Fluoxetine targets early progenitor cells in the adult brain

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Antidepressant drugs of the selective serotonin reuptake inhibitor (SSRI) class (e.g., fluoxetine) are commonly used to treat a wide spectrum of mood disorders in adults (1); they are also increasingly prescribed to children and adolescents (2, 3). However, the cellular basis for the action of SSRIs is not clear. In addition to its effects on neurotransmission, SSRI fluoxetine increases generation of new neurons in the dentate gyrus (DG) of the adult brain (4–9). Importantly, recent findings suggest that this increase may be a causative factor in the behavioral effects of this class of antidepressants (7). These discoveries may provide a novel framework for understanding depression and designing new therapeutic drugs.

One of the problems in defining SSRIs targets within the neuronal proliferation-differentiation cascade is the imprecision in quantifying the changes in each class of neural precursor cells in the brain. Accurate enumeration of changes in distinct subpopulations of neuronal precursors by immunocytochemistry is problematic: High cell density, complex cell morphology, and uncertainties in defining distinct boundaries between subcellular classes of cells reduces the precision of evaluating changes in particular subclasses of neuronal precursors (e.g., in contrast to BrdU- or thymidine labeling of cell nuclei). Fig. 1 A–F compares the structures of the SVZ and DG as revealed by immunocytochemistry for nestin and by expression of nestin-CFPnuc or nestin-GFP (13). Whereas we were unable to generate accurate counts of nestin- or nestin-GFP-positive cells, we were able to unambiguously enumerate (by using confocal stereology) all of the labeled nuclei in the SVZ and DG of the nestin-CFPnuc mice. We have used this nestin-CFPnuc reporter line to define discrete steps in the neuronal differentiation cascade in the DG (leading from stem/progenitor cells to differentiated granule neurons), based on the morphology of the cells, the marker proteins that they express, and their mitotic activity (measured by BrdU incorporation). We identify six classes of cells in the neuronal lineage in the DG of nestin-CFPnuc mice (Figs. 1 and 2).

The first class is represented by glial fibrillary acidic protein (GFAP)-positive nestin-CFPnuc cells. The triangular soma and the nuclei of these cells reside in the subgranular zone (SGZ); they extend a single- or double-apical process radially across the granule

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Abbreviations: ANP, amplifying neuroprogenitor; DG, dentate gyrus; Dcx, doublecortin; GCL, granule cell layer; GFAP, glial fibrillary acidic protein; IN, immature neuron; NB 1, type 1 neuroblasts; NB 2, type 2 neuroblasts; NeuN, neuronal nuclei; Prox-1, homeobox prospero-like protein; PSA-NCAM, polysialic-acid neural cell adhesion molecule; QNP, quiescent neuroprogenitor; SGZ, subgranular zone; SSRI, selective serotonin reuptake inhibitor; SVZ, subventricular zone.

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cell layer (GCL), terminating with elaborated arbors of very fine leaf-like processes in the molecular layer (Fig. 1 G–K; see also ref. 13). This characteristic apical process is easily visualized with antibodies to GFAP, nestin, vimentin, and brain fatty acid-binding protein. Cells of this class have been described in detail (13, 17–21), and they correspond to the most primitive, stem-like population in the DG; note, however, that not all of the criteria of stem cells, e.g., ability to self-renew, have been demonstrated for these cells (22–24). Only a small fraction of these cells (<2%) can be labeled by BrdU after a short (2-h) pulse, indicating their low rate of division and consistency with the quiescent state of these cells (18, 20); we therefore designate these cells as quiescent neural progenitors (QNP). We have not been able to detect instances of symmetric division of such cells (i.e., generating two similar cells or keeping the plane of division perpendicular to the SGZ); however, these cells can be seen undergoing asymmetric divisions (below).

