Development/Plasticity/Repair

# Dopamine $D_2$ Receptor Activity Modulates Akt Signaling and Alters GABAergic Neuron Development and Motor Behavior in Zebrafish Larvae

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**An imbalance in dopamine-mediated neurotransmission is a hallmark physiological feature of neuropsychiatric disorders, such as schizophrenia. Recent evidence demonstrates that dopamine $D_2$ receptors, which are the main target of antipsychotics, modulate the activity of the protein kinase Akt, which is known to be downregulated in the brain of patients with schizophrenia. Akt has an important role in the regulation of cellular processes that are critical for neurodevelopment, including gene transcription, cell proliferation, and neuronal migration. Thus, it is possible that during brain development, altered Akt-dependent dopamine signaling itself may lead to defects in neural circuit formation. Here, we used a zebrafish model to assess the direct impact of altered dopamine signaling on brain development and larval motor behavior. We demonstrate that $D_2$ receptor activation acutely suppresses Akt activity by decreasing the level of pAkt(Thr308) in the larval zebrafish brain. This $D_2$-dependent reduction in Akt activity negatively regulates larval movement and is distinct from a $D_1$-dependent pathway with opposing affects on motor behavior. In addition, we show that $D_2$-dependent suppression of Akt activity causes a late onset change in GSK3b activity, a known downstream target of Akt signaling. Finally, altered $D_2$ receptor signaling, or direct inhibition of Akt activity, causes a significant decrease in the size of the GABAergic neuron population throughout most of the brain. Our observations suggest that $D_2$ receptor signaling suppresses Akt-GSK3b activity, which regulates GABAergic neuron development and motor behavior.**

## Introduction

The neurotransmitter dopamine (DA) mediates numerous adult brain functions (Di Chiara, 2005; Dunnet, 2005), and DA-mediated neurotransmission imbalances are associated with several psychiatric disorders, notably schizophrenia (Hurd and Hall, 2005). DA binds to five-specific receptors in the mammalian brain, which are classified into two subtypes: $D_1$-like ($D_1$, $D_5$) and $D_2$-like ($D_2$, $D_3$, $D_4$) (Neve et al., 2004). The $D_1$ receptor is coupled to the stimulatory G protein (Gs), activating adenylyl cyclase (AC), leading to an activation of protein kinase A (PKA). The $D_2$ receptor is coupled to the inhibitory G protein (Gi), which inhibits the activation of AC (Missale et al., 1998). It was recently shown that signaling through the $D_2$ receptors can also modulate the activity of the Akt pathway through a complex formation with $\beta$-arrestin and PP2A (Beaulieu et al., 2007). This complex inhibits phosphorylation of Akt and modulates the reward system and motor behavior of rodents (Beaulieu et al., 2004, 2005).

Components of the DA signaling pathway are expressed during mammalian development (Shearman et al., 1997; Araki et al., 2007) and modulation of DA signaling alters the proliferation of embryonic neural progenitor cells (for review, see Souza and Tropepe, 2011). Furthermore, it was recently shown that a balance between $D_1$ and $D_2$ receptor signaling could control the migration of newly generated GABAergic neurons in the forebrain (Crandall et al., 2007). These findings have important implications for understanding the neurodevelopmental basis of several behavioral disorders, for which alterations in DA and GABAergic neuronal systems have been implicated (Benes and Berretta, 2001; Seeman, 2006; Souza et al., 2006). However, the molecular mechanisms underlying DA modulation of GABAergic neuron development remain to be elucidated, and its direct impact on behavior has not yet been firmly established.

Here, we report on the use of a zebrafish model to further examine DA signaling, GABAergic neuron development, and the emergence of simple movement behavior. Orthologous DA $D_1$-like and $D_2$-like receptor expression is detected in specific embryonic and early larval brain regions (Boehmler et al., 2004, 2007; Li et al., 2007). Furthermore, behavioral studies indicate that the neural cells are competent to respond to DA signaling at a time when simple motor behaviors are emerging in the young larvae (Boehmler et al., 2007; Bretaud et al., 2007; Thirumalai and Cline,

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2008). This provides an excellent model to examine the immediate impact that alterations in DA signaling have on GABAergic neuron development and behavior.

We show for the first time that $D_2$-dependent modulation of Akt activity is conserved in the larval zebrafish brain and that the $D_2$-Akt dependent pathway has a functional role in regulating larval movement that is distinct from a $D_1$-dependent pathway. In addition, we demonstrate that imbalances in DA signaling significantly influence the size of the GABAergic neuron population that forms in specific regions of the brain. Our observations suggest that DA signaling imbalance can have a profound effect on GABAergic development and motor behavior within a narrow developmental time window.

## Materials and Methods

*Zebrafish strains and drug exposure.* Adult zebrafish (*Danio rerio*) used in this study were maintained at 28°C on a 14 h light/10 h dark cycle and housed in an automated recirculating system (Aquaneering). Adult males and females were used for breeding only. Animals were treated in accordance with the regulations on animal experimentation established by the Canadian Council on Animal Care. The experimental procedures were approved by the University of Toronto Animal Care Committee. Embryos were collected after natural spawning, staged as described by Kimmel et al. (1995) and reared in embryo medium (EM) at 28°C according to standard procedures (Westerfield, 2007). The experiments performed on 3–5 d larval zebrafish were before sex determination, which normally occurs at ~1 month of age. The wild-type strain used was AB (Zebrafish International Resource Center) and the *Tg(dlx5a/6aIG:GFP)* transgenic strain was a kind gift from Dr. Marc Ekker (University of Ottawa, Ottawa, ON, Canada). For drug exposures, the chemical was added directly to embryo medium. In experiments in which the larvae were exposed to a drug for 10–60 min, the larvae were transferred to a 24-well plate (Nunc) with the drug dissolved in embryo medium at working concentrations. In experiments in which the larvae were exposed to a drug for 2 d, fresh embryo media containing the drug(s) of interest were replaced every 24 h. Dopamine (DA; H8502), quinpirole (Qui; Q111), eticlopride (Etic; E101), SCH-23390 (SCH; D054), SKF-38393 (SKF; S101), fostriecin (Fost; F4425), and MPTP (M0896) were purchased from Sigma-Aldrich. The Akt inhibitor (Akti-1/2) was purchased from EMD-Calbiochem.

*Preparation of tissue sample for protein analysis* in vitro. After drug exposure, 3 d post fertilization (dpf) or 5 dpf larvae were first washed with embryo medium and then immobilized in ice-cold PBS. The larvae were decapitated, and the brains were dissected and incubated in Western blot ice-cold lysis buffer [20 mM MOPS, pH 7, 2 mM EGTA, 5 mM EDTA, protease inhibitor cocktail (P 8340, Sigma), Halt phosphatase inhibitor cocktail (78420, Pierce), and 0.5% Nonidet P40, final pH 7.2] or ELISA buffer (0.01N HCl, 1 mM EDTA, 4 mM sodium metabisulfite).

*DA immunoassay.* Fifty control and DA-treated 5 dpf zebrafish larval brains were dissected, rinsed in EM, and placed in 100 $\mu$l of ELISA buffer. Measurement of total DA was performed using a DA enzyme immunoassay kit (BA 10–0300, Rocky Mountain Diagnostics). The data were acquired in duplicate.

*Immunoblot.* Lysates were quickly sonicated on ice before centrifugation at 15,000 × *g* for 30 min at 4°C. Supernatants were stored at −20°C or prepared for electrophoresis with sample buffer NuPAGE LDS (Invitrogen) plus 5% $\beta$-mercaptoethanol and incubated at 70°C for 10 min. The samples were loaded into bis-Tris NuPAGE 4–12% gels (Invitrogen) and submitted to electrophoresis followed by transfer to PVDF membrane (Hybond ECL, GE Healthcare Pharmacia Biotech). Membrane blots were incubated with antibodies anti-pAkt(Thr308) (1:1000, #9275 Cell Signaling Technology), anti-pAkt(Ser473) (1:1000, #9271 Cell Signaling Technology), anti-pCREB(Ser133) (1:1000, #9198 Cell Signaling Technology), anti-pGSK3$\beta$(Ser9) (1:1000, #9323 Cell Signaling Technology), and anti-pERK (1:1000, #9101 Cell Signaling Technology) diluted in TBS Tween 20 0.1% overnight at 4°C, and anti-Akt (1:4000, #9272 Cell Signaling Technology), anti-CREB (1:2000, #MAB1501 Millipore Bioscience Research Reagents), anti-tyrosine hydroxylase (1:1000, MAB318 Millipore Bioscience Research Reagents), anti-GSK3$\beta$ (1:1000, #9315 Cell Signaling Technology), anti-ERK (1:1000, #9102 Cell Signaling Technology), and anti-actin (1:5000, #MAB1501 Millipore Bioscience Research Reagents) diluted in PBS Tween 20 0.1% overnight at 4°C. Thereafter, membranes were washed and incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies, goat anti-rabbit IgG (1:20,000), and goat anti-mouse IgG (1:7000) (Invitrogen). Membranes were probed by chemiluminescent detection with ECL Plus (GE Healthcare) and developed. Films were scanned and densitometric analysis was performed using NIH ImageJ version 1.43u.

*Immunocytochemistry.* Embryos were fixed overnight at 4°C in 4% paraformaldehyde in PBS. The samples were infiltrated with gradients of sucrose, and cryostat sections were refixed in 4% paraformaldehyde in PBS. For the HuC labeling samples, antigen retrieval was performed using 50 mM Tris buffer, pH 8.0, at 75–80°C for 30 min. The sections were preincubated in dilution buffer (PBS, 3% goat serum, 5% BSA, 0.3% Triton X-100 and, for phosphorylation labeling, 5% Halt phosphatase inhibitor cocktail) for 1 h, followed by overnight incubation at 4°C with antibodies anti-pAkt(Thre308) (1:100, #9275 Cell Signaling Technology), anti-tyrosine hydroxylase (1:100, #MAB318 Millipore Bioscience Research Reagents), anti HuC/D (1:400, A-21271 Invitrogen), anti-glutamine synthetase (1:600, #MAB302 Millipore), or anti-GFP (1:1000, #A21311 Invitrogen). Sections were subsequently washed four times in PBS and incubated overnight at 4°C with Cy3 anti-rabbit (1:500, Jackson ImmunoResearch) or Cy5 anti-rabbit (1:500, Jackson ImmunoResearch). The sections were counter-stained with Hoechst (Sigma, 861405) or propidium iodide (Sigma, 81845), washed three times in PBS for 10 min, and covered by mounting with antifade (Invitrogen).

*Image acquisition and cell counting.* Immunostained sections were documented with a confocal microscope (Leica TCS SP5) using the 40× oil-immersion objective. Cells were counted using the IMARIS 7.0 software, separately in each channel on multiple optical sections (1 $\mu$m thickness). Stacks of 10 images (10 $\mu$m) are shown in Figures 1 and 4–8. The proportions of distinct cell types were calculated in several brains, normalized by the total number of cells (Hoechst labeling) and average ±SD are given as a result. Methods for grouped data (repeated measurements data) were applied in the statistical analysis, taking into account data collected from several sections of several brains.

*Recording movement episodes.* For the experiments investigating the involvement of DA signaling in movement behavior, 3 dpf and 5 dpf larvae were treated with drugs for 30 min, washed, and transferred to embryo medium for 20 min using clear 24-well tissue culture plates (single larva/well) at 28°C (Nunc). During this time, larvae were recorded using a digital camera (JVC–Everio). For the experiments investigating the effects of long-term treatment with drugs, 5 dpf-treated larvae were transferred to embryo medium for 30 min, rinsed, and transferred to fresh embryo medium, where they were recorded for 30 min. Movement episodes, defined here as the number of movements initiated by the larvae regardless of the distance traveled per movement, were recorded during the specified time intervals. The larvae were recorded in a clear 24-well tissue culture dish (single larva/well) at 28°C.

*Statistical analysis.* All data were analyzed by either Student's *t* tests or one-way ANOVA using SigmaPlot/SigmaStat. Values were expressed as percentage of control, mean ± SD. Differences were considered significant when $p < 0.05$.

