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Short communication

Antidepressants alter cell proliferation in the adult brain in vivo and in neural cultures in vitro

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Abstract

The action of antidepressants on cell proliferation {bromodeoxyuridine (BrdU) or [^{3}H]thymidine incorporation} was studied in the adult rat hippocampus in vivo and in neural precursors (immature rat cerebellar granule cells) in vitro. In vivo, prolonged (21 days) but not acute (single) intraperitoneal treatment with fluoxetine (5 mg/kg) resulted in a 3.4-fold increase of bromodeoxyuridine-positive cells in the subgranular zone of the dentate gyrus. In cell cultures, at 1 and 10 days in vitro, 48-h fluoxetine exposure (1 μM, which is comparable to therapeutic plasma concentrations) reduced thymidine incorporation when initiated at 1 day in vitro, but increased cell proliferation when initiated at 10 days in vitro. Clomipramine and imipramine produced similar action in vitro; desipramine was ineffective.

Keywords: Antidepressant; Fluoxetine; Hippocampus; Neurogenesis; Cerebellar granule

1. Introduction

Jacobs et al. (2000) recently reviewed the current state of knowledge regarding adult brain neurogenesis, and proposed that clinical depression could be one of the psychiatric illnesses in which impaired neurogenesis could play a significant role. Their article also stresses the possible stimulatory role of the neurotransmitter serotonin on neurogenesis. Although attractive, the proposed concept still lacks experimental support. Nevertheless, some preliminary data indeed suggest that antidepressant drugs that inhibit serotonin reuptake (including fluoxetine), when administered chronically to rats, increase neurogenesis in the hippocampus of adult animals (Jacobs and Fornal, 1999; Malberg et al., 1999). The exact molecular mechanisms of how antidepressants stimulate cell proliferation are still unknown, however. In non-neuronal cells in culture, fluoxetine was capable of both *inhibiting* and *stimulating* cell proliferation, depending on the status of intracellular signaling pathways (Edgar et al., 1999; Genaro et al., 2000).

In this work, we have studied the action of antidepressants on cell proliferation, both in the adult hippocampus in vivo and in primary cultures of rat cerebellar granule cells in vitro. Previously, it was shown that a pharmacological treatment that increases cell proliferation/neurogenesis in the adult rat hippocampus in vivo, e.g., with insulin-like growth factor I (IGF-I) (Aberg et al., 2000), is also mitogenic to cerebellar granule cells in vitro (Cui and Bulleit, 1998); *reduction* of neurogenesis was observed after opiate administration both in the adult rat hippocampus in vivo (Eisch et al., 2000), and in cerebellar granule cells in vitro (Hauser et al., 2000).

2. Materials and methods

2.1. Animals, cell cultures, and drug treatment

Brown–Norway rats (217 BNRIJ; Harlan, Indianapolis, IN) weighing 240–260 g were housed three per cage under a 14-h light/10-h darkness cycle (darkness commenced at 18:00). They were injected with fluoxetine (5 mg/kg; gift from Dr. Alessandro Guidotti) or its vehicle (1% dimethylsulfoxide, DMSO, in saline) 1 h prior to darkness, and with bromodeoxyuridine (BrdU) before sacrifice. Rats were sacrificed 18 h after either the 1st injection (acute treatment) or after the 21st injection (chronic treatment). Four

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to 5 h before sacrifice, all rats received BrdU (intraperitoneally, 60 mg/kg) to label the dividing cells.

Primary cultures of rat cerebellar granule cells were prepared from the cerebella of 7-day-old rat pups (Sprague–Dawley); they were prepared and grown in a serum-free medium and in 24-well plates (300,000 cells/0.5 ml medium/well) as described elsewhere (Manev and Uz, 1999). This culture consists mostly of neuronal precursors that differentiate into neurons within about a week in vitro (Fiszman et al., 2000). We tested the following compounds: fluoxetine (RBI; F-132), clomipramine (RBI; C-129), imipramine (RBI; I-111), desipramine (RBI; D-125), and serotonin (Sigma; H-4511). All compounds were dissolved in DMSO (Sigma); 1 μl/well was added directly from stock solutions; controls were treated with DMSO. Two experimental designs were used: (a) treatment initiated 24 h after plating (1 day in vitro; DIV), and (b) treatment initiated 10 days after plating (10 DIV). Cell proliferation was assayed 48 h later (i.e., at 3 and 13 DIV, respectively), as the incorporation of [^{3}H]thymidine/well. Previous in vitro studies have established an effect on cell proliferation of 1 μM fluoxetine, a concentration comparable to plasma levels of therapeutic concentrations of this antidepressant (Edgar et al., 1999). Thus, 1 μM concentrations were used in most of our experiments.

