Chronic Fluoxetine Increases Extra-Hippocampal Neurogenesis in Adult Mice

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Abstract

Background: Chronic treatment with antidepressants has been shown to enhance neurogenesis in the adult mammalian brain. Although this effect was initially reported to be restricted to the hippocampus, recent work has suggested that fluoxetine, a selective serotonin reuptake inhibitor, also promotes neurogenesis in the cortex. However, whether antidepressants target neural progenitor cells in other brain regions has not been examined.
Methods: Here, we used BrdU labeling and immunohistochemistry with a transgenic mouse line in which nestin+ neural progenitor cells can be inducibly labeled with the fluorescent protein, Tomato, following tamoxifen administration. We investigated the effects of chronic fluoxetine on cell proliferation and nestin+ progenitor cells in periventricular areas in the medial hypothalamus and medial habenula, two brain areas involved in stress and anxiety responses.
Results: Our data provide the first in vivo evidence that fluoxetine promotes cell proliferation and neurogenesis and increases the mRNA levels of BDNF in the hypothalamus and habenula.
Conclusions: By identifying novel cellular targets of fluoxetine, our results may provide new insight into the mechanisms underlying antidepressant responses.

Keywords: antidepressant, habenula, hypothalamus, neurogenesis

Introduction

Identifying the precise cellular targets of antidepressants could inform the mechanism of action of these drugs and may facilitate the development of novel therapeutic approaches for the treatment of depression and anxiety disorders. Previous work has shown that chronic treatment with the antidepressant fluoxetine (FLX) increases the proliferation of neural progenitor cells (NPCs) in the subgranular zone (SGZ) of the hippocampus but not in the subventricular zone of the lateral ventricles (Malberg et al., 2000). Research over the past decade has led to the realization that adult neurogenesis also occurs in several brain regions outside of these two canonical niches, most notably in periventricular areas surrounding the third ventricle, including the hypothalamus (Chouaf-Lakhdar et al., 2003; Kokoeva et al., 2005; Lee et al., 2012) and circumventricular organs (Bennett et al.,

2009; Hourai and Miyata, 2013). However, the effects of chronic FLX treatment on these non-canonical NPC populations *in vivo* have not been reported. Interestingly, one recent report has revealed that FLX increases adult neurogenesis in the frontal cortex *in vivo* (Ohira et al., 2013), and a second study has recently shown that FLX regulates the proliferation and differentiation of hypothalamic NPCs *in vitro* (Sousa-Ferreira et al., 2014). Here, we used BrdU labeling and Nestin-Cre-ER-Tomato (NCerT) mice (Benner et al., 2013) to examine the *in vivo* effects of chronic FLX on cell proliferation and nestin+ cells surrounding the dorsal and ventral third ventricle (i.e., the medial habenula and medial hypothalamus, respectively). Importantly, the FLX administration paradigm utilized here has been shown to be effective in achieving antidepressant-like responses in several paradigms,

such as the novelty-suppressed feeding and tail suspension tests (Sachs, Jacobsen, et al., 2013).

The administration of tamoxifen (TMX) to NCerT mice results in the irreversible labeling of nestin-expressing cells with the fluorescent protein Tomato, and allows for fate-mapping experiments (Benner et al., 2013). Using NCerT animals, our results confirm the pro-neurogenic effect of FLX in the SGZ and identify cell populations in the hypothalamus and habenula as novel targets of FLX. Our data provide a potential cellular mechanism whereby FLX could influence the activity of the habenula and the hypothalamus, two brain regions thought to play important roles in depression and anxiety (Raadsheer et al., 1994; Swaab et al., 2000; Savitz, Bonne, et al., 2011; Savitz, Nugent et al., 2011; Carlson et al., 2013).

Materials and Methods

Animals

Nestin-Cre-ER-Tomato (NCerT) mice have been described previously (Benner et al., 2013). Male mice on a mixed background (c57BL6/J and 129S6/SvEvTac) were used for all studies. Animals were eight weeks old at the start of FLX treatment. All experiments were conducted in compliance with the guidelines set forth in the Guide for the Care and Use of Laboratory Animals, and all animal experiments were covered on a protocol that was approved by the Duke University Institutional Animal Care and Use Committee.

Drugs and Drug Treatments

FLX was obtained from Spectrum Chemical Corporation, and TMX and bromodeoxyuridine (BrdU) were obtained from Sigma. Animals were treated with FLX in the drinking water (155 mg/L) as described previously (Sachs, Jacobsen et al., 2013; Siesser et al., 2013). TMX was injected (50 mg/kg, intraperitoneal [IP]) every other day for two weeks, and mice were sacrificed four weeks following the start of TMX and FLX administration. BrdU labeling was performed as described previously (Sachs, Jacobsen, et al., 2013; Sachs, Rodriguiz, et al., 2013). For proliferation experiments, mice were injected with BrdU (100 mg/kg, IP) 24, 18, and 4 h prior to sacrifice. For fate-mapping experiments, mice were treated with BrdU in the drinking water (1 g/l) for one week, after which they were administered FLX in the drinking water and sacrificed four weeks following the start of BrdU administration, as described previously (Sachs, Jacobsen, et al., 2013).

Immunohistochemistry

Immunohistochemistry was performed as described previously (Sachs, Jacobsen, et al., 2013; Sachs, Rodriguiz, et al., 2013). Antibodies used were rat anti-BrdU (Accurate Chemical Corporation, 1:200), chicken anti-GFAP (Aves Labs), mouse anti-NeuN (EMD Millipore, 1:200), chicken anti-vimentin (EMD Millipore, 1:200), rabbit anti-doublecortin (Abcam, 1:200), and rabbit anti-mCherry (EnCor Biotechnology, 1:200). For BrdU and NeuN immunohistochemistry, sodium citrate antigen retrieval was performed by boiling the slides in a sodium citrate buffer for 20 min. Following antigen retrieval, all endogenous Tomato fluorescence was lost, and thus Tomato was detected by immunohistochemistry (using the mCherry antibody listed above) for double-labeling experiments. Alexa Fluor 488- and 568-conjugated secondary antibodies and TOTO-3 iodide (a nuclear stain)

were obtained from Molecular Probes (Life Technologies) and used as recommended by the manufacturer.

Images were taken on a Zeiss Axiovert fluorescence microscope or an LSM510 confocal fluorescence microscope. Quantifications were performed by an individual blinded to the treatment condition. The density of BrdU+ cells was determined by measuring the total area of each brain region in each section and multiplying by the section thickness and the number of sections. Because Nestin/Tomato+ cells were spatially restricted to a thin layer of cells within a larger area (i.e., the ependymal layers of the third ventricle or the SGZ), data are expressed as the number of positive cells divided by the length of the perimeter of the third ventricle for the hypothalamus and habenula, or the length of the SGZ for the hippocampus.

Real-Time PCR

Tissue punches (1 mm in thickness and diameter) from the habenula and medial hypothalamus were obtained from mice chronically treated with FLX in the drinking water for 25 days. RNA extraction and real-time PCR was performed as described previously (Sachs, Jacobsen, et al., 2013). The following primer pairs were used: GAPDH, forward, 5' -CAT GTT CCA GTA TGA CTC CAC TC – 3', and reverse, 5' - GGC CTC ACC CCA TTT GAT GT – 3'; brain-derived neurotrophic factor (BDNF), forward, 5' -CAA TGC CGA ACT ACC CAA – 3', and reverse, 5' - AAC ATA AAT CCA CTA TCT TCC CC – 3'; and CREB, forward, 5' - AGC CGG GTA CTA CCA TTC TAC -3', and reverse, 5' - GCA GCT TGA ACA ACA ACT TGG – 3'.