## Results

### DA modulates Akt(Thr308) phosphorylation in the larval zebrafish brain

To examine the modulation of Akt signaling by DA in the zebrafish larval brain, we first tested whether brain DA levels could increase as a result of treating live whole larvae with exogenous DA dissolved in EM. We treated 5 dpf larvae ($n = 50$) with 100 $\mu$M DA for 30 min. After extensive rinsing in EM, brain samples were pooled and DA concentrations were evaluated by the ELISA method. Based on three separate sample inputs, we observed that exogenous DA treatment resulted in ~60–90% more DA in the

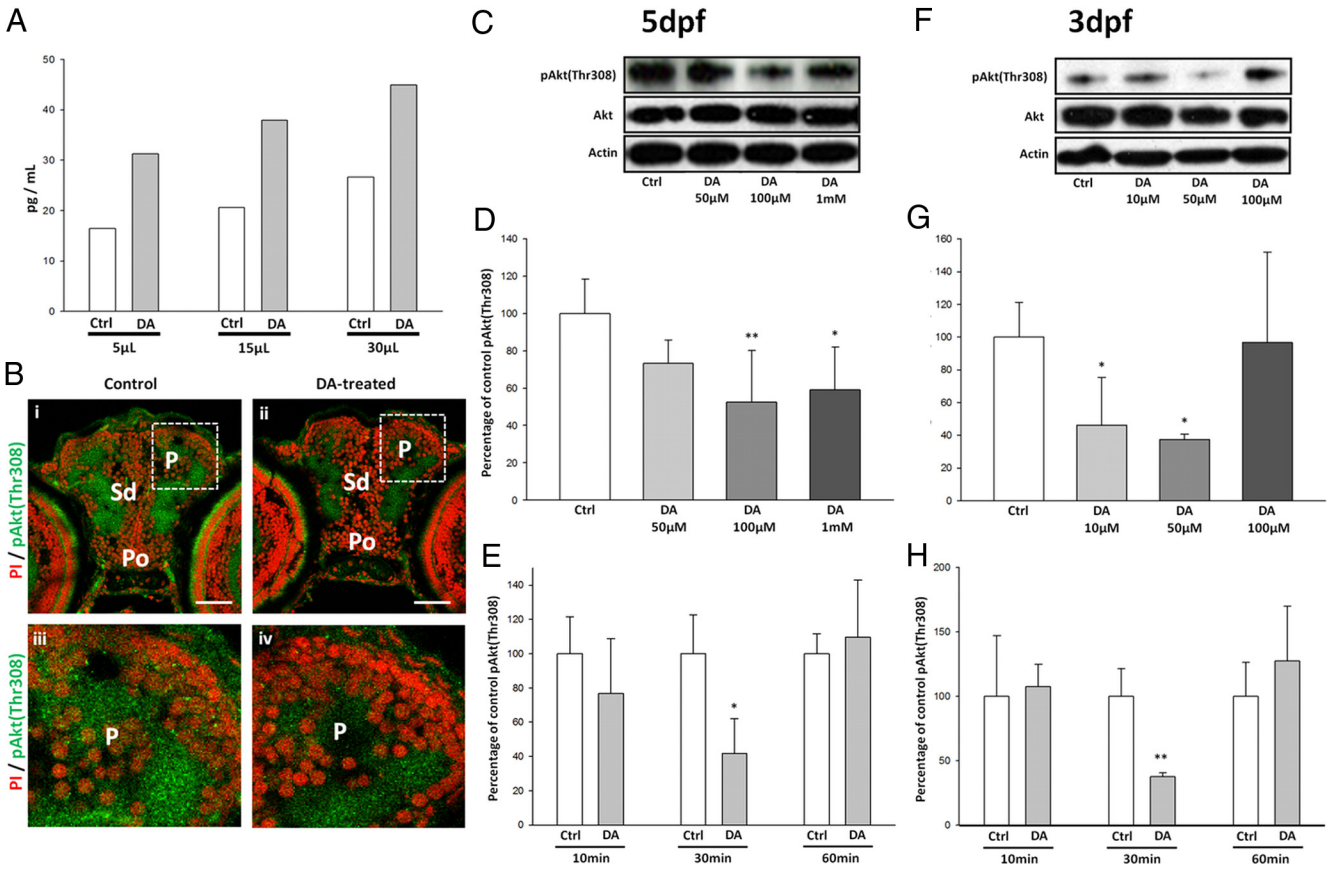



**Figure 1.** DA regulates phosphorylation of Akt in the developing brain of zebrafish. ***A***, ELISA assay for DA levels in extracts from the brain of zebrafish nontreated and bath treated with DA for 30 min. Samples of different volumes (5, 15, and 30 $\mu$l) were analyzed. ***B***, Representative confocal images of pAkt in the 5 dpf larvae treated with DA and immunostained with anti-pAkt(Thr308) (green) antibodies and counterstained with PI (red). The levels of pAkt(Thr308) are decreased in the brains of DA-treated larvae (***ii***, ***iv***) compared with control (***i***, ***iii***). ***C–H***, Representative Western blot (***C***, ***F***) and densitometric (***D***, ***E***, ***G***, ***H***) analyses of extracts prepared from brains of 5 dpf (***C–E***) and 3 dpf (***F–H***) larvae nontreated and DA-treated for 30 min. The modulation of pAkt(Thr308) is DA-concentration-dependent (***D***, ***G***), and 3 dpf larval brain is more sensitive for lower concentrations of DA (***G***) than 5 dpf larval brain (***D***). DA similarly modulates pAkt(Thr308) in both 3 dpf and 5 dpf larval brain at 30 min. P, Pallium; Sd, dorsal subpallium; PI, propidium iodide; Po, preoptic region; Ctrl, control. Scale bar, 50 $\mu$m. $N = 4–5$ larvae per group; data are average ± SD. $^*p \leq 0.05$, $^{**}p \leq 0.01$.

brain of 5 dpf larvae (Fig. 1*A*). This result suggests that a significant amount of exogenous DA could be transported directly to the brain through whole animal incubation.

We next investigated whether exogenous DA can regulate Akt signaling in the zebrafish larval brain. To address this question, we treated 5 dpf larvae with different concentrations of DA for 30 min. We observed a DA concentration-dependent modulation of phosphorylation of Akt(Thr308) (Fig. 1*C*,*D*). There is a slight decrease of pAkt(Thr308) levels at 50 $\mu$M [$N = 3–4$; one-way ANOVA, Fisher least significant difference (LSD); $p = 0.135$], and a significant decrease of 47% at 100 $\mu$M ($N = 4$; one-way ANOVA, Fisher LSD; $p = 0.01$) and 41% at 1 mM ($N = 4$; one-way ANOVA, Fisher LSD; $p = 0.022$) (Fig. 1*D*). These findings are consistent with previous experiments demonstrating that DA results in Akt(Thr308) dephosphorylation in the mouse striatum, which is critical for inhibiting its kinase activity (Beaulieu et al., 2005). To assess the kinetics of DA-dependent alterations in Akt phosphorylation, we exposed 5 dpf larvae to 100 $\mu$M DA for 10, 30, and 60 min (Fig. 1*E*). We observed a slight, but nonsignificant decrease of pAkt(Thr308) levels after 10 min of treatment ($N = 5$; Student's $t$ test; $p = 0.216$) and a significant decrease of 58% after 30 min of treatment ($N = 4$; Student's $t$ test; $p = 0.03$). After 60 min of 100 $\mu$M DA treatment, pAkt(Thr308) returned to control levels ($N = 5$; Student's $t$ test; $p = 0.601$) (Fig. 1*E*). The kinetics of the Akt response in the 5 dpf larval brain appears to be in line with studies in the mammalian brain suggesting a relatively prolonged Akt response to DA compared with the cAMP/PKA response (Beaulieu et al., 2007). Using immunohistochemistry, we observed that the reduction in pAkt(Thr308) levels in 5 dpf larvae treated with 100 $\mu$M DA for 30 min affected several brain regions that are known targets of endogenous DA signaling, including those in the forebrain (Fig. 1*B*) and midbrain (data not shown).

The zebrafish DA brain circuitry at 5 dpf resembles the adult brain pattern (Schweitzer and Driever, 2009) and mediates simple visuomotor behaviors (Li and Dowling, 2000). We asked whether DA-dependent modulation of Akt also occurs at an earlier stage of development after most DA neurons are generated, but before the DA circuitry has fully matured. Thus, we treated 3 dpf larvae with three different concentrations of DA for 30 min. We found that DA modulates pAkt(Thr308) at lower concentrations at this earlier stage of development (Fig. 1*F*,*G*). DA caused a 54% decrease of pAkt(Thr308) levels at 10 $\mu$M ($N = 5$; one-way ANOVA, Fisher LSD; $p = 0.037$) and a 63% decrease at 50 $\mu$M ($N = 3–4$; one-way ANOVA, Fisher LSD; $p = 0.027$). However, 100 $\mu$M DA treatment was more variable and did not result in any change in the levels of pAkt(Thr308) compared with controls ($N = 3–5$; one-way ANOVA, Fisher LSD; $p = 0.898$) (Fig. 1*G*). Because the optimal DA concentration was 50 $\mu$M, we treated 3 dpf larvae with this concentration for different periods of time (Fig. 1*H*). We did not find any changes in the levels of pAkt(Thr308) after 10 min of treatment ($N = 5$; Student's $t$ test; $p = 0.803$), but there was a significant decrease of 63% after 30 min

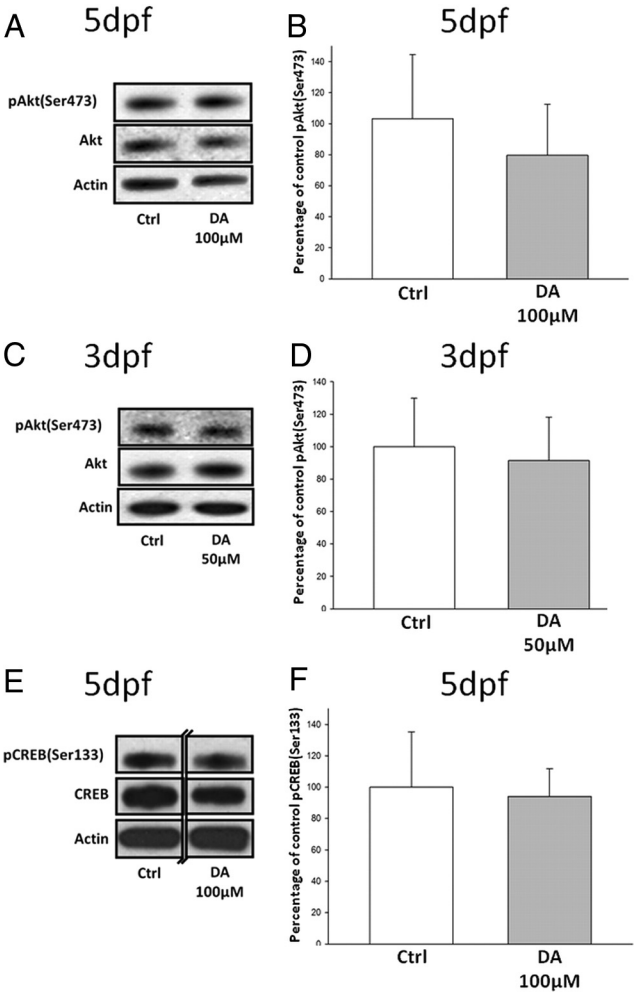



**Figure 2.** DA specifically regulates pAkt(Thr308). ***A–F***, Representative Western blot (***A***, ***C***, ***E***) and densitometric (***B***, ***D***, ***F***) analyses of extracts prepared from brains of 5 dpf (***A***, ***B***, ***E***, ***F***) and 3 dpf (***C***, ***D***) larvae nontreated and DA-treated for 30 min. There is no modulation of pAkt(Ser473) in the brain of 5 dpf (***A***, ***B***) and 3 dpf (***C***, ***D***) larvae treated with DA. Also, there are no changes in the levels of pCREB(Ser133) in the brain of 5 dpf larvae treated with DA (***E***, ***F***). Ctrl, Control. $N = 3–6$ larvae per group; data are average ± SD.

of treatment ($N = 4–5$; Student's $t$ test; $p = 0.004$). Similar to 5 dpf larvae, after 60 min of 50 $\mu$M DA treatment, the levels of pAkt(Thr308) returned to control levels ($N = 4–5$; Student's $t$ test; $p = 0.376$) (Fig. 1*H*). Thus, the magnitude and kinetics of the pAkt modulation by DA was comparable at 3 dpf and 5 dpf, although the exogenous DA concentration required to elicit the response was twofold greater at 5 dpf. This could be due to the efficiency of DA transport to the brain at older stages.

Previous studies demonstrated that DA receptor activation regulates pAkt(Thr308) through a complex with βArrestin2 and protein phosphatase 2A (PP2A) that functions independently of changes in pAkt(Ser473) (Beaulieu et al., 2005). Thus, we investigated whether DA regulates pAkt(Ser473) in the developing zebrafish brain. Using our optimal DA treatment parameters, we did not find any alterations in the levels of pAkt(Ser473) in 5 dpf larval brains ($N = 8$; Student's $t$ test; $p = 0.563$) (Fig. 2*A*,*B*) or 3 dpf larval brains ($N = 3$; Student's $t$ test; $p = 0.729$) (Fig. 2*C*,*D*). DA receptor signaling is also mediated through the cAMP/PKA and phospholipase C (PLC) pathways (Lee et al., 2004; Neve et al., 2004). Thus, we investigated whether levels of pCREB(Ser133), which is a substrate of PKA function, were altered in the 5 dpf larval brains treated with 100 $\mu$M DA for 30 min. However, we did not observe significant changes in the pCREB(Ser133) levels after DA treatment compared with control ($N = 5–7$; Student's $t$ test; $p = 0.768$) (Fig. 2*E*,*F*). Because DA can activate calcium signaling by PKA and/or PLC (Lee et al., 2004; Neve et al., 2004), we also investigated whether the ERK pathway, which is sensitive to calcium modulation by PKA and crucial for normal brain development, was altered in 3 dpf larvae treated with 50 $\mu$M DA for 30 min. As with pCREB, we found no alterations in the levels of pERK(Thr202/Tyr204) ($N = 3$; Student's $t$ test; $p = 0.555$) (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). Together, these data suggest a conserved role for DA-dependent regulation of Akt function through the modulation of pAkt(Thr308) levels at the onset of postembryonic brain development in zebrafish.