The second class is represented by small (somatic diameter ~10 μm) round or oval cells located in the SGZ (Fig. 1 H–K). Similar to QNPs, these cells express nestin-CFPnuc, brain fatty acid-binding protein, and Sox2, but they do not stain for GFAP or vimentin and stain very weakly for nestin (which may indicate that CFPnuc protein persists in these cells longer than nestin, or that nestin is unequally distributed during cell division); they also do not stain for doublecortin (Dcx), polysialic acid neural cell adhesion molecule (PSA-NCAM), or for markers of differentiated neurons [homeobox prospero-like protein (Prox-1), βIII-tubulin, neuronal nuclei (NeuN), or calbindin]. These cells are labeled with BrdU at high frequency (20–25% 2 h after a single injection of BrdU), indicating that most of them are involved in mitotic activity; we designate these cells as amplifying neural progenitors (ANP). They are often seen in clusters extending along the SGZ (Fig. 1 L); when the plane of division of cells in these clusters is visible, it is most often perpendicular to the SGZ, such that the daughter cells remain in the SGZ (Fig. 1 L). Importantly, a fraction of these cells are seen separating from QNPs after mitosis; in each case, the division plane is parallel or slightly oblique to the SGZ such that the daughter cell is deposited beneath the QNP cell (Fig. 1 H–K) (the plane of division may explain why these cells do not inherit GFAP or nestin, which are predominantly localized to the apically positioned processes of the QNPs but not to their soma). Together, our results suggest that QNP cells, by undergoing asymmetric divisions, give rise to ANP cells, which then propagate in the SGZ through a series of symmetric divisions.

The next class of precursor cells, still located in the SGZ, ceases to express nestin, Sox2, brain fatty acid-binding protein, or CFPnuc and starts to express Dcx and PSA-NCAM (Fig. 1 M and N). A small subclass (~1% of cells in this class) morphologically resembles ANPs, carries short (1–5 μm) horizontal processes (Fig. 1 M), and is the final population in the differentiation cascade that is labeled by BrdU (19). Most of the cells in this class are represented by larger (10–15 μm somatic diameter) cells that extend longer (10–30 μm) horizontal processes in the plane of the SGZ and do not incorporate BrdU (Fig. 1 N). These cells stain for Dcx, PSA-NCAM, Prox-1, and βIII-tubulin but do not express NeuN. Thus, the bulk of this class is represented by postmitotic neuronal precursors; we designate them as type 1 neuroblasts (NB1).
Cells of the next class, type 2 neuroblasts (NB2), are larger than NB1 cells (somatic diameter ~15 μm) and remain confined to the SGZ. They extend longer (20–40 μm) processes horizontally and obliquely to the plane of the SGZ (Fig. 1O). They do not express QNP or ANP markers (nestin, GFAP, vimentin, Sox2, brain fatty acid-binding protein, or CFPnuc), and express Dcx, PSA-NCAM, Prox-1, βIII-tubulin, and NeuN.

The next class of cells corresponds to immature neurons (IN). They are larger than the cells of the previous classes (somatic diameter 15–20 μm), and their morphology resembles that of mature granule cells of the DG (Fig. 1P). Their soma is round or oval and can be found both in the SGZ and, mainly, in the GCL. These cells carry a single apical process that branches in its distal part located in the molecular layer. They express Dcx, PSA-NCAM, Prox-1, βIII-tubulin, and NeuN.

The next class represents differentiated granule neurons, with developed apical dendrites and axons forming the mossy fiber. They cease to express PSA-NCAM and Dcx but express Prox-1, βIII-tubulin, NeuN, and markers of mature granule neurons (e.g., calbindin; ref. 25).

The differentiation cascade in the DG of nestin-CFPnuc mice thus can be divided into discrete steps based on the expression of markers, morphology, and mitotic activity (Fig. 2).

Fluoxetine Increases Symmetric Divisions of Early Progenitors in the DG. Chronic treatment with fluoxetine increases the number of new neurons in the DG (4–9). Note, however, that these observations do not reveal the identity of cells targeted by the drug; this increase can potentially reflect changes in stem/progenitor cells, advanced neuroblasts, immature neurons, or in some combination of these classes. We used our nestin-CFPnuc reporter line to investigate changes induced by fluoxetine in each of the classes we identified in the DG. We treated the animals with fluoxetine for 15 days, labeled dividing cells with BrdU, and monitored selected cell populations in the DG after 24 h by using confocal stereology (Fig. 3). The number of BrdU-labeled cells in the DG was increased by 40.9% (538 ± 51 vs. 758 ± 58; P = 0.013) after fluoxetine administration, in line with previous reports on the effects of chronic treatment with the drug (refs. 4 and 7; Fig. 3A–C). We also found that after treatment, the number of CFPnuc-positive cells (i.e., QNPs and ANPs together) increased by 24.7% (8,356 ± 622 vs. 10,422 ± 646; P = 0.037; Fig. 3 D–F). When these cells were divided into QNP and ANP classes based on expression of GFAP, the QNP class showed no change (4,516 ± 582 vs. 4,675 ± 518; Fig. 3G), whereas the number of ANP cells increased by 49.6% (3,840 ± 431 vs. 5,745 ± 506; P = 0.012; Fig. 3H). No change was detected in the volume of the GCL, including the SGZ, between the control and experimental animals (0.468 ± 0.052 vs. 0.483 ± 0.052 mm³).