Results

Consistent with previous reports that FLX increases adult hippocampal neurogenesis (Malberg et al., 2000; Sachs, Jacobsen, et al., 2013), our data confirm that chronic FLX treatment (for four weeks) increases BrdU incorporation in the SGZ (n = 10 CON, 11 FLX, t-ratio = 2.88, p = 0.0133, Figure 1A–C). Interestingly, chronic FLX also significantly increased BrdU incorporation in the medial hypothalamus (n = 15 per group, t-ratio = 3.188, p = 0.0042, Figure 1D–F) and in the medial habenula (n = 10 per group, t-ratio = 2.29, p = 0.0375, Figure 1G–I), two regions in which FLX-induced increments in neurogenesis have not been previously reported. However, even following chronic treatment with FLX, the overall amount of BrdU incorporation in the hypothalamus and habenula was much lower than that observed in the SGZ, which is consistent with prior studies (Perez-Martin et al., 2010).

We next performed fate-mapping experiments on BrdU-labeled cells in the hippocampus, habenula, and hypothalamus to determine whether FLX influenced the survival and neuronal differentiation of dividing cells in each area. Consistent with our previous results (Sachs, Jacobsen, et al., 2013), approximately 80% of BrdU+ cells in the hippocampus stain positive for the neuronal marker, NeuN, four weeks following the start of BrdU administration (Figure 2A). FLX treatment did not lead to any significant changes in the total number of BrdU+/NeuN+ neurons in the hippocampus four weeks after the start of BrdU labelling (Figure 2B). The percentage of BrdU+ cells in the hippocampus that acquired a neuronal phenotype was also not significantly affected by FLX treatment (79.3±0.3% for controls vs. 79.5±0.5% for FLX-treated animals), which is also consistent with our previous findings and suggests that FLX does not influence the survival or differentiation of NPCs (Sachs, Jacobsen, et al., 2013). Notably, for these survival/differentiation experiments, FLX treatment was initiated after BrdU labeling was complete, thus
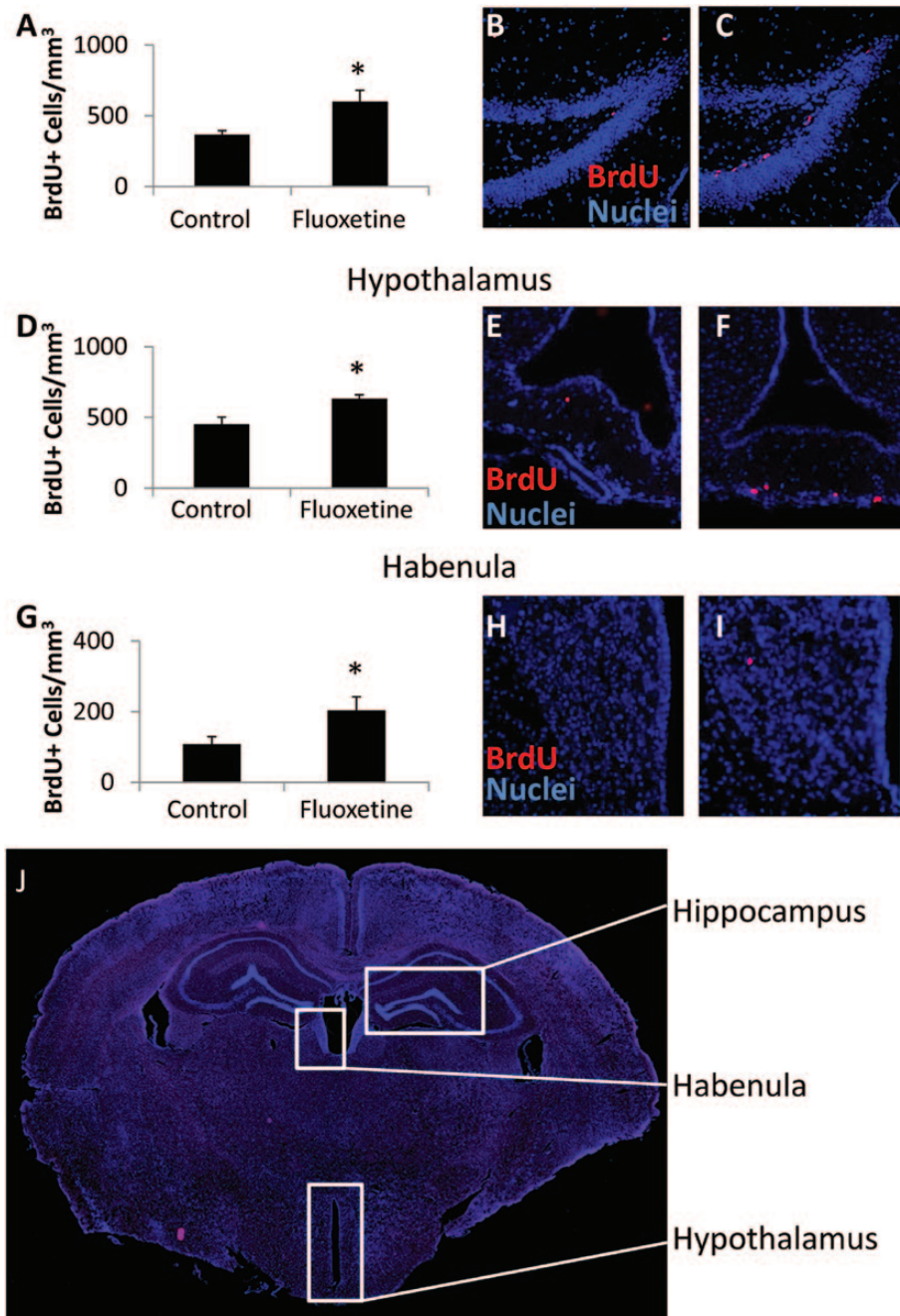
Figure 1. Fluoxetine leads to increased cell proliferation in the brain. (A) Quantification of BrdU incorporation data in the hippocampus of control and FLX-treated animals. Representative images from the hippocampus of control (B) and FLX-treated (C) mice are also shown. (D) Quantification of BrdU incorporation data in the hypothalamus of control and FLX-treated animals. Representative images from the hypothalamus of control (E) and FLX-treated (F) mice are also shown. (G) Quantification of BrdU incorporation data in the habenula of control and FLX-treated animals. Representative images from the hippocampus of control (H) and FLX-treated (I) mice are also shown. (J) A whole brain image with the hippocampus, habenula, and hypothalamus marked is shown for reference. BrdU is shown in red, nuclei are shown in blue. n = 10 control and 11 FLX for A–C; n = 15 per group for D–F; n = 10 per group for G–I. *p < 0.05 by t-test.

avoiding the potentially confounding influence of FLX on cell proliferation (Figure 1). In the hypothalamus, 38.7±4.7% of the BrdU-labeled cells in untreated animals stained positive for the neuronal marker, NeuN, while 45.3±4.0% of BrdU-labeled cells were NeuN+ in FLX-treated mice (no significant differences). No significant differences in the total number of BrdU+/NeuN+ cells in the hypothalamus were observed (Figure 2C and D). Similar results were observed in the medial habenula, where 39.7±5.1% of BrdU+ cells were NeuN+ in untreated mice and 45.8±4.1% of