### Modulation of Akt activity by DA receptor $D_2$, but not $D_1$, in the larval zebrafish brain

To examine which family of DA receptors is involved in the regulation of Akt in the early larval zebrafish brain, we treated 5 dpf larvae for 30 min with 100 $\mu$M DA, 10 $\mu$M quinpirole ($D_2$ receptor agonist), 10 $\mu$M eticlopride ($D_2$ receptor antagonist), and 10 $\mu$M SCH-23390 ($D_1$ receptor antagonist) (Fig. 3*A*). Consistent with our previous results, there was a 45% decrease in the level of pAkt(Thr308) in the brains of larvae treated with DA ($N = 7–9$; one-way ANOVA, Fisher LSD; $p = 0.001$). Interestingly, we observed a similar significant reduction (43%) in pAkt(Thr308) levels in the brains of larvae treated with quinpirole ($N = 7–8$; one-way ANOVA, Fisher LSD; $p = 0.003$). However, we found no change in the level of pAkt(Thr308) in the brains of those larvae treated with eticlopride ($N = 3–7$; one-way ANOVA, Fisher LSD; $p = 0.706$) or SCH-23390 ($N = 3–7$; one-way ANOVA, Fisher LSD; $p = 0.201$). Although 30 min of eticlopride treatment was not sufficient to alter the levels of pAkt(Thr308), we did observe that pAkt(Thr308) levels were significantly increased in the brains of larvae treated with both DA and eticlopride when compared with DA alone ($N = 4–7$; one-way ANOVA, Fisher LSD; $p < 0.001$), and this effect was similar to control levels ($N = 4–7$; one-way ANOVA, Fisher LSD; $p = 0.273$) (Fig. 3*A*). It was previously shown that the inhibition of PP2A, which modulates Akt activity, by fostriecin could oppose the regulation of pAkt(Thr308) by DA (Beaulieu et al., 2005). We tested whether this modulation could be recapitulated in the larval zebrafish brain by cotreating 5 dpf larvae with 10 $\mu$M quinpirole and 10 $\mu$M fostriecin to see whether we could block the effect observed with quinpirole alone. Indeed, we found no significant difference in the quinpirole+fostriecin group when compared with the control ($N = 3–7$; one-way ANOVA, Fisher LSD; $p = 0.088$) (Fig. 3*A*). Therefore, DA modulation of pAkt(Thr308) is $D_2$-receptor-dependent and likely functions through a complex that has PP2A activity.

### $D_2$ receptor-Akt signaling suppresses motor behavior of 5 dpf zebrafish larvae

It has been recently shown that DA regulates the behavior of zebrafish larvae (Boehmler et al., 2007; Bretaud et al., 2007; Thirumalai and Cline, 2008). In particular, Thirumalai and Cline (2008) showed that endogenous DA was required for movement initiation. Therefore, we were interested in examining the role of DA-dependent Akt signaling in motor behavior. To begin to address this question, we treated 5 dpf larvae with different DA agonists and antagonists for 30 min, then rinsed the larvae in EM and incubated them in fresh EM for a subsequent 30 min. We quantified the number of times that the larvae initiated a move-

ment (regardless of distance traveled during each movement), referred to here as a movement episode, during this timeframe using digital recording. Larval movement episodes in the first 10 min with 100 $\mu$M DA exposure were not significantly different from nontreated controls ($N = 6–7$; Student's $t$ test; $p = 0.443$). However, we observed a sharp 90% decrease in movement episodes between 10 and 30 min of DA exposure ($N = 6–8$; Student's $t$ test; $p = 0.007$), and this behavior was recovered during the 30 min after washing and replacing the EM without DA ($N = 8$; Student's $t$ test; $p = 0.536$) (Fig. 3*B*). This result indicates that exogenous DA treatment of 5 dpf larvae and 3 dpf larvae (supplemental Fig. S2, available at www.jneurosci.org as supplemental material) causes a robust and reversible decrease in movement episodes that occurs within 30 min of exposure, which correlates with the kinetics of the pAkt(Thr308) modulation by DA.

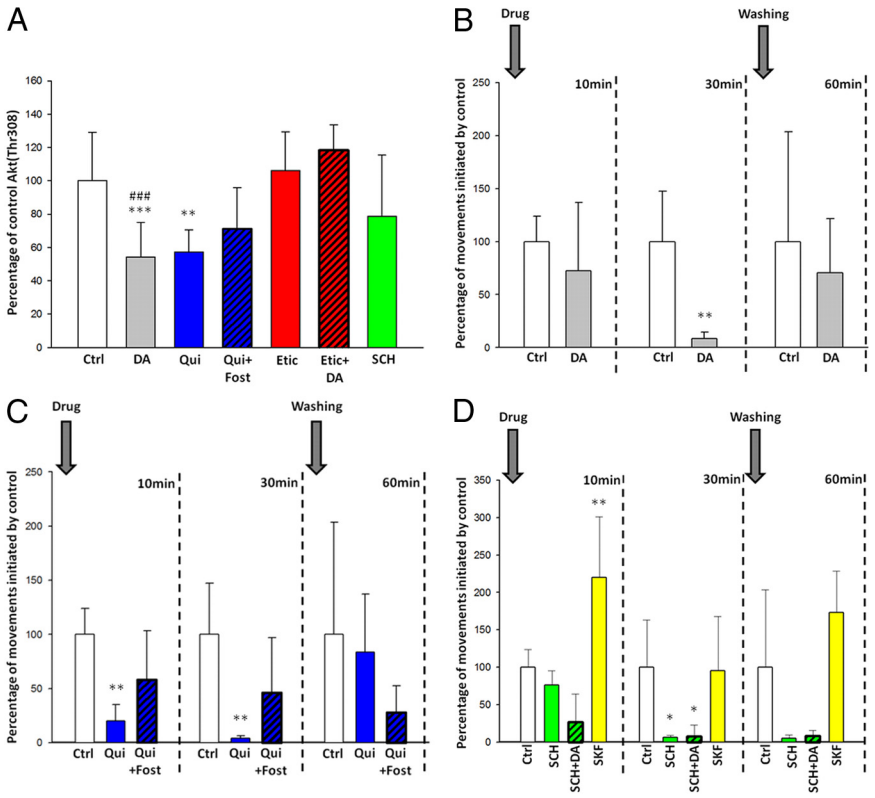



**Figure 3.** DA receptor $D_2$ regulates Akt phosphorylation and motor behavior of 5 dpf larvae. ***A***, Representative Western blot densitometric analyses of extracts prepared from brains of 5 dpf larvae treated for 30 min. DA decreases levels of pAkt(Thr308). Qui decreases pAkt(Thr308) levels, which is partially rescued when treated with Fost. Etic does not change levels of pAkt(Thr308), but rescues the DA-dependent modulation of pAkt(Thr308) to the control (Ctrl) levels. There are no changes in the pAkt(Thr308) levels in the brain of larvae treated with SCH. ***B–D***, Number of movements episodes by 5 dpf larvae during 30 min of treatment and during 30 min after drug washing. ***B***, There are no changes in larvae in the first 10 min of DA treatment. There is a decrease in larval movement during the 10–30 min period of DA treatment. Movement episodes are rescued to control levels during 30 min after drug washing. ***C***, There is a decrease in larval movement during the first 10 min and 10–30 min of Qui treatment. Movement episodes are rescued to control levels during the 30 min after drug washing. This modulation of motor control by Qui is partially rescued with Fost treatment. ***D***, SCH modulates movement during 10–30 min of treatment, but not in the first 10 min. This modulation is intensified when the larvae are treated with SCH + DA. There is no rescue of movement episodes after drug washing. SKF increases movement episodes in the first 10 min. During the 10–30 min period, the movement episodes are similar to control levels. There is an increase in movement episodes during the 30 min after drug washing. $N = 3–8$ larvae per group; data are average ± SD. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ compared with control. $^{\#\#\#}p \leq 0.001$ compared with Etic + DA.

## Opposing roles for $D_1$ and $D_2$ receptor signaling in regulating the motor behavior of 5 dpf zebrafish larvae

We tested whether a $D_2$ receptor-Akt signal transduction pathway mediates the DA-dependent decrease in movement episodes. Larvae (5 dpf) treated with 10 $\mu$M quinpirole resulted in an 80% decrease in movement episodes within the first 10 min of exposure ($N = 5–6$; one-way ANOVA, Fisher LSD; $p = 0.005$), and this effect was exacerbated to a 94% decrease after 30 min ($N = 5–6$; one-way ANOVA, Fisher LSD; $p = 0.011$) (Fig. 3*C*). Movement episodes were recovered during the 30 min after washing ($N = 5–6$; one-way ANOVA; $p = 0.469$) (Fig. 3*C*). To further examine the involvement of the Akt pathway in response to $D_2$-receptor-specific activation, we cotreated the larvae with 10 $\mu$M quinpirole and 10 $\mu$M fostriecin, which we previously showed could significantly block the reduction of pAkt(Thr308) caused by quinpirole alone. Compared with nontreated controls, the larvae cotreated with quinpirole and fostriecin did not result in a significant decrease in movement episodes after 10 min ($N = 5–6$; one-way ANOVA, Fisher LSD; $p = 0.086$) or 30 min ($N = 5–6$; one-way ANOVA, Fisher LSD; $p = 0.201$). These data indicate that Akt modulation is critical for $D_2$-receptor-dependent larval motor behavior. During the 30 min after drug washout, the quinpirole+fostriecin group showed no significant behavioral difference when compared with the nontreated control group ($N = 5–6$; one-way ANOVA; $p = 0.469$) (Fig. 3*C*), consistent with the overall reversible effects of $D_2$-receptor-specific modulation.

The $D_1$ and $D_2$ DA receptors function to activate distinct signal transduction pathways. $D_1$ receptors activate the cAMP/PKA pathway and, when dimerized with $D_2$ receptors, activate the PLC pathway (Lee et al., 2004). On the other hand, $D_2$ receptors modulate both cAMP/PKA and Akt pathways (Lee et al., 2004). Our results demonstrate that $D_2$ receptor, but not $D_1$ receptor, activation can modulate Akt activity in the larval zebrafish brain and that $D_2$-specific signaling can alter larval motor behavior. However, this does not preclude a role for $D_1$ receptor activation in mediating motor behavior independently of Akt signaling. Thus, to test a role for $D_1$ receptor activation in the modulation of motor behavior, we treated 5 dpf larvae with 10 $\mu$M SCH-23390 ($D_1$ receptor antagonist), 10 $\mu$M SCH-23390 + 100 $\mu$M DA, or 10 $\mu$M of the-specific $D_1$ receptor agonist SKF-38393 (Fig. 3*D*). After 10 min of drug exposure, we observed no difference in the number of movement episodes between the control and SCH-23390 groups ($N = 4–6$; one-way ANOVA, Fisher LSD; $p = 0.624$) and a slight, but nonsignificant decrease in the SCH-23390+DA group ($N = 4–6$; one-way ANOVA, Fisher LSD; $p = 0.119$). However, after 30 min, movement episodes were reduced by 94% in the SCH-23390 group ($N = 4–6$; one-way ANOVA; $p = 0.015$) and 93% in the SCH-23390+DA group ($N = 4–6$; one-way ANOVA, Fisher LSD; $p = 0.011$). Thirty minutes after the washing out the drug, the number of movement episodes remained considerably reduced in both the SCH-23390 ($N = 4–6$; one-way ANOVA; $p = 0.099$) and SCH-23390+DA groups

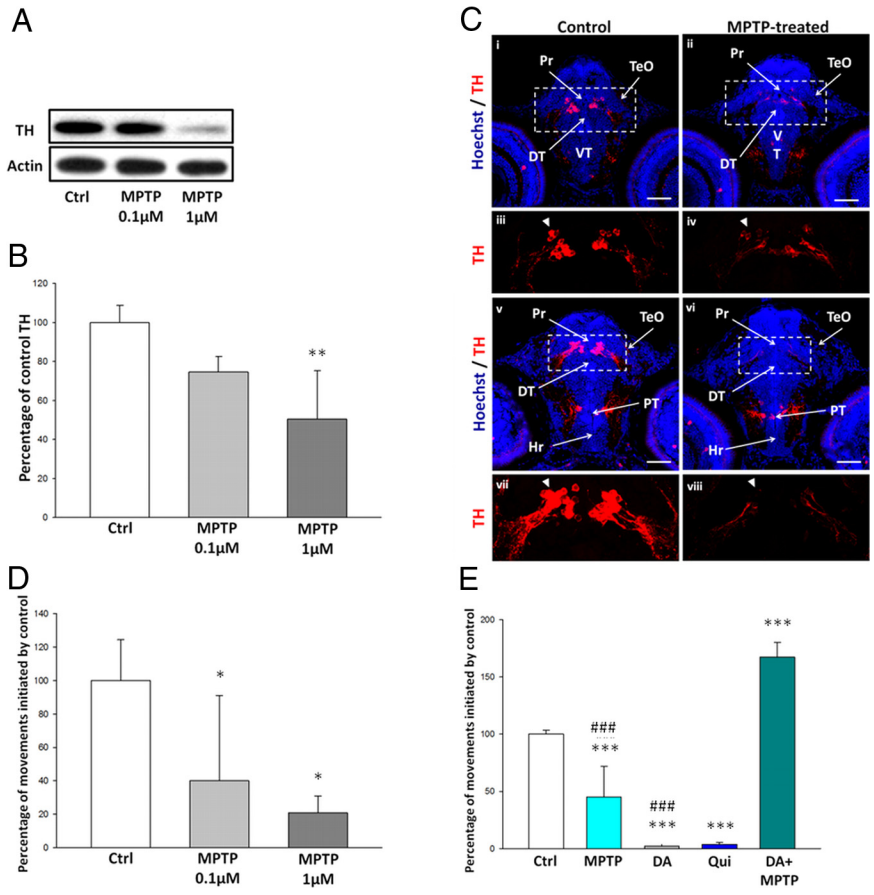



**Figure 4.** Chronic DA treatment changes larval motor behavior. ***A***, ***B***, Representative Western blot (***A***) and densitometric analyses (***B***) of extracts prepared from brains of 5 dpf larvae incubated with MPTP for 2 d. The decrease of TH expression is MPTP concentration dependent. ***C***, Representative confocal images confirm the decrease of TH levels in the brain of 5 dpf larvae treated with MPTP. Samples were immunostained with TH (red) and counterstained with Hoechst (blue). There is a decrease of TH labeling in the Pr of MPTP-treated larvae (***ii***, ***iv***, ***vi***, ***viii***) when compared with control (***i***, ***iii***, ***v***, ***vii***). ***D***, ***E***, Number of movements initiated during 30 min after drug washing, by 5 dpf larvae drug treated for 2 d. ***D***, The depression in the motor behavior of MPTP-treated larvae is concentration dependent. ***E***, MPTP, DA, and Qui treatment suppresses movement episodes. Larvae treated with DA+MPTP result in increased movement episodes. Pr, Pretectum; TeO, optic tectum; DT, dorsal thalamus; VT, ventral thalamus; PT, posterior tuberculum; Hr, rostral hypothalamus; Ctrl, control. Scale bar, 50 $\mu$m. $N = 3–4$ larvae per group; data are average ± SD. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ compared with control. $^{\#\#\#}p \leq 0.001$ compared with DA+MPTP.