2.2. BrdU assay of cell proliferation

The brains were fixed in 4% p-formaldehyde, followed by post-fixation overnight and infiltration with 30% sucrose/phosphate buffer. Frozen coronal sections were taken serially every 30 μm throughout the entire extent of the hippocampal formation. They were maintained in PBS at 4°C until assay. Sections were first treated with HCl (2N) for 1 h at room temperature and neutralized with sodium borate (0.1 M) for 30 min. After rinsing with PBS, sections were bleached with 0.3% hydrogen peroxide/10% methanol/PBS for 30 min at room temperature to inactivate endogenous tissue peroxidases. Sections were rinsed, then blocked for at least 1–2 h with 3% normal goat serum/0.1% Triton X-100/PBS. Anti-BrdU antibody (clone G3G4, hydridoma supernatant, developed by Dr. S. Kaufman and obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa) was applied overnight at 4°C at a dilution of 1:300. After rinsing, biotinylated anti-mouse immunoglobulin G (IgG; adsorbed to remove cross-reactivity with rat serum proteins) was added at 1:750 for 2 h at room temperature. After rinsing, an avidin–biotin–peroxidase conjugate was added (Vector Labs Vectastain Elite kit, Burlingame, CA) for 2 h, then rinsed. A permanent brown reaction product was visualized using 3,3′-diaminobenzidine (0.33 mg/ml) and hydrogen peroxide (0.0024%), 15 min at room temperature. As negative controls, some sections were processed without BrdU injection, omitting acid/base treatment, or omitting the primary or secondary antibodies; no stained nuclei were seen under these conditions.

Control and fluoxetine-treated brains were processed in batches in parallel under identical conditions; counts of BrdU-labeled cells were made by an observer who was unaware of group identity. In preliminary studies, upon examining every 12th section of the entire hippocampal formation, we found that the effect of chronic fluoxetine was statistically significant in both the dorsal and the ventral hippocampus, but since the overall extent of labeling and the magnitude of the fluoxetine effect were both greater in the dorsal hippocampus, this region was examined in further detail. For scoring each brain, a series of six to nine sections was chosen at random for scoring within the dorsal hippocampal region defined by Paxinos and Watson as the region spanning from Bregma-2.56 to -3.80. BrdU-labeled nuclei were counted in all subfields of the dentate gyrus, and assigned to one of four compartments: hilus, subgranular zone (border of hilus and granule cell layer; highly enriched in neural progenitor cells), granule cell layer, or molecular layer. Excluded were labeled nuclei associated with blood vessels or the upper surface of the molecular layer. For each animal, the mean value of BrdU-positive cells per section was calculated and used for group analyses. Qualitatively, the pattern of BrdU-labeling was similar to that reported by other investigators, and was similar in both control and fluoxetine-treated groups: about 65% of the total labeled cells resided in the subgranular zone, as single cells or small clusters.

2.3. [^{3}H]thymidine assay of cell proliferation

The cerebellar granule cell cultures were grown in 24-multiwell dishes and were treated with 1 μCi/ml [^{3}H]thymidine (Amersham; TRK120) for 6 h (Fig. 2). After incubation, the medium with radioactive thymidine was removed and cultures were washed twice with Locke's buffer (buffer as in: Manev et al., 1989). Cells were detached using a trypsin/EDTA solution (Sigma), collected in individual tubes, and processed on a cell harvester equipped with Whatman GF/C filters. Samples were washed six times. Filters were allowed to dry and the radioactivity was counted in a liquid scintillation β counter (Fiszman et al., 2000), and is expressed as a percentage of the corresponding DMSO-treated control.

