i.e., in the absence of a functional spinal neuronal network. However, indirect effects through other cells in neurospheres could not be excluded. Direct action of dopamine on progenitor cells has also been suggested for adult neurogenesis in the cerebral cortex (Höglinger et al., 2004).

Downstream Mechanisms of D4a Activation

We propose that dopamine acts at least in part through activating the hedgehog pathway, the major signaling pathway involved in the developmental generation (Lewis and Eisen, 2001; Varga et al., 2001) and adult regeneration of motor neurons (Reimer et al., 2009). Importantly, dopamine agonists cannot rescue inhibition of motor neuron generation after knockdown of the hedgehog effector *gli2b*, suggesting convergence at the level of Gli proteins. Moreover, dopamine agonists increase expression of direct hedgehog target and downstream genes, *patched2*, *olig2*, and *Nkx6.1*. Mechanistically, the D4 receptor signals by reducing cAMP levels (Asaumi et al., 2006; Wang et al., 2002), and we show that manipulations of cAMP mimic and override effects of dopamine agonists and antagonists on motor neuron development. PKA is the main target of cAMP and a known negative regulator of the hedgehog pathway and motor neuron development in zebrafish (Hammerschmidt et al., 1996; Varga et al., 2001) and mammals (Epstein et al., 1996) via Gli proteins (Pan et al., 2009; Tuson et al., 2011), suggesting that dopamine activates hedgehog signaling by lowering activity of cAMP-dependent PKA (Figure 5G). This could explain the

partial rescue observed in the weak *smu* mutant, in which some hedgehog signaling is retained (Varga et al., 2001).

Other actions of dopamine agonists are consistent with an action on the hedgehog pathway. For example, direct manipulations of hedgehog signaling in chick explants lead to a similar balanced change in V2 and motor neuron numbers as we observe here for dopamine agonists (Karunaratne et al., 2002). Shortening of the G2 phase in progenitor cells, which we observe after dopamine agonist treatment, has also been found for hedgehog signaling (Peco et al., 2012). The reduction in the number of *vsx1:GFP⁺/HB9⁺* hybrid cells after dopamine agonist treatment might be a consequence of hedgehog-dependent sharpening of progenitor domain boundaries, as it has been shown that some p2 progenitors transiently express *olig2* (Chen et al., 2011) and sustained hedgehog signaling is necessary for boundary sharpening (Dessaud et al., 2010, 2007). However, transfating of hybrid cells into motor neurons is also a possibility. Overall, the late-coming dopaminergic axons are likely to elicit a prolonged higher activity of the hedgehog pathway, thus promoting motor neuron generation at the expense of V2 interneurons.

We conclude that descending axons strongly influence the plasticity of spinal progenitor cells during development and after adult lesion in a vertebrate. We identify dopamine as a positive signal for motor neuron generation. This opens up avenues toward understanding concerted development of descending projections and spinal targets and offers clues as to the signals that could potentially influence proliferative activity of progenitor cells in other vertebrates (Meletis et al., 2008; Shihabuddin et al., 2000).

EXPERIMENTAL PROCEDURES

Animals

All fish are kept and bred in our laboratory fish facility according to standard methods (Westerfield, 2000), and all experimental procedures have been approved by the British Home Office. A list of the lines used can be found in the Supplemental Experimental Procedures.

Drug Application in Embryos

Compounds were purchased from Sigma. With the exception of NPA, for which the standard concentration was 0.5 μ M, compounds were applied at 10 μ M unless stated otherwise (for further details, see the Supplemental Experimental Procedures).

HPLC

Dopamine levels were detected by HPLC in whole embryos at 24 hpf and in heads and trunks at 48 hpf as previously published (Sallinen et al., 2009).