($N = 4–6$; one-way ANOVA, Fisher LSD; $p = 0.087$) (Fig. 3*D*). Thus, inhibition of the $D_1$ receptor was sufficient to cause a decrease in movement episodes, regardless of the presence of exogenous DA.

It is possible that blocking endogenous DA signaling through the $D_1$ receptor results in increased DA availability for signaling via other receptors, including $D_2$. However, treatment with SCH-23390 alone does not significantly alter pAkt(Thr308) levels (Fig. 3*A*), suggesting that $D_2$ signaling is not directly affected. Alternatively, $D_1$ receptor signaling may have the opposite effect on motor behavior compared with $D_2$ receptor signaling and does not rely on pAkt(Thr308) modulation. Consistent with this hypothesis, treatment with the $D_1$ receptor agonist SKF-38393 resulted in a strong increase in movement episodes by ~120% after 10 min ($N = 4–6$; one-way ANOVA, Fisher LSD; $p = 0.005$). However, by 30 min of exposure to SKF-38393 ($N = 4–6$; one-way ANOVA, Fisher LSD; $p = 0.900$) and after washing out the drug ($N = 4–6$; one-way ANOVA, Fisher LSD; $p = 0.151$), motor behavior returned to levels observed in the control (Fig. 3*D*). Overall, our findings support the hypothesis that activation of the $D_1$ receptor results in a general increase in zebrafish larval motor behavior, while activation of the $D_2$ receptor has the opposite effect of decreasing motor behavior in an Akt-dependent manner.

### Reducing endogenous DA levels in the larval zebrafish brain suppresses motor behavior

Our studies with exogenous DA agonist and antagonist treatment reveal a complex regulation of zebrafish motor behavior whereby activation of the $D_2$ receptors lead to a strong suppression of movement episodes, while activation of the $D_1$ receptor leads to an enhancement of movement episodes. We next asked whether reducing the levels of endogenous DA would result in an increase or decrease in movement episodes. To address this question we used the DA neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which can either cause a defect in DA function by decreasing overall levels of tyrosine hydroxylase (TH) expression (Anichtchik et al., 2004) or by killing DA neurons (Lam et al., 2005; McKinley et al., 2005; Panula et al., 2010). Using our experimental parameters, we treated 3 dpf zebrafish with MPTP for 2 d (replenished once per day). After 2 d of treatment, we incubated the larvae in fresh EM to wash out the drug and then transferred the larvae to a new embryo medium to assess their motor behavior. We performed Western blot analysis (Fig. 4*A*,*B*) and demonstrated an MPTP concentration-dependent decrease in TH expression by ~25% in the brains of 5 dpf larvae treated with 0.1 $\mu$M MPTP ($N = 3$; one-way ANOVA, Fisher LSD; $p = 0.097$) and by 50% in the brains of larvae treated with 1 $\mu$M MPTP ($N = 3$; one-way ANOVA, Fisher LSD; $p = 0.009$). Immunohistochemical analysis demonstrated that the pretectal cluster of TH-expressing neurons in the 5 dpf larvae was primarily affected by the neurotoxicity of MPTP (Fig. 4*C*). In contrast, the diencephalic group of DA neurons was relatively spared using this low dose and short duration of MPTP, although we cannot rule out the possibility that subtle changes in TH expression occurred that are not readily detectable using standard confocal microscopy.

We next treated 3 dpf zebrafish larvae for 2 d with MPTP followed by 30 min rinsing and transferring larvae to fresh EM to evaluate the number of movement episodes. We observed a 60% decrease in motor behavior of larvae treated with 0.1 $\mu$M MPTP ($N = 6$; one-way ANOVA, Fisher LSD; $p = 0.002$) and a 79% decrease in those treated with 1 $\mu$M MPTP ($N = 6$; one-way ANOVA, Fisher LSD; $p = 0.018$) (Fig. 4*D*). To confirm the specificity of the MPTP-induced motor deficit, we tested in a separate experiment whether cotreatment with MPTP and DA would rescue the effect (Fig. 4*E*). Consistent with previous result, 1 $\mu$M MPTP-treated larvae showed a ~55% decrease in movement episodes ($N = 4$; one-way ANOVA, Fisher LSD; $p < 0.001$). Moreover, there was a 97% decrease in movement episodes in DA-treated larvae ($N = 4$; one-way ANOVA, Fisher LSD; $p < 0.001$) and a 96% decrease in quinpirole-treated larvae ($N = 4$; one-way ANOVA, Fisher LSD; $p < 0.001$). Importantly, we observed a significant rescue in the number of movement episodes in larvae cotreated with MPTP and DA compared with the

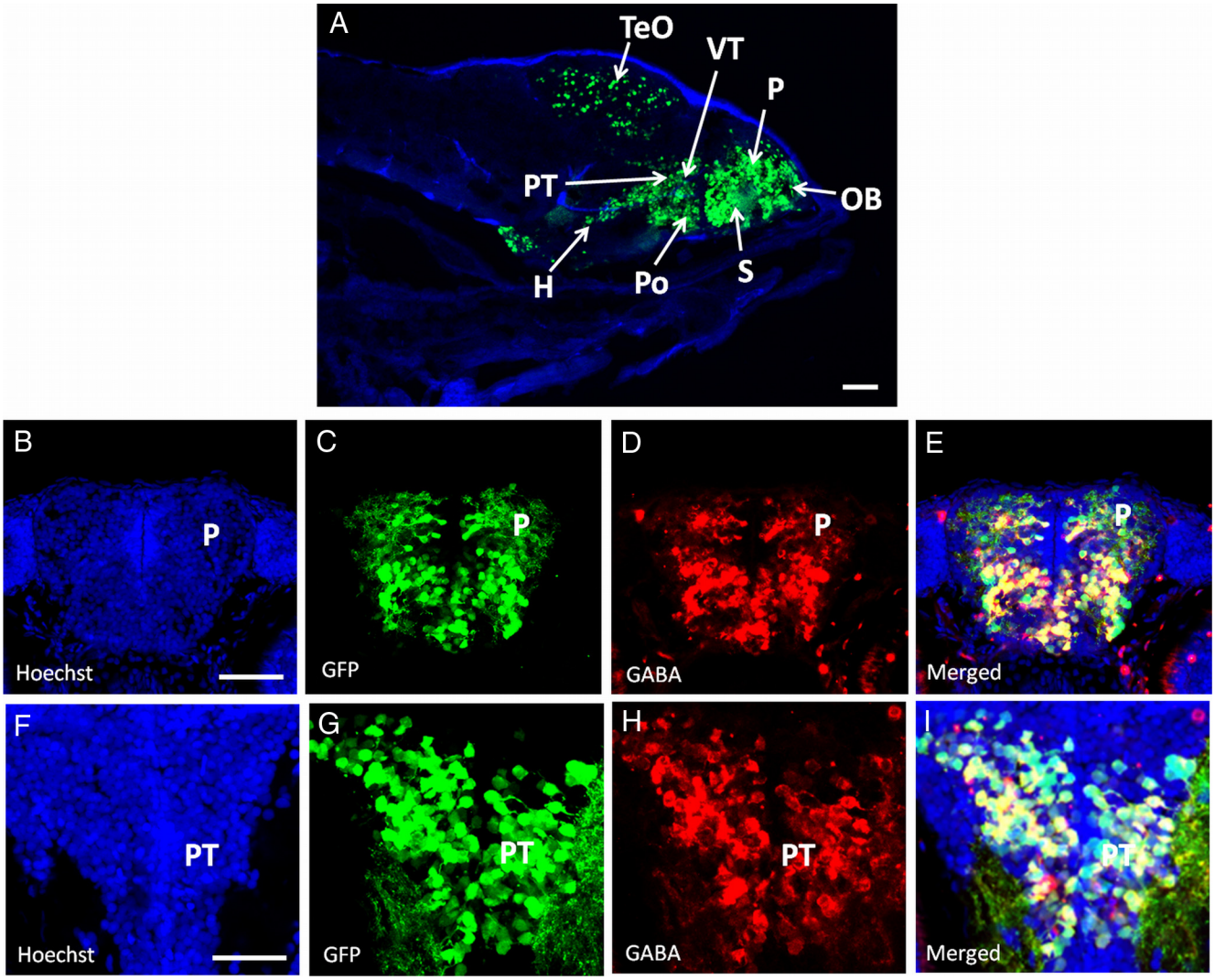



**Figure 5.** Neurons expressing the *Tg(dlx5a/6aIG:GFP)* transgene in the developing brain are GABAergic. Representative confocal images of immunostained sections show that most GFP+ neurons also express GABA. ***A***, Sagittal view of endogenous expression of GFP (green) in 5 dpf *Tg(dlx5a/6aIG:GFP)* transgenic zebrafish head. There are GFP+ neurons in the 5 dpf brain regions: S, Po, VT, PT, H, and TeO. ***B–D***, ***F–H***, Transversal sections of 5 dpf *Tg(dlx5a/6aIG:GFP)* transgenic zebrafish brains were immunostained with GFP (***C***, ***G***; green) and GABA (***D***, ***H***; red) antibodies, and counterstained with Hoechst (***B***, ***F***; blue). ***E***, ***I***, There is colocalization of GFP and GABA in the brain of 5 dpf larvae. P, Pallium; S, subpallium; VT, ventral thalamus; Po, preoptic region; PT, posterior tuberculum; TeO, optic tectum; H, hypothalamus. Scale bar, 50 $\mu$m. $N = 3$ larvae per group.

MPTP-only group ($N = 4$; one-way ANOVA, Fisher LSD; $p < 0.001$), or the DA-only group ($N = 4$; one-way ANOVA, Fisher LSD; $p < 0.001$). Thus, a reduction in endogenous DA, primarily within the pretectal DA neurons, reduces larval motor behavior. Acute DA and quinpirole treatments suppressed the larval motor behavior (Fig. 3*B*,*C*), which were rescued after 30 min of drug washing. However, the motor behavior was still decreased in the larvae chronically treated even after washing for 30 min in EM. Therefore, we wondered whether DA signaling was directly affecting motor behavior or whether DA was affecting the development of neuronal circuits that might be involved in the motor behavior.

### DA modulates GABAergic neuron development in the early larval brain

GABAergic circuitry is involved in motor behavior (Kawaguchi and Hirano, 2002; McCairn et al., 2009). The *Dlx* genes regulate neurogenesis and are essential for the development of GABAergic neurons in the forebrain (Ellies et al., 1997; Stühmer et al., 2002). We used a zebrafish transgenic line *Tg(dlx5a/6aIG:GFP)*, which expresses GFP in GABAergic neurons in the embryonic and postembryonic brain (Zerucha et al., 2000; Mione et al., 2008; MacDonald et al., 2010). We tested whether neurons expressing GFP in the 3–5 dpf *Tg(dlx5a/6aIG:GFP)* transgenic zebrafish brain coexpress GABA by using immunohistochemistry with anti-GFP and anti-GABA antibodies, and confocal microscopy. Consistent with previous findings (Mione et al., 2008), GFP+ neurons were found in the 5 dpf subpallium, preoptic region, thalamus, as well as the region encompassing the posterior tuberculum and hypothalamus (Fig. 5*A*). We also observed GFP+ neurons in the optic tectum (Fig. 5*A*). GFP and GABA colocalized to cell bodies in these regions (Fig. 5*B–I*) as well as processes, which was evident from the expression in neuron tracts such as the anterior commissure, postoptic commissure, or lateral forebrain bundle (Fig. 5*B–I*). The vast majority of GFP+ neurons were GABAergic, but there were GABAergic neurons that did not express the transgene.

