

# Enhanced Sensitivity of the MRL/MpJ Mouse to the Neuroplastic and Behavioral Effects of Chronic Antidepressant Treatments

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Chronic administration of antidepressant drugs produce changes in neuroplasticity and behavior in rodents, effects that may be associated with the slow emergence of clinical therapeutic effects. Owing to the uncertainty over the effects of chronic antidepressant treatments in mice, these experiments compared the regulation of neurogenesis, neurotrophin levels, and behavior produced by chronic antidepressant treatments between two inbred mouse strains, MRL/MpJ and C57BL/6J. The MRL/MpJ strain is associated with enhanced wound healing and tissue regeneration, whereas C57BL/6J mice are used commonly for behavioral studies. Proliferation and survival of hippocampal progenitor cells were measured using flow cytometry, a new platform that rapidly quantifies the incorporation of 5-bromo-2-deoxyuridine (BrdU). Hippocampal cell proliferation was increased significantly after chronic administration of fluoxetine (FLX: 5, 10 mg/kg, intraperitoneal (i.p.), b.i.d.) or desipramine (DMI: 5, 10 mg/kg, i.p., b.i.d.) for 21 days in MRL/MpJ mice, but not in C57BL/6J mice. Hippocampal progenitor cells born prior to chronic antidepressant treatments were not affected in either mouse strain. Protein levels of brain-derived neurotrophic factor (BDNF) in MRL/MpJ mice were elevated significantly in the frontal cortex, hippocampus, and amygdala after chronic FLX treatment, but increased only in the frontal cortex by chronic DMI. In contrast, BDNF levels in C57BL/6J mice were decreased in the hippocampus and increased in the amygdala after chronic FLX, and were decreased in the brain stem after chronic DMI. Novelty-induced hypophagia (NIH) was used to examine a behavioral effect produced by chronic antidepressant treatment. MRL/MpJ mice, chronically administered FLX or DMI, had significantly shorter latencies to consume food when exposed to a novel environment than untreated mice, whereas there were no effects on the behavior of C57BL/6J mice. In conclusion, robust effects of chronic antidepressant treatments on hippocampal cell proliferation and BDNF levels paralleled the ability of these drugs to produce changes in NIH behavior in MRL/MpJ, while none of these effects were produced in C57BL/6J mice. The greater responsiveness of MRL/MpJ mice may be important for drug discovery, for genetic studies, and for understanding the neural mechanisms underlying the physiological and behavioral effects of chronic antidepressant treatments.

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## INTRODUCTION

The primary effect of the most currently available pharmacologic antidepressants is to enhance the transmission of brain monoamine systems, principally the neurotransmitters serotonin and norepinephrine (Frazer, 2001). One of their major clinical limitations is that 2–6 weeks of treatment is usually necessary before therapeutic effects develop, even though monoaminergic signaling is altered

shortly after the beginning of treatment. The temporal onset of clinical antidepressant effects has led to the hypothesis that the ultimate causes of therapeutic effects of chronic antidepressant drug administration arise from molecular and cellular adaptations requiring persistent drug exposure (Nestler *et al*, 2002; Duman and Monteggia, 2006).

Two specific mechanisms by which chronic antidepressant treatments may produce their effects are increases in adult hippocampal neurogenesis (Dranovsky and Hen, 2006) and mobilization of neurotrophic factors (Duman and Monteggia, 2006). Chronic, but not acute, administration of different classes of antidepressant treatments to rats have been shown to increase adult hippocampal neurogenesis (Malberg *et al*, 2000; Manev *et al*, 2001). Similarly, chronic administration of antidepressant treatments to rats,

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from different classes, have been reported to commonly increase the expression of brain-derived neurotrophic factor (BDNF) mRNA in the hippocampus (Nibuya *et al*, 1995; Coppel *et al*, 2003; Dias *et al*, 2003) and frontal cortex (Nibuya *et al*, 1995), as well as BDNF protein levels in the frontal cortex (Altar *et al*, 2003; Balu *et al*, 2008b).

