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Discovery of Novel Hippocampal Neurogenic Agents By Using an *in vivo* Stable Isotope Labeling Technique

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Abstract

Neurogenesis occurs in discrete regions of adult mammalian brain, including the subgranular zone of the hippocampus. Hippocampal neurogenesis is enhanced by different classes of antidepressants, but screening for neurogenic actions of novel antidepressants has been inefficient due to limitations of BrdU labeling techniques. We describe an efficient *in vivo* method for measuring hippocampal neurogenesis, involving incorporation of the stable isotope, ^2H, into genomic DNA during labeling with ^2H_2O (heavy water). Male rodents received 8-10% ^2H_2O in drinking water; DNA was isolated from hippocampal progenitor cells or neurons. Label incorporation into progenitor cells of Swiss Webster mice revealed subpopulation kinetics: 16% divided with $t_{1/2}$ of 2.7 weeks; the remainder did not divide over 1 year. Progenitor cell proliferation rates in mice were strain-dependent. Chronic antidepressant treatment for 3 weeks, with ^2H_2O administered during the final week, increased progenitor cell proliferation across all strains tested. Fluoxetine treatment increased ^2H incorporation into DNA of gradient-enriched neurons or flow-sorted neuronal nuclei, 4 weeks after ^2H_2O labeling, representing the survival and differentiation of newly divided cells into neurons. By screening 11 approved drugs for effects on progenitor cell proliferation, we detected previously unrecognized, dose-dependent enhancement of hippocampal progenitor cell proliferation by two statins and by the anticonvulsant, topiramate. We also confirmed stimulatory activity of other anticonvulsants and demonstrated inhibition of progenitor cell proliferation by isotretinoin and prednisolone. In conclusion, stable isotope labeling is an efficient, high-throughput in vivo method for measuring hippocampal progenitor cell proliferation that can be used to screen for novel neurogenic drugs.

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Introduction

In rodents, adult neurogenesis occurs in discrete regions of the brain, particularly in the subventricular zone, giving rise to granule cells in the olfactory bulb, and in the subgranular zone, generating new granule cells in the dentate gyrus of the hippocampus (Altman and Das, 1965; Kaplan and Hinds, 1977; Kuhn et al., 1996). Hippocampal neuronal cells in the adult are formed through replication and differentiation from pluripotent neural progenitor cells (Gage et al., 1998). Immature neurons migrate to the granule cell layer and mature over a period of weeks into granule cells that form functional connections (van Praag et al., 2002).

Hippocampal neurogenesis has emerged as a central therapeutic target for anti-depressant agents, based on accumulating evidence supporting the neurogenic theory of depression (Jacobs et al., 2000; Kempermann, 2002). All known classes of clinical antidepressant drugs, including tricyclics, monoamine oxidase inhibitors and selective serotonin reuptake inhibitors (SSRIs), have been shown to increase cell proliferation in the hippocampus (Malberg et al., 2000; Santarelli et al., 2003). Moreover, Santarelli et al. (2003) provided evidence, by irradiation of the hippocampus, that hippocampal neurogenesis is required to achieve the behavioral effects of antidepressants in animal models. Hippocampal cell proliferation is decreased under conditions of chronic stress, and this effect is reversed by antidepressant treatment (reviewed by Dranovsky and Hen, 2006; Warner-Schmidt and Duman, 2006). Imaging studies have also shown that depressed human subjects exhibit volume loss and atrophy of the hippocampus (Sheline, 2003). In addition to a role in mood disorders, increased neurogenesis may be a repair mechanism in stroke (Lichtenwalner and Parent, 2006) and traumatic brain injury (Emery et al., 2005) and also contribute to learning and memory (Shors et al., 2001).

A number of factors and intersecting pathways influence the drug-induced stimulation of neurogenesis in the hippocampus. For example, antidepressant drugs activate intracellular second messenger systems, leading to the activation of transcription factors and neurotrophic

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factors, finally culminating in increased numbers of new neurons in the hippocampus (Warner-Schmidt and Duman, 2006). The complexity of neurogenic regulation opens the possibility that multiple therapeutic targets may exist, on the one hand, but also makes predicting the effect of any agent acting on a particular target difficult and complicates the interpretation of *in vitro* screening approaches based on receptor binding or modulation of enzyme activities. It is therefore important to validate *in vivo* the neurogenic activity of agents identified through *in vitro* screens; moreover, *in vivo* screening might uncover novel mechanisms that contribute to neurogenesis arising from unanticipated connectivity relationships in the whole organism.

Measurement of neurogenesis *in vivo* has been problematic, however. Early studies used [^3H]-thymidine to label dividing cells (Altman and Das, 1965; Kaplan and Hinds, 1977), while the most commonly used method currently for measuring cell proliferation in the hippocampus involves injecting animals with bromodeoxyuridine (BrdU) 24 hours before sacrifice (Kuhn et al., 1996; Cameron and McKay, 2001). The hippocampus is then serially sectioned for immunohistochemical detection of BrdU, as well as double-immunohistochemical labeling with neuronal markers to establish the phenotype of BrdU-positive cells. BrdU-labeling has limitations, however, such as a short half-life and rapid clearance of BrdU from the brain, variable efficiency of BrdU entry into cellular precursor pools, and the requirement for high doses to accurately estimate the number of proliferating cells (Cameron and McKay, 2001; Gould and Gross, 2002). Moreover, immunohistochemical enumeration of BrdU-labeled cells in the entire hippocampus is labor intensive, so that throughput with BrdU-labeling is not sufficient for use in broad screening or testing of potential neurogenic agents.

Here, we describe the application of heavy water (^2H_2O) as a DNA label for quantifying the proliferation of hippocampal progenitor cells as well as their survival and maturation into neurons. We have previously shown in humans and experimental animals that deuterium (^2H) from ^2H_2O is incorporated constitutively into the deoxyribose moiety of purine deoxyribonucleotides in genomic DNA through the *de novo* nucleotide synthesis pathway (Fig 1)

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and exhibits several advantages as a labeling approach, compared to the use of pyrimidine nucleoside labels like BrdU (Neese et al., 2002). Heavy water is easily administered at a constant level for extended periods, without toxicity, and thereby allows quantitative measurement of cell proliferation over the period of label administration. Since hippocampal cell proliferation is increased by antidepressant treatments, we validated the method by measuring the effects of different classes of antidepressants. In addition, we tested retinoids and glucocorticoids as potential inhibitors of hippocampal progenitor cell proliferation. We then used this biomarker as a relatively high-throughput *in vivo* screening tool to discover previously unrecognized neurogenic stimulatory actions of approved drugs.

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Methods

Animals

All animal studies were carried out within NIH guidelines for the care and use of laboratory animals and received approval from the institutional animal use committee. 10-12 week old male C57Bl/6, Swiss Webster, or Balb/c mice were obtained from Charles River Laboratories (Wilmington, MA) and 10-12 week old male 129SvEv mice were obtained from Taconic (Oxnard, CA). The outbred Swiss Webster strain of mice was used for the initial drug-screening experiments. Subsequent experiments were performed in the inbred 129SvEv strain, as these showed the least inter-animal variability. Male Sprague-Dawley rats (250 g) were obtained from Charles River Laboratories (Wilmington, MA). Animals were housed in a climate-controlled environment and fed standard rodent chow and water *ad libitum*.

^2H_2O Labeling

For ^2H_2O labeling, animals received a priming intraperitoneal bolus of 49 ml/kg >99% ^2H_2O (Spectra Stable Isotopes, Columbia, MD) containing 0.9% NaCl and were maintained on 10% ^2H_2O in drinking water for the duration of the labeling period. In previous studies (reviewed in Jones and Leatherdale, 1991), intake of up to 20% ^2H_2O has no apparent phenotypic or behavioral effects. For protocols involving label incorporation in hippocampal progenitor cells, mice were labeled continuously for up to 1 year. In a separate study, total hippocampal tissue was isolated after 3, 7, or 14 days of labeling. Animals that received drug treatment were labeled during the final 7-10 days of treatment. For studies that assessed proliferation of mature neurons, animals were labeled with 10% ^2H_2O for 3 weeks, after which time the label was discontinued and animals were sacrificed 4 weeks later.