BrdU+ cells were NeuN+ in FLX-treated animals (no significant differences). As in the hippocampus and hypothalamus, no significant effects of FLX treatment on the total number of BrdU+/NeuN+ cells in the medial habenula were observed (Figure 2E and F). These findings suggest that FLX enhances neurogenesis in the habenula and hypothalamus primarily by increasing proliferation, rather than altering the survival of newly-generated cells or the percentage of dividing cells that commits to a neuronal lineage.
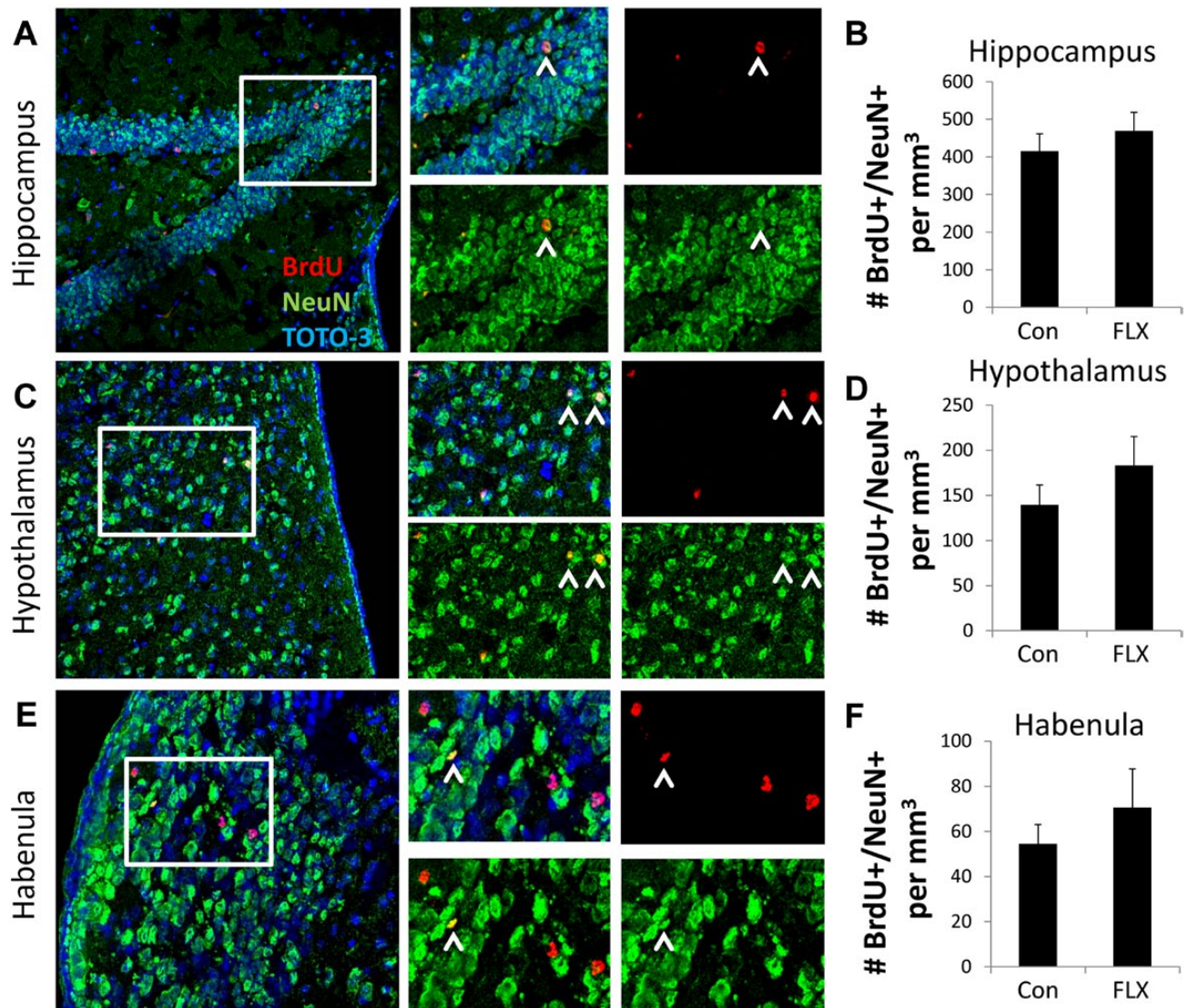
Figure 2. Fluoxetine does not affect the percentage of BrdU+ cells that become neurons. A representative image from the hippocampus (A), hypothalamus (C), and habenula (E) of a FLX-treated mouse is shown, and quantification is shown for the hippocampus (B), hypothalamus (D), and habenula (F). For all images, BrdU is shown in red, NeuN is shown in green, and nuclei are shown in blue. Arrowheads indicate double-positive cells. n = 9–11 per group.

Based on our observation that less than 50% of the dividing cells in the habenula and hypothalamus became NeuN+ within four weeks of BrdU labeling, we hypothesized that a subset of the dividing cells in these areas might differentiate into other cell types, such as glia. In the hippocampus, 7.8±1.8% of BrdU+ cells in untreated mice were immunopositive for the glial marker, GFAP, four weeks after the start of BrdU labeling. Following FLX treatment, 7.4±2.7% of BrdU+ cells in the hippocampus were double positive for GFAP (no significant differences, Figure 3A and B). In the hypothalamus, 11.7±2.9% of the BrdU+ cells were also GFAP+ in untreated animals, whereas 8.5±2.9% of the BrdU+ cells were also GFAP+ in FLX-treated mice (no significant differences, Figure 3C and D). Finally, in the medial habenula, 11.5±2.0% of BrdU+ cells were also GFAP+ in untreated animals and 17.4±6.9% of BrdU+ cells were double positive for GFAP in FLX-treated animals (no significant differences, Figure 3E and F).

In addition to increasing BrdU incorporation, chronic FLX also significantly increased the number of Tomato+ cells per unit length of the dentate gyrus of the hippocampus (n = 8 per

group, t-ratio = 3.08, p = 0.014, Figure 4A), and the ependymal layers of the ventral third ventricle (n = 8 per group, t-ratio = 4.07, p = 0.0025, Figure 4B) and dorsal third ventricle (n = 8 per group, t-ratio = 3.46, p = 0.007, Figure 4C) in NCerT mice. However, the localization of Nestin+ cells was quite distinct from the localization of BrdU+ cells four weeks after the start of cell labeling, at least in the habenula and hypothalamus. In the hypothalamus, virtually all of the nestin/Tomato+ cells of NCerT mice remained localized to the ependymal layer four weeks after the start of labeling (96.1±2.0% in control animals and 98.3±0.3% in FLX-treated mice, no significant difference). In contrast, less than 15% of the BrdU+ cells in the hypothalamus were localized to the ependymal layer at this time point (14.6±3.1% in control animals and 12.2±2.4% in FLX-treated animals, n = 15 per group, no significant difference).