Studies of antidepressants in mice are important because of their ability to establish the generality of findings, the reduced costs of drug discovery, and the development of targeted genetic modifications. However, chronic antidepressants have produced inconsistent results in mice. Behavioral effects of antidepressants in mice in the forced-swim test or tail-suspension test vary according to genetic background (Lucki *et al*, 2001; Crowley *et al*, 2005). Although chronic antidepressant drug administration increased hippocampal neurogenesis in 129SvEv mice after imipramine and fluoxetine (FLX) (Santarelli *et al*, 2003) or mixed 129SvEv and C57BL/6 mice after desipramine (DMI) (Gur *et al*, 2007), similar effects were not produced in the other mouse strains (Holick *et al*, 2008; Miller *et al*, 2008). Chronic, but not acute, treatment with imipramine or DMI elevated BDNF mRNA levels in the hippocampus and frontal cortex in C57BL/6J mice (Tsankova *et al*, 2006) and mice with a mixed genetic background (129SvEv  $\times$  C57BL/6) (Conti *et al*, 2002). However, few studies have examined the effects of chronic antidepressant treatments on BDNF protein levels in the mouse brain.

There are few behavioral tests in rodents that are responsive only after the chronic administration of antidepressant drugs. Behavioral hyponeophagia paradigms, that measure reduction of the consumption of a palatable food by exposure to a novel environment, have been shown to respond to antidepressant treatments only after chronic administration (Bechtholt *et al*, 2008; Dulawa and Hen, 2005; Merali *et al*, 2003). Novelty-induced hypophagia (NIH), which does not require food deprivation, is sensitive to chronic antidepressant treatments in a number of mouse strains (Merali *et al*, 2003; Dulawa *et al*, 2004; Gur *et al*, 2007). Using novelty suppression of feeding, a version of the test requiring food deprivation, the behavioral effects of chronic antidepressants were related to increased hippocampal neurogenesis in studies showing that ablation of adult neurogenesis by hippocampal-directed X-irradiation blocked the antidepressant behavioral response in mice (Santarelli *et al*, 2003) and rats (Jiang *et al*, 2005).

Owing to the uncertainty of the effects of chronic antidepressant drug treatments in mice, this study compared the response to chronic antidepressant drug treatments between two mouse strains, MRL/MpJ and C57BL/6J. The MRL/MpJ mouse strain was selected for its enhanced wound healing and regenerative response to injury (Clark *et al*, 1998; Leferovich *et al*, 2001; Heber-Katz *et al*, 2004) compared with C57BL/6J mice, the control strain for these studies. Moreover, MRL/MpJ mice displayed increased proliferation in the neurogenic subventricular zone compared with other mouse strains (Baker *et al*, 2006). C57BL/6J mice are used commonly in behavioral and pharmacological studies, and constitute a portion of the MRL/MpJ genetic background. The effects of chronically administered FLX and DMI on the proliferation and survival of hippocampal progenitor cells, BDNF protein levels in

different brain regions, and behavior in the NIH test were measured in both of the strains. The effects of chronic antidepressant drug treatments on hippocampal neurogenesis were measured in this study using flow cytometry, a technique that measures 5-bromo-2-deoxyuridine (BrdU) incorporation in cells more rapidly and objectively than immunohistochemistry. The augmented cytogenic, neurotrophic, and behavioral responses to chronic antidepressant drug treatments of MRL/MpJ mice compared with C57BL/6 mice, suggest them to be an attractive platform for antidepressant drug discovery based on chronic treatment effects.

## MATERIALS AND METHODS

### Animals

Adult male C57BL/6J and MRL/MpJ (Jackson Laboratories, Bar Harbor, ME) were 8–9 weeks old at the beginning of all studies. The animals (non-littermates) were housed in groups of five in polycarbonate cages and maintained in a 12 h light–dark cycle (lights on at 07:00 h) in a temperature (22°C)- and humidity-controlled colony. The animals were given free access to food and water. Animal procedures were conducted in accordance with the guidelines published in the NIH Guide for Care and Use of Laboratory Animals, and all protocols were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

### Drug Treatments

MPJ/MpJ and C57BL/6J mice were administered intraperitoneal (i.p.) injections of saline (0.9% NaCl), FLX hydrochloride (2.5, 5, 10 mg/kg; Anawa, Zurich), or DMI hydrochloride (2.5, 5, 10 mg/kg; Sigma St Louis, MO) twice daily for 21 days ( $n = 10\text{--}15/\text{group}$ ). All of the mice in each cage received the same drug treatment. The doses were calculated according to the base weight of the drug and administered in a volume of 10 ml/kg.

To measure the effects of chronic antidepressant drug treatments on hippocampal cell proliferation, animals were injected with BrdU (100 mg/kg i.p.; Roche Applied Sciences Indianapolis, IN) once daily during the last 4 days of antidepressant treatment (1 h after the morning antidepressant injection), and were killed 24 h after the last drug treatment. To measure the effects of antidepressants on hippocampal cell survival, mice were first loaded with BrdU (100 mg/kg, i.p.) for 4 days, and chronic antidepressant treatment for 21 days was initiated 24 h after the last BrdU injection (Malberg *et al*, 2000). Mice were killed 24 h after the last antidepressant treatment. BrdU was dissolved in warm physiological saline and injected i.p. in a volume of 10 ml/kg.