To determine the role of DA signaling in the development of GABAergic neurons, we treated 3 dpf *Tg(dlx5a/6aIG:GFP)* embryos for 2 d with 50 $\mu$M DA, 1 $\mu$M MPTP, MPTP+DA, or 10 $\mu$M quinpirole. At 5 dpf, larval brain sections we analyzed by immu-

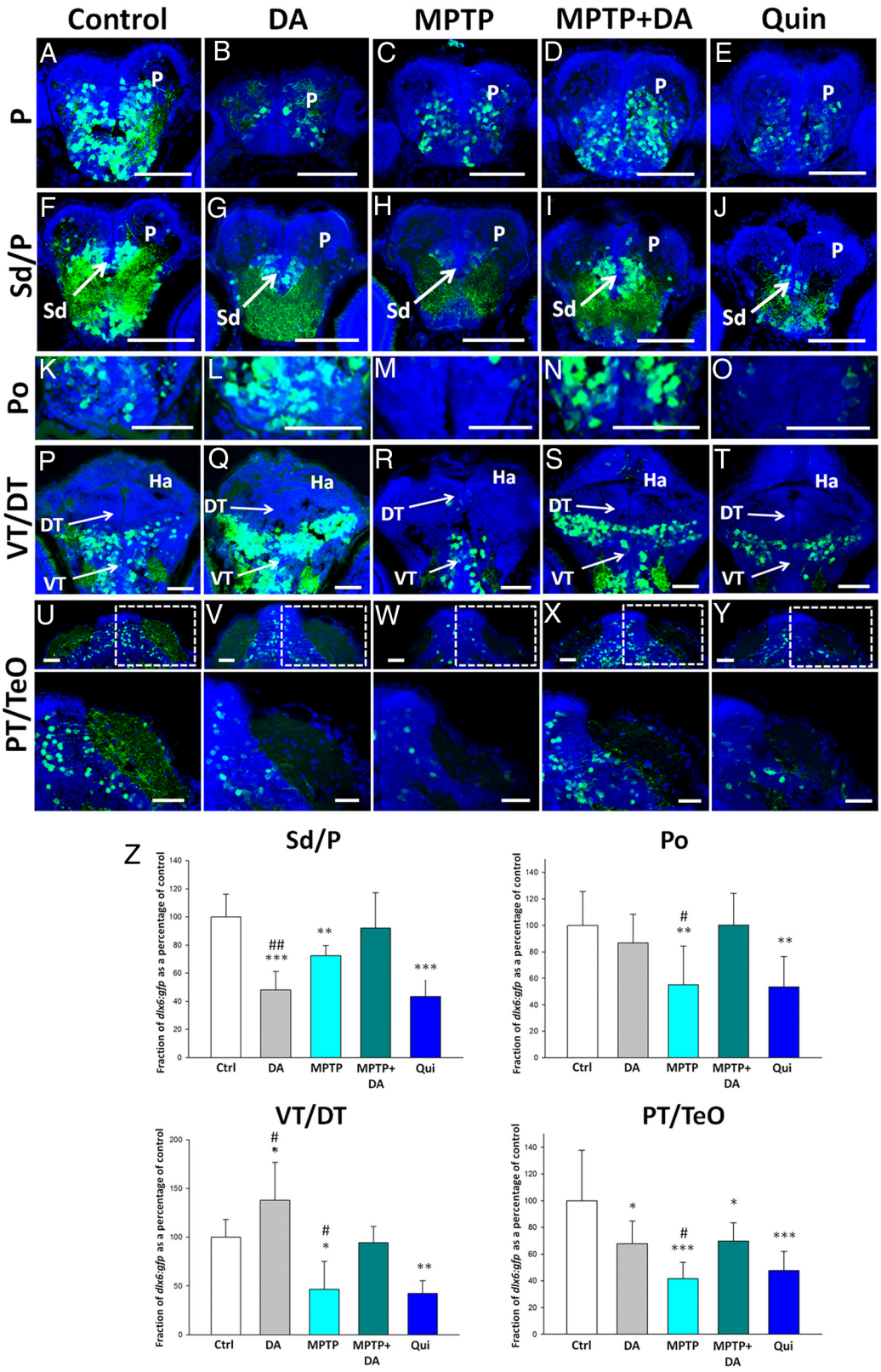



**Figure 6.** Imbalance in the DA signaling disturbs development of GABAergic system. Confocal images were studied to evaluate the effects of DA imbalance on the number of GFP+ neurons generated during 3–5 dpf. Transverse sections of brains of 5 dpf *Tg(dlx5a/6aIG:GFP)* transgenic zebrafish treated with drugs for 2 d. Samples were immunostained with GFP (green) antibodies and counterstained with Hoechst (blue). ***A–J***, ***Z***, There is a decrease in the number of GFP+ neurons in both P and Sd of larvae treated with DA, MPTP, and Qui. This decrease is rescued to control levels in the P and Sd of larvae treated with MPTP+DA. ***K–O***, ***Z***, DA treatment does not change the number of GFP+ neurons in the Po, but there is a decrease in the Po of larvae treated with MPTP and Qui. Also, MPTP+DA rescue the number of GFP+ neurons to the control levels. ***P–T***, ***V***, There is an increase of GFP+ neurons in the VT/DT of larvae treated with DA, but a decrease in the VT/DT of larvae treated with MPTP and Qui. There is also a rescue to the control levels of the number of GFP+ neurons in the brain of MPTP+DA-treated larvae. ***U–Z***, All the treatments (DA, MPTP, MPTP+DA, and Qui) diminished the number of GFP+ neurons in the PT/TeO. P, Pallium; Sd, dorsal subpallium; VT, ventral thalamus; DT, dorsal thalamus; Po, preoptic region; PT, posterior tuberculum; TeO, optic tectum; Hr, rostral hypothalamus; Ctrl, control. Scale bar, 50 $\mu$m. $N = 3–9$ larvae per group; data are average ± SD. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ compared with control. $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$, compared with DA+MPTP.

nohistochemistry with anti-GFP antibodies, and the numbers of GFP+ neurons were quantified using confocal imaging. We focused our attention on five distinct brain regions, from the telencephalon rostrally to the optic tectum caudally, based on the neuroanatomical subdivisions described by Mueller and Wullimann (2005).

### Subpallium

We first examined the population of GFP+ neurons within the region of the subpallium, focusing on coronal sections that included the anterior commissure as an anatomical landmark. There was a decrease in the number of GFP+ neurons in larvae treated with DA ($N = 3–4$; one-way ANOVA, Fisher LSD; $p < 0.001$) or in larvae treated with MPTP ($N = 3–4$; one-way ANOVA, Fisher LSD; $p = 0.04$) (Fig. 6*A–C*,*F–H*). In contrast, we did not observe any changes in the number of GFP+ cells in the MPTP+DA-treated group compared with the untreated control group ($N = 4$; one-way ANOVA, Fisher LSD; $p = 0.516$) (Fig. 6*A*,*D*,*F*,*I*). These data suggest that an imbalance in DA signaling during the 3–5 dpf developmental window reduces the number of GABAergic neurons. $D_2$-receptor-dependent signaling was sufficient to elicit this effect, since the quinpirole-treated group resulted in a similar decrease in GFP+ neurons in the subpallium ($N = 4$; one-way ANOVA, Fisher LSD; $p < 0.001$) (Fig. 6*A*,*E*,*F*,*J*).

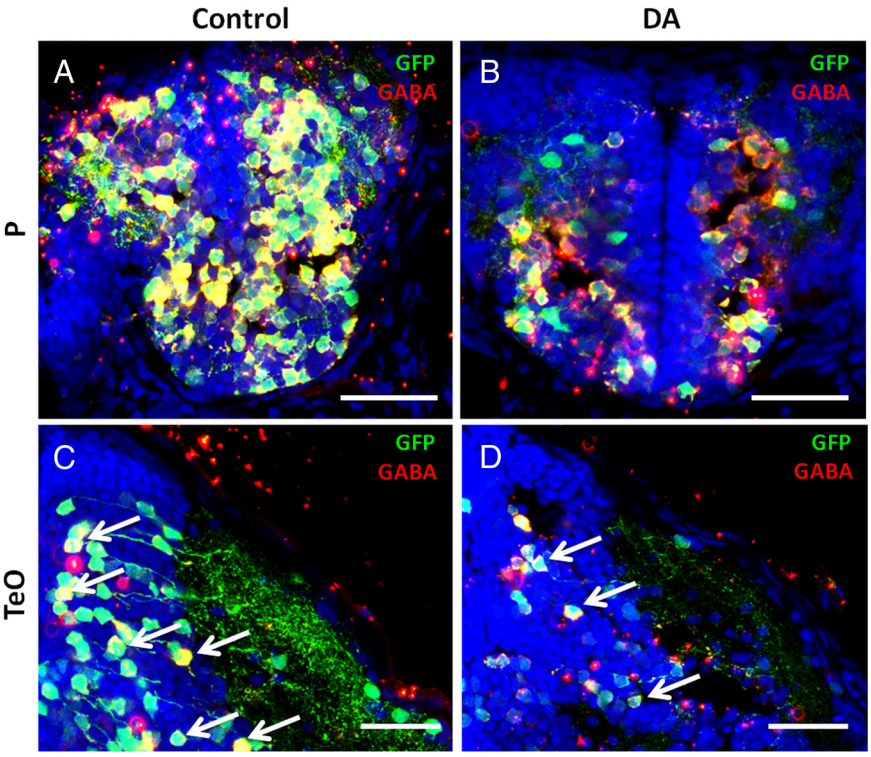



**Figure 7.** Loss of GFP+ cells corresponds to a loss of GABA-expressing neurons. ***A***, ***C***, ***D***, Confocal images of sections double immunolabeled with antibodies against GFP and GABA reveal that the vast majority of GFP+ cells coexpress GABA (***C***, ***D***, arrows) under control conditions in the region of the pallium (***A***; P) and optic tectum (***C***; TeO). ***B***, ***D***, After a 2 d treatment with DA, the number of GFP+/GABA+ cells decreases in both regions of the brain. Images counterstained with Hoechst (blue). Scale bar, 50 $\mu$m.

### Preoptic area

Treatment with exogenous DA did not alter GABAergic neuron development in the preoptic region, ventral to the lateral forebrain bundle ($N = 4–6$; one-way ANOVA, Fisher LSD; $p = 0.424$) (Fig. 6*K*,*L*). Nonetheless, we did observe a significant decrease in the number of preoptic GFP+ cells in the quinpirole-treated larvae ($N = 4–5$; one-way ANOVA, Fisher LSD; $p = 0.013$) and MPTP-treated larvae ($N = 4–8$; one-way ANOVA, Fisher LSD; $p = 0.009$), consistent with our observation in the subpallium (Fig. 6*K*,*M*,*O*). As before, larvae-treated with MPTP and DA showed no change in the number of GFP+ cells in the preoptic region of the forebrain compared with untreated controls ($N = 3–4$; one-way ANOVA, Fisher LSD; $p = 0.996$) (Fig. 6*K*,*N*).

### Thalamus

In the more caudal sections incorporating the dorsal and ventral thalamus at the level of the habenula (dorsally) and the postoptic commissure (ventrally), we observed an increase in the number of GFP+ neurons in DA-treated larvae compared with untreated controls ($N = 6–9$; one-way ANOVA, Fisher LSD; $p = 0.032$) (Fig. 6*P*,*Q*). In contrast, the number of GFP+ neurons in the MPTP-treated larvae ($N = 4–6$; one-way ANOVA, Fisher LSD; $p = 0.012$) (Fig. 6*P*,*R*) and quinpirole-treated larvae ($N = 6$; one-way ANOVA, Fisher LSD; $p = 0.004$) (Fig. 6*P*,*S*) was significantly reduced, comparable to effects observed in the subpallium and preoptic region. As before, MPTP+DA does not alter the number of GFP+ cells compared with untreated controls ($N = 5–6$; one-way ANOVA, Fisher LSD; $p = 0.771$) tending to apparently rebalance DA levels (Fig. 6*P*,*T*).

### Hypothalamus, posterior tuberculum, and caudal dorsal thalamus

The number of GFP-expressing neurons in the region encompassing the rostral hypothalamus, posterior tuberculum, and dorsal thalamus was investigated. Since relatively few GFP+ neurons were distributed across this general region, we included all of the GFP+ cells for quantification, without separating in specific subregions. In contrast to the effects in more rostral regions of the diencephalon, we did not find any significant alterations in the number of GFP+ cells in the larvae treated with DA, MPTP, or quinpirole (data not shown). Thus, although altering DA signaling primarily results in a widespread effect on GABAergic neuron development, there are region-specific effects as well.