Fluorescence-Activated Cell Sorting

For purification of *olig2:dsRed⁺/HB9:GFP⁺* cells, we dissociated 200 embryos that were double transgenic for *olig2:dsRed* and *HB9:GFP* (head removed) at 26 hpf, yielding 17,000 *dsRed⁺/GFP⁺* and 25,000 *dsRed⁺/GFP⁺* cells. For full description, see the Supplemental Experimental Procedures.

Adult Spinal Lesion Experiments

As described previously (Reimer et al., 2008), fish were anesthetized and the spinal cord was completely transected under visual control 4 mm caudal to the junction between brain stem and spinal cord. For full description, including intraperitoneal drug injections, see the Supplemental Experimental Procedures.

Human Embryonic Stem Cell Culture

Motor neurons were generated from human embryonic stem cells using a modified standard protocol including fibroblast growth factor, retinoic acid, and purmorphamine for stepwise differentiation (Li et al., 2005b). For a full description, see the Supplemental Experimental Procedures.

Immunohistochemistry

Immunohistochemistry on whole-mounted embryos (Feldner et al., 2007), adult spinal cord sections (50 μ m in thickness) (Reimer et al., 2008), and embryonic stem cell cultures (Patani et al., 2011) has been described. A list of antibodies used can be found in the Supplemental Experimental Procedures.

In Situ Hybridization

In situ hybridization on adult spinal sections (Reimer et al., 2009) and whole-mounted embryos (Chen et al., 2009) have been described. A list of probes can be found in the Supplemental Experimental Procedures.

Reverse Transcriptase-PCR

We used standard PCR to amplify products from cDNA generated from embryos, adult spinal cord or cells in culture. For full details and a list of primers, see the Supplemental Experimental Procedures.

Morpholino Treatment

Morpholinos were injected into embryos at the one-to-four cell stage, as described elsewhere (Feldner et al., 2007). Control morpholino injections were carried out with every experiment on embryos taken from the same clutch or pool of clutches as the embryos receiving specific morpholinos, to control for any influences of developmental differences. For full details, see the Supplemental Experimental Procedures.

EdU Label in Embryos

Embryos were incubated in 10 mM EdU solution (containing 15% DMSO in 0.3 Danieau's) on ice for 20 min. After 2 hr, embryos were fixed and pH3 immunohistochemistry was performed. Finally, the Invitrogen Click-iT reaction was conducted according to manufacturer's instructions.

Tests of Swimming Behavior

Tail Touch Response

The D4 receptor antagonist L-745870 was applied at 24 hpf and washed out at 72 hpf. At 4 dpf, the startle response was determined by touching the lower trunk of the larvae with a blunt insect needle ten times. The frequency with which the larvae reacted to the touch by swimming away was recorded. The experimenter was blinded to the treatment.

Spontaneous Swimming

The D4 receptor antagonist L-745870 was applied at 24 hpf and washed out at 72 hpf. At 9 dpf, the larvae were transferred into 12 well plates and the total distance swum and high mobility frequency (episodes of fast swimming; >60% of maximal velocity) in 10 min were determined in triplicate using a Noldus behavior analysis setup and EthoVision software (version 7).

Quantification of Spinal Cell Types

Embryos

To determine the number of *HB9⁺*, *HB9:GFP⁺*, *vsx1:GFP⁺*, and *pax2a:GFP⁺* neurons, labeled cells in trunk segments 6 and 7 were counted in confocal image stacks. Live islet-1:GFP embryos were visually scored for the presence of continuous band of fluorescent cells in the trunk, which was confirmed by cell counts and intensity measurements (for further details, see Supplemental Experimental Procedures).

Adults

Small *HB9:GFP⁺* or *HB9* immunoreactive cells, representing newly generated motor neurons (Reimer et al., 2008) were quantified using stereological methods in the vicinity of the lesion site (± 750 μ m); full details are given in the Supplemental Experimental Procedures. For statistical analysis, we used the Mann-Whitney U test or analysis of variance (ANOVA) with Bonferroni/Dunn post hoc test for multiple comparisons, unless indicated differently.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.devcel.2013.04.012>.

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