### BrdU Incorporation Using Flow Cytometry

The majority of the experiments studying the effects of chronic antidepressant drug treatments on neurogenesis measured BrdU incorporation in the hippocampus using flow cytometry. This method is more rapid and objectively quantitative than immunohistochemistry, and produces a pattern of experimental results that are similar to those

reported using immunohistochemistry (Bilsland *et al*, 2006; Balu *et al*, 2008a).

Mice were decapitated, their brains removed quickly, and dissected on ice. The right hippocampal lobe was removed, placed in Hank's Balanced Salt Solution (Gibco Grand Island, NY) and finely minced. Prior studies showed no differences between the right and left lobes of the hippocampus. An enzymatic cocktail (0.5 ml, 1 mg/ml papain, Roche Applied Sciences Indianapolis, IN; 0.1 M L-cysteine, Sigma St Louis, MO) was added to that tube and incubated in a dry heat block at 37°C for 15 min. An equal amount of Hibernate-A (Brain Bits Springfield, IL) containing 10% heat-inactivated fetal bovine serum (Gibco Grand Island, NY) was added to each tube to stop the enzymatic digestion. The tissue was triturated mechanically to form a single-cell suspension and then centrifuged at 300 g for 5 min.

The supernatant was removed and the resultant cell suspension was stained using the FITC BrdU Flow Kit (BD Biosciences San Jose, CA). The cells were fixed and permeabilized by resuspension in 100 µl of the Cytotfix-Cytoperm buffer at room temperature for 30 min, followed by the addition of 1 ml of wash buffer. The samples were spun at 300 g for 5 min, and the supernatant was aspirated. The cells were further permeabilized by resuspension in 100 µl of the Cytoperm Plus buffer on ice for 10 min. After washing and centrifuging, the cells were refixed in 100 µl of the Cytotfix-Cytoperm buffer at room temperature for 5 min. The cells were then resuspended in 100 µl of DNase (30 µg; stock from kit was diluted in DPBS (Ca<sup>2+</sup>/Mg<sup>2+</sup> free) containing 0.1 mM CaCl<sub>2</sub> and 10 mM MgCl<sub>2</sub>) in a dry heat block at 37°C for 1 h. Following washing and centrifuging, the cells were labeled with 50 µl of FITC-conjugated anti-BrdU (1:50 dilution) in the dark, at room temperature for 20 min. After the samples were washed, they were labeled with 20 µl of the nuclear marker, 7-AAD, at room temperature in the dark. The cells were then resuspended in staining buffer (PBS, 3% fetal bovine serum, 0.09% sodium azide). Prior to analysis, cells were filtered through a cell strainer cap (30 µm) to remove debris. The data were collected the same day, on a BD FACS Canto system at the University of Pennsylvania Flow Cytometry Core Facility, and analyzed using the Winlist 6.0 software.

### BrdU Incorporation Using Immunohistochemistry

As flow cytometry has not been used frequently to measure neurogenesis, the effects of chronic antidepressant treatments on hippocampal cell proliferation were compared directly with immunohistochemistry. Separate cohorts of MRL/MpJ mice were treated with saline or FLX (5 mg/kg) or with saline or DMI (5 mg/kg) for 21 days. The brains were bisected with the left hemisphere used for immunohistochemistry and the right hippocampus used for flow cytometry. The left hemispheres were post-fixed in 4% paraformaldehyde for 7 days. The brains were then transferred to a 30% sucrose solution for 5 days, at which point the brains were removed and stored at -80°C until they were sliced.

Coronal sections (40 µm) from the entire rostrocaudal extent of the dentate gyrus were cut from a single hemisphere on a cryostat at -20°C. Every sixth slice was mounted in groups of 10-12 per slide (Superfrost plus),

dried overnight, and processed for BrdU using peroxidase methods. BrdU staining and quantification was performed according to methods outlined in Shors *et al*, 2007. Cells stained for BrdU were counted in the combined SGZ and GCL areas, avoiding cells in the outermost focal plane. The number of counted cells was multiplied by 12 (number of intervening slices × number of hemispheres) to give an estimate of the total number of BrdU-labeled cells per hippocampus. Only animals with 8-14 countable sections were included for analysis. Harmonic means were generated for each subject to adjust for the number of tissue sections.