Similar results were observed in the habenula. Adjacent to the dorsal third ventricle, 76.8±3.5% of the nestin/Tomato+ cells in control animals and 86.9±3.8% of the Tomato+ cells in FLX-treated mice remain localized in the ependymal layer four weeks
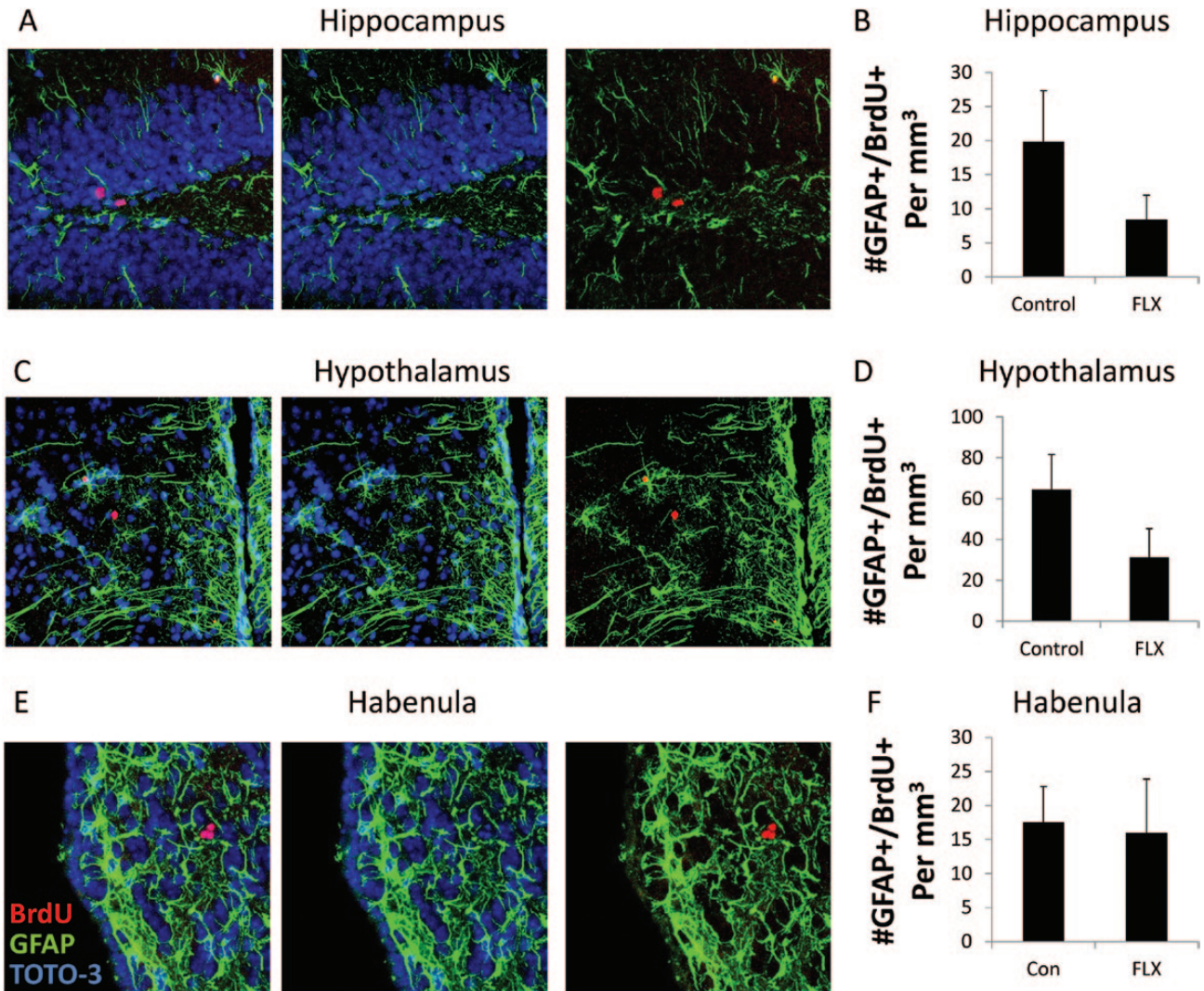
Figure 3. Fluoxetine does not affect the percentage of BrdU+ cells that become astrocytes. Representative images from the hippocampus (A), hypothalamus (C), and habenula (E) of a FLX-treated mouse are shown. Quantification of these results is presented for the hippocampus (B), hypothalamus (D), and habenula (F). For all images, BrdU is shown in red, GFAP is shown in green, and nuclei are shown in blue. n = 8–10 per group.

following the start of TMX administration (no significant differences). In contrast, less than 10% of BrdU+ cells in the medial habenula were localized to the ependymal layer (2.9±2.1% for control animals and 9.0±4.1% for FLX-treated mice; no significant differences). In addition, whereas the hippocampus had higher levels of BrdU incorporation than either the hypothalamus or habenula (Figure 1), the hippocampus had lower numbers of nestin-Tomato+ cells than either of these other two areas (Figure 4). Taken together, these results suggest that FLX may target at least two distinct cell populations (i.e., a BrdU+ parenchymal population and a Nestin+ ependymal population) in the hypothalamus and medial habenula.

In contrast to the hippocampus, where most Tomato+ cells become immunopositive for the mature neuronal marker, NeuN, within four weeks of the start of TMX labeling (Figure 5A), virtually all of the Tomato+ cells surrounding the third ventricle (Figure 5B) and dorsal third ventricle (Figure 5C) remain in the ependymal layer and do not stain positive for NeuN. However, a very small subpopulation of Tomato+ cells in the hypothalamus (<1%) is present in the parenchyma and is NeuN+ (Figure 5B), suggesting that Nestin+/Tomato+ cells can give rise to mature neurons.

Also in contrast to the hippocampus, where we have previously shown that FLX leads to a significant increase in the number of doublecortin (DCX)+ immature neurons (Sachs, Jacobsen, et al., 2013), we observed no DCX+ immature neurons in the habenula (Figure 6A) or hypothalamus (Figure 6B), despite observing robust DCX immunoreactivity in the hippocampus (Figure 6C) and subventricular zone of the lateral ventricles (Figure 6D).

Consistent with their differentiation into neurons, most of the Tomato+ cells in the hippocampus do not stain with the glial marker, GFAP (Figure 7A). However, over 95% of the Tomato+ cells in the hypothalamus remain localized to the ependymal layer and many of these cells stain positive for GFAP (Figure 7B). These Tomato+ and GFAP+ cells in the ependymal layer display the morphological features of tanycytes, which is consistent with the fact that tanycytes are known to express the astrocyte marker, GFAP (Redecker, 1989). Interestingly, tanycytes have been reported to be the NPCs of the hypothalamus (Lee et al., 2012). Although less than 5% of all Tomato+ cells in the hypothalamus were localized in the parenchyma, several Tomato+ cells outside of the ependymal layer were also GFAP+, suggesting that at least
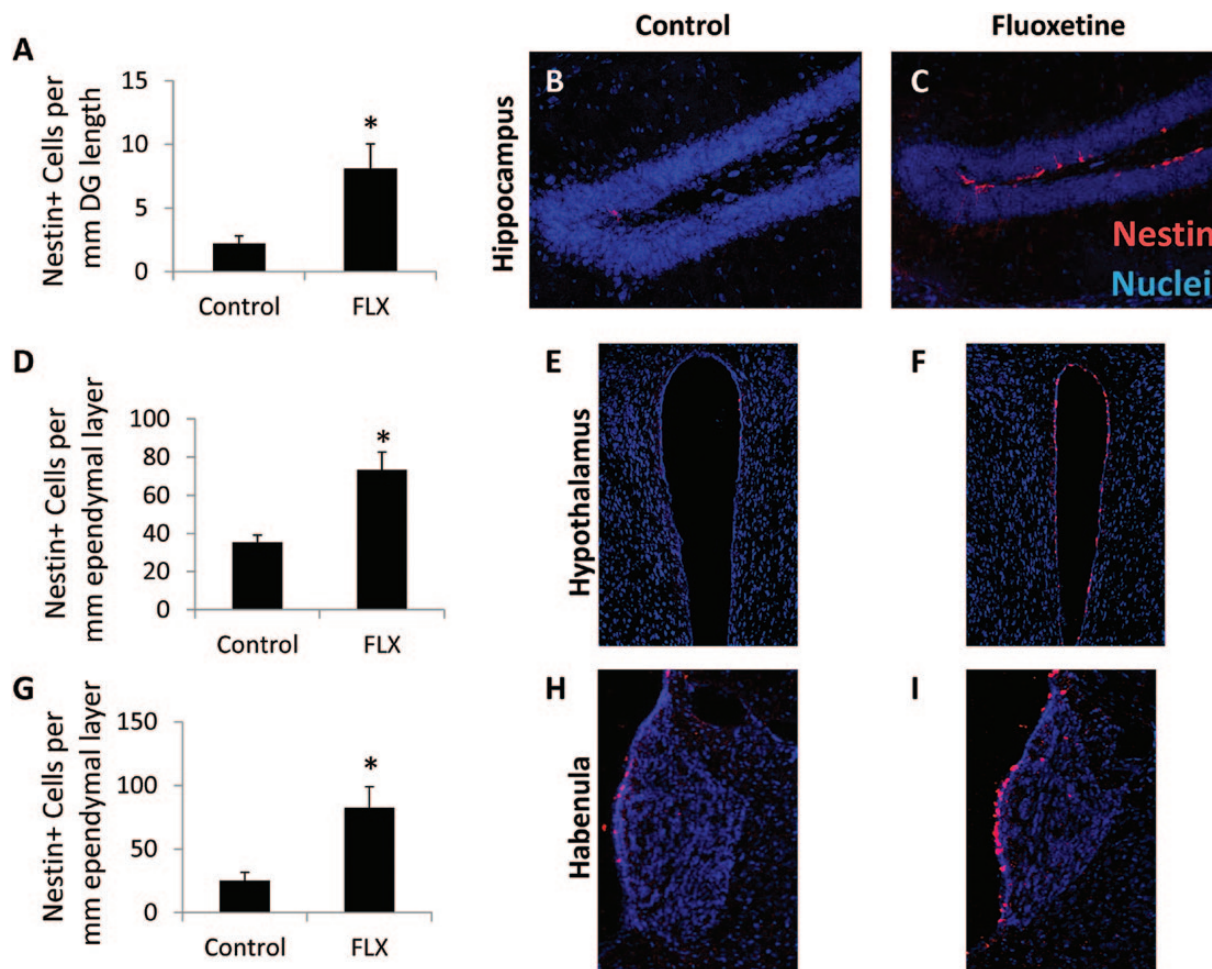
Figure 4. Fluoxetine leads to increased numbers of nestin+ cells in the brain. (A) Quantification of the number of Tomato+ cells in the hippocampus with representative images from control (B) and FLX-treated (C) animals. (D) Quantification of the number of Tomato+ cells in the hypothalamus, with representative images from control (E) and FLX-treated (F) animals. (G) Quantification of the number of Tomato+ cells in the habenula with representative images from control (H) and FLX-treated (I) animals. Tomato expression is shown in red, nuclei are shown in blue. n = 8 per group. *p < 0.05 by t-test.