The number of PSA-NCAM-positive cells (which include NB1, NB2, and IN cells, Fig. 4 A and B) was increased by 26.5% (8,936 ± 577 vs. 11,298 ± 719; P = 0.022) (identical changes were seen for Dcx-positive cells; note that Dcx and PSA-NCAM colocalized in both control and fluoxetine-treated animals; data not shown). When these cells were subdivided further by using the criteria described above, the number of NB1 cells was increased by 42.1% (4,918 ± 418 vs. 6,988 ± 538; P = 0.089; Fig. 4C), and the number of NB2 and IN cells remained unchanged (3,110 ± 209 vs. 3,452 ± 413 and 908 ± 11 vs. 858 ± 88, respectively) (Fig. 4 D and E), compatible with the notion that the wave of increased proliferation and differentiation has not reached those cell classes.

Thus, the earliest class affected by fluoxetine is the ANP cells which are progeny of stem-like QNP cells. Importantly, the QNPs themselves do not increase in number, consistent with the lack of symmetrical divisions in this class. The increase in ANPs can be due to either (i) an increased rate of asymmetric divisions of QNPs (i.e., QNPs may be dividing more often under the influence of fluoxetine but only give rise to daughter ANP cells while keeping their own number constant) or (ii) increased symmetric division of ANP cells (i.e., the same number of ANPs may be born from QNPs, but they then divide more frequently). To distinguish between these possi-
abilities, we counted the number of BrdU-labeled QNPs and ANPs. We used triple labeling (CFPnuc, BrdU, and GFAP) to discriminate between QNPs and ANPs and to quantify their mitotic activity (Fig. 5). The number of BrdU-labeled QNPs was not affected by fluoxetine treatment \((83 \pm 22\) vs. \(90 \pm 16\); \(P = 0.8\); Fig. 5A), whereas the number of BrdU-labeled ANPs was increased \(46.4\% (280 \pm 36\) vs. \(410 \pm 33\); \(P = 0.023\); Fig. 5B); the fraction of dividing cells among QNPs (Fig. 5C) and ANPs (Fig. 5D) did not change. These results indicate that the rate of QNP cell division is unchanged and that fluoxetine increases symmetric divisions of ANP cells. When considered together with the data on other cell classes, these results suggest that ANPs are the only class of precursor cells in the DG that directly respond to fluoxetine.

We also analyzed the changes in the SVZ, another major neurogenic region (Fig. 7A), which is published as supporting information on the PNAS web site. We did not observe changes in the number of BrdU-labeled cells \((10,058 \pm 766\) vs. \(9,550 \pm 769\); Fig. 7B, D, and E), in agreement with the previous observations in rats (4). Furthermore, we did not find any significant changes either in the number of nestin-CFPnuc cells, \((454 \pm 52\) vs. \(473 \pm 55 \times 10^3\); Fig. 7C–E), or in their density \((648 \pm 55\) vs. \(687 \pm 64 \times 10^3\) mm\(^3\)), or in the volume \((0.648 \pm 0.058\) vs. \(0.603 \pm 0.051\) mm\(^3\)) of the SVZ. Together, our data indicate that the fluoxetine-induced increase in the number of early progenitor cells is specific for the DG and does not affect the SVZ.

To investigate whether the fluoxetine-induced increase in progenitor cells is manifested later as an increase in the number of new neurons in the DG and whether the increase is maintained after the Fig. 3. Fluoxetine increases cell proliferation in the adult DG. Chronic (15 days) fluoxetine treatment of adult (7 months) mice, analyzed 1 day after BrdU administration. Fluoxetine increases the number of BrdU-positive cells (A). (B and C) Representative photomicrographs of DG sections from animals treated with vehicle (B) and fluoxetine (C); dashed lines in B, C, E, and F outlines the external limits of the DG. Exposure to fluoxetine also increases the number of nestin-CFPnuc cells in the SGZ (D, histogram; E, section of the DG of a control animal; F, section of the DG of a fluoxetine-treated animal). Within total nestin-CFPnuc cells, the number of ANPs (H), but not QNPs (G), increases in response to fluoxetine. (Scale bars: 50 \(\mu\)m.) In all histograms, white bars correspond to the vehicle injections (V), and gray bars to the fluoxetine injections (F). Error bars show SEM. The results for individual animals (\(n = 8\) per group in this figure) are shown as black dots. *, \(P < 0.05\).