When the results of the DMI study were analyzed, we saw that there was a large increase in the basal levels of BrdU incorporation in MRL/MpJ mice, compared with the companion FLX study done earlier and also with the characterization studies done earlier in the laboratory. A second DMI comparison between FACS and IHC was done several months later, and this study also measured an increased baseline rate of proliferation and more modest drug response. After contacting Jackson Laboratories, it was learned that the housing conditions of the MRL/MpJ mice were changed prior to the DMI studies (around October 2007; Dr Peter Kelmenson, personal communication), and that the time of this change corresponded to the date when the MRL/MpJ mice started showing higher rates of BrdU incorporation. The results of the two DMI studies were combined.

### BDNF Protein

Twenty-four hours after the last antidepressant treatment, mice were decapitated and their brains removed quickly for dissection into the following regions: hippocampus, frontal cortex, amygdala, and brain stem. Each region was flash frozen in isopentane and placed in -80°C until analysis. BDNF protein levels were quantified using a commercially available sandwich ELISA kit (Promega, Madison, WI). The tissue was homogenized in 0.75 ml of lysis buffer (100 mM PIPES pH 7.0, 500 mM NaCl, 2 mM EDTA, 0.1% sodium azide, 2% bovine serum albumin, 0.2% Triton X-100, 5 µg/ml aprotinin, 0.1 µg/ml pepstatin A, 0.5 µg/ml antipain). The homogenate was centrifuged at 14 000 g for 30 min at 4°C. The supernatant was removed, and the amount of BDNF protein was analyzed by ELISA in duplicate samples according to the manufacturer's instructions. BDNF levels were normalized to the wet tissue weight.

### Novelty-Induced Hypophagia

Mice were housed in pairs upon arrival in the animal facility in polycarbonate cages (11 × 7 × 5 inches). They were allowed to acclimate to these conditions for 1 week prior to training. The training consisted of daily sessions (15 min for C57BL/6J mice and 5 min for MRL/MpJ mice) in which mice were given access to a highly palatable food (peanut butter chips; Nestle, Glendale, CA) delivered in a clear plastic petri dish. Opaque, black, plastic dividers (7 × 5 inches) were placed inside each cage to separate the mice during training and home cage testing periods. Mice were acclimated to the dividers for 1 h before the introduction of food. Although training sessions were conducted during daylight hours, the room was illuminated by red light to

facilitate ingestion. The latency to approach and initiate ingestion and the amount of food consumption during the session were measured. For C57BL/6J mice, a stable mean latency to approach the food was obtained by the ninth day, such that the variability of means over three sessions was <20%. For MRL/MpJ mice, this criterion was reached by the fourth day. Mice that did not meet this criterion were not included in the study. Training was then suspended after the stability criterion was reached.

Mice received injections of 0.9% saline ( $n=10$ ), DMI (5 mg/kg,  $n=9$ ), or FLX (5 mg/kg,  $n=10$ ) twice daily for 21 days. The doses were chosen because they produced the peak effect on cell proliferation in the MRL/MpJ mice. During days 18–20 of treatment, mice were re-exposed to the peanut butter chips under the same home cage training conditions described earlier, except that the room was illuminated in visible fluorescent light. Testing at home and in novel environments occurred on the last 2 days of treatment, 1 h after their injection. Testing in the novel environment occurred in a different room in which the animals were trained. Mice were removed from their home cage and placed in an empty, clear polycarbonate cage (11 × 7 × 5 inches) without bedding. The novel cage was placed on a table with white cardboard paper placed underneath and on two sides of the cage. Bright illumination was placed directly overhead (60 W light bulb). There was no acclimation period prior to testing in the novel environment. The novel test session was videotaped and the latency to ingestion and the amount consumed during the test sessions were measured.

### Data Analysis

Measurement of BrdU incorporation using flow cytometry, BDNF protein levels, and NIH latencies between saline-injected controls and antidepressant treatments were compared using one-way analysis of variance (ANOVA). Dunnett's *post hoc* test was used to compare the means of individual treatment groups with the common control group. Unpaired two-tailed Student's *t*-test was used to compare results between flow cytometry and manual counting. The results from the two DMI comparisons were not significantly different from each other and were therefore combined for analysis. Linear regression analysis was performed to determine the correlation between methods. For all tests,  $P<0.05$  was considered statistically significant.