Drug Treatments

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Male rats and mice were treated with antidepressants of different classes such as an SSRI - fluoxetine, a tricyclic – imipramine, or a serotonin-norepinephrine reuptake inhibitor (SNRI) - venlafaxine, administered in drinking water. The drug solutions were dissolved at a concentration of 100, 200 and 100 mg/L, for fluoxetine, imipramine and venlafaxine, respectively. These concentrations were calculated to achieve a dose of 10 mg/kg/day for fluoxetine and venlafaxine and 20 mg/kg/day for imipramine, based on the average cage consumption of water (3 ml / 30g mouse or 25 ml / 250g rat). Treatment with anti-depressant drugs was continued for 3-5 weeks.

Various approved drugs were screened for neurogenic activity (Table 1). These agents were selected based on long-standing use in humans and on the recognition of having several therapeutic actions (i.e., pleiotropic effects). This effort was intended to be a proof-of-concept study to demonstrate 'indications discovery', i.e. ability to find unexpected actions of agents with potential use in a new indication. The doses selected were based on use in published preclinical studies with these agents, and the concentrations in food or water were calculated based on the average cage consumption of food (3 g / 30g mouse) and water (3 ml / 30g mouse). As a follow-up study after initial screening, a potential 'class' effect for one of the agents (topiramate) tested in the initial screen was investigated by treating mice orally for 3 weeks with one of 11 other anticonvulsants: valproate (1 g/kg), clonazepam (3 mg/kg), gabapentin (100 mg/kg), carbamazepine (30 mg/kg), ethosuximide (300 mg/kg), levetiracetam (30 mg/kg), oxcarbazepine (100 mg/kg), phenytoin (100 mg/kg), primidone (100 mg/kg), tiagabine (30 mg/kg) or zonisamide (50 mg/kg).

Dose response studies were also done for topiramate (10, 30, 100, 150 mg/kg, p.o.), atorvastatin (1, 3, 10 and 30 mg/kg in diet), simvastatin (1, 3, 10 and 30 mg/kg, p.o.). In addition, mice received chronic treatment with potential inhibitors of neurogenesis such as isotretinoin (1 and 3 mg/kg, i.p.) or prednisolone (5 and 40 mg/kg, in diet).

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Isolation of hippocampal progenitor cells and neurons

Animals were euthanized by CO_2 asphyxiation, brains were immediately removed and the hippocampus was dissected out. Progenitor cells and neurons were isolated by a modification of methods described previously (Palmer et al., 1999). Briefly, tissues were finely minced and digested in a solution of papain (4 U/ml; Worthington Biochemical Corporation, Lakewood, NJ) and DNase (250 U/ml; Roche Applied Science, Indianapolis, IN) dissolved in Hibernate-A (BrainBits LLC, Springfield, IL). The digested tissue was then mechanically triturated and thoroughly mixed with an equal volume of Percoll solution, made by mixing nine parts of Percoll (Amersham Biosciences, Piscataway, NJ) with one part 10X PBS. The cell suspension was fractionated by centrifugation for 30 min, 18°C at 20,000 X g. Density beads were run in parallel and the progenitor cells fractionated between 1.064 – 1.075 g/ml, whereas neuronal cells fractionated at densities ≤1.035 g/ml. The progenitor and neuronal cell fractions was collected, washed free of Percoll, and stored frozen at -20°C until isolation of DNA. A flow chart of the method is shown in Fig 1A.

Characterization of progenitor cells and neurons by flow cytometry

The gradient-purified progenitor cells were immunofluorescently stained for intracellular markers, nestin and vimentin, and analyzed by flow cytometry. The cells were fixed and permeabilized with IntraCyte™ buffers (Orion Biosolutions, Vista, CA) and incubated overnight at 4°C with mouse anti-nestin (1:50, Rat401, BD Pharmingen, San Diego, CA) or mouse anti-vimentin (1:50, BD Pharmingen, San Diego, CA) primary antibodies. Gradient-purified hippocampal neurons were stained for tetanus toxin C fragment (TTX), a cell surface marker for neurons. The cells were fixed with 4% paraformaldehyde and incubated with tetanus toxin C fragment followed by anti-TTX mouse monoclonal antibody (Roche applied Science, Indianapolis, IN). After washing, cells were incubated with ALEXA488-conjugated goat anti-

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mouse IgG secondary antibody (Molecular Probes, Eugene, OR). Propidium iodide (PI) was used to stain DNA, and ALEXA488 staining of PI-positive single cells (gated on plots of forward scatter peak area vs. peak height) was analyzed on a Coulter EpicsXL cytometer (Beckman Coulter).

Isolation of nuclei from mature neurons and sorting NeuN positive nuclei by flow cytometry

Neuronal nuclei were isolated from frozen hippocampal tissue by a modification of a recently-described method (Spalding et al., 2005). Briefly, tissue was homogenized in 1 ml lysis buffer (0.32 M sucrose, 5 mM $CaCl_2$, 3 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris-HCl [pH 8.0], 0.1% Triton X and 1mM DTT). Homogenized samples were gently suspended in 1.8 ml of sucrose solution (1.8 M sucrose, 3 mM magnesium acetate, 1 mM DTT, 10 mM Tris-HCl [pH 8.0]), layered onto a cushion of 1 ml sucrose solution, and centrifuged at 30,000 g for 2.5 hr at 4º C. The isolated nuclei were resuspended in 1 ml PBS and stored overnight at 4º C.

To identify neuronal nuclei, anti-NeuN antibodies were directly conjugated with Zenon mouse IgG labeling reagent (Alexa 488, Molecular Probes) by mixing 10 μl of ALEXA 488 conjugate in 100 μl blocking buffer (PBS / 0.5% BSA / 10% normal goat serum) with 300 μl NeuN antibody (1 mg/ml; diluted 1:250 in PBS) and incubating at room temperature for 5 minutes. The suspension of nuclei (1 ml) was added and incubated at 4°C for 1 hour. The nuclei were then washed twice with 3 ml PBS by centrifuging at 1000 x g for 10 min. Propidium iodide was used to stain DNA, and neuronal nuclei were sorted as a homogeneous population of $NeuN^{bright}$ cells, using a Coulter Epics Elite sorter (Beckman Coulter) with gates set for PI-positive single nuclei.

Measurement of cell proliferation

Gas chromatographic/mass spectrometric (GC/MS) analyses were performed as described previously (Neese et al., 2002; Busch et al., 2004) to measure ^2H incorporation from ^2H_2O into

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purine deoxyribonucleotides in genomic DNA. Briefly, DNA was isolated from isolated progenitor cells, sorted neurons or hippocampal tissue using a DNEasy tissue kit (Qiagen, Valencia, CA) and hydrolyzed enzymatically to free deoxyribonucleosides. The deoxyribose moiety of purine deoxyribonucleosides was converted to the pentafluorobenzyl tetraacetate derivative and analyzed by GC/MS in the negative chemical ionization mode, using an Agilent (Palo Alto, CA) model 5973 mass spectrometer and a 6890 gas chromatograph fitted with a db-225 column. Selected ion monitoring was performed with mass-to-charge ratios (*m/z*) of 435 for the M0 and 436 for M1 mass isotopomer, respectively. Incorporation of ^2H from ^2H_2O into purine deoxyribose was quantified as the molar excess fraction M1 (EM1), i.e. the increase over natural abundance (background) defined as the fractional M1 value for an unlabeled DNA standard from calf thymus.

$$EM1 = \frac{(\text{abundance } m/z \text{ } 436)_{sample}}{(\text{abundance } m/z \text{ } 435 + 436)_{sample}} - \frac{(\text{abundance } m/z \text{ } 436)_{standard}}{(\text{abundance } m/z \text{ } 435 + 436)_{standard}}$$

The fraction of newly labeled cells was calculated as the ratio of excess ^2H enrichment (EM1) in isolated cells to the corresponding enrichment in bone marrow DNA (an essentially fully turned-over tissue after 7-10 days of labeling, thereby representing an asymptotic enrichment value for comparison to other tissues), as described previously (Neese et al., 2002). For studies involving shorter labeling periods than 7-10 days, ^2H incorporation in fully turned-over tissue DNA was estimated from body water ^2H enrichments at sacrifice, based on previously established relationships between body water ^2H enrichments and asymptotic labeling in tissues (Neese et al., 2002).

Statistical Analysis

For label incorporation curves, the data were fit by nonlinear regression analysis (SigmaPlot). Student's t-test was used with a 95% confidence interval for comparison between two groups.

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For comparison between multiple groups, one way ANOVA was used, with a post-hoc Student-Newman-Keul's test for all pairwise multiple comparisons or Dunnett's test for comparisons with a negative control (SigmaStat). Data were considered significant at $p < 0.05$.

