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A rapid method for the quantification of mouse hippocampal neurogenesis *in vivo* by flow cytometry Validation with conventional and enhanced immunohistochemical methods

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Abstract

Neural stem cells reside in the subventricular zone and the dentate gyrus of the hippocampus in adult mammalian brain. In the hippocampus, a number of factors are reported to modulate the rate of neural progenitor proliferation in the hippocampus, such as exercise, corticosteroids, and many pharmacological agents including several classes of antidepressants. It is currently unclear whether this increased proliferation is physiologically relevant, but it provides a potentially useful biomarker to assess novel antidepressant compounds.

Changes in neurogenesis are typically quantified by administration of bromodeoxyuridine (BrdU) *in vivo*, and subsequent quantification of labelled nuclei. A robust and rapid means of quantifying BrdU labelling in adult hippocampus *in vivo* would allow higher throughput screening of potential antidepressant compounds. In this study we describe a FACS-based method for quantification of BrdU labelled cells in fixed cell suspensions from BrdU-treated adult mouse hippocampus. A variety of experimental conditions known to modulate proliferation were tested, including administration of corticosterone and the antidepressants imipramine and fluoxetine. The robust changes compared to control groups observed in these models were similar to previously reported studies, thus offering a more rapid and streamlined means to quantify effects of compounds on hippocampal proliferation.

Keywords: Neurogenesis; Bromodeoxyuridine; BrdU; Corticosterone; Antidepressant; Flow cytometry; FACS; Dentate gyrus

1. Introduction

Neurogenesis persists in the murine brain in the subventricular zone of the anterior lateral ventricle and the subgranular zone of the hippocampal dentate gyrus. These germinal zones give rise to new neurones throughout adulthood; precursors from the subventricular zone migrate along the rostral migratory stream to the olfactory bulb, whereas precursors from the dentate gyrus migrate into the granule cell layer of the dentate gyrus and integrate into the hippocampal circuitry.

Since the discovery of ongoing adult hippocampal neurogenesis, there has been intense study of the regulation of this system. Hippocampal neurogenesis is typically analysed using the thymidine analogue bromodeoxyuridine (BrdU); this is taken up by dividing cells in S-phase and labels DNA of the dividing cells and their progeny, allowing both quantification of numbers of dividing cells and analysis of migration and cell fate. A large number of factors are reported to modulate hippocampal neurogenesis; in rodents, factors shown to increase proliferation in the hippocampus include running wheel exercise (van Praag et al., 1999; Brown et al., 2003), cytokines such as IGF (Aberg et al.,

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2000) or VEGF (Jin et al., 2002), adrenalectomy (Cameron and McKay, 1999), estrogen (Tanapat et al., 1999), neurotransmitters such as 5-HT and noradrenaline, and various insults such as ischaemia and seizure activity (reviewed in Zhao et al., 2004). Similarly, many factors are reported to decrease proliferation in the hippocampus; these include ageing (Kuhn et al., 1996), chronic stress (Gould et al., 1997), glutamate (Cameron et al., 1995), glucocorticoids (Cameron and Gould, 1994), and opiates (Eisch et al., 2000).

Recently there has been a great deal of interest in regulation of hippocampal precursor cell proliferation by antidepressants and by stress. An increasing body of data indicates that antidepressants from different classes, such as the tricyclic imipramine and the selective serotonin reuptake inhibitor fluoxetine, can increase hippocampal cell proliferation (Santarelli et al., 2003). Conversely, chronic stress and stress related glucocorticoids such as corticosterone can decrease hippocampal precursor proliferation. The physiological relevance of these changes in hippocampal neurogenesis remain unclear, though evidence from a recent study blocking hippocampal cell proliferation by irradiation indicates that the increased neurogenesis is relevant for antidepressant efficacy (Santarelli et al., 2003). Regardless of the requirement of neurogenic changes in the clinical effects of antidepressants, changes in hippocampal neurogenesis may constitute a useful biomarker for identifying novel antidepressant compounds.

To measure cell proliferation in the hippocampus, BrdU uptake is typically quantified using immunohistochemical labelling. Brains are sectioned, then immunostained for expression of BrdU and quantified by counting the number of BrdU immunoreactive cells in the dentate in a number of sections and stereological estimation of the total number of BrdU cells in the hippocampus. This technique provides a robust measure of the number of BrdU expressing cells in the hippocampus, but it is laborious and intrinsically low-throughput, taking typically several weeks to complete a large study. A robust and rapid higher throughput methodology to evaluate hippocampal neurogenesis would be useful in increasing the rate at which compounds can be tested for effects on neurogenesis, a necessary requirement in a pharmaceutical setting in particular. In this study, we describe the development of a rapid means to quantify hippocampal neurogenesis by flow cytometry; in this case, hippocampi are dissected from BrdU labelled adult mice and dissociated. DNA is digested using DNase I, and BrdU expression visualised using a conjugated primary antibody. Nuclei are labelled using the nuclear dye 7-AAD, and the BrdU expression quantified by flow cytometry. The data from these methodologies are robust, and studies in corticosterone or antidepressant treated mice are in line with both previously reported studies and direct comparison by immunohistochemistry. Flow cytometry allows analysis of large numbers of samples within days, and provides a useful methodology to allow screening of potential antidepressant compounds *in vivo*.

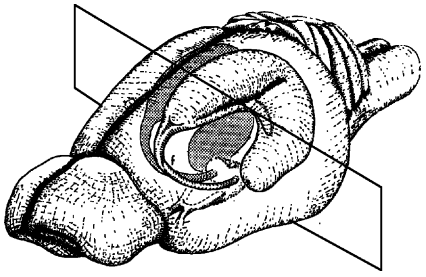
2. Materials and methods

2.1. Compound administration to mice

CD1 mice were purchased from Harlan (Hull, UK) and maintained on a 12 h light:dark cycle with food and water freely available. Animals were 25–30 g at the start of studies. In all experiments described, BrdU was prepared in saline and administered via the i.p. route. Results from BrdU dose titration indicated that BrdU 100 mg/kg could result in less spread of data than 50 mg/kg, so this dose was used for all experiments described. BrdU was administered for four consecutive days prior to euthanasia of animals; in experiments with chronic dosing, BrdU was administered for the last 4 days of the dosing schedule. Animals were euthanased the day following last BrdU or compound dose. All compounds described were prepared in physiological saline daily and administered via the i.p. route in a volume of 10 ml/kg. For all chronic dosing studies, compounds were administered for 14 consecutive days. For corticosterone implantation experiments, slow release corticosterone pellets were purchased from Innovative Research of America. Animals were anaesthetised, and four pellets implanted subcutaneously—pellets were implanted into the flanks of each animal to minimise disruption during compound administration. All experiments were carried out in accordance with local guidelines and the Animals (Scientific Procedures) Act.