### Optic tectum

Finally, we examined the number of GFP+ neurons in sections of the rostral optic tectum just caudal to the pretectum and likely including part of the tegmentum ventrally. We observed a significant decrease in the number of GFP+ cells in DA-treated ($N = 4–6$; one-way ANOVA, Fisher LSD; $p = 0.015$) (Fig. 6*U*,*V*), MPTP-treated ($N = 4–5$; one-way ANOVA, Fisher LSD; $p < 0.001$) (Fig. 6*U*,*W*), and quinpirole-treated ($N = 4–8$; one-way ANOVA, Fisher LSD; $p < 0.001$) (Fig. 6*U*,*Y*) larvae. Although the cotreatment of MPTP and DA resulted in a decrease in the number of GFP+ cells compared with untreated controls ($N = 4–6$; one-way ANOVA, Fisher LSD; $p = 0.022$) (Fig. 6*U*,*X*), we did observe a significant increase in GFP+ cells in this group com-

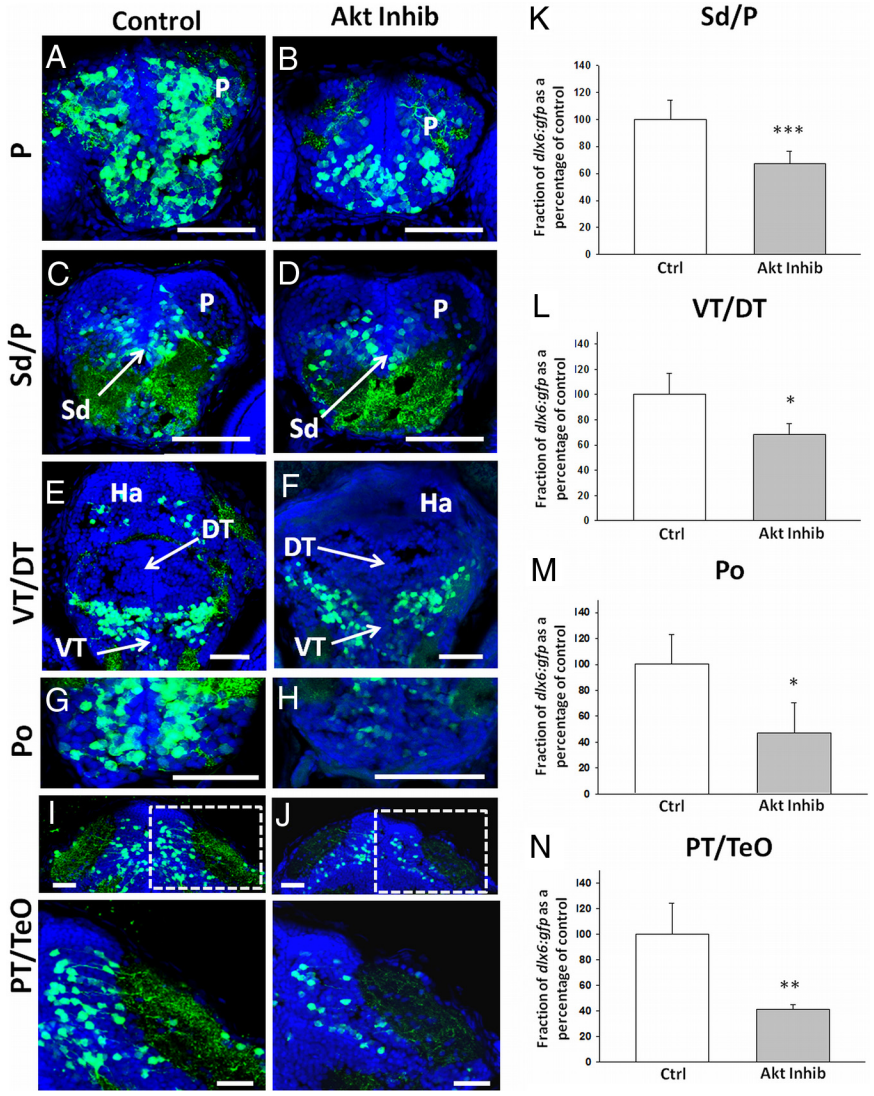



**Figure 8.** Akt inhibition causes a decrease in GFP+ GABAergic neurons. Confocal images were studied to evaluate the effects of Akt inhibition on the number of GFP+ neurons generated during 3–5 dpf. Transverse sections of brains of 5 dpf *Tg(dlx5a/6aIG:GFP)* transgenic zebrafish treated with drugs for 2 d. Samples were immunostained with GFP (green) antibodies and counterstained with Hoechst (blue). ***A–D***, ***K***, There is a decrease in the number of GFP+ neurons in both P and Sd of larvae treated with Akti-1/2 (Akt Inhib). ***E***, ***F***, ***L***, There is a decrease of GFP+ neurons in the VT/DT of larvae treated with Akt Inhib. ***G***, ***H***, ***M***, There is a decrease in the number of GFP+ neurons in the Po of larvae treated with Akt Inhib. ***I***, ***J***, ***N***, There is a decrease in the number of GFP+ neurons in the optic tectum of larvae treated with Akt Inhib. P, Pallium; Sd, dorsal subpallium; VT, ventral thalamus; DT, dorsal thalamus; Po, preoptic region; PT, posterior tuberculum; TeO, optic tectum; Hr, rostral hypothalamus; Ctrl, control. Scale bar, 50 $\mu$m. $N = 3–7$ larvae per group; data are average ± SD. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ compared with control.

prevent the loss of GFP+ GABAergic neurons due to MPTP treatment, suggesting that MPTP does not have an independent neurotoxic effect on GABAergic neurons in our assay. To further support this notion, we treated 4 dpf larvae for 1 d with 1 $\mu$M MPTP, which we showed was sufficient to result in similar neurotoxic effects in the pretectal dopaminergic neuronal cluster (supplemental Fig. S3*C*,*D*, available at www.jneurosci.org as supplemental material), but had no effect on GFP+ GABAergic neurons in the pallium/subpallium region of the brain (supplemental Fig. S3*A*,*B*, available at www.jneurosci.org as supplemental material). Together with the evidence mentioned above, these data indicate that the loss of DA neurons (or reduced DA signaling) precedes the loss of GABAergic neurons, which would normally occur when exposed to MPTP from 3–5 dpf, and that MPTP does not directly kill GABAergic neurons.

DA is known to modulate neurogenesis in the embryonic brain (Ohtani et al., 2003; Popolo et al., 2004; McCarthy et al., 2007). Given that our treatments occurred over the course of 2 d of development, it is possible that exogenous DA, quinpirole, or MPTP affects the survival of neurons that were postmitotic at the onset of treatment or the differentiation of progenitor cells during the 2 d interval. We investigated whether there were any major changes in the number of differentiated cells that might account for the changes in the number of GFP+ neurons in the 5 dpf brains from larvae treated with DA for 2 d. Immunohistochemistry was performed using specific antibodies for glutamine synthetase, as glial marker, or HuC/D, a differentiated neuronal marker. Compared with untreated controls, the pattern of expression of these two markers was virtually indistinguishable. In controls, glutamine synthetase-expressing glial cell bodies were present in the periventricular zones with long radial processes that were perpendicular to the ventricle (supplemental Fig. S4*Ai–iii*, available at www.jneurosci.org as supplemental material). This pattern of expression was the same in DA-treated larvae (supplemental Fig. S4*Aiv-vi*, available at www.jneurosci.org as supplemental material). Similarly, many HuC/D+ neurons were present in the brain parenchyma (excluding the periventricular zones) in both the control (supplemental Fig. S4*Bi-iii*, available at www.jneurosci.org as supplemental material) and DA-treated (supplemental Fig. S4*iv-vi*, available at www.jneurosci.org as supplemental material) groups. Similar observations were made in other brain regions where changes in the number of GFP+ neurons occurred as a result of DA modulation (data not shown). Finally, DA treatment itself has no effect on the number of TH+ neurons in the brain (supplemental Fig. 5, available at www.

pared with MPTP-only treated group ($N = 5–6$; one-way ANOVA, Fisher LSD; $p = 0.022$) (Fig. 6*W*,*X*), consistent with the above results showing that the effects of DA and MPTP on GABAergic development are counteracting.

To confirm that the loss of GFP+ cells corresponded to a loss of GABA-expressing neurons, rather than a downregulation of transgene expression, we used double-immunolabeling to detect GFP and GABA in 3 dpf larvae treated with 50 $\mu$M DA for 2 d. Our results showed that there is a loss of GFP+/GABA+ neurons in the pallium/subpallium (Fig. 7*A*,*B*) and optic tectum (Fig. 7*C*,*D*), confirming the loss of GABAergic neurons.

We used relatively low MPTP concentrations that are expected to be selectively neurotoxic to DA neurons with few, if any, off-target effects. Consistent with this expectation, our experiments in Figure 6 show that exogenous DA alone is sufficient to

jneurosci.org as supplemental material). Therefore, overall viability of differentiated cells appears unaffected by DA modulation, suggesting that there may be a cell type-specific effect on GABAergic cell fate during neurogenesis. However, further detailed cell lineage experiments in the context of modulated DA signaling are required to confirm this hypothesis.

### Direct inhibition of Akt activity affects GABAergic neuron development

Our results implicate Akt activity as a strongly correlated component of the $D_2$ signaling pathway regulating GABAergic neuron development. To test whether Akt activity is required for GABAergic neuron development, we treated 3 dpf larvae for 2 d with 100 nM of an Akt inhibitor (Akti-1/2). This experiment showed that Akt inhibition caused a significant decrease in the number of GFP+ GABAergic neurons in the pallium/subpallium ($N = 4$–7; Student's $t$ test; $p = 0.003$) (Fig. 8*A–D*,*K*), the region of the thalamus ($N = 3$; Student's $t$ test; $p = 0.045$) (Fig. 8*E*,*F*,*L*), the preoptic area ($N = 3$–5; Student's $t$ test; $p = 0.020$) (Fig. 8*G*,*H*,*M*), and the optic tectum ($N = 3$–6; Student's $t$ test; $p = 0.005$) (Fig. 8*I*,*J*,*N*). Therefore, Akt inhibition is sufficient to phenocopy the effect on GABAergic neurons that we observed with exogenous quinpirole. Together with our previous results, these data suggest that Akt signaling is required downstream of $D_2$ receptor activation to promote GABAergic neuron development.

### Chronic DA treatment alters GSK3$\beta$ activity in the larval brain

Our data indicates that modulation of DA signaling during the 3–5 dpf developmental window affects GABAergic neuron development in specific brain regions and that these neuroanatomical changes are correlated with alterations in motor behavior. Since we observed that DA $D_2$ signaling acutely modulates Akt activity in the brain at both 3 dpf and 5 dpf (Fig. 1), we hypothesized that the Akt pathway might be disrupted after chronic DA treatment (between 3 and 5 dpf). However, after washing out DA for 30 min with EM, we did not find any difference in brain pAkt(Thr308) levels in larvae treated with 50 $\mu$M DA after two consecutive days compared with untreated controls ($N = 4$; Student's $t$ test; $p = 0.873$) (Fig. 9*A*,*B*). Although alterations in pAkt levels may provide an immediate readout of $D_2$ receptor signaling, it is possible that the longer term effects of sustained activation of this signaling pathway is manifested by changes in components that are downstream of Akt. Thus, we examined the level of GSK3$\beta$, which is a downstream target of Akt and has an important role in neurodevelopment (Harwood, 2001). First, we tested whether the level of pGSK3$\beta$(Ser9) in the 5 dpf larval brain was altered due to an acute treatment with 100 $\mu$M DA for 30 min. Western blot analysis showed that there is no change in pGSK3$\beta$(Ser9) levels between the groups ($N = 7$; Student's $t$ test; $p = 0.166$) (Fig. 9*C*,*D*). In contrast, even after washing out DA for 30 min with EM, a 2 d treatment of 50 $\mu$M DA (between 3 and 5 dpf) resulted in a 49% decreased in brain pGSK3$\beta$(Ser9) levels compared with untreated controls ($N = 3$–4; Student's $t$ test; $p = 0.042$) (Fig. 9*E*,*F*). These data indicate that sustained, but not acute, DA treatment affects the activity of GSK3$\beta$ in the larval brain.