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Results

Flow cytometric analysis of gradient-enriched hippocampal progenitor cells

Measurement of ^{2}H incorporation into the DNA of hippocampal progenitor cells requires isolation of this cell population from intact tissue. To this end, progenitor cells were isolated from the hippocampus of Swiss Webster mice by Percoll gradient fractionation (Fig. 2A); the isolated cells were fixed and permeabilized, stained for specific intracellular markers of progenitor cells, nestin and vimentin, and analyzed by flow cytometry after gating on nucleated (propidium iodide-stained) cells and excluding doublets. A majority of cells stained positively for either nestin (65% positive in Fig. 2B) or vimentin (72% positive), as compared to isotype controls (2.1% positive). A broad distribution of fluorescence intensity was observed, and the cells remaining in the negative region of the dot plot appeared to be weakly stained.

Label incorporation kinetics in progenitor cells

In order to characterize the proliferation kinetics of gradient-enriched hippocampal progenitor cells, Swiss Webster mice were labeled continuously with 10% ^2H_2O in drinking water, starting at 10 weeks of age; hippocampal progenitor cells were isolated after various labeling times, and ^{2}H incorporation into DNA, analyzed by GC/MS, was used to determine the fraction of cells that had incorporated the label through cell division (Fig. 2C). Approximately 16% of cells incorporated label at plateau, with a half-life within this dividing population of about 2.7 weeks; the majority of cells continued to be unlabeled over the course of a year. This is consistent with a “kinetic subpopulation” pattern wherein a preponderance of non-dividing precursors is present with a subset of actively dividing cells that enters and exits the pool (by differentiation or death). The baseline rate of progenitor proliferation was strain-dependent (Fig 2D). The initial rate of labeling, measured after 1 week of ^2H_2O intake, varied about threefold among four mouse strains tested and were significantly ($p < 0.001$) different from each other. The highest

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proliferation rate was observed in C57Bl/6 mice, whereas the 129SvEv mice had the lowest rate of proliferation.

The fraction of new cells in whole hippocampal tissue of C57Bl/6 mice after 3, 7 or 14 days of labeling was 0.7 ± 0.01%, 1.3 ± 0.3% and 2.9 ± 0.6% respectively. Thus, gradient isolation enriched for proliferating cells, and the consistency of the labeling results indicated that the cell isolation method was highly consistent in this regard.

Effects of anti-depressant drugs on progenitor cell proliferation in rodent hippocampus

Male Swiss-Webster mice (Fig. 3A) or Sprague-Dawley rats (Fig. 3B) were treated for 3 weeks with anti-depressant drugs of different classes: an SSRI (fluoxetine, 10 mg/kg/day), a tricyclic, (imipramine, 20 mg/kg/day) or an SNRI (venlafaxine, 10 mg/kg/day). After labeling with ^2H_2O during the last week of treatment, antidepressant-treated animals from all groups showed a significant increase in the progenitor cell proliferation rate in the hippocampus (Fig 3A and 3B). Both baseline proliferation and the magnitude of the drug effects were somewhat different between mice and rats, however. Fluoxetine treatment produced a significant ($p < 0.01$) increase in the hippocampal progenitor cell proliferation in C57Bl/6, 129SvEv, Swiss Webster, and Balb/c mice (Fig 3C). The percent stimulation of progenitor cell proliferation by fluoxetine was similar across strains.

Effects of anti-depressant treatment on survival and differentiation of newly divided cells

A “pulse-chase” protocol was used to assess the survival and differentiation of progenitor cells into neurons in vehicle- and fluoxetine-treated animals. Male 129SvEv mice were treated with fluoxetine (10 mg/kg/day) or vehicle for 5 weeks and labeled with 10% ^2H_2O for the last 3 weeks of treatment. A longer labeling time was used in this protocol, to allow greater numbers of newly divided progenitor cells to be labeled, allowing for some label loss due to death of labeled cells and differentiation into non-neuronal progeny. The antidepressant treatment and ^2H_2O intake

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were then discontinued, and the animals were followed for another 4 weeks, after which time intact neurons or neuronal nuclei were isolated for analysis of DNA labeling. Fig. 4A shows that > 80% of gradient-enriched hippocampal neurons stained positively for tetanus toxoid (TTX), a surface marker for neuronal cells. Further purification of neurons by flow cytometric sorting proved difficult, however, because of the large size range of neuronal cell bodies and large amount of axonal debris present in these gradient fractions (data not shown). Successful resolution of $NeuN^{bright}$ and $NeuN^{dim}$ nuclei obtained from frozen mouse brain was possible (Fig. 4B), allowing sorting of brightly stained nuclei of mature neurons to > 98% purity upon reanalysis (not shown). GC/MS analysis of extracted DNA from both preparations showed that fluoxetine treatment produced a significant increase in the fraction of ^{2}H-labeled neurons, 4 weeks after the end of a 3-week labeling period (Fig 4, A and B). ^{2}H incorporation into DNA of gradient-enriched neurons exceeded that of $NeuN^{bright}$ neuronal nuclei; although Percoll gradients efficiently resolve proliferating neural progenitors from neuronal cells, the gradient-enriched population may include a greater proportion of immature neurons derived from dividing precursors that do not yet express high levels of NeuN. Taken together, these results confirm that differentiation of recently divided precursors into mature neurons is enhanced by fluoxetine and show that this antidepressant drug effect is detectable by $^{2}H_2O$ labeling.

Effect of retinoid or glucocorticoid administration on progenitor cell proliferation

We also evaluated the activity of a retinoid and a glucocorticoid on hippocampal progenitor cell proliferation. Male 129SvEv mice were treated with isotretinoin (1 or 3 mg/kg, i.p.) for 3 weeks and labeled with 10% $^{2}H_2O$ in the final 10 days of treatment. There was a dose-dependent decrease in hippocampal progenitor cell proliferation following retinoid treatment (Fig 5A). Treatment with 3 mg/kg dose of isotretinoin produced a 38% reduction over baseline which was statistically significant ($p < 0.05$) compared to vehicle-treated controls.

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Male Swiss Webster mice were treated with prednisolone (5 or 40 mg/kg in diet) and labeled with 8% ^2H_2O for 4 weeks. These mice received a slightly different treatment and labeling protocol since they were part of a study investigating the effects of glucocorticoid treatment on several metabolic pathways concurrently. Prednisolone treatment produced a dose-dependent significant ($p < 0.01$) decrease in hippocampal progenitor cell proliferation (Fig 5B). Progenitor cell proliferation was significantly (52%) reduced at the 40 mg/kg dose, compared to untreated controls, and showed a trend toward reduction at the 5 mg/kg dose.

Use of the hippocampal progenitor cell proliferation assay to screen drugs

Next, we explored the feasibility of using hippocampal progenitor cell proliferation as a screen for candidate neurogenic agents. To this end, a panel of approved drugs with known or suspected pleiotropic actions (Table 1), but not previously known to have neurogenic activity in normal murine hippocampus, was tested for their effects on proliferation of hippocampal progenitor cells in Swiss Webster mice (Fig. 6). Two of these agents were found to increase the rate of progenitor cell proliferation. The hydroxymethyl-glutaryl CoA (HMGCoA) reductase inhibitor (statin), atorvastatin (10 mg/kg p.o.), and the anticonvulsant, topiramate (100 mg/kg in diet), each given daily for 3 weeks, significantly increased progenitor cell proliferation, measured by ^2H_2O labeling during the third week. The increases were of a similar magnitude as observed with the positive controls, fluoxetine and imipramine (Fig. 6). No stimulatory effects were seen for 9 other drugs given by various routes (diet, drinking water, oral gavage, or i.p.) at daily doses reported in the literature to be pharmacologically active for other actions (Table 1).

In follow-up studies, we assessed dose-response relationships of the compounds with stimulatory activity and tested other agents in their classes (statins and anticonvulsants). Topiramate produced a dose-dependent increase in the proliferation of hippocampal progenitor cells of 129SvEv mice (Fig 7A). Oral treatment with 30 mg/kg/day produced an approximately 70% increase over baseline proliferation, approaching a plateau at higher doses (100 and 150

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mg/kg/day). We also screened 11 other anticonvulsant drugs at single doses known to have anticonvulsant activity in animals (Fig 7B). Treatment of Swiss Webster mice for 3 weeks with either valproate (1 g/kg in diet) or oxcarbazepine (100 mg/kg, p.o.) significantly increased progenitor cell proliferation. Treatment with valproate doubled the number of proliferating cells as compared to vehicle, while oxcarbazepine treatment produced a 67% increase over baseline proliferation. A valproate stimulatory effect on neurogenesis in the hippocampus has been previously reported (Hao et al., 2004), but the activity of oxcarbazepine is a novel finding. The other anticonvulsants either had no effect on progenitor cell proliferation or were slightly inhibitory at the single doses tested. We conclude that a subset of anticonvulsants is neurogenic, as judged by DNA labeling of hippocampal progenitors.