2.2. Preparation of samples for flow cytometry

Mice were euthanased by decapitation, and brains removed. Typically, brain was halved along the midline with one half being immersion fixed for immunohistochemical validation and follow up with additional cytochemical markers of cell fate. For flow cytometry, hippocampal lobes (avoiding the rostral 1/3 of the lobe to avoid the subventricular zone and rostral migratory stream proliferative pools—see Fig. 1) were removed into Hank's BSS, with as much white matter as possible being scraped from the surface of the hippocampus using a flat spat-



1. Brain cut along axis shown, removing rostral tip of hippocampus.
2. Using fine forceps, cortex peeled back to expose hippocampus.
3. White matter trimmed from sides of hippocampus, and scraped from exposed surface.
4. Hippocampus dissected into Hank's BSS.

Fig. 1. Illustrative diagram demonstrating the dissection of hippocampus for use in flow cytometry assay.

ula or the side of the forceps. Once hippocampi were dissected from all animals, samples were transferred separately to 1.5 ml Eppendorf tubes, minced with fine bowspring scissors and each sample incubated in 0.5 ml of an enzymatic solution comprising papain (2.5 U/ml), dispase (1 U/ml) and DNase I (250 U/ml) (all purchased from Sigma–Aldrich Ltd., Poole, UK) for 30 min at 37 °C. Samples were then triturated using a 1 ml pipette tip, washed with 0.5 ml DMEM/10% fetal bovine serum and centrifuged at 3000 rpm. Supernatant was aspirated and samples resuspended in 1 ml DMEM/10% fetal bovine serum and triturated again. Samples were spun once more, then washed in Dulbecco's PBS with light trituration. This yielded dissociates with few large aggregates of tissue. Samples were centrifuged, then fixed and permeablised using cytofix/cytoperm buffer from the BD Biosciences BrdU flow cytometry kit, comprising 4% paraformaldehyde and saponin.

For labelling of samples, the BD Biosciences FITC-BrdU flow cytometry kit was used (BD Biosciences, Oxford, UK). This kit contains BrdU, DNase I, FITC-BrdU, 7-AAD, and various buffers for fixation, permeablisation, and washing of samples. Briefly, samples were fixed and permeablised using cytofix/cytoperm buffer, then further permeablised using Cytoperm plus buffer, which contains 10% dimethylsulfoxide. DNA was denatured by incubating for 45 min in DNase I (Sigma–Aldrich) prepared in HBSS with Ca^{2+} and Mg^{2+} at 50 U/ml. Samples were washed, and FITC conjugated anti-BrdU added for 20 min at room temperature in the dark at a dilution of 1:50. Samples were washed once more, then resuspended in 20 μl 7-AAD solution to label DNA; to each sample 1 ml D-PBS was then added and samples were independently filtered through a 70 μm cell strainer into flow cytometry tubes.

2.3. Flow cytometry protocol

Flow cytometry was undertaken using the FACS Canto flow cytometry analysis system. 7-AAD cells were first gated on a histogram; the expressing cells were visualised on a forward/side scatter plot. 7-AAD cells were 'back-gated' on the forward/side scatter plot to eliminate debris prior to analysis; this also eliminated autofluorescence of the sample. An analysis plot was generated with FITC fluorescence on the *Y*-axis and 7-AAD fluorescence on the *X*-axis. Gates were always set using dissociates from animals which did not receive BrdU *in vivo*, but which were processed and stained alongside the experimental samples. Ten thousand 7-AAD expressing cells were gated, and the number of these cells expressing BrdU analysed. Data were expressed as BrdU cells per 10,000 cells.

2.4. Immunohistochemistry protocol

Following euthanasia by decapitation, brains were removed, partitioned into lateral hemispheres along the midline and one half of the brain was immersion-fixed in 10% neutral buffered formalin and paraffin embedded. The entire rostral-caudal extent of the hippocampus was microtome sectioned at 6 μm onto X-tra™ slides (Surgipath, Runcorn, UK) and stored at room temperature. Experimental conditions for immunolabelling of BrdU-positive cells involved an improved single day procedure utilising robotic immunostainers. Briefly, samples were deparaffinised in xylene and rehydrated through a graded ethanol series using an automated slide processor (Leica ST5020 Multistainer, Wetzlar, Germany). Sections were subsequently quenched of endogenous peroxidase using 0.3% hydrogen peroxide in PBS and then microwaved in 0.01 M citrate buffer, pH 6.0 for 15 min to retrieve antigenicity as previously described (Oliver et al., 2000). Sections were then blocked in 5% normal rabbit serum in OptiMax buffer (Biogenex, Menarini Diagnostics, Wokingham, UK) for 60 min, washed twice in OptiMax buffer, incubated for 30 min in rat anti-BrdU antiserum (MCA2060, Serotec, Kidlington, UK) at an IgG concentration of 2.5 μg/ml, washed twice in OptiMax buffer and immunoreactivity visualised using ABC (Vector Labs., Peterborough, UK) and DAB/H_2O_2 (prepared as described by manufacturer (Biogenex)). Steps from blocking onwards were carried out on Optimax robotic immunostainers (Biogenex). Sections were then counterstained with haematoxylin, dehydrated in a graded ethanol series, cleared in xylene and coverslipped with DPX. Pretreatments of dewaxing and rehydration and post-treatments including dehydration and counterstaining and coverslipping were carried out on a Leica ST5020 Multistainer and CV5030 coverslipper, respectively. Every 40th section was counted using a 40× objective through the extent of the hippocampus, and stereology-based techniques used to estimate total hippocampal BrdU-positive cell number.

2.5. Statistical analysis

Statistical analyses were carried out using Graphpad Prism analysis package. For comparison of groups, one way analysis of variance was carried out, followed by Dunnett's test comparing all groups to control.