a subset of the Tomato+ cells might become mature GFAP+ astrocytes (Figure 7B). In contrast to the hypothalamus, the ependymal cells in the dorsal third ventricle were GFAP-, and thus most of the Tomato+ cells, which remained in the ependymal layer, were GFAP- (Figure 7C). GFAP+/Tomato+ cells were observed in the stria medullaris (Figure 7D) of the thalamus but not in the medial habenula (Figure 7C). Thus, our results (Figures 3 and 7) suggest that FLX leads to increased gliogenesis, in addition to increased neurogenesis, in the hypothalamus and habenula.

Given the tanycyte-like morphology and localization of the Tomato+ cells in the hypothalamus, we sought to confirm the identity of these cells as tanycytes by staining for vimentin, a protein that is commonly used as a tanycyte marker (Rodriguez et al., 2005; Lee et al., 2012). In the hippocampus, the processes of a subset of the Tomato+ cells stain positive for vimentin four weeks after the start of tamoxifen administration (Figure 8A). However, virtually all of the Tomato+ cells adjacent to the third ventricle and dorsal third ventricle remain in the ependymal layer and are immunoreactive for vimentin (Figures 8B and C), suggesting that these FLX-responsive cells are tanycytes and may represent a NPC population in the hypothalamus and habenula.

Prior work has shown that infusions of BDNF into the ventricles can lead to increased cell proliferation in the medial hypothalamus and the habenula (Pencea et al., 2001). To evaluate whether BDNF might be involved in the effects of FLX on cell proliferation and neurogenesis in these regions, real-time PCR was performed. Our results indicate that chronic FLX significantly increases the mRNA levels of BDNF in the hypothalamus (Figure 9A) and the habenula (Figure 9B), as we have previously shown for the hippocampus (Sachs, Jacobsen, et al., 2013). In contrast, no significant effects of FLX on CREB mRNA expression were observed in either the hippocampus or the habenula (Figure 9).

Discussion

The enhanced cell proliferation and increased numbers of nestin+ NPCs in the hypothalamus and habenula in response to chronic FLX treatment represent a potential mechanism whereby FLX could modulate the activity of these structures, an effect that could have important implications for depression- and anxiety-like behaviors. Interestingly, patients with major depression and bipolar disorder have been reported to exhibit smaller habenula volumes than healthy controls (Ranft et al., 2010; Savitz, Nugent, et al., 2011), as well as smaller hypothalamic volumes and/or larger third ventricles (Schindler et al., 2012). In addition, dysregulation of the hypothalamic-pituitary-adrenal
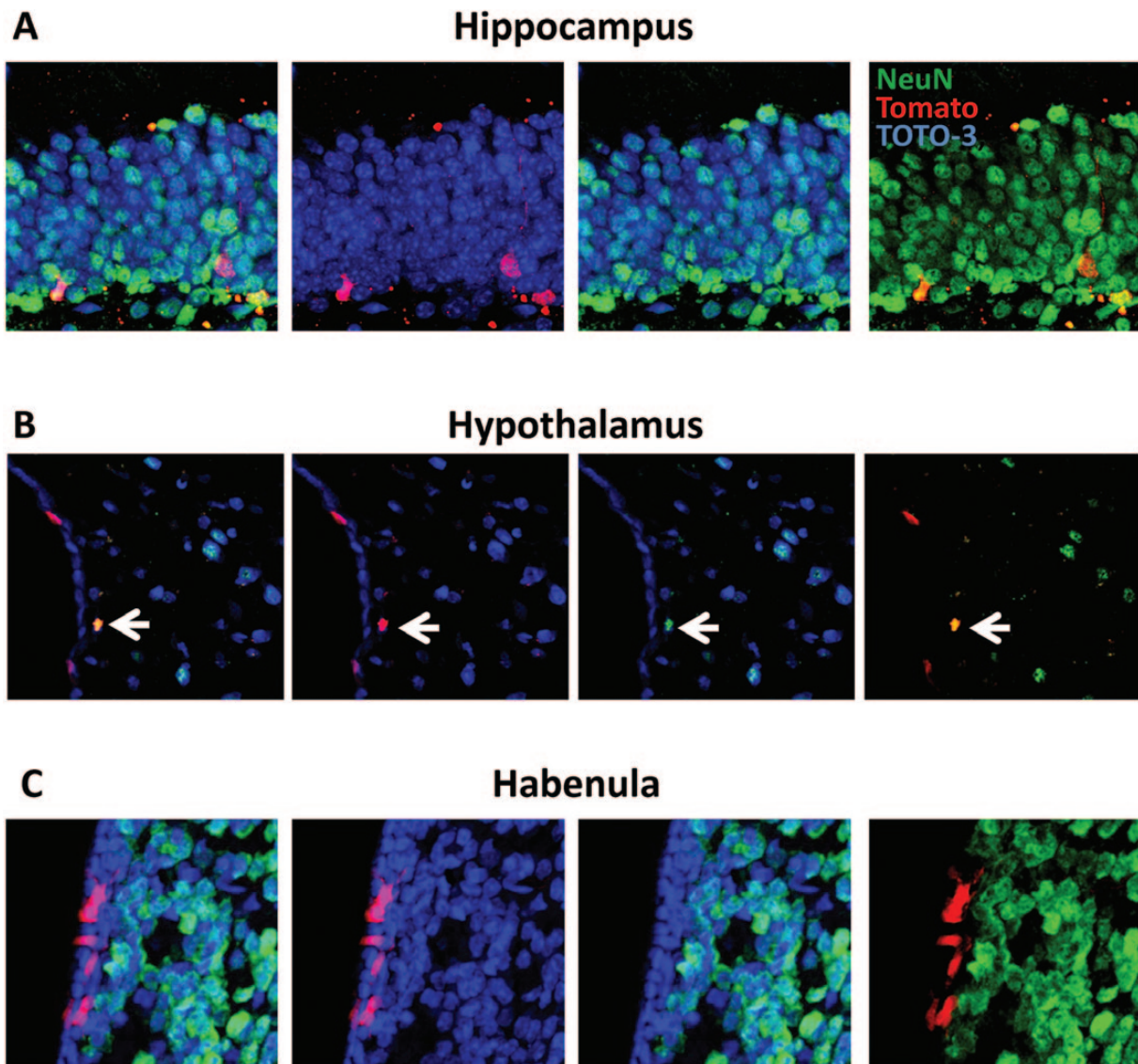
Figure 5. Neurogenesis from nestin+ precursors. Representative images of nestin-Tomato+/NeuN+ neurons in the hippocampus (A), hypothalamus (B), and (C) habenula. Tomato expression is shown in red, NeuN expression is shown in green, and nuclei are shown in blue. Arrows indicate double-positive cells.