Fig. 4. Fluoxetine increases NB1 cells in the adult DG. (A and B) Immunostaining for PSA-NCAM (green), and nestin-CFPnuc (red). Two cell types are distributed throughout the SGZ, often in close apposition to each other; however, they do not overlap, as illustrated in B (PSA-NCAM cell is red, and nestin-CFPnuc nuclei are green; note that colors are switched at low magnification for better visualization). (C–G) Postmitotic precursors in the fluoxetine-treated DG of adult mice, analyzed 1 day after BrdU labeling. Fluoxetine increases the number of NB1 (C) but not of more advanced NB2 (D) or IN (E) cells. V, vehicle; F, fluoxetine. \(n = 8\) per group. **, \(P < 0.01\). F and G are representative photomicrographs of DG from control (injected with vehicle) (F) and fluoxetine-treated (G) animals. (Scale bars: A, 20 \(\mu\)m; B, 5 \(\mu\)m; F and G, 10 \(\mu\)m.)
cessation of treatment with fluoxetine, we performed the fluoxetine treatment and BrdU labeling as described above but killed the animals 30 days (instead of 1 day) later. In this setting, the number of BrdU-labeled cells was 46.2% higher in the fluoxetine-treated group (234 vs. 342; \( P = 0.037; \) Fig. 6A). The number of BrdU-labeled NeuN-positive neurons also was higher, by 46.3%, in the fluoxetine group (216 vs. 316; \( P = 0.033; \) Fig. 6B–E).

The fraction of BrdU NeuN cells among total BrdU-positive cells did not change (92.7% vs. 92.8%; Fig. 6C); note that the high percentage of BrdU-labeled cells that also stain for NeuN indicates that with or without fluoxetine, the majority of surviving newborn cells in the DG become granule neurons. No change was detected in the volume of the GCL, including the SGZ, between the control and experimental animals (0.496 vs. 0.512 mm\(^3\)).

We also examined changes in the defined classes of precursor cells in mice killed 30 days after the end of the treatment with fluoxetine. Neither the total number of nestin-CFPnuc cells, nor the number of cells in QNP, ANP, NB1, NB2, or IN classes was changed (Fig. 6 F–J), suggesting that once the exposure to fluoxetine ends,
the rate of stem/progenitor cell division returns to its baseline rate. Together, these results suggest that the fluoxetine-induced increase in the number of ANP precursors in the DG later translates into an increase in the number of new neurons. They further suggest that the fate of the newborn cells remains unaltered, i.e., the vast majority of the surplus cells become granule neurons.

Discussion

We here present an approach for the quantitative dissection of the neurogenesis cascade and use this approach to show that fluoxetine targets a defined group of neuronal precursors in the DG. Our results link early progenitor cells to the action of SSRI antidepressants in the adult brain and suggest a strategy to investigate the changes induced by other antidepressant treatments.

Our approach circumvents several obstacles in assessing changes in cell number during neurogenesis, e.g., high cell density, which hinders precise counts, or uncertainty in attributing precursor cells to a particular class. It reduces the complex distribution pattern of precursor cells to a readily quantifiable punctate pattern of labeled nuclei. It allows unambiguous enumeration of cells in a particular precursor class and can be used to analyze changes induced by a wide range of stimuli in the developing or adult brain (8, 10, 11).

By using this approach, we identified six distinct classes of cells that comprise discrete steps in the differentiation cascade between neural stem cells and fully differentiated granule neurons; these classes can be distinguished easily by a combination of expressed markers and by morphology. They encompass and partially overlap with the categories of neuronal precursors defined by other approaches (13, 18–20, 25–28). For instance, QNP cells correspond to a readily quantifiable punctate pattern of labeled nuclei. It allows unambiguous enumeration of cells in a particular precursor class and can be used to analyze changes induced by a wide range of stimuli in the developing or adult brain (8, 10, 11).

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