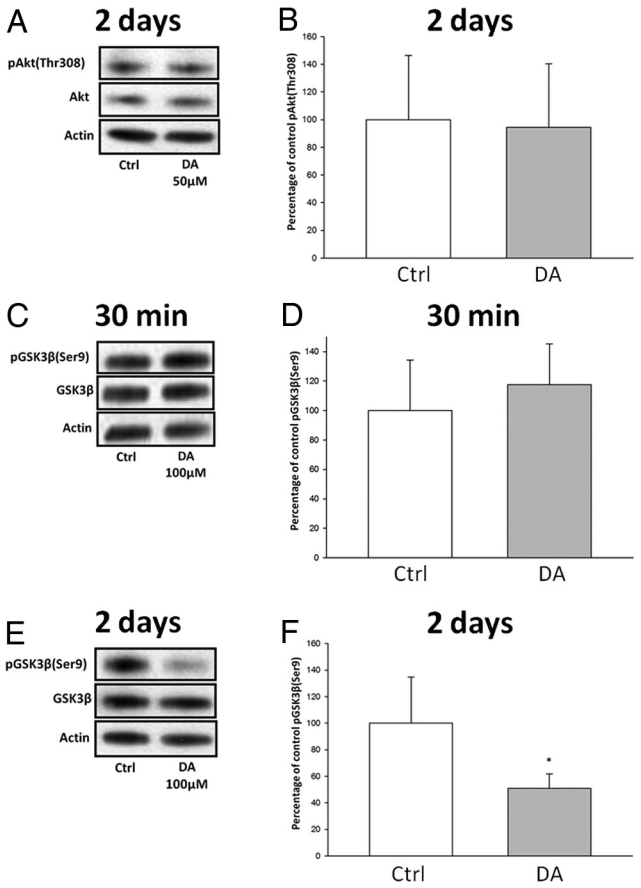
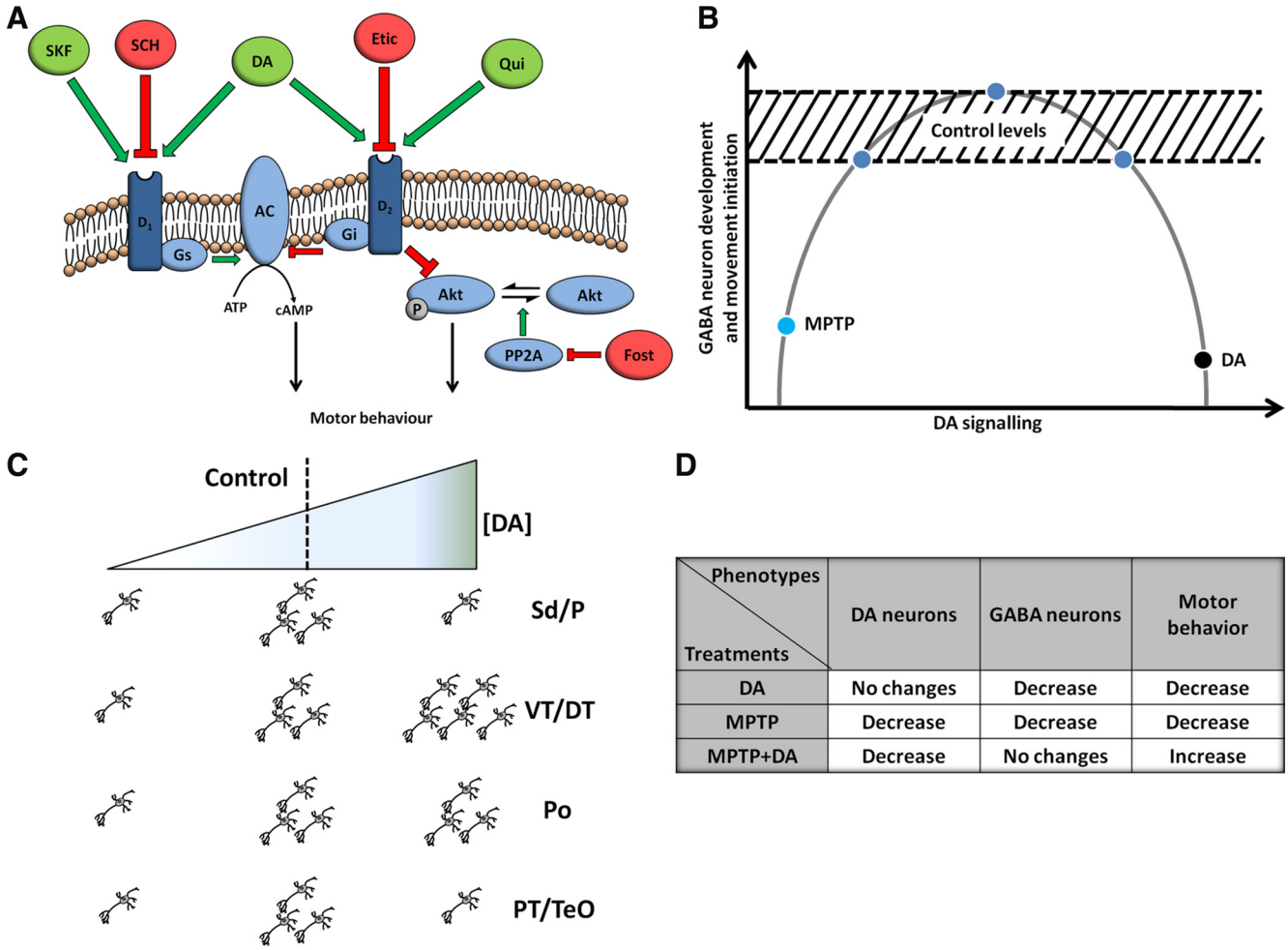



**Figure 9.** DA treatment for 2 d downregulates GSK3$\beta$ activity. ***A–F***, Representative Western blot (***A***, ***C***, ***E***,) and densitometric (***B***, ***D***, ***F***) analyses of extracts prepared from brains of 5 dpf larvae DA-treated for 2 d. ***A***, ***B***, There is no change in the levels of pAkt(Thr308) in the 5 dpf larvae treated with DA over 2 d. ***C***, ***D***, DA does not modulate pGSK3$\beta$(Ser9) after 30 min of treatment. ***E***, ***F***, The level of GSK3$\beta$(Ser9) is reduced in the brain of 5 dpf larvae treated with DA for 2 d. Ctrl, Control. $N = 3$–4 larva per group; data are average ± SD. *$p \leq 0.05$.

## Discussion

Several components of the DA biosynthetic and signaling pathways are expressed in the zebrafish larval brain (Holzschuh et al., 2001; Boehmler et al., 2004, 2007; McLean and Fetcho, 2004; Anichtchik et al., 2006; Li et al., 2007), but which intracellular pathways are modulated by DA and its role in mediating behavior, as well as how DA signaling regulates zebrafish brain development, is not well characterized. Previous studies have demonstrated that DA is involved in larval motor regulation and that this is mediated, in part, by cAMP signaling (Boehmler et al., 2007; Thirumalai and Cline, 2008). Here, we extend these observations by demonstrating that DA also modulates Akt signaling in the zebrafish forebrain and midbrain, primarily through $D_2$ receptors. Furthermore, we show that Akt modulation by $D_2$ receptor signaling is involved in controlling larval movement. Recent studies demonstrated that DA modulates Akt signaling in the adult rodent and primate striatum, which is associated with regulating motor control (Beaulieu et al., 2004, 2005). Although our study focused on early larval stages, the larval pattern of DA circuitry in zebrafish is comparable to the adult pattern (McLean and Fetcho, 2004), suggesting that this pattern of DA-dependent Akt modulation is maintained in adulthood. However, future investigations are necessary to confirm this hypothesis. DA-dependent regulation of Akt signaling may be an evolutionarily conserved mechanism for controlling motor behavior in young and older animals.

GSK3$\beta$, a downstream component of Akt signaling, is important for neurodevelopment (Harwood, 2001). Beaulieu et al. (2004) demonstrated that DA regulates Akt signaling and, consequently, GSK3$\beta$ activity, and this appears to be comparable to the regulation of this pathway that we observed. For example, acute DA treatment led to a decrease in pAkt levels, but had no effect on the




| Phenotypes / Treatments | DA neurons | GABA neurons | Motor behavior |
|---|---|---|---|
| DA | No changes | Decrease | Decrease |
| MPTP | Decrease | Decrease | Decrease |
| MPTP+DA | Decrease | No changes | Increase |

**Figure 10.** Model of DA function in the developing zebrafish larval brain. ***A***, DA decreases both motor behavior and pAkt(Thr308) in the brain of 5 dpf larvae. $D_2$ antagonist eticlopride (Etic) blocks the modulation of pAkt(Thr308) and motor behavior. $D_2$ agonist quinpirole (Qui) decreases pAkt(Thr308) and motor behavior. This modulation is partially rescued by the inhibitor of phosphatase 2A (PP2A) fostriecin (Fost). $D_1$ antagonist SCH-23390 (SCH) slightly decreases the level of pAkt(Thr308) and motor behavior. On the other hand, the $D_1$ agonist SKF-38393 (SKF) increases motor behavior. Thus, the DA-dependent modulation of pAkt(Thr308) is through $D_2$ receptors. ***B***, The theoretical inverted-U model for DA effects in 5 dpf zebrafish GABAergic neuron development and larval motor behavior. ***C***, DA regulates the development of GABAergic neurons in the brain of zebrafish larvae. Decreases and increases in DA levels modulate the number of GABA cells in the Sd/P and PT/TeO. The number of GABAergic neurons in VT/DT is correlated with the levels of DA. In the case of Po, DA levels modulate the number of GABA cells, with a plateau in the control levels. DA levels do not affect the number of GABAergic neurons in Hr/PT/DT. ***D***, Table summarizing the overall effects of DA imbalance on development and behavior in 5 dpf zebrafish larvae. P, Pallium; Sd, dorsal subpallium; VT, ventral thalamus; DT, dorsal thalamus; Po, preoptic region; PT, posterior tuberculum; TeO, optic tectum; Hr, rostral hypothalamus.

level of GSK3$\beta$ activation. In contrast, even after washing out the excess of DA for 30 min, chronic DA treatment decreased the levels of pGSK3$\beta$ in the brain of 5 dpf zebrafish, while the same chronic DA treatment had no effect on the levels of pAkt. Thus, at least one downstream consequence of DA-dependent Akt modulation is a change in the activity of GSK3$\beta$, which may play a role in mediating GABAergic neuron development and/or function. However, the complexity of the signaling and the duration over which these changes take place indicates that multiple additional pathways are likely required to mediate this effect.

DA circuits have a complex role in the manifestation of motor behavior (Bentivoglio and Morelli, 2005). Here, we demonstrate that, in 5 dpf zebrafish, acute activation of $D_1$ receptors increase movement episodes over the first 10 min of stimulation independently of Akt activity. On the other hand, a $D_2$-Akt-dependent decrease in movement occurs within 10–30 min of treatment. These observations are consistent with known DA receptor response kinetics in mammals. Namely, that $D_1$ receptor activation is faster than $D_2$ receptor activation, and that $D_2$ receptors have higher affinity to DA than $D_1$ receptors (Beaulieu et al., 2007). Thus, acute $D_1$ and $D_2$ receptor activation appear to have opposing roles in mediating movement in zebrafish larvae, suggesting that a balance in signaling between these two receptors is required for normal motor behavior (Fig. 10*A*).

DA is known to regulate mammalian neural progenitor proliferation (Popolo et al., 2004; McCarthy et al., 2007) and migration of GABAergic neurons in the telencephalon (Crandall et al., 2007). In addition, deficiencies and specific polymorphisms in Akt1 affect the structure of mammalian cortical regions that are modulated by DA pathways (Lai et al., 2006; Tan et al., 2008). In a recent study, Oishi and colleagues (2009) demonstrated that Akt activity [pAkt(Thr308)-dependent] mediates exogenous growth factor signaling to promote the differentiation of mouse ganglionic eminence neural progenitor cells into GABAergic neurons, but not glutamatergic neurons. This study also revealed that the Akt-induced GABAergic neuronal differentiation requires the function of the transcriptional regulator *Mash1* (Oishi et al., 2009). Thus, Akt signaling has an important role in GABAergic neuronal differen-

tiation and our present data extend these findings by showing that one of the signaling pathways that promotes GABAergic neurogenesis during development is the DA signaling pathway.

In our chronic DA treatments we demonstrated that excessive DA signaling caused a decrease in the number of GABAergic neurons in specific brain regions, such as the pallium, subpallium, and optic tectum. The reduced number of GABAergic neurons was extended to other brain regions, such as the preoptic area and thalamus, when the specific $D_2$ agonist quinpirole was used. Interestingly, MPTP treatment, which can kill DA neurons in zebrafish larvae (Panula et al., 2010), resulted in a widespread defect in GABAergic neuron development that was comparable to that observed with quinpirole treatment. Thus, in general, there is an inverted-U effect of chronic DA signaling on GABAergic neuron development (Fig. 10*B*). We can also conclude that much of the DA signaling that follows this pattern of activation/suppression is mediated through $D_2$ receptors. However, the effects of DA and quinpirole were not identical. For example, chronic DA treatment resulted in a significant increase in GABAergic neurons in the region of the thalamus, whereas chronic quinpirole or MPTP treatment resulted in a decrease in GABAergic neurons in the same region (Fig. 10*C*). Furthermore, DA had no effect on the development of GABAergic neurons in the preoptic area, whereas quinpirole or MPTP caused a decrease in the number of GABAergic neurons in this region. One hypothesis to account for these complex observations is that DA signaling through $D_1$ and $D_2$ receptors has subtle differential consequences on GABAergic neuron development that is also region specific. For instance, antagonistic $D_1$ and $D_2$ receptor signaling might account for the difference in thalamic GABAergic neuron development observed between DA treatment (increase in GABAergic neurons) and quinpirole treatment (decrease in GABAergic neurons). However, more detailed experiments are required to address this hypothesis.

It is well known that both DA and GABAergic neurons are involved in motor behavior and motivation (Bentivoglio and Morelli, 2005; Breakefield et al., 2008). However, the interaction between the different circuits involved in these behaviors is complex. Here, we treated 3 dpf zebrafish for 2 d with a concentration of MPTP (1 $\mu$M) that is lower than what is reported in most previous studies and observed a decrease in TH expression, primarily confined to pretectal DA neurons, which are known to project to the telencephalon, thalamus, and optic tectum (Ma, 2003). Our main findings are summarized in Figure 10*D*. MPTP alone or DA alone significantly decreased movement episodes at 5 dpf, which correlated with the loss of endogenous GABAergic neurons in the brain. The numbers of endogenous TH+DA neurons are not changed in larvae treated with DA. This suggests a model whereby a decrease in the number of GABAergic neurons reduces the overall GABA-dependent modulation that promotes movement downstream of dopamine signaling. We were also able to test the effects of reduced numbers of endogenous DA neurons while maintaining the overall numbers of GABAergic neurons similar to controls by performing a combined MPTP+DA chronic treatment. In this case, there was an increase in movement initiation. We posit that the regulation of movement initiation in the 5 dpf zebrafish larvae is a complex interaction of DA and GABA circuits. A reduced level of endogenous DA increases movement initiation when GABAergic neuronal circuitry is relatively normal, suggesting that DA negatively regulates this aspect of motor behavior. In contrast, reduced numbers of GABAergic neurons, regardless of the state of endogenous DA signaling, causes a decrease in movement initiation. Thus, while DA likely regulates motor behavior through a combination of direct and indirect pathways, our findings suggest that GABAergic modulation is downstream of DA signaling and has an overall positive role in regulating movement initiation, or perhaps a form of motivation. The neuroanatomical loci mediating these effects are not entirely clear. Our evidence suggests that GABAergic neurons throughout the telencephalon, diencephalon, and optic tectum are regulated by DA neurons, especially those within the pretectal cluster. The precise interconnections that ultimately modulate glutamatergic neurons for motor output remain to be determined, but we suggest that DA-dependent GABAergic modulation of these effector (presumably glutamatergic) neurons is a central feature of larval motor behavior.