3. Results

3.1. Detection of BrdU cells in hippocampal sections and hippocampal dissociates

Immunohistochemistry for BrdU-positive cells was typically carried out using a paraffin section-based methodology, involving thin (6 μm) slide mounted sections with microwave-based antigen retrieval. This method is advantageous as it is considerably quicker than conventional HCl-denaturation immunohistochemistry (single day versus 2–3 days), semi-automated rather than manually labour intensive and does not require the use of formamide, HCl or borax. Additionally, the use of thin slide-mounted paraffin sections has further advantages; firstly, sections can be stored at room temperature; secondly, more sections are obtainable per brain; and thirdly, consecutive (or even same) sections can be more readily used for co-localization analyses using multi-label immunohistochemistry or *in situ* hybridisation (Oliver et al., 1997). Qualitatively, the paraffin section method resulted in strong labelling of BrdU-positive cells in the subgranule zone of the dentate gyrus (Fig. 2A).

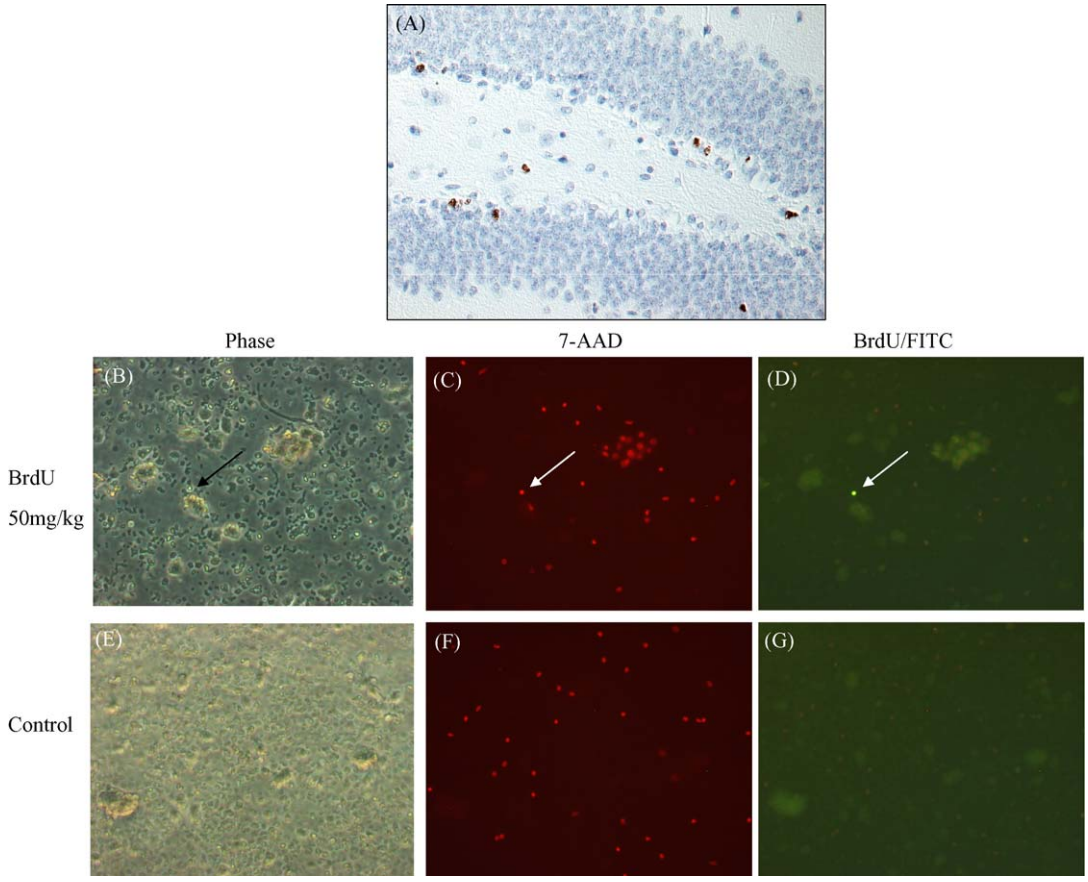
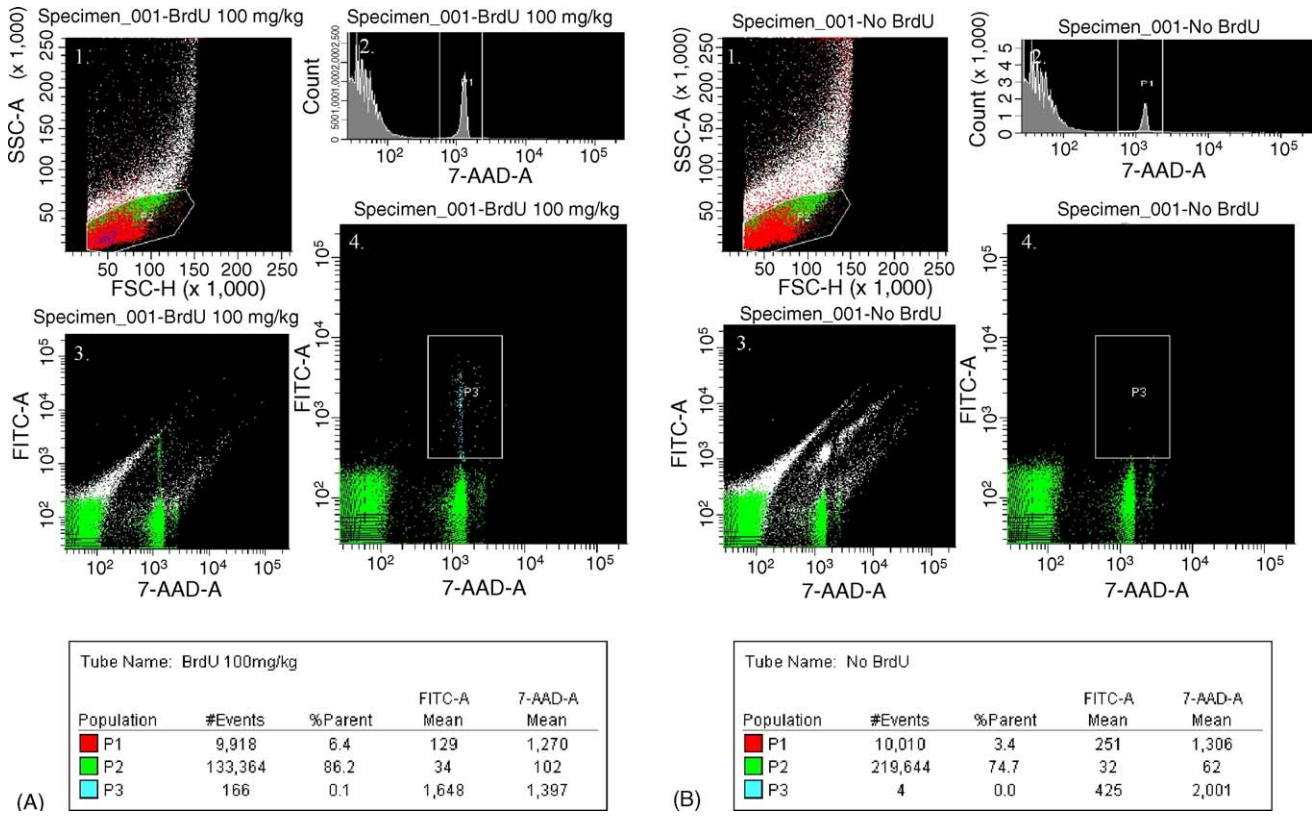