The stimulatory action of statins on hippocampal progenitor cell proliferation was also confirmed and extended in dose-response studies, using atorvastatin or simvastatin. Treatment of 129SvEv mice with increasing doses of atorvastatin (1, 3, 10, 30 mg/kg in diet) produced a dose-dependent increase in hippocampal progenitor cell proliferation, with the maximal (68% increase over baseline) effect seen at the 3 mg/kg dose (Fig 8A). Chronic oral treatment with another HMGCoA reductase inhibitor, simvastatin, also produced a dose-dependent increase in the proliferation of precursor cells (Fig 8B) in the hippocampus of 129SvEv mice. This more lipophilic statin showed significant stimulatory activity at the 10 and 30 mg/kg doses.

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Discussion

Neurogenesis has been difficult to exploit as a drug target for therapeutic actions such as anti-depression or cognitive enhancement, because of methodological and practical limitations in measuring proliferation of brain cells *in vivo* by BrdU immunohistochemistry (cf. Introduction). Besides other methodological and technical concerns, perhaps the most important practical limitation of the BrdU approach for *in vivo* screening is the need for manual counting of multiple tissue sections, which limits throughput. In contrast, mass spectrometric analysis of DNA labeling after oral ^2H_2O labeling has advantages of automated sample processing and data analysis. Measurements are objective, reproducible, quantitative, and amenable to rigorous quality control, thus overcoming the limited throughput of counting $BrdU^+$ cells on serial sections and subjectivity of visual scoring of positive cells. We previously developed a mass spectrometric technique for quantifying proliferation of slowly dividing cells, based on cumulative ^2H incorporation into DNA during continuous labeling with ^2H_2O in rodents and humans, and have applied this approach to a variety of cell types (Neese et al., 2002). We have also demonstrated a significant correlation between BrdU labeling index and fractional synthesis of DNA measured by ^2H_2O method in the same animal (Kim et al., 2005). Here, we have for the first time applied this technique to rodent brain, and shown that it affords sufficient sensitivity, reliability, and throughput (processing of 40-50 samples from cell isolation to GC/MS analysis can be done by a technician in 5 days) to enable the discovery of novel neurogenic agents by *in vivo* screening.

Overall, our ^2H_2O labeling results are consistent with measurements of hippocampal neurogenesis by BrdU labeling. Assuming that the hippocampus has ≈ 2 × 10^6 total cells (Abusaad et al., 1999), the labeling rate of 0.2% per day in total C57Bl/6 hippocampus that we observed with ^2H_2O would be equivalent to ≈ 4000 labeled cells/day, a value comparable to estimates by 12-24-hour saturation labeling with BrdU (Hayes and Nowakowski, 2002). Palmer et al. (1999) reported that 0.7% of progenitor cells were labeled after daily BrdU injection for 6

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days in a confocal analysis of BrdU incorporation into gradient-isolated progenitor cells from rat hippocampus, a similar value as we measured by ^2H_2O labeling on similarly isolated rat hippocampal progenitors (1% per week). In mice, the hierarchy of baseline progenitor cell proliferation across different mouse strains in our study agreed well with strain effects on total hippocampal cell proliferation obtained by BrdU labeling (C57Bl/6 > Balb/c > Swiss Webster > 129/Sv; (Kempermann et al., 1997; Hayes and Nowakowski, 2002) and further supports the view that genetic background strongly influences hippocampal neurogenesis. Interestingly, genetic differences in hipocampal neurogenesis have been shown to correlate with hippocampal function (Kempermann et al. 1998), and recently, using recombinant inbred strain of mice, Kempermann at al. (2006) have identified several genes that control adult neurogenesis. In terms of survival and differentiation of proliferated cells, 1% new NeuN^{+ve} neurons were observed in the hippocampus of 129SvEv mice, 1 month after a 3 week ^2H_2O labeling protocol, similar to the estimation of BrdU-labeled hippocampal granule cells in the closely related 129/SvJ strain of mice (Kempermann et al., 1997). Most importantly for drug studies, ^2H_2O labeling qualitatively and quantitatively reproduced the known enhancing effects of antidepressants of various classes on hippocampal progenitor cell proliferation (Malberg et al., 2000; Santarelli et al., 2003) as well as the known inhibitory effect of isotretinoin on hippocampal progenitor cell proliferation (Crandall et al., 2004), and the pro-neurogenic activity of valproate (Hao et al. 2004). We conclude that, ^2H_2O labeling generates similar results and reveals the same neurogenic drug actions as BrdU labeling.

For screening purposes, we chose to analyze progenitor cells, rather than mature neurons, even though labeling of both was increased by antidepressants. Progenitor cells comprise the majority of proliferating cells in the hippocampus, and sufficient ^2H label for quantification is incorporated into DNA of gradient-enriched progenitors after only 7-10 days of ^2H_2O exposure, whereas label detection in mature neurons takes additional 4 weeks, and the new neurons are diluted into a much larger number of non-dividing cells. Since standard

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antidepressants exhibit a lag period of about 10 days before stimulating neurogenesis in rodent hippocampus (Santarelli et al., 2003), we designed our screening experiments for 3 weeks of treatment, with ^2H_2O labeling for the last 7-10 days. The lag between drug treatment and labeling can be shortened for detecting early onset of action of novel agents.

Using ^2H_2O labeling, we were able to explore aspects of hippocampal cell dynamics that would not have been readily accessible by BrdU labeling. First, analysis of gradient-enriched hippocampal progenitors after up to 1 year of *in vivo* ^2H_2O labeling revealed kinetic heterogeneity in this population: only about 16% of these cells turned over during this time. This finding suggests that high-density hippocampal gradient fractions harbor a mixture of proliferating progenitors and resting cells. ^2H_2O labeling might be valuable in searching for cellular markers associated with the proliferating progenitor cell subset.

Secondly, we were able to exploit the increased throughput of the ^2H_2O progenitor cell proliferation assay to screen a sizable panel of approved drugs for previously unknown neurogenic effects in the hippocampus. Two of the 11 agents tested initially, atorvastatin and topiramate, increased progenitor cell proliferation, and these findings were robust in follow-up dose-response studies. Moreover, the proliferative activity of these agents appeared to be shared by other drugs of their class: a second statin, simvastatin, showed stimulatory activity, as did another two of 12 structurally diverse anticonvulsants tested, oxcarbazepine and valproate. This surprisingly high "hit" rate supports our notion, discussed in detail elsewhere (Turner and Hellerstein, 2005), that unanticipated cross-talk of drug actions on apparently off-target pathways *in vivo* might be more common than previously thought, due to unappreciated connectivity relationships in complex metabolic networks. In the case of neurogenesis, this makes sense in view of the recognized complex regulation of neurogenesis through sensory input, neurotransmitters, hormones, and neurotrophic factors such as brain derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), insulin growth factor (Warner-Schmidt and Duman, 2006), and several other known and unknown pathways.

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The drug activities that we uncovered also seem plausible, after the fact, based on published literature. Atorvastatin has been reported to boost neurogenesis in the hippocampus and subventricular zone after middle cerebral artery occlusion , a model for stroke (Chen et al., 2003), but drug effects in uninjured brain were not measured in that study. Increased expression of VEGF and BDNF may mediate the neurogenic effect of atorvastatin in both stroke-induced (Chen et al., 2005) and in unmanipulated animals. Moreover, statins also alter expression of genes associated with apoptosis, cell growth and signaling (Johnson-Anuna et al., 2005). The proliferation-stimulating activities of oxcarbazepine and topiramate are plausible, given published results with valproate, although these results would have been difficult to predict, as many anticonvulsants did not increase progenitor cell proliferation. Although the exact mechanism of neurogenic activation by some anticonvulsants remains unknown, these drugs influence several neurotransmitter systems, as well as intracellular signaling cascades, such as the extracellular signal regulated kinase pathway which has been shown to be activated by the neurogenic anticonvulsant valproate (Hao et al. 2004). The inhibition of hippocampal progenitor cell proliferation by the synthetic corticosteroid analogue, prednisolone, confirms the suppressive effect of glucocorticoids, since similar effects have been reported for corticosterone and dexamethasone (Cameron and Gould, 1994; Kim et al., 2004).