## RESULTS

### Effects of Chronic Fluoxetine and Desipramine Treatments on Hippocampal Cell Proliferation

In MRL/MpJ mice, chronic administration of FLX (Figure 1a) or DMI (Figure 1b) for 21 days dose-dependently increased cell proliferation in the hippocampus, as measured with flow cytometry. The lowest dose (2.5 mg/kg) of FLX or DMI failed to elevate cell proliferation. A peak 3-fold elevation of proliferative activity was achieved with the 5 mg/kg dose of FLX, whereas the highest dose tested (10 mg/kg) produced a 2-fold increase. The peak effect for DMI, a 2-fold elevation of proliferation, was achieved with the 5 mg/kg dose, whereas the highest tested

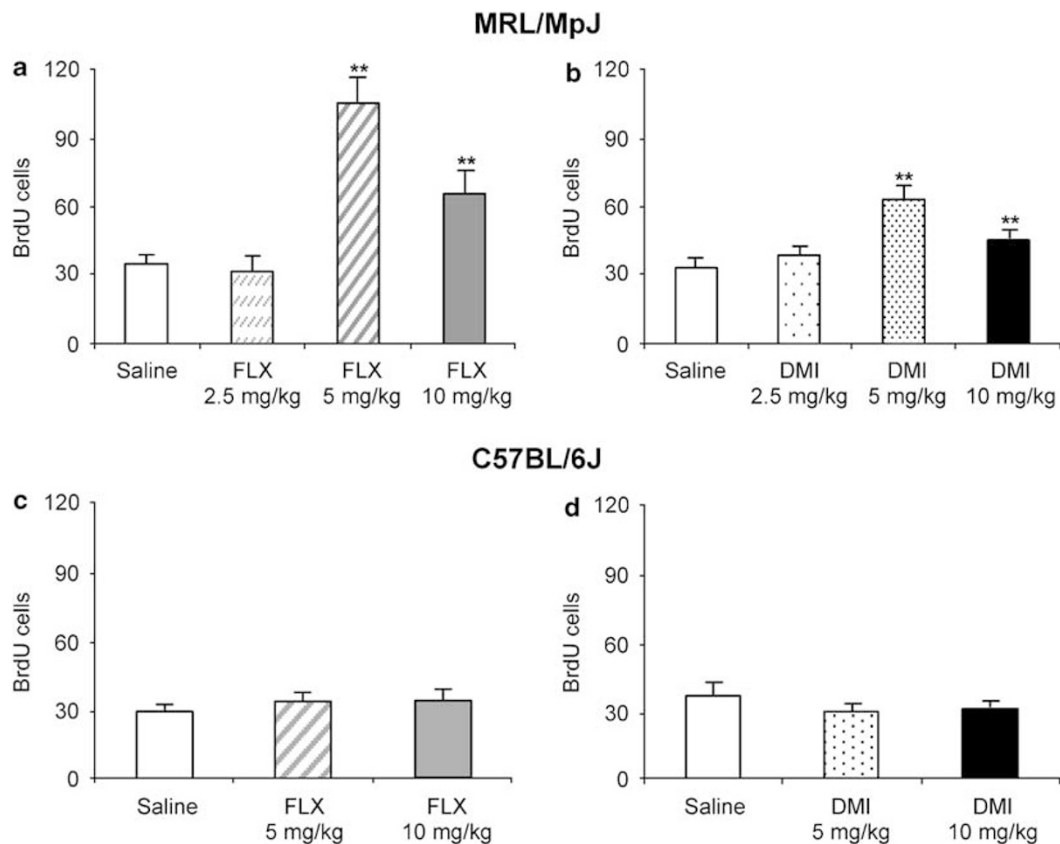
dose (10 mg/kg) produced a 50% increase. Contrary to the results obtained with MRL/MpJ mice, chronic treatment of C57BL/6J mice with FLX (Figure 1c) or DMI (Figure 1d), at any of the tested doses, failed to increase hippocampal cell proliferation.

In order to validate the findings obtained by flow cytometry, two separate cohorts of MRL/MpJ mice were treated chronically with 5 mg/kg of FLX or DMI for 21 days. Hippocampal cell proliferation was measured in the same animal by bisecting the brain and quantifying BrdU incorporation by flow cytometry in one hippocampal lobe and immunohistochemistry on the contralateral hemisphere. The effects of FLX (Figure 2a and b) and DMI (Figure 2d and e) on cell proliferation were equivalent in magnitude for both methods. Moreover, there was a significant correlation between the results obtained with flow cytometry and manual counting in both the FLX (Figure 2c;  $r(15)=0.62$ ,  $P=0.007$ ) and DMI (Figure 2f;  $r(33)=0.65$ ,  $P<0.001$ ) experiments. The effect sizes were calculated for both drug treatments to compare the precision of the two methods of analysis. The effect size correlations (FLX: flow cytometry = 0.77, immunohistochemistry = 0.85; DMI: flow cytometry = 0.35, immunohistochemistry = 0.42) showed that immunocytochemistry was slightly more precise than flow cytometry, by providing greater average differences between groups relative to the overall variance. FLX produced a similar magnitude of effect in MRL/MpJ mice between the first and second study (a.a., Figures 1 and 2). The effect of DMI was smaller in the second study than in the first, due to changes in rearing conditions for MRL/MpJ mice imposed by Jackson Laboratories around October 2007 (D Balu, personal communication).