Fig. 2. Bromodeoxyuridine administered for 4 consecutive days in adult mice results in selective labelling of a subpopulation of cells in the subgranular zone of the hippocampal dentate gyrus, visualised using the enhanced immunocytochemistry protocol (A). Hippocampi were dissected from BrdU 50 mg/kg (B–D) or non-BrdU treated mice (Control; E–G), dissociated, fixed and permeablised. DNA was denatured using DNase I, and BrdU visualised using FITC conjugated monoclonal anti-BrdU. Nuclei were counterstained using 7-AAD. Phase contrast images (B and E) show a large amount of debris and myelin fragments in these crude dissociates; 7-AAD labelling reveals the cellular population (C and F) in which a small subpopulation of brightly labelled BrdU expressing cells are evident in BrdU treated (D) but not control (G) samples. Arrow highlights one BrdU expressing cell in the BrdU treated dissociate.

Preparation of hippocampal dissociates from whole hippocampal lobes results in a sample comprising single cells, small clumps of tissue (removed by straining prior to FACS analysis) and cell debris and myelin fragments. These dissociates were DNase I digested and labelled using directly FITC-conjugated BrdU antibody and the nuclear marker 7-AAD. Fig. 2 shows photomicrographs of sample BrdU treated (B–D) and non-BrdU treated (E–G) dissociates; to take the images, 0.5 ml of dissociates in suspension were pipetted into wells in a 6-well culture cluster, and allowed to settle for 3 h. Phase contrast images (B and E) show the composition of the suspension; it is difficult to identify cells amongst the cellular debris and myelin fragments. Addition of the nuclear marker 7-AAD allows the cellular population to clearly be identified from the debris (C and F). The directly conjugated FITC-anti-BrdU antibody labels a population of cells in the BrdU-treated dissociates (D), but not in the non-BrdU-treated dissociates (G). Thus, BrdU labelled cells can be detected in dissociated cell suspensions prepared from the hippocampus of mice labelled with BrdU *in vivo*, making possible the use of flow cytometry to analyse the number of labelled cells.

3.2. Flow cytometer acquisition parameters for BrdU labelled cells and titration of in vivo BrdU dose

The suspensions obtained from hippocampal dissociation were analysed using flow cytometry. Fig. 3 shows the plots for cytometer setup using the optimised protocol with back-gating on the FACS Canto system, for hippocampal dissociates from a CD1 mouse dosed with BrdU 100 mg/kg (A) and a non-BrdU treated control mouse (B). The following plots were displayed for each sample: forward scatter (a measure of event size) against side scatter (a measure of event granularity), a histogram with 7-AAD fluorescence on the *X*-axis with counts on the *Y*-axis, and two plots with 7-AAD fluorescence on the *X*-axis against FITC fluorescence on the *Y*-axis. On the forward/side scatter plots (A1, B1), the samples contain a large range of side scatter and forward scatter events. This reflects the composition of these crude dissociates, with presumed clumps of myelin and cellular debris. There is also a high degree of autofluorescence, possibly as a result of the aldehyde fixation protocol, which can be observed as the diagonal lines streaming from the main event populations in Fig. 3A and B. In order to eliminate some of this



Tube Name: BrdU 100mg/kg

Population	#Events	%Parent	FITC-A Mean	7-AAD-A Mean
P1	9,918	6.4	129	1,270
P2	133,364	86.2	34	102
P3	166	0.1	1,648	1,397

Tube Name: No BrdU

Population	#Events	%Parent	FITC-A Mean	7-AAD-A Mean
P1	10,010	3.4	251	1,306
P2	219,644	74.7	32	62
P3	4	0.0	425	2,001

Fig. 3. Cytometer setup for flow cytometry BrdU analysis. Shown are the setup parameters used for the FACS Canto system, as these gave optimal results. The panels on the left (A) show scatter plots obtained from a hippocampal dissociate from a CD1 mouse dosed for 4 days with BrdU 100 mg/kg; the panels on the right (B) show scatter plots obtained with a non-BrdU treated mouse. In both cases (1) shows the physical parameters of the cells, with cell size (forward scatter, FSC) on the *X*-axis and cell granularity (side scatter, SSC) on the *Y*-axis and (2) shows a histogram of 7-AAD fluorescence, with peaks corresponding to the cellular debris (on the left) and the 7-AAD labelled cells (in the centre of the plot, gated by P1). Ten thousand events falling within the P1 gate were collected for each sample. Cells from the P1 gate on (2) are highlighted in red on (1); they predominantly form a discrete population low in forward and side scatter. These were 'back-gated' on (1)—the P2 gate. (3) Shows the total event population, with FITC fluorescence on the *Y*-axis and 7-AAD fluorescence on the *X*-axis; much cellular debris and diagonal lines of autofluorescence are apparent. Events falling within the P2 gate are shown in green; when only the P2 gated events are shown on a similar plot (4), the debris is much reduced and the autofluorescence is eliminated. This allows the visualisation of a discrete population of FITC (BrdU labelled) events to be observed in the BrdU treated dissociate (A4, gated in P3), which are absent in the non-BrdU treated dissociate (B4, gated in P3). Quantification of events in each gate is shown below.