Intriguingly, the novel pro- and anti-neurogenic drug effects that we discovered closely parallel the known behavioral effects of these drugs. Retinoid therapy may cause depression in humans as a side-effect of treatment (Hull and D'Arcy, 2003). Similarly, conditions exhibiting hypercortisolism (Cushing’s syndrome, stress, glucocorticoid therapy) are also associated with clinical depression (Mitchell and O'Keane, 1998). Conversely, anticonvulsants have been used as augmentation therapy in depression (Hantouche et al. 2005); differential neurogenic activity may guide the choice of anticonvulsants for this therapeutic use. Moreover, there is increasing evidence that statins may have utility in the treatment of neurological diseases (Menge et al., 2005). Long-term use of statins has been shown to be associated with reduced risk of anxiety,

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depression and hostility (Young-Xu et al., 2003). In a trial for Alzheimer's disease, patients treated with atorvastatin for 1 year showed significant improvement of depression (Sparks et al., 2005). Together with our observations of progenitor cell stimulatory activity of these drugs, these findings provide further support for the neurogenic theory of depression and indicate that *in vivo* screens for neurogenesis may be useful in the discovery and preclinical validation of novel antidepressants.

Finally, hippocampal neurogenesis is involved in other biological processes besides depression. Since neurogenesis plays a significant role in synaptic plasticity, disorders of learning and memory may be amenable to discovery efforts using this as a biomarker. Neurogenesis may also be a therapeutic target for other conditions, such as traumatic brain injury, stroke, and Alzheimer's disease.

In conclusion, ^2H_2O labeling represents a quantitative, reproducible and relatively high-throughput *in vivo* method for measuring hippocampal progenitor cell proliferation, which is closely linked to neurogenesis and is useful for screening and discovering novel neurogenic stimulatory drugs. Statins and certain anticonvulsant agents were discovered to have potent stimulating activity and may have therapeutic uses based on this activity.

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Footnotes

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Legends for figures

Figure 1: Pathways which incorporate ^2H into ribose during de novo purine nucleoside synthesis. Potential sites of ^2H incorporation from ^2H_2O into deoxyribose moiety in replicating DNA are shown in color. Abbreviations: PPP, pentose phosphate pathway; GNG, gluconeogenesis; DNPS, de novo purine synthesis pathway; DNNS, de novo nucleotide synthesis pathway, RR, ribonucleoside reductase, ^3H-dT, tritiated thymidine, and BrdU, bromodeoxyuridine.

Figure 2: Phenotype and proliferative characteristics of hippocampal progenitor cells. (A): Schematic illustration of Percoll gradient isolation method. (B): Flow cytometric analysis of fixed and permeabilized progenitor cells incubated with primary antibodies for nestin and vimentin, followed by ALEXA-488 conjugated secondary antibody; propidium iodide (PI) was used to label nuclei. (C): Fractional synthesis of hippocampal progenitor cell DNA in male Swiss Webster mice labeled for either 3 days, 1, 2, 4, 16, 28 or 52 weeks with 10% ^2H_2O. (D): Progenitor cell proliferation rate in male C57/Bl6, Swiss Webster, Balb/c or 129SvEv mice labeled with 10% ^2H_2O for 1 week. *$p < 0.05$ for comparisons between C57Bl/6 and Balb/c, as well as Swiss Webster and 129SvEv, **$p < 0.01$ for comparison between C57Bl/6 and Swiss Webster, ***$p < 0.001$ for comparisons of 129SvEv with C57Bl/6 or Balb/c. Data represent mean ± SEM of 6 animals per group.

Figure 3: Antidepressants increase hippocampal progenitor cell proliferation. (A): Fractional synthesis of hippocampal progenitor cell DNA in male Swiss Webster treated with vehicle, fluoxetine (10 mg/kg/day), imipramine (20 mg/kg/day) or venlafaxine (10 mg/kg/day) in drinking water for 3 weeks and labeled with 10% ^2H_2O during the final week of treatment. *$p < 0.05$ compared to 'Vehicle', data represent mean ± SEM of 8 mice per group. (B): Fractional synthesis of hippocampal progenitor cell DNA in male Sprague-Dawley rats treated with vehicle,

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fluoxetine (10 mg/kg/day), imipramine (20 mg/kg/day) or venlafaxine (10 mg/kg/day) in drinking water for 3 weeks and labeled with 10% ^2H_2O during the final week of treatment. *$p < 0.05$ compared to 'Vehicle', data represent mean ± SEM of 6 rats per group. (C): Fractional synthesis of hippocampal progenitor cell DNA in male C57Bl/6, Swiss Webster, Balb/c or 129SvEv mice treated with fluoxetine (10 mg/kg/day) in drinking water for 3 weeks and labeled with 10% ^2H_2O in the final week of treatment. **$p < 0.01$ compared to 'Vehicle' for each of the strain, data represent mean ± SEM of 6-8 mice per group.

Figure 4: Flow cytometric analysis and label incorporation in neuronal DNA. Male 129SvEv mice were treated with fluoxetine (10 mg/kg/day) in drinking water for 5 weeks with 10% ^2H_2O labeling for the last 3 weeks of treatment, and then sacrificed 4 weeks after the end of treatment and label. (A): (Left) Hippocampal neuronal cells isolated by Percoll gradient fractionation were fixed and incubated with tetanus toxin C fragment (TTX) and anti-TTX primary antibody, followed by ALEXA-488 conjugated secondary antibody, propidium iodide (PI) was used to identify nuclei. (Right) Fractional synthesis of neuronal cell DNA from fluoxetine-treated animals was significantly (**$p < 0.01$) different from vehicle-treated animals, data represent mean ± SEM of 8 mice per group. (B): (Left) Hippocampal nuclei were isolated by ultracentrifugation, incubated with ALEXA-488 conjugated anti-NeuN primary antibody and sorted after gating on PI^{+ve} events. (Right) Fractional synthesis of neuronal nuclear DNA from fluoxetine-treated animals was significantly (***$p < 0.001$) different from vehicle-treated animals, data represent mean ± SEM of 8 mice per group.

Figure 5: Isotretinoin and prednisolone decrease hippocampal progenitor cell proliferation. (A): Fractional synthesis of hippocampal progenitor cell DNA in male 129SvEv mice treated with either vehicle or isotretinoin (1 and 3 mg/kg/day, i.p.) for 3 weeks and labeled with 10% ^2H_2O in the final week of treatment. *$p < 0.05$ compared to 'Vehicle', data represent mean ± SEM of 6

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mice per group. (B): Fractional synthesis of hippocampal progenitor cell DNA in male Swiss Webster mice treated with either vehicle or prednisolone (5 and 40 mg/kg/day) in diet, and labeled with 10% ^2H_2O for 4 weeks. **$p < 0.01$ compared to 'Vehicle', data represent mean ± SEM of 6 mice per group.

Figure 6: Screening of pluripotent drugs for hippocampal progenitor cell proliferation. Fractional synthesis of hippocampal progenitor cell DNA in male Swiss Webster mice treated for 3 weeks with various drugs (details are given in Table 1) and labeled with 10% ^2H_2O in the final week of treatment. *$p < 0.05$ compared to 'Vehicle', data represent mean ± SEM of 6 mice per group.

Figure 7 (A): Dose response of topiramate for hippocampal progenitor cell proliferation. Male 129SvEv mice were treated with vehicle or topiramate (10, 30, 100, 150 mg/kg/day, p.o.) for 3 weeks and labeled with 10% ^2H_2O in the final week of treatment. *$p < 0.05$ compared to 'Vehicle', data represent mean ± SEM of 6 mice per group. (B): Screening of anticonvulsants for hippocampal progenitor cell proliferation. Male Swiss Webster mice were treated with different anticonvulsants (details given in 'Methods') for 3 weeks and labeled with 10% ^2H_2O in the final week of treatment. *$p < 0.05$ compared to 'Vehicle', data represent mean ± SEM of 6 mice per group.

Figure 8: Dose response of statins for hippocampal progenitor cell proliferation. (A): Male 129SvEv mice were treated with atorvastatin (1, 3, 10 or 30 mg/kg/day) in diet for 3 weeks and labeled with 10% ^2H_2O in the final week of treatment. *$p < 0.05$ compared to 'Vehicle', data represent mean ± SEM of 6 mice per group. (B): Male 129SvEv mice were treated with simvastatin (1, 3, 10 or 30 mg/kg/day p.o.) for 3 weeks and labeled with 10% ^2H_2O in the final week of treatment. *$p < 0.05$ compared to 'Vehicle', data represent mean ± SEM of 6 mice per group.