### Effects of Chronic Fluoxetine and Desipramine Treatments on BDNF Protein Levels

Chronic treatment of MRL/MpJ mice with FLX elevated BDNF protein levels in several brain regions (Figure 3a). In the hippocampus, both the 5 and 10 mg/kg doses significantly increased BDNF protein levels by 2-fold. In the frontal cortex, the lower dose of FLX elevated BDNF levels by 20% (NS), whereas the higher dose caused a significant 3-fold elevation in BDNF protein. FLX at 10 mg/kg also increased BDNF levels in the amygdala by 3-fold. Neither dose of FLX affected BDNF protein levels in the brain stem. Chronic treatment of MRL/MpJ mice with 5 and 10 mg/kg of DMI (Figure 3b) significantly elevated BDNF protein levels in the frontal cortex by 15%, without effect in any of the other examined brain regions.

Chronic treatment of C57BL/6J mice with FLX (Figure 3c) or DMI (Figure 3d) produced different effects on BDNF protein in various brain regions. At the 10 mg/kg dose, FLX caused an almost 2-fold elevation of BDNF levels in the amygdala, whereas BDNF levels were reduced by 30% in the hippocampus. FLX at 5 mg/kg did not affect BDNF levels in any of the examined brain regions. In contrast to the results with MRL/MpJ mice, chronic treatment of C57BL/6J mice with DMI at both doses did not increase BDNF protein levels in any region of the forebrain, but selectively reduced BDNF levels by 50% in the brainstem.



**Figure 1** Chronic fluoxetine (FLX) and desipramine (DMI) treatments elevated cell proliferation in MRL/MpJ mice but not C57BL/6J mice. (a) MRL/MpJ mice were administered saline ( $n = 15$ ) or FLX (2.5, 5, 10 mg/kg, b.i.d.;  $n = 10$ /group) for 21 days. The 5 and 10 mg/kg doses of FLX increased cell proliferation ( $F(3,38) = 21.89, P < 0.0001$ ) (b) MRL/MpJ mice were administered saline ( $n = 15$ ) or DMI (2.5, 5, 10 mg/kg, b.i.d.;  $n = 10$ /group) for 21 days. DMI (5 and 10 mg/kg) significantly increased cell proliferation ( $F(3,38) = 8.77, P < 0.001$ ). (c) C57BL/6J mice were administered saline ( $n = 10$ ) or FLX (5 or 10 mg/kg, b.i.d.;  $n = 10$ /group) for 21 days. FLX did not alter cell proliferation ( $F(2,21) = 0.60, P = 0.56$ ). (d) C57BL/6J mice were administered saline ( $n = 10$ ) or DMI (5 or 10 mg/kg, b.i.d.;  $n = 10$ /group) for 21 days. DMI did not increase cell proliferation ( $F(2,22) = 1.17, P = 0.32$ ). Values are expressed as the number of BrdU-positive cells per 10 000 7-AAD events. Bars represent mean values + SEM. Asterisks (\*\*) indicate groups that differed significantly from control ( $P < 0.005$ ) according to Dunnett's test.

### Effects of Chronic Antidepressant Treatment on the Survival of Hippocampal Progenitors in the MRL/MpJ and C57BL/6J Mouse Strains

The effects of antidepressant drug treatments on cell survival were examined by administering BrdU prior to chronic treatment for 21 days. For these experiments, only the 5 mg/kg dose of FLX and DMI, which produced the peak effects on cell proliferation, were tested. However, neither chronic administration of FLX nor DMI altered the survival of newly born hippocampal progenitors in either the MRL/MpJ strain (Figure 4a) or the C57BL/6J strain (Figure 4b).