cellular debris and autofluorescence, a 'back-gating' approach was used. In the 7-AAD histogram (A2 and B2), two peaks can be observed. The peak of low 7-AAD fluorescence represents myelin fragments and debris which is not labelled with the nuclear marker. The peak of higher fluorescence reflects the nuclei labelled with 7-AAD, together with some of the autofluorescent events with equivalent fluorescence. When the 7-AAD labelled cells are gated (gate P1 on 7-AAD histogram) and visualised on the forward scatter/side scatter dot plots (A1, B1, shown as red population) it is clear that the majority of the cells have low side scatter and low-mid forward scatter. A polygonal gate (P2) was drawn around these cells; this was kept constant across all samples analysed. Events from the P2 gate are shown in green on the 7-AAD/FITC plots. In panels 2-A3 and -B3, where all events are shown, it is clear that the diagonal lines of autofluorescence and much of the cellular debris falls outwith the P2 gate (non-gated cells are shown in white). Panels 2-A4 and -B4 show the events from the P2 gate, which eliminates some of the debris, and almost all the autofluorescent diagonal lines. Remaining debris is seen as a large population low in both 7-AAD and FITC fluorescence, located in the lower left hand region of the scatter plot. The 7-AAD expressing events comprise two subpopulations, one large population and a much smaller population with higher DNA content, possibly representing doublets or dividing cells with double DNA content; voltage settings in the FITC channel were adjusted using non-BrdU labelled sample to a point where the 7-AAD cells formed a compact group, and kept constant across samples within each experiment. In the BrdU treated hippocampal dissociate (A4), there is a clear population of cells which are higher in the FITC channel, shown in blue by the P3 gate; this population is absent in the non-BrdU treated sample (B4), apart from a few sporadic events. Compensations settings were adjusted such that this population was aligned vertically above the 7-AAD population. The quantification of these two sample hippocampal dissociates is shown below each set of graphs; in the P3 gate in the non-BrdU treated sample there are four events from 10,010 7-AAD expressing cells gated. In the BrdU 100 mg/kg treated sample, there are 166 FITC positive events, from 9918 gated. Thus, this BrdU treated sample contains a population of events

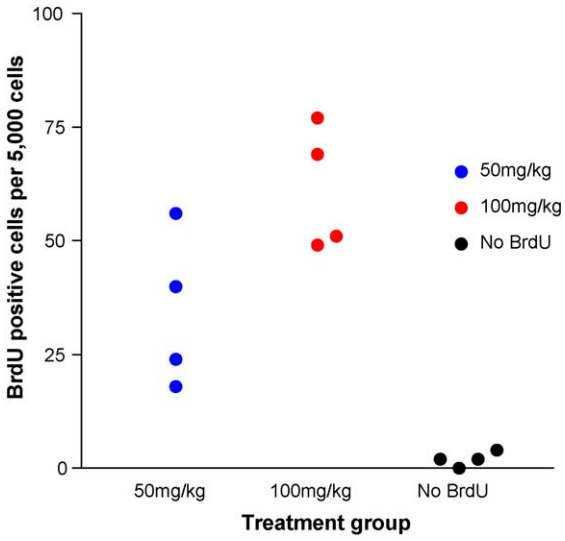


Fig. 4. CD1 mice were dosed i.p. with BrdU at 50 mg/kg or 100 mg/kg, or not BrdU treated ($n = 4$ per treatment group). Hippocampi were dissociated, fixed and labelled for flow cytometry. Samples were analysed using the FACS Canto analysis cytometer, with back gating of 7-AAD labelled cells on physical parameters. 7-AAD labelled cells were gated, and the number of BrdU positive cells in 5000 7-AAD labelled cells quantified. Results show a population of BrdU positive cells, and with a smaller spread of data in animals dosed with BrdU 100 mg/kg than 50 mg/kg. Animals which did not receive BrdU had only sporadic events in the BrdU gate.

high in FITC labelling which is absent in the non-BrdU treated dissociate, indicating that the flow cytometry protocol can detect a population of cells labelled with BrdU *in vivo* and antibody stained as a fixed hippocampal dissociate.

In order to determine the inter-animal variability, a small dose titration study was undertaken. Four animals were dosed with BrdU 50 mg/kg, four with BrdU 100 mg/kg and four were not treated with BrdU. The results are shown in Fig. 4. Samples were prepared as described above and quantified using flow cytometry. Only sporadic events were observed in the non-BrdU treated animals. In animals treated with BrdU 50 mg/kg or 100 mg/kg, a population of BrdU cells was apparent, averaging around 75 cells/5000 7-AAD expressing cells gated in the 100 mg/kg sample. The spread of data was less in animals dosed with BrdU 100 mg/kg.

3.3. *Effects of antidepressants on hippocampal neurogenesis; quantification of BrdU labelled cells by flow cytometry and immunohistochemistry*

Experiments were undertaken to evaluate the sensitivity of the flow cytometry methodology to detect changes in hippocampal neurogenesis induced by antidepressants. It is well established in the literature that the tricyclic antidepressant imipramine and the selective serotonin reuptake inhibitor fluoxetine cause increases in neurogenesis (Santarelli et al., 2003). Both antidepressants