axis is commonly observed in psychiatric populations (Pariante and Lightman, 2008). Although the lateral habenula has received considerable recent attention as a potential target for antidepressant treatment (Sartorius et al., 2010) and for its role in mediating depression- and anxiety-like behavior (Sartorius et al., 2010; Li et al., 2011; Stamatakis and Stuber, 2012), preclinical studies in animals have also revealed important roles for the medial habenula in fear and anxiety responses (Agetsuma et al., 2010; Lee et al., 2010; Yamaguchi et al., 2013), thus highlighting the potential importance of the observed effects of FLX on neurogenesis in the medial habenula. In the future, it will be important to determine the exact types of hypothalamic (and habenular) neurons produced and to define the subregions of the hypothalamus where they integrate. These types of studies will shed new light on the mechanisms whereby adult neurogenesis in the hypothalamus and/or habenula could play a role in the development or treatment of psychiatric disease.

The relationships between adult neurogenesis, psychiatric disease, and antidepressant responses remain controversial, although most of the work investigating the potential role of

adult neurogenesis within the context of depression and/or anxiety has focused exclusively on the hippocampus. Our documentation of extra-hippocampal neurogenic responses to chronic FLX may inform this controversy. Indeed, it is possible that at least some of the therapeutic and/or side effects of FLX result from changes in cell proliferation and/or neurogenesis in the hypothalamus or medial habenula. For example, adult hippocampal neurogenesis has been reported to play an important role in buffering stress responses (Snyder et al., 2011) and in mediating behavioral responses to FLX (Santarelli et al., 2003), but these effects and the mechanisms underlying them remain contentious (Bessa et al., 2009; Anacker and Pariante, 2012). Given the prominent roles of the hypothalamus and habenula in mediating responses to stressful and aversive stimuli (Herman and Cullinan, 1997; Gold and Chrousos, 2002; Matsumoto and Hikosaka, 2007; Hikosaka, 2010), it is possible that neurogenesis in these structures could also contribute to these effects. Alternatively, given the fact that inhibiting hypothalamic neurogenesis inhibits weight gain (Lee et al., 2012), it is possible that increasing hypothalamic neurogenesis could contribute to the increased weight gain that is observed
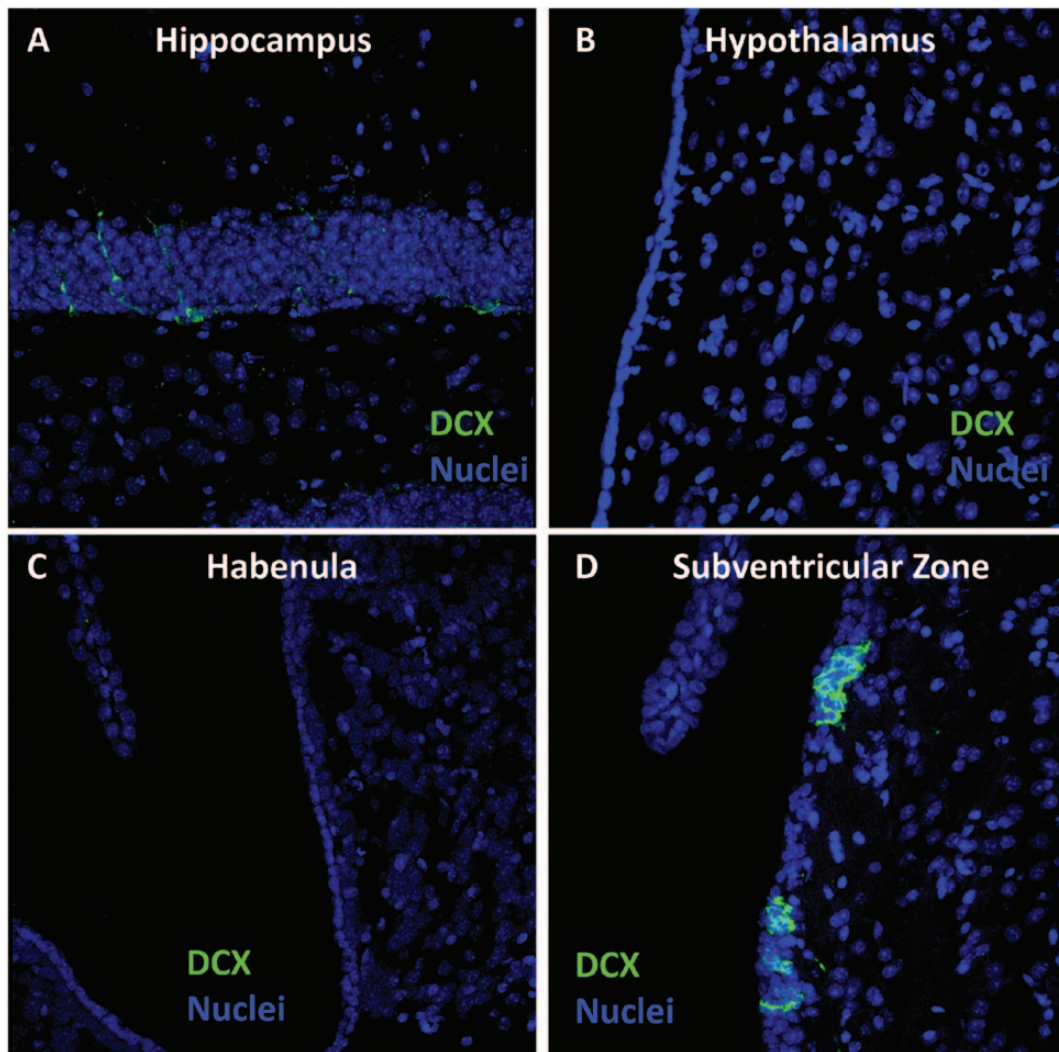
Figure 6. Doublecortin immunoreactivity in adult mouse brain. Representative images from the hippocampus (A), hypothalamus (B), habenula (C), and subventricular (D) zone of the lateral ventricle. Nuclei are shown in blue, and doublecortin (DCX) is shown in green.

following treatment with some antidepressant medications (Serretti and Mandelli, 2010). Future research into the behavioral and physiological consequences of stress- or antidepressant-induced changes in hypothalamic and habenular neurogenesis could shed light on this topic.

Towards this end, it will be critical for future studies to engineer more selective ways to prevent FLX-induced cell proliferation within specific niches of progenitor cells in the brain. Most of the classical methods to inhibit neurogenesis are largely non-selective and involve the administration of anti-mitotic agents (Shors et al., 2002) or exposure to irradiation (Santarelli et al., 2003). The use of a lead shield to restrict radiation exposure to certain brain regions has led to some degree of selectivity (Santarelli et al., 2003), but given the anatomical localization of the hippocampus, habenula, and hypothalamus, it would be virtually impossible to use this technique to obliterate proliferating cells in the hippocampus while preserving cell proliferation in the habenula and hypothalamus (see Figure 1J). More recently, stereotactic approaches to deliver x-ray irradiation locally to the hypothalamus have been used (Lee et al., 2012), and these techniques provide a significant improvement over earlier irradiation methods, but the development of novel, less invasive genetic methods is warranted.