### Effects of Chronic Antidepressant Treatments on Novelty-Induced Hypophagia Behavior

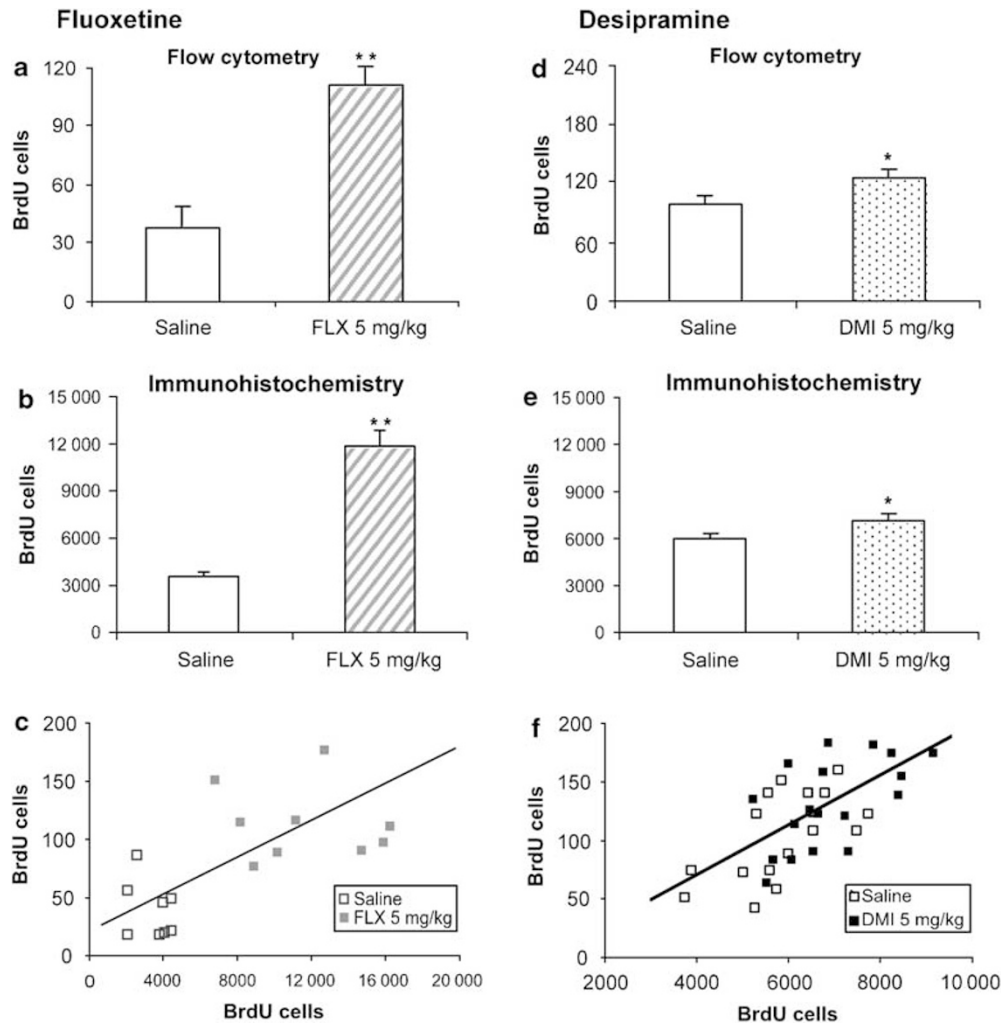
The exposure of MRL/MpJ mice to a novel environment increased their latency to consume peanut butter chips 6-fold, compared with their home cage latency times of 5 s, to a mean value of 37 s (Figure 5a). Chronic treatment of these mice with FLX (5 mg/kg) or DMI (5 mg/kg) significantly reduced the consumption latencies to 11 and 5 s, respectively (Figure 5a). When compared with the home cage, consumption in the novel environment was suppressed by 50%. However, there were no differences in

home cage or novel cage consumptions between the drug treatment groups (data not shown).

During training, C57BL/6J mice were given more training sessions and a longer exposure time to the food than MRL/MpJ mice, in order to develop stable home cage response latencies. C57BL/6J mice had stable home cage latencies of 13 s, compared with MRL/MpJ mice that were stable at 5 s. Exposure to the novel environment produced an increase in approach latency and decrease in food consumption (data not shown) in the C57BL/6J mice. Approach latencies were increased to a greater extent than MRL/MpJ mice (Figure 5b). Unlike MRL/MpJ mice, chronic antidepressant drug treatments did not reduce their latency to eat in the novel cage (Figure 5b). The drug treatments also did not alter their home cage or novel cage consumptions (data not shown).