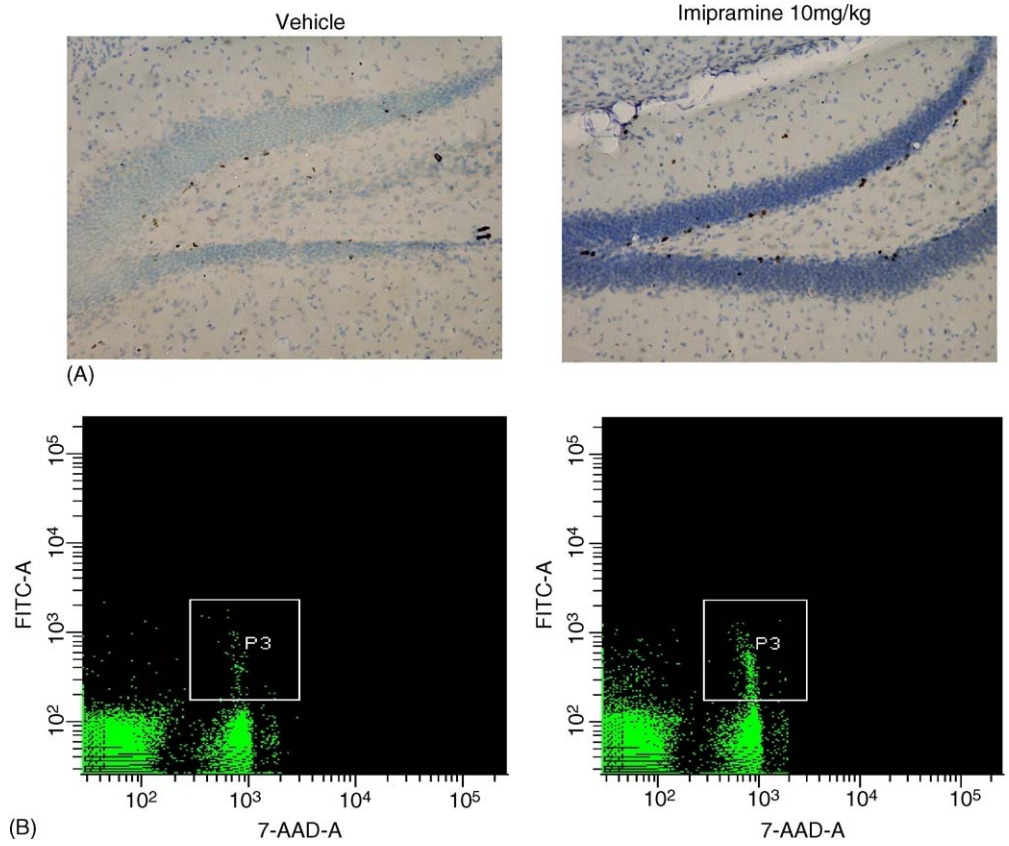


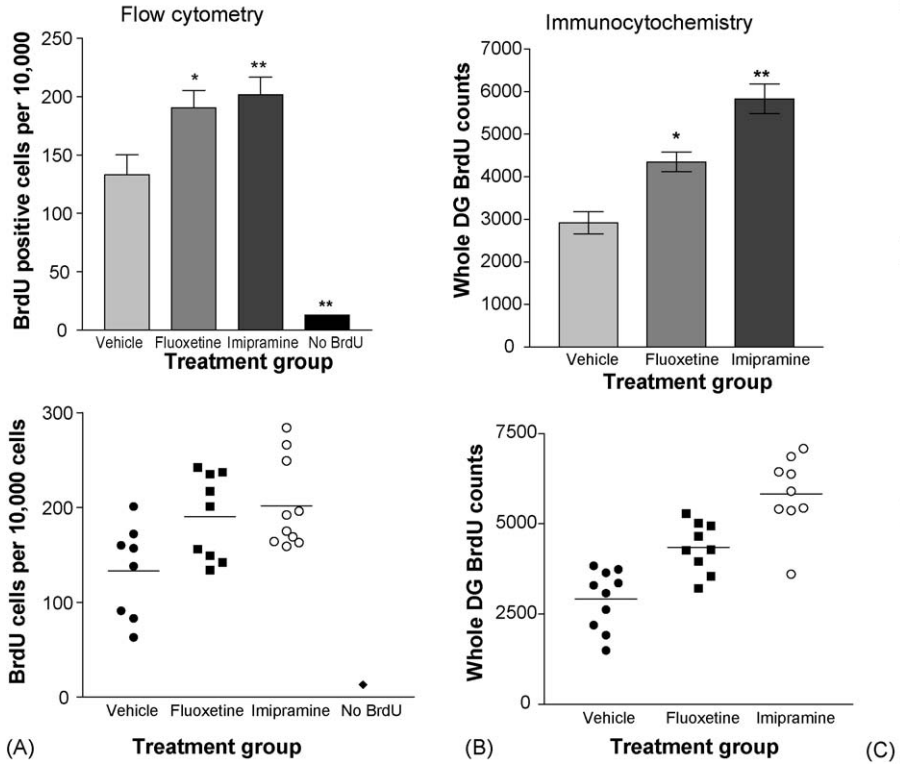
Fig. 5. Illustrative images of quantification strategies for BrdU labelling in vehicle (left hand panels) or imipramine 10 mg/kg (right hand panels) treated mouse hippocampus. (A) shows immunostaining of sections for BrdU. (B) shows illustrative flow cytometer scatter plots. In both cases, more BrdU labelled events are apparent in the imipramine treated mice than in the vehicle treated mice.

were tested for effects using the flow cytometry methodology; hippocampus was dissected from one hemisphere for flow cytometry quantification and paraffin section immunohistochemistry was used on the other hemisphere to quantify the number of labelled cells in the dentate gyrus, allowing a direct comparison with the flow cytometry data.

To evaluate effects of antidepressants, fluoxetine 10 mg/kg, imipramine 10 mg/kg or vehicle were dosed daily in CD1 mice for 14 days, with BrdU administered daily at 100 mg/kg for the last 4 days of the dosing schedule; animals were euthanased the day after the last dose.

Fig. 5 shows illustrative images of methods used to quantify BrdU uptake. The panels on the left hand side show results from vehicle treated animals, while those on the right show imipramine 10 mg/kg treated animals. Fig. 5A shows illustrative images of BrdU labelling in hippocampus visualised by staining 6 μm paraffin sections. There is a clear increase in BrdU labelling in the imipramine treated animal compared to the vehicle treated. Sample scatter plots from flow cytometry analysis, with back-gating as described above, are shown in Fig. 5B—in these plots, there are higher numbers of cells in the P3 gate in the imipramine treated animal compared to the control. The quantification of BrdU labelling by flow cytometry and immunohistochemistry is shown in Fig. 6.