2.4. Statistics

Analysis of variance (ANOVA) was followed by the Student's *t*-test or the Dunnett's test for multiple comparison with control. $^*P < 0.05$ was taken as significant.

3. Results

Chronic fluoxetine administration in vivo resulted in a 3.4-fold increase of BrdU-positive cells (control = 2.11 ±

0.52; fluoxetine = 7.16 ± 0.68; see Fig. 1 for details) whose position in the subgranular zone (border of hilus and granule cell layer) of the dentate gyrus (Fig. 1) corresponds to the granule cell precursors described by others (Jacobs and Fornal, 1999; Malberg et al., 1999). The single fluoxetine injection did not significantly alter their numbers (Fig. 1).

In vitro, the 48-h exposure of cerebellar granule cells to fluoxetine concentration-dependently decreased cell proliferation assayed at 3 DIV, but increased it at 13 DIV (24-h exposure was ineffective; not shown) (Fig. 2A). Interestingly, clomipramine and imipramine produced a similar effect, whereas desipramine was ineffective in both conditions (Fig. 2B). Higher concentrations of desipramine (5 and 10 μM) also failed to stimulate proliferation at 13 DIV (not shown). Serotonin (5-HT) was also tested. At 1 μM, it slightly decreased proliferation at 3 DIV (not shown) but increased it at 13 DIV: 1 μM 5-HT = 149 ± 19*; 5 μM 5-HT = 181 ± 21*; 10 μM 5-HT = 198 ± 17* (mean ± S.E., expressed as percent control; *$P < 0.05$ vs. control; $n = 8$/group).

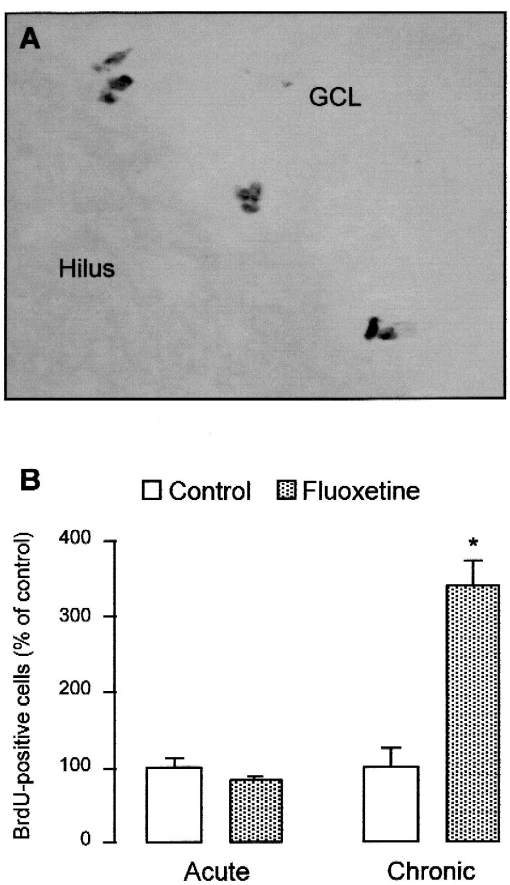

Fig. 1. Stimulator effect of fluoxetine on cell proliferation in the adult rat hippocampus. (A) Example of BrdU-positive nuclei (detected with the anti-BrdU antibody G3G4; Developmental Studies Hybridoma Bank, University of Iowa) in the subgranular zone [the border between the hilus and the granule cell layer (GCL)] of the dentate gyrus of a rat chronically treated with fluoxetine. (B) Quantitative analysis of the number of BrdU-positive cells revealed a significant (*$P < 0.001$; ANOVA) increase due to chronic fluoxetine treatment ($n = 7$) vs. the vehicle administration ($n = 7$) (21 daily injections). Results (mean ± standard error) are expressed as percentage of corresponding controls.