The current study did not directly explore the mechanisms through which FLX administration leads to increased proliferation in the habenula and hypothalamus. However, our current results indicate that chronic FLX can increase BDNF mRNA expression in the habenula and hypothalamus, similar to what has been reported previously in the hippocampus (Nibuya et al., 1995; Sachs, Jacobsen, et al., 2013). Prior research has shown that infusions of BDNF can promote cell proliferation and neurogenesis in the hypothalamus and the habenula (Pencea et al., 2001). It is possible that the observed increase in BDNF following FLX plays a role in FLX-induced increments in cell proliferation within the hypothalamus and medial habenula, but future research will be required to evaluate this.

In the hypothalamus, NCERT mice exhibit Tomato expression primarily in tanycytes following TMX administration. Thus, our current results are the first to demonstrate that FLX targets hypothalamic tanycytes. Several prior studies have suggested that ependymal cells (Johansson et al., 1999) or tanycytes (Lee et al., 2012) represent the resident NPC populations in the adult mammalian hypothalamus. Whether FLX leads to changes in tanycyte function has remained unknown, but the increased number of nestin/Tomato+ cells following chronic FLX
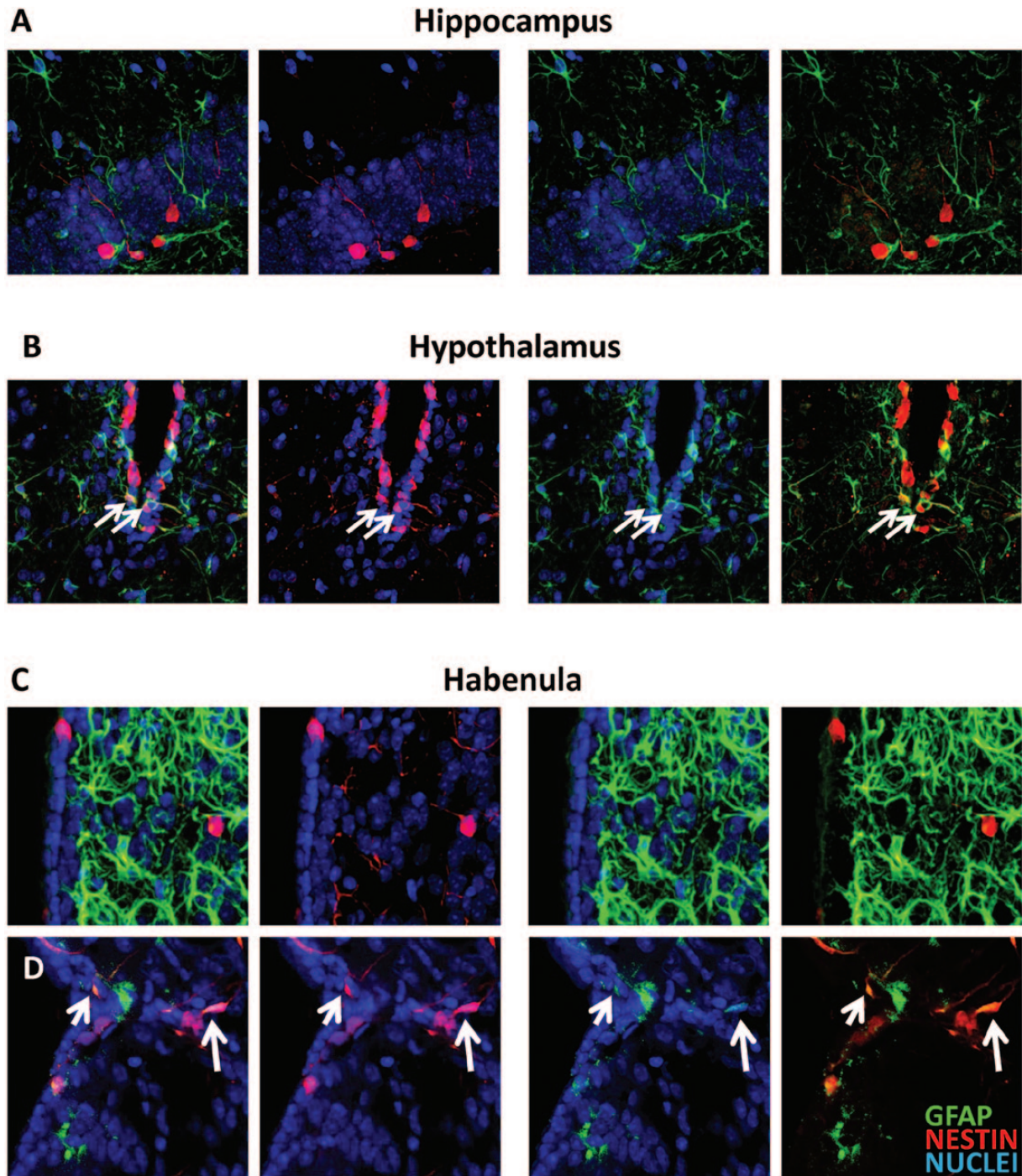
Figure 7. Gliogenesis from nestin+ precursors. Representative images of nestin-Tomato+ /GFAP+ glia in the hippocampus (A), hypothalamus (B), and habenula (C, D) in NCERT mice. Tomato expression is shown in red, NeuN expression is shown in green, and nuclei are shown in blue. C demonstrates the medial habenula itself, whereas D shows the region immediately dorsal to the medial habenula, the stria medullaris of the thalamus. Tomato expression is shown in red, GFAP expression is shown in green, and nuclei are shown in blue. Arrows indicate double-positive cells.

treatment suggest that tanycyte-dependent processes, including neurogenesis, might be influenced by antidepressants.

Prior work has suggested that the nestin+ cells in the walls of the lateral ventricle and those populating the ependymal layer of the third ventricle represent at least two distinct cell types (Ernst and Christie, 2005). Our current work, showing that the ependymal cells of the third ventricle stain positive for GFAP, whereas those in the dorsal third ventricle do not, also suggests