By both methods of quantification, both antidepressants significantly increased BrdU labelled cells in the hippocampal dentate gyrus. The results by flow cytometry are shown in Fig. 6A. The top panel shows the mean results from all animals, the bottom panel shows the spread of data within groups. Fluoxetine increased BrdU positive cells to around 140% of vehicle levels, while the response to imipramine was roughly 150% of vehicle. The direct immunohistochemistry comparison of the same brains is shown in Fig. 6B, again with the mean data on the top panel and the spread of data on the lower panel; here again both antidepressants significantly increased BrdU labelled cells. By this method of quantification, fluoxetine caused an increase to 148% of vehicle, while imipramine increased BrdU cells to 200% of vehicle. In both cases, the increase in BrdU cells by imipramine treatment is more pronounced than that induced by fluoxetine treatment, and in all cases the spread of data points are similar within groups. Fig. 6C shows a direct comparison on an animal by animal basis of the numbers obtained from the flow cytometry and immunocytochemistry analyses. The numbers obtained do not directly correlate on an animal by animal basis. Given that both the methods tested give merely an estimation of total hippocampal BrdU cell number, estimated either by sampling a fixed number of cells in the flow cytometry protocol or by stereology-based estimation of total hippocampal cell number in



(C) Count comparison

Vehicle	Counts	FACS
1	3360	83
2	3840	160
3	3740	172
4	1920	138
5	3648	91
6	1496	157
7	3080	
8	3300	201
9	2200	63
10	2628	
Fluoxetine	**Counts**	**FACS**
1	4280	237
2	5280	242
3	3212	217
4		156
5	5016	235
6	3960	201
7	4944	149
8	4268	134
9	4656	
10	3552	142
Imipramine	**Counts**	**FACS**
1	5440	196
2	6440	266
3	6380	249
4	7084	175
5		164
6	6864	169
7	5412	159
8	5368	192
9	3608	163
10	5904	284

Fig. 6. Quantification of hippocampal BrdU labelling in CD1 mice treated for 14 days with vehicle, fluoxetine 10 mg/kg or imipramine 10 mg/kg (all i.p.) by flow cytometry and immunohistochemical methodologies. (A) shows quantification by flow cytometry; the top panel shows the mean data, the bottom panel the spread of data. (B) shows the results using immunohistochemistry on the same animals described in (A), again with the mean data on the top panel and the spread on the bottom panel. In both cases, both antidepressants increased proliferating cells, with imipramine being the more potent of the two. (C) shows a direct comparison on an animal by animal basis of the two quantification methodologies. Statistical analysis was by one-way ANOVA followed by Dunnett's test ($^{*}p < 0.05$, $^{**}p < 0.01$).

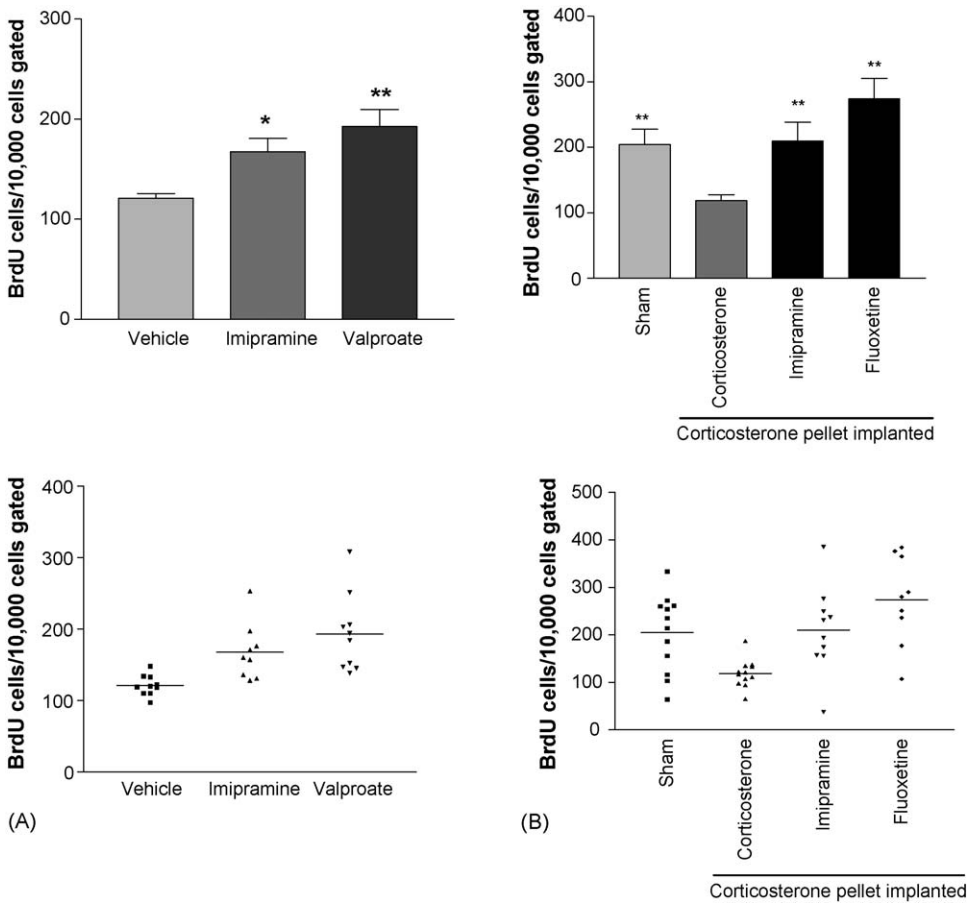


Fig. 7. Increased hippocampal precursor proliferation induced by chronic administration of imipramine and sodium valproate (A). Corticosterone pellet implantation decreased hippocampal precursor proliferation (B), an effect reversed by treatment with either fluoxetine or imipramine. Statistical analysis was by one-way ANOVA followed by Dunnett's test ($^{*}p < 0.05$, $^{**}p < 0.01$).

the immunohistochemistry protocol, some differences in quantification may be expected. Additionally, the methods compare different hippocampal lobes and so some variance between the two pieces of tissue is also likely even though both derive from the same animal. Overall, both detection methods highlighted significant increases in BrdU cell counts, indicating that the flow cytometry methodology is a viable strategy for the quantification of *in vivo* neurogenesis.

We have also carried out a number of other experiments evaluating the use of the flow cytometry strategy for the analysis of hippocampal precursor proliferation. The results of two of these studies are shown in Fig. 7. In Fig. 7A, mice were dosed with imipramine 10 mg/kg or sodium valproate 400 mg/kg for 14 days, then culled and hippocampal proliferation analysed using the flow cytometry assay. The results again demonstrate significantly increased proliferation with imipramine, reinforcing the previous data, and also demonstrate increased proliferation with sodium valproate. In Fig. 7B, mice were implanted with slow-release corticosterone pellets, then dosed either with vehicle (sham and corticosterone groups) or with fluoxetine 10 mg/kg or imipramine 10 mg/kg as shown. The results demonstrate a pronounced decrease in hippocampal BrdU labelling with corticosterone implantation, which was reversed by administration of the antidepressants. Together, these additional studies further highlight the utility of this rapid, unbiased method for quantification of hippocampal cell proliferation.