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Table 1

Drug	Class	Dose	Route
Aspirin	NSAID	60 mg/kg/day	Diet
Atorvastatin	HMG CoA Reductase Inhibitor	10 mg/kg/day	Oral gavage
Calcitriol	Vitamin D analog	2.5 μg/kg/day	Diet
Clofibrate	PPAR-α agonist	50 mg/kg/day	Diet
Enalapril Maleate	ACE Inhibitor	2.5 mg/kg/day	Drinking water
Etiocholanedione	Steroid Analog	1.5 g/kg/day	Diet
Flurbiprofen	NSAID	50 mg/kg/day	Intraperitoneal
Ketoconazole	Anti-fungal	100 mg/kg/day	Diet
Methotrexate	Folate Inhibitor	20 mg/kg/2days	Intraperitoneal
Rosiglitazone	PPAR-γ agonist	6 mg/kg/day	Diet
Topiramate	Anti-convulsant	100 mg/kg/day	Diet

Abbreviations: HMG CoA, hydroxyl-methylglutaryl coenzyme A; NSAID, non-steroidal anti-inflammatory drug; PPAR, peroxisome proliferator-activated receptor; ACE, angiotensin converting enzyme.

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Figure 1

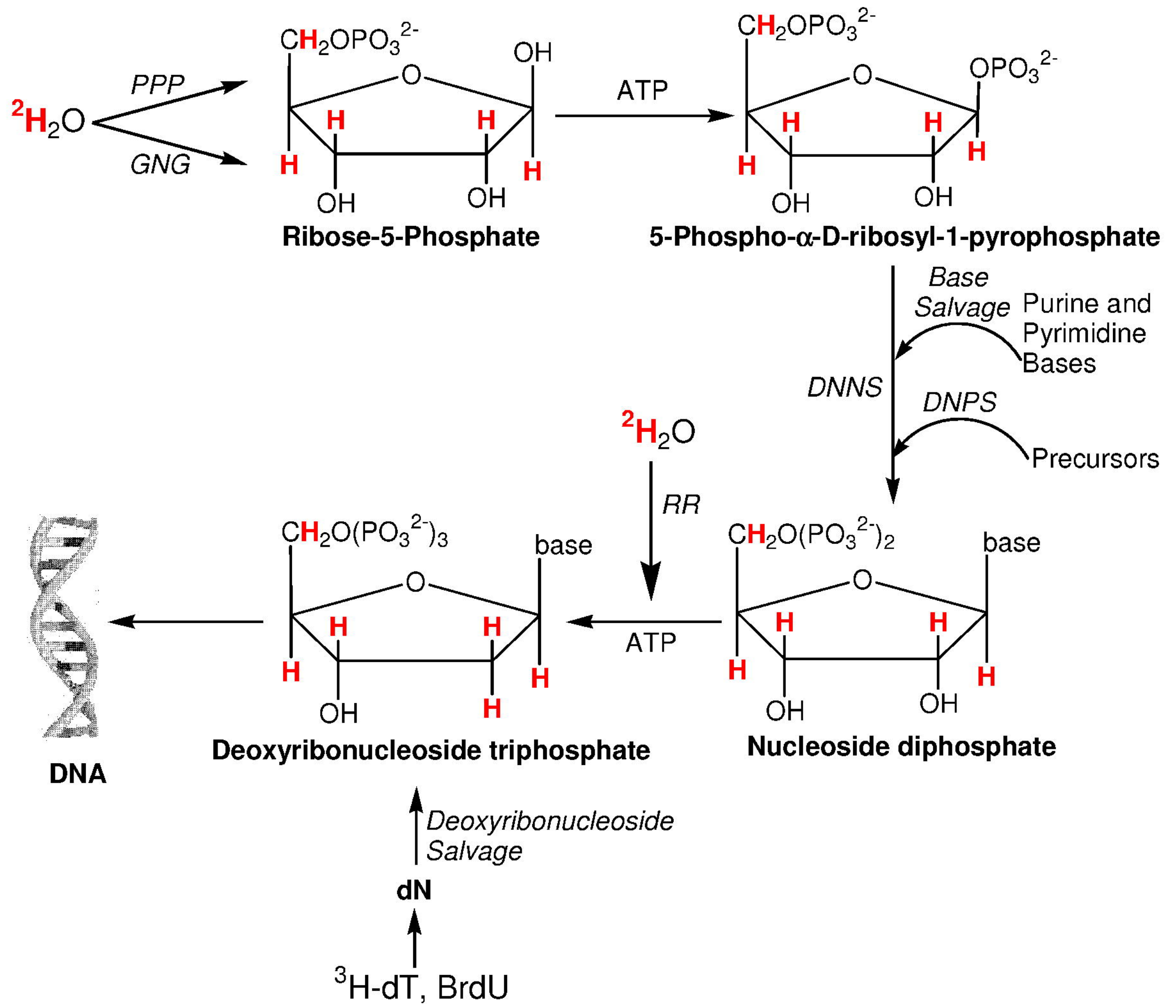

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Figure 2

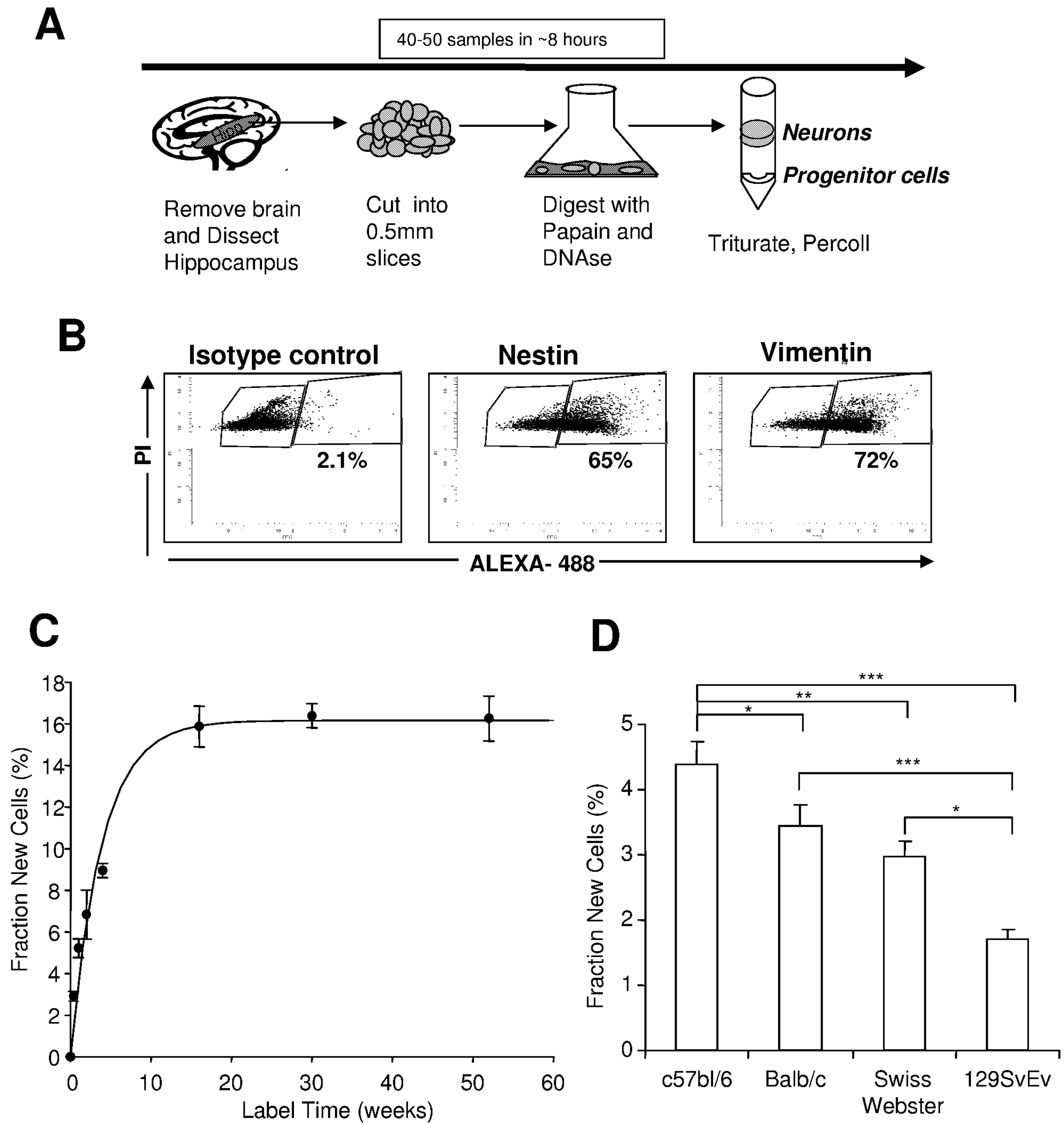

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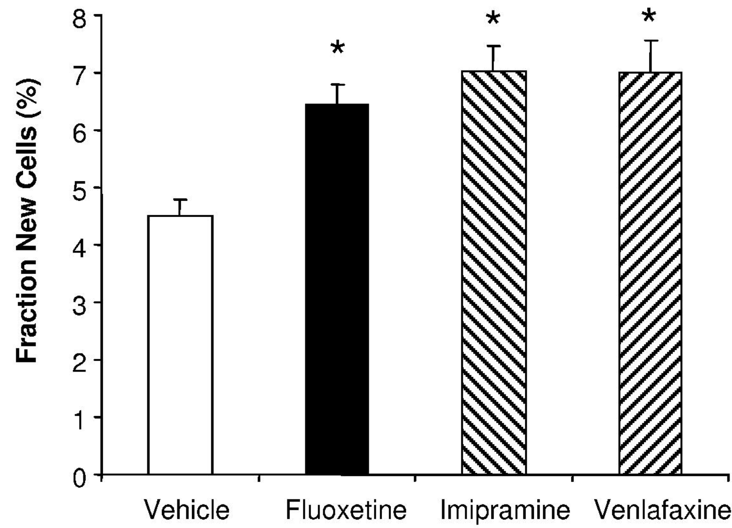
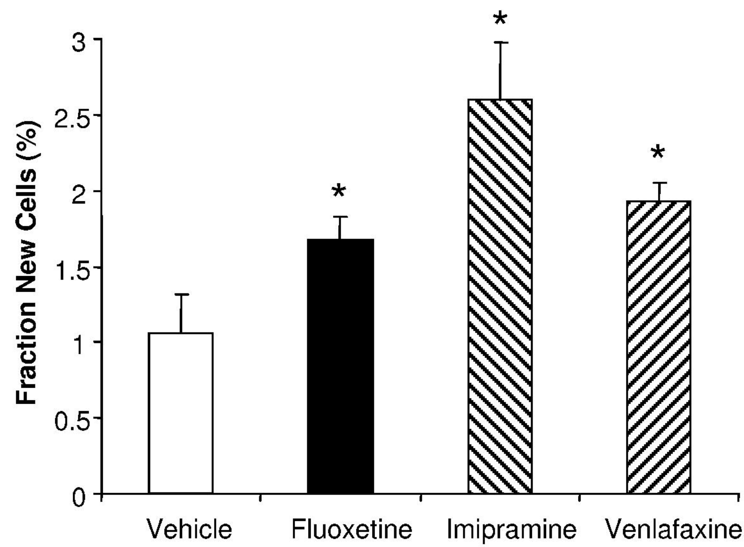
Figure 3

A



B



C

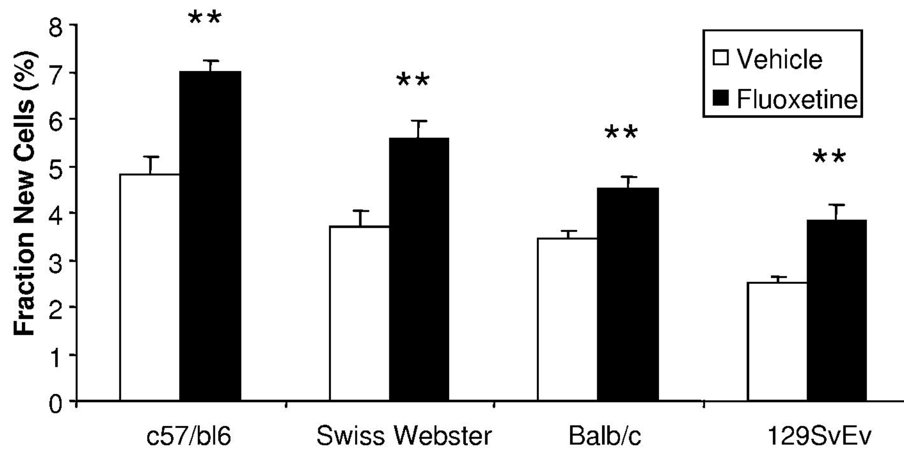


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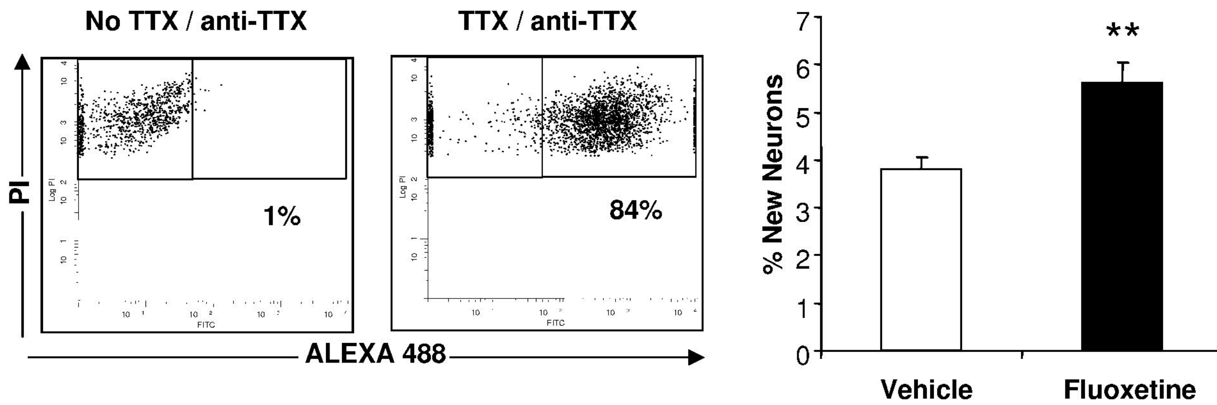
Figure 4