that the ependymal cells in these two regions also represent distinct cell types. Nonetheless, our data show that nestin+ cells in both regions are responsive to chronic FLX treatment. Similar to at least one prior report (Ernst and Christie, 2005), we were unable to identify any nestin+/BrdU+ cells in the third ventricle or dorsal third ventricle (not shown), and the spatial localization of the nestin+ cells and the BrdU+ cells appeared to be almost exclusively non-overlapping. Our findings suggest the
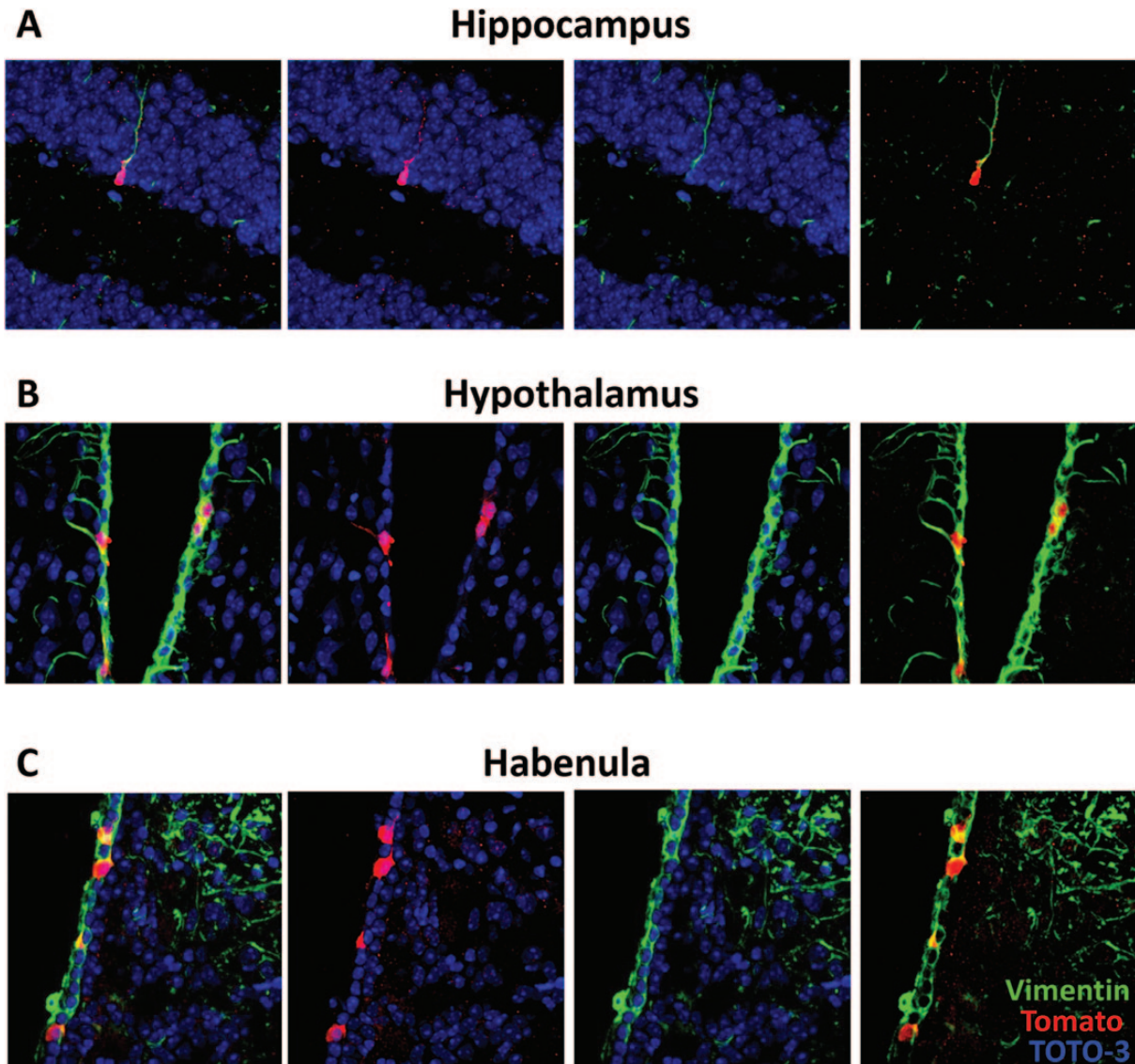
Figure 8. Vimentin expression in nestin-Tomato+ cells in NCERT mice. Representative images of nestin-Tomato+ cells (red) in the hippocampus (A), hypothalamus (B), and habenula (C) that stain positive with vimentin (green). Nuclei are shown in blue.

nestin+ cells either do not proliferate or proliferate at extremely low rates. These findings are in contrast to some previous work, which did identify nestin+/BrdU+ cells in the ependymal and subependymal layers of the third ventricle in rats (Chouaf-Lakhdar et al., 2003).

One prior study demonstrated that progenitor cells in the hypothalamus give rise to neurons via immature neuronal intermediates that are DCX negative, suggesting that DCX may not be involved in adult hypothalamic neurogenesis (Guo et al., 2009). Several groups have reported finding no evidence of DCX expression in the hypothalamus, except for at low levels within the suprachiasmatic nucleus (Geoghegan and Carter, 2008). However, doublecortin-like, a protein that is highly homologous to DCX, is reportedly expressed in the hypothalamus (Saaltink et al., 2012). In contrast, at least one group has reported DCX immunoreactivity within the hypothalamus (Kokoeva et al., 2007), which is not consistent with our current results. It is possible that differential levels of cross-reactivity to DCX-like observed with various DCX antibodies could explain the reported

differences in DCX expression in the hypothalamus, but future studies would be required to address this. Future research will be required to define the mechanisms through which adult-generated neurons in the hypothalamus and habenula mature and become integrated into existing circuits. In addition, it will be important to provide a more comprehensive fate mapping of all adult-generated cells in these brain regions.

Overall, our study demonstrates that chronic treatment with FLX is sufficient to increase neurogenesis and enhance BDNF expression in the adult mammalian hypothalamus and habenula. Our results may provide new insight into the mechanism of action of antidepressant medications and could help to further refine the cellular targets for molecular intervention in the treatment of mood and anxiety disorders.
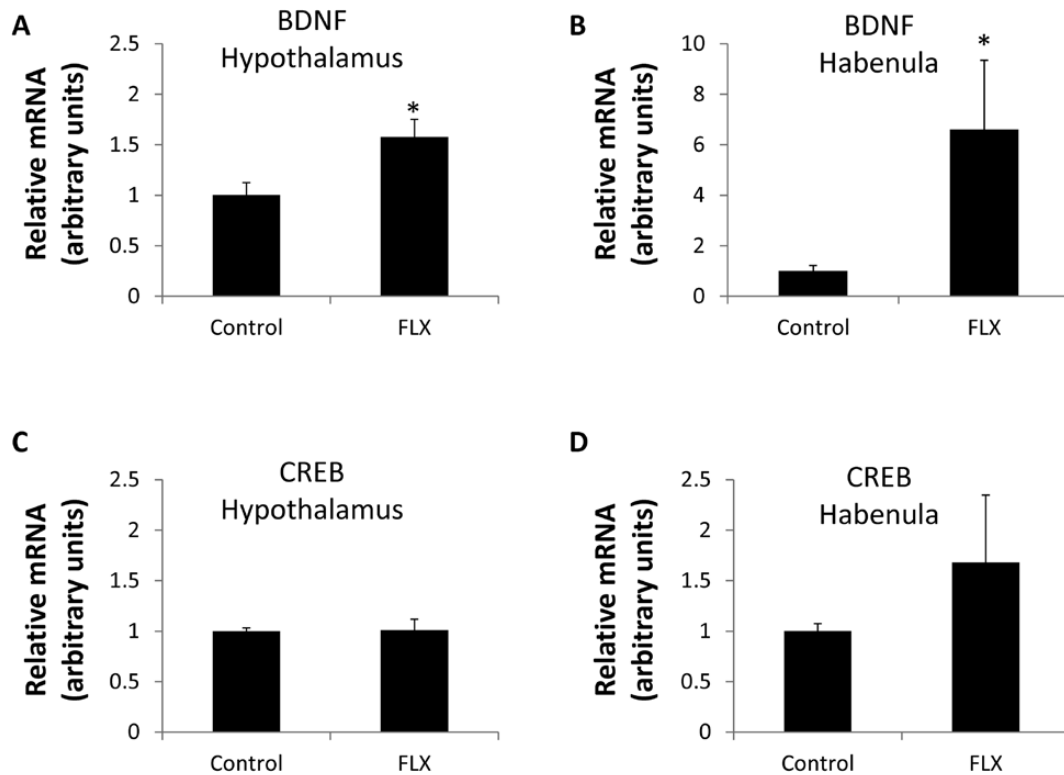
Acknowledgments

Figure 9. BDNF and CREB mRNA expression in the hypothalamus and habenula following chronic FLX. Quantification of real-time PCR data reveals that chronic FLX leads to an increase in the expression of BDNF in the hypothalamus (A) and habenula (B), but not to an increase in CREB in these brain regions (C and D). *p < 0.05 by t-test. n = 7 per group.

Statement of Interest

Dr Caron has received compensation from Lundbeck as a member of their Psychopharmacology Advisory Board and is a consultant for Omeros Corp. Dr Sachs declares no conflicts of interest.

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