4. Discussion

In this study we describe a novel method for the quantification of hippocampal BrdU uptake in mice by labelling with BrdU *in vivo* followed by dissociation of the individual hippocampi, immunolabelling and quantification using flow cytometry. We have compared this method with standard immunohistochemical methods on an animal by animal basis and demonstrated statistically significant changes *in vivo* upon administration of steroids, antidepressants and other paradigms known to regulate progenitor proliferation.

The primary aim for developing these methodologies was to decrease the time taken to analyse the effects of pharmacological agents on hippocampal neurogenesis. Typically, hippocampal neurogenesis is quantified by immunostaining free floating 30–50 μm sections or paraffin sections. Undoubtedly, both of these immunohistochemistry approaches are limited by the time taken for sections to be cut and for manual, microscope-based quantification (though the former in particular can be streamlined to some degree, such as increasing the number of samples per paraffin block and by reducing the number of sections col-

lected). Using the flow cytometry approach, samples can be prepared and fixed in hours; these fixed samples can then be stored or can be stained and analysed. Staining of the samples takes typically around 4–5 h for 40 samples. Running the samples takes approximately 3–5 min per sample; thus, the flow cytometry protocol can be carried out from euthanasia of the animals to completion in 72 h or less, comparing favourably with a typical 10–15 day procedure for the histology techniques in a large study.

Thus, the principal advantage that the flow cytometry approach has over the immunohistochemistry based approaches is the speed and relative ease to run the assay. Further purification steps, such as density sedimentation, could be added to the existing method to eliminate some of the debris. This would, however, add a lot of complexity and time to the protocol when large numbers of samples are monitored—thus, we have not pursued these steps. When optimising the assay, it is critical that each experiment incorporates some non-BrdU labelled animals to allow the setting of both the voltage and compensation settings, and to allow the analysis gates to be drawn, as shown in Fig. 3. The flow cytometry assay results in the loss of the positional information within the dentate gyrus and morphological information needed for lineage studies. There is also the possibility of BrdU incorporation into non-neurogenic dividing cells, such as endothelial cells or reactive microglia (Ehninger and Kempermann, 2003). Endothelial cells comprise around 17% of all nestin-positive cells; the design of the flow cytometry assay allows firstly for determination of significant changes in proliferation which can then be analysed in more detail at a cellular level by immunohistochemistry. To minimise this non-specific labelling, it is crucial during the dissection to clear as much white matter as possible from the hippocampal lobes as they are dissected, in particular ensuring that none of the overlying corpus callosum – rich in oligodendrocyte progenitor cells – is included, and to ensure that none of the tissue underlying the hippocampus is dissected; it is also important to avoid the rostral tip of the hippocampus, where some contamination with SVZ precursors may occur. The ability to separately immersion fix the half of the brain not used for flow cytometry and to section this for immunohistochemistry provides a useful means of post validating the results obtained from the flow cytometry assay; it also provides a stock of sections which can be used for further analyses of lineage or plasticity markers. Thus, where there are a large number of conditions/compounds to be tested, the flow cytometry assay can be used to generate data rapidly and act as a triage to highlight those treatments which may be interesting for further immunohistochemical validation. In spite of these caveats, the results have correlated well in terms of the magnitude of response between groups based on our direct comparisons.

The data generated by the flow cytometry methodology compares with previously reported data in the literature. The effects of antidepressants on neurogenesis have been described by a number of different groups. In our hands, both the tricyclic antidepressant imipramine and the selective 5-HT reuptake inhibitor fluoxetine significantly increased neurogenesis after 2 weeks chronic dosing, quantified by flow cytometry on one hippocampal lobe with direct comparison using the optimised immunohistochemistry protocol on the other half of the brain. The data generated in these experiments are comparable to those from previously reported studies. Chronic administration of fluoxetine to rats for 14 or 28 days resulted in a significant increase in hippocampal neurogenesis to around 140% of control (Malberg et al., 2000); in this study, electroconvulsive seizure, the monoamine oxidase inhibitor tranylcypromine and the noradrenaline selective reuptake inhibitor reboxetine were also effective in increasing hippocampal neurogenesis. A recent paper (Santarelli et al., 2003) showed increases in dentate neurogenesis by fluoxetine and imipramine; these increases were blocked by X-irradiation to kill dividing cells in the dentate, and, importantly, so were the behavioural effects of the antidepressants. This supports the hypothesis that the upregulation of neurogenesis by antidepressants may indeed be of physiological significance in the efficacy of these drugs. Hippocampal precursor proliferation has also been shown to be decreased by chronic stress and social defeat; these deficits are restored by antidepressants. Increased neural precursor proliferation was also observed with 3-week chronic antidepressant treatment in CD1 mice, and 2- and 3-week treatment in C57Bl6/J mice, with 7-day corticosterone treatment, and with running wheel exercise using the flow cytometry assay (data not shown).

Regulation of hippocampal precursor proliferation by stress and depression is thus an area of increasing interest. Although the physiological relevance of neurogenesis to the clinical efficacy of antidepressants has not been categorically demonstrated, it is a potentially useful biomarker for antidepressant activity, and a higher throughput strategy for the quantification of hippocampal neurogenesis would facilitate the testing of potential novel antidepressant or anxiolytic compounds. It must be borne in mind that antidepressants are not the only factors which regulate hippocampal neurogenesis, and higher throughput strategy would obviously be of benefit for testing other compounds, effects of genetic mutations or effects of environmental modification on hippocampal neurogenesis. The BrdU flow cytometry assay we have developed is rapid and robust, yielding data equivalent to immunocytochemical analysis. The method could also be adapted to develop a quantitative readout of cell survival after a pulse-chase study with BrdU and a lag time of several weeks to monitor newborn neuronal integration. In addition, the flow cytometry methodology can be further refined to visualise subpopulations of BrdU positive cells by co-labelling other markers of progenitors in addition to BrdU. The ability to couple the flow cytometry assay to immunocytochemical analysis by fixing and sectioning the half of the brain not used for flow cytometry adds flexibility to the technique and allows other immunocytochemical markers to be followed. This method is thus of potentially great utility in analysing the effects of pharmacological or other manipulations on mouse hippocampal neurogenesis.

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