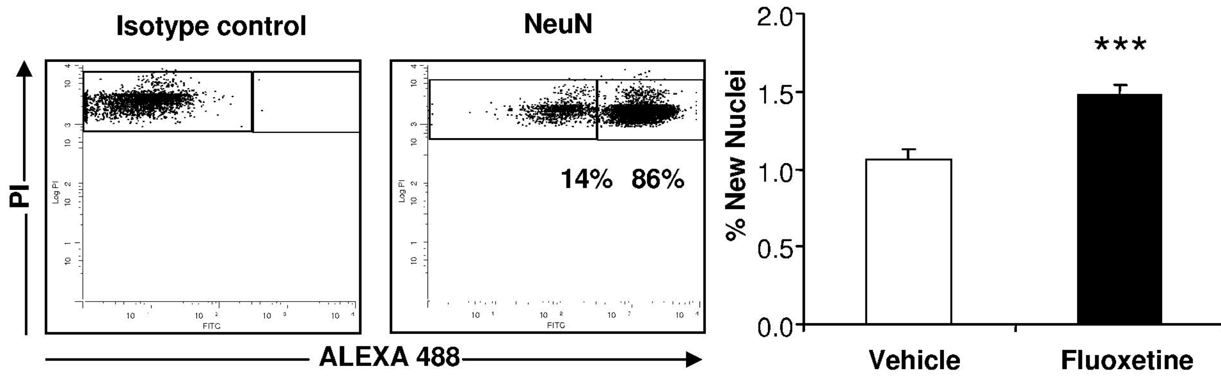


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Figure 5

A

B

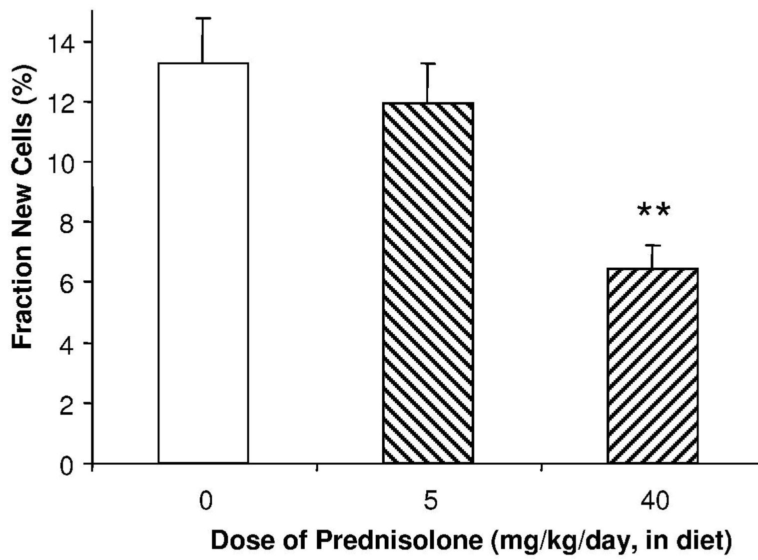

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Figure 6

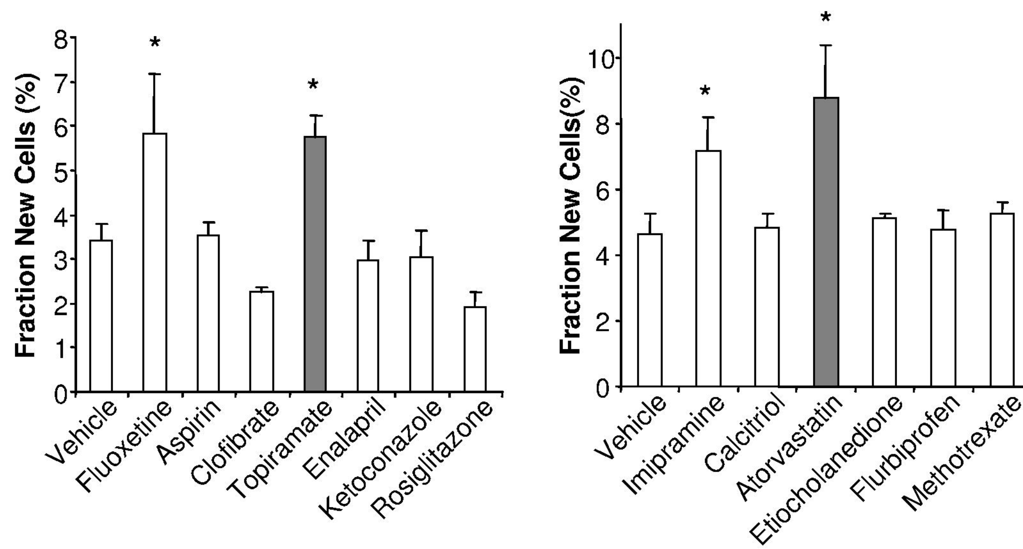

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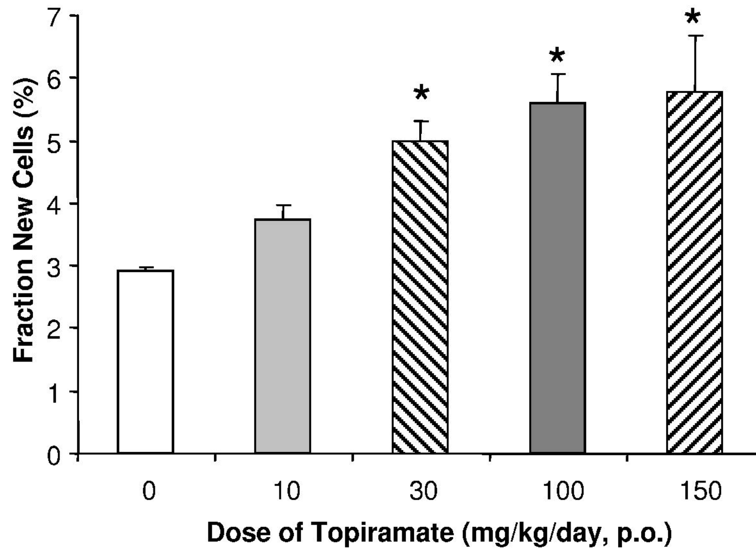
Figure 7

A



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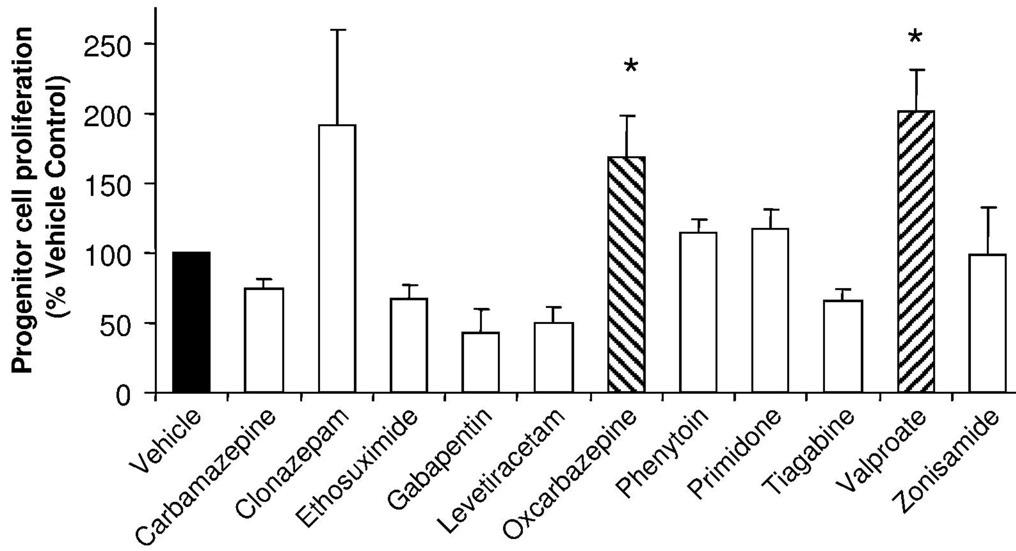

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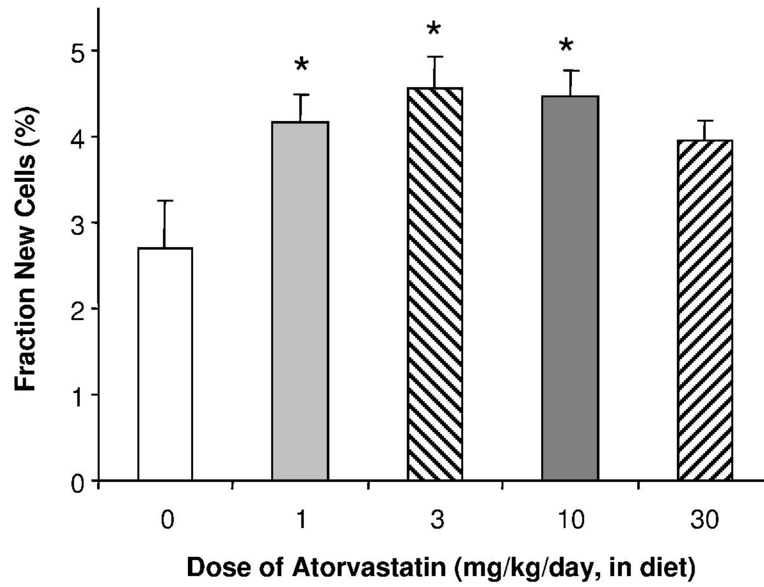
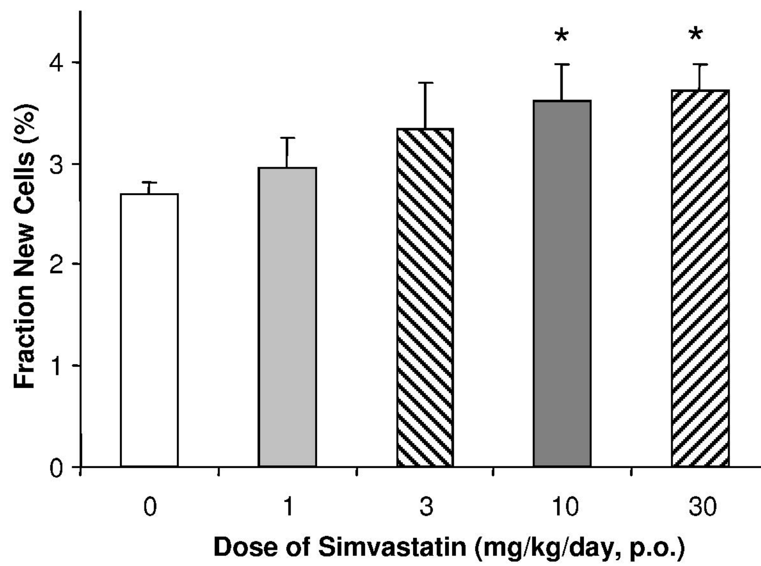
Figure 8

A



B



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