Several studies have shown that disturbances in both DA regulation and GABAergic neural circuitry occur in the brain of patients with neuropsychiatric disorders, such as schizophrenia (Hurd and Hall, 2005; Souza et al., 2006; Uhlhaas and Singer, 2010). It is suggested that alterations in the DA system in the brain of schizophrenics occurs during development and may not be due to secondary effects of drug treatment (Albert et al., 2002; Koh et al., 2003; Souza et al., 2008, 2010). The main target for antipsychotics, the DA receptor $D_2$, modulates the Akt pathway in mammals (Lai et al., 2006; Beaulieu et al., 2007; Tan et al., 2008), and our data show that this effect is conserved in zebrafish. Furthermore, genetic associations of Akt1 with schizophrenia and alterations in the levels of Akt in the brains of schizophrenics (Emamian et al., 2004) also emphasize the importance of this signaling pathway in the development of psychiatric disorders. To our knowledge, our study is the first to show modulation of Akt signaling by DA in the developing brain. Also, our findings support the view that DA has an important role in GABAergic neuron development and function. Since the GABAergic system in the zebrafish brain is highly conserved with that of the mammalian brain (Mueller et al., 2006), we suggest that imbalances of DA might lead to disturbances in the development of the GABAergic neurons that are reported in the brains of schizophrenic patients. Thus, our study emphasizes the utility of zebrafish larvae as a tool for uncovering the mechanisms of neurodevelopmental disorders that affect brain structure and function in a defined developmental time window.

## References


Albert KA, Hemmings HC Jr, Adamo AI, Potkin SG, Akbarian S, Sandman CA, Cotman CW, Bunney WE Jr, Greengard P (2002) Evidence for decreased DARPP-32 in the prefrontal cortex of patients with schizophrenia. Arch Gen Psychiatry 59:705–712.

Anichtchik OV, Kaslin J, Peitsaro N, Scheinin M, Panula P (2004) Neurochemical and behavioural changes in zebrafish *Danio rerio* after systemic administration of 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. J Neurochem 88:443–453.

Anichtchik O, Sallinen V, Peitsaro N, Panula P (2006) Distinct structure and activity of monoamine oxidase in the brain of zebrafish (*Danio rerio*). J Comp Neurol 498:593–610.

Araki KY, Sims JR, Bhide PG (2007) Dopamine receptor mRNA and protein expression in the mouse corpus striatum and cerebral cortex during pre- and postnatal development. Brain Res 1156:31–45.

Beaulieu JM, Sotnikova TD, Yao WD, Kockeritz L, Woodgett JR, Gainetdinov RR, Caron MG (2004) Lithium antagonizes dopamine-dependent behaviors mediated by an AKT/glycogen synthase kinase 3 signaling cascade. Proc Natl Acad Sci U S A 101:5099–5104.

Beaulieu JM, Sotnikova TD, Marion S, Lefkowitz RJ, Gainetdinov RR, Caron MG (2005) An Akt/beta-arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior. Cell 122:261–273.

Beaulieu JM, Gainetdinov RR, Caron MG (2007) The Akt-GSK-3 signaling cascade in the actions of dopamine. Trends Pharmacol Sci 28:166–172.

Benes FM, Berretta S (2001) GABAergic interneurons: implications for understanding schizophrenia and bipolar disorder. Neuropsychopharmacology 25:1–27.

Bentivoglio M, Morelli M (2005) The organization and circuits of mesencephalic dopaminergic neurons and the distribution of dopamine receptors in the brain. In: Dopamine, Chap I. New York: Elsevier.

Boehmler W, Obrecht-Pflumio S, Canfield V, Thisse C, Thisse B, Levenson R

(2004) Evolution and expression of D2 and D3 dopamine receptor genes in zebrafish. Dev Dyn 230:481–493.

Boehmler W, Carr T, Thisse C, Thisse B, Canfield VA, Levenson R (2007) D4 Dopamine receptor genes of zebrafish and effects of the antipsychotic clozapine on larval swimming behaviour. Genes Brain Behav 6:155–166.

Breakefield XO, Blood AJ, Li Y, Hallett M, Hanson PI, Standaert DG (2008) The pathophysiological basis of dystonias. Nat Rev Neurosci 9:222–234.

Bretaud S, Li Q, Lockwood BL, Kobayashi K, Lin E, Guo S (2007) A choice behavior for morphine reveals experience-dependent drug preference and underlying neural substrates in developing larval zebrafish. Neuroscience 146:1109–1116.

Crandall JE, McCarthy DM, Araki KY, Sims JR, Ren JQ, Bhide PG (2007) Dopamine receptor activation modulates GABA neuron migration from the basal forebrain to the cerebral cortex. J Neurosci 27:3813–3822.

Di Chiara G (2005) Dopamine, motivation and reward. In: Dopamine, Chap VI, pp. 303–394. New York: Elsevier.

Dunnet SB (2005) Motor function(s) of the nigrostriatal dopamine system: studies of lesions and behavior. In: Dopamine, Chap V, pp. 237–301. New York: Elsevier.

Ellies DL, Stock DW, Hatch G, Giroux G, Weiss KM, Ekker M (1997) Relationship between the genomic organization and the overlapping embryonic expression patterns of the zebrafish dlx genes. Genomics 45:580–590.

Emamian ES, Hall D, Birnbaum MJ, Karayiorgou M, Gogos JA (2004) Convergent evidence for impaired AKT1-GSK3beta signaling in schizophrenia. Nat Genet 36:131–137.

Harwood AJ (2001) Regulation of GSK-3: a cellular multiprocessor. Cell 105:821–824.

Holzschuh J, Ryu S, Aberger F, Driever W (2001) Dopamine transporter expression distinguishes dopaminergic neurons from other catecholaminergic neurons in the developing zebrafish embryo. Mech Dev 101:237–243.

Hurd YL, Hall H (2005) Human forebrain dopamine systems: characterization of the normal brain and in relation to psychiatric disorders. In: Dopamine, Chap IX, pp. 525–571. New York: Elsevier.

Kawaguchi SY, Hirano T (2002) Signaling cascade regulating long-term potentiation of GABA(A) receptor responsiveness in cerebellar Purkinje neurons. J Neurosci 22:3969–3976.

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF (1995) Stages of embryonic development of the zebrafish. Dev Dyn 203:253–310.

Koh PO, Undie AS, Kabbani N, Levenson R, Goldman-Rakic PS, Lidow MS (2003) Upregul ation of neuronal calcium sensor-1 (NCS-1) in the prefrontal cortex of schizophrenic and bipolar patients. Proc Natl Acad Sci U S A 100:313–317.

Lai WS, Xu B, Westphal KG, Paterlini M, Olivier B, Pavlidis P, Karayiorgou M, Gogos JA (2006) Akt1 deficiency affects neuronal morphology and predisposes to abnormalities in prefrontal cortex functioning. Proc Natl Acad Sci U S A 103:16906–16911.

Lam CS, Korzh V, Strahle U (2005) Zebrafish embryos are susceptible to the dopaminergic neurotoxin MPTP. Eur J Neurosci 21:1758–1762.

Lee SP, So CH, Rashid AJ, Varghese G, Cheng R, Lança AJ, O'Dowd BF, George SR (2004) Dopamine D1 and D2 receptor Co-activation generates a novel phospholipase C-mediated calcium signal. J Biol Chem 279:35671–35678.

Li L, Dowling JE (2000) Effects of dopamine depletion on visual sensitivity of zebrafish. J Neurosci 20:1893–1903.

Li P, Shah S, Huang L, Carr AL, Gao Y, Thisse C, Thisse B, Li L (2007) Cloning and spatial and temporal expression of the zebrafish dopamine D1 receptor. Dev Dyn 236:1339–1346.

Ma PM (2003) Catecholaminergic systems in the zebrafish. IV. Organization and projection pattern of dopaminergic neurons in the diencephalon. J Comp Neurol 460:13–37.

MacDonald RB, Debiais-Thibaud M, Talbot JC, Ekker M (2010) The relationship between dlx and gad1 expression indicates highly conserved genetic pathways in the zebrafish forebrain. Dev Dyn 239:2298–2306.

McCairn KW, Bronfeld M, Belelovsky K, Bar-Gad I (2009) The neurophysiological correlates of motor tics following focal striatal disinhibition. Brain 132:2125–2138.

McCarthy D, Lueras P, Bhide PG (2007) Elevated dopamine levels during gestation produce region-specific decreases in neurogenesis and subtle deficits in neuronal numbers. Brain Res 1182:11–25.

McKinley ET, Baranowski TC, Blavo DO, Cato C, Doan TN, Rubinstein AL (2005) Neuroprotection of MPTP-induced toxicity in zebrafish dopaminergic neurons. Brain Res Mol Brain Res 141:128–137.

McLean DL, Fetcho JR (2004) Ontogeny and innervation patterns of dopaminergic, noradrenergic, and serotonergic neurons in larval zebrafish. J Comp Neurol 480:38–56.

Mione M, Baldessari D, Deflorian G, Nappo G, Santoriello C (2008) How neuronal migration contributes to the morphogenesis of the CNS: insights from the zebrafish. Dev Neurosci 30:65–81.

Missale C, Nash SR, Robinson SW, Jaber M, Caron MG (1998) Dopamine receptors: from structure to function. Physiol Rev 78:189–225.

Mueller T, Vernier P, Wullimann MF (2006) A phylotypic stage in vertebrate brain development: GABA cell patterns in zebrafish compared with mouse. J Comp Neurol 494:620–634.

Mueller T, Wullimann MF (2005) Atlas of early zebrafish brain development: a tool for molecular neurogenetics, Ed 1. San Diego: Elsevier Science.

Neve KA, Seamans JK, Trantham-Davidson H (2004) Dopamine receptor signaling. J Recept Signal Transduct Res 24:165–205.

Ohtani N, Goto T, Waeber C, Bhide PG (2003) Dopamine modulates cell cycle in the lateral ganglionic eminence. J Neurosci 23:2840–2850.

Oishi K, Watatani K, Itoh Y, Okano H, Guillemot F, Nakajima K, Gotoh Y (2009) Selective induction of neocortical GABAergic neurons by the PDK1-Akt pathway through activation of Mash1. Proc Natl Acad Sci U S A 106:13064–13069.

Panula P, Chen YC, Priyadarshini M, Kudo H, Semenova S, Sundvik M, Sallinen V (2010) The comparative neuroanatomy and neurochemistry of zebrafish CNS systems of relevance to human neuropsychiatric diseases. Neurobiol Dis 40:46–57.

Popolo M, McCarthy DM, Bhide PG (2004) Influence of dopamine on precursor cell proliferation and differentiation in the embryonic mouse telencephalon. Dev Neurosci 26:229–244.

Schweitzer J, Driever W (2009) Development of the dopamine systems in zebrafish. Adv Exp Med Biol 651:1–14.

Seeman P (2006) Targeting the dopamine D2 receptor in schizophrenia. Expert Opin Ther Targets 10:515–531.

Shearman LP, Zeitzer J, Weaver DR (1997) Widespread expression of functional D1-dopamine receptors in fetal rat brain. Brain Res Dev Brain Res 102:105–115.

Souza BR, Souza RP, Rosa DV, Guimarães MM, Correa H, Romano-Silva MA (2006) Dopaminergic intracellular signal integrating proteins: relevance to schizophrenia. Dialogues Clin Neurosci 8:95–100.

Souza BR, Motta BS, Rosa DV, Torres KC, Castro AA, Comim CM, Sampaio AM, Lima FF, Jeromin A, Quevedo J, Romano-Silva MA (2008) DARPP-32 and NCS-1 expression is not altered in brains of rats treated with typical or atypical antipsychotics. Neurochem Res 33:533–538.

Souza BR, Torres KC, Miranda DM, Motta BS, Scotti-Muzzi E, Guimarães MM, Carneiro DS, Rosa DV, Souza RP, Reis HJ, Jeromin A, Romano-Silva MA (2010) Lack of effects of typical and atypical antipsychotics in DARPP-32 and NCS-1 levels in PC12 cells overexpressing NCS-1. J Negat Results Biomed 9:4.

Souza BR, Tropepe V (2011) The role of dopaminergic signalling during larval zebrafish brain development: a tool for investigating the developmental basis of neuropsychiatric disorders. Rev Neurosci 22:107–119.

Stühmer T, Puelles L, Ekker M, Rubenstein JL (2002) Expression from a Dlx gene enhancer marks adult mouse cortical GABAergic neurons. Cereb Cortex 12:75–85.

Tan HY, Nicodemus KK, Chen Q, Li Z, Brooke JK, Honea R, Kolachana BS, Straub RE, Meyer-Lindenberg A, Sei Y, Mattay VS, Callicott JH, Weinberger DR (2008) Genetic variation in AKT1 is linked to dopamine-associated prefrontal cortical structure and function in humans. J Clin Invest 118:2200–2208.

Thirumalai V, Cline HT (2008) Endogenous dopamine suppresses initiation of swimming in prefeeding zebrafish larvae. J Neurophysiol 100:1635–1648.

Uhlhaas PJ, Singer W (2010) Abnormal neural oscillations and synchrony in schizophrenia. Nat Rev Neurosci 11:100–113.

Westerfield M (2007) The zebrafish book; a guide for the laboratory use of zebrafish (*Danio rerio*), Ed 5. Eugene, OR: University of Oregon.

Zerucha T, Stühmer T, Hatch G, Park BK, Long Q, Yu G, Gambarotta A, Schultz JR, Rubenstein JL, Ekker M (2000) A highly conserved enhancer in the Dlx5/Dlx6 intergenic region is the site of cross-regulatory interactions between Dlx genes in the embryonic forebrain. J Neurosci 20:709–721.