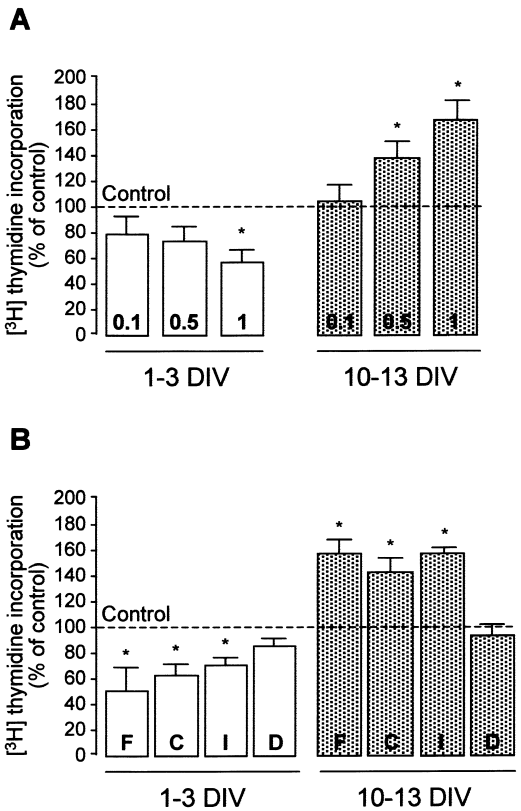

Fig. 2. Dual effects of antidepressants on the proliferation (thymidine incorporation) of rat cerebellar granule cell cultures: (A) fluoxetine (48-h treatment started at 1 or 10 DIV) triggers a concentration-dependent inhibitory effect in younger cultures and stimulatory effect in older cultures (numbers in the bars indicate concentrations in μM). (B) The drugs (indicated by capital letters in individual bars; 1 μM each) fluoxetine (F), clomipramine (C), imipramine (I), or desipramine (D) were added to cerebellar granule cells for 48 h, starting at 1 or 10 DIV. Results (mean ± standard error) are expressed as percentage of corresponding control values; *$P < 0.05$ compared with corresponding control ($n = 6$–12 wells/per group).

4. Discussion

Our in vivo findings are compatible with the in vivo results reported earlier in abstract form by others, and might be indicative of fluoxetine-stimulated neurogenesis in the adult brain (Jacobs and Fornal, 1999; Malberg et al., 1999). A prolonged fluoxetine treatment was required to increase hippocampal cell proliferation, suggesting that the mechanisms involved might correspond to those responsible for therapeutic effect (Jacobs et al., 2000). A small decrease in BrdU labeling ($p = 0.18$) was observed after the single fluoxetine injection ($n = 4$), in comparison with corresponding controls ($n = 5$); this could possibly be caused by a transient increase in glucocorticoid secretion,

elicited by fluoxetine, which normalizes after repeated drug administration (Duncan et al., 1998).

Our in vitro findings of a dual effect of antidepressants, i.e., inhibition of cell proliferation in younger cerebellar granule cell cultures and stimulation in older cultures, may be related to differences in intracellular signaling systems at different DIV (Edgar et al., 1999), or may be due to a possible heterogeneity of cells in the cerebellar granule cell cultures. Young cultures proliferate at a much higher rate and are mostly comprised of neuronal precursors; proliferation in older cultures might originate in non-neuronal cells. Although further experiments are needed to clarify these possibilities, it is interesting that antidepressants that predominantly affect the serotonergic system (fluoxetine, clomipramine) or both serotonergic and noradrenergic systems (imipramine) stimulated cell proliferation, whereas desipramine (affects mostly the noradrenergic system) was ineffective. It is unlikely that the effects of the drugs in cerebellar granule cell cultures were mediated by their action on endogenous serotonin; namely, serotonin was not added to the culture medium, and cerebellar granule cells do not synthesize it. The fact that serotonin also stimulated proliferation in older cultures suggests that some serotonin-related target, possibly the 5-HT 1A receptor (Jacobs et al., 1998), may be involved. Although further research is needed to identify this target, our results point to antidepressants as a class of clinically used drugs that appear to exert a profound effect on proliferation of cells in the central nervous system in vivo and in vitro. Whether this action of antidepressants affects neurogenesis (a complex process comprised of both cell proliferation and cell survival), and whether it is involved in their therapeutic efficacy and/or in their side-effects, remains to be clarified.

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