## DISCUSSION

The results of this study showed the existence of important strain differences in the responses of mice to chronic administration of antidepressant drugs. MRL/MpJ mice showed robust increases in hippocampal cell proliferation, increased cortico-limbic BDNF protein levels, and

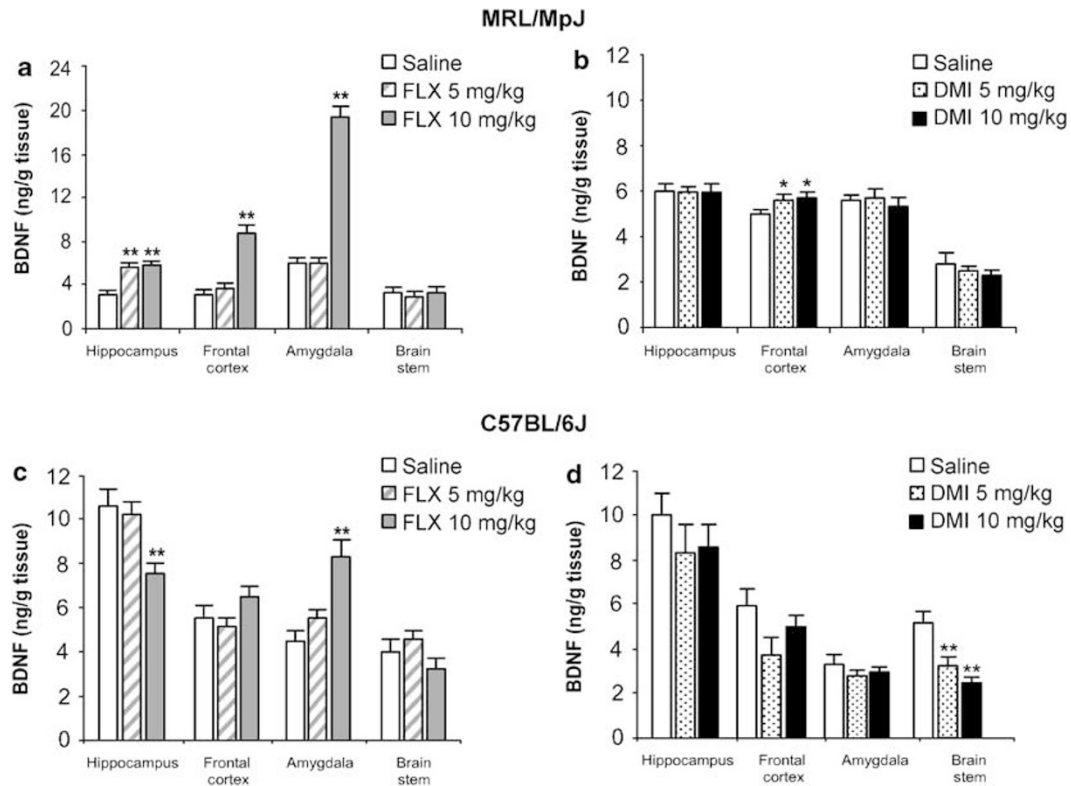


**Figure 2** Comparison of BrdU incorporation in the hippocampus measured by flow cytometry and immunohistochemistry after chronic antidepressant treatments. One cohort of MRL/MpJ mice (a–c) was administered saline ( $n = 9$ ; open bars) or fluoxetine (FLX; 5 mg/kg, b.i.d.;  $n = 9$ ; striped bars), whereas another (d–f) was administered saline ( $n = 17$ ; open bars) or desipramine (DMI; 5 mg/kg, b.i.d.;  $n = 18$ ; dotted bars) for 21 days. (a, d) Cell proliferation was measured in one hippocampal lobe by flow cytometry. Values are expressed as the number of BrdU-positive cells per 10 000 7-AAD events. FLX ( $t(18) = 5.94, P < 0.001$ ) and DMI ( $t(33) = 2.12, P = 0.04$ ) significantly increased cell proliferation. (b, e) The contralateral hemisphere was sectioned and BrdU-labeled cells in the hippocampus were counted using immunohistochemistry. Values are expressed as the number of BrdU-positive cells per hippocampus. FLX ( $t(15) = 6.47, P < 0.001$ ) and DMI ( $t(33) = 2.66, P = 0.01$ ) significantly increased cell proliferation. Bars represent mean values + SEM. Asterisks indicate groups that differed significantly from saline (\* $P < 0.05$ , \*\* $P < 0.005$ ), according to the unpaired Student's *t*-test. (c, f) Scatter plots showing the correlation between results obtained from individual mice using flow cytometry and immunohistochemistry (FLX:  $r(15) = 0.62, P = 0.007$ ;  $r(33) = 0.65, P < 0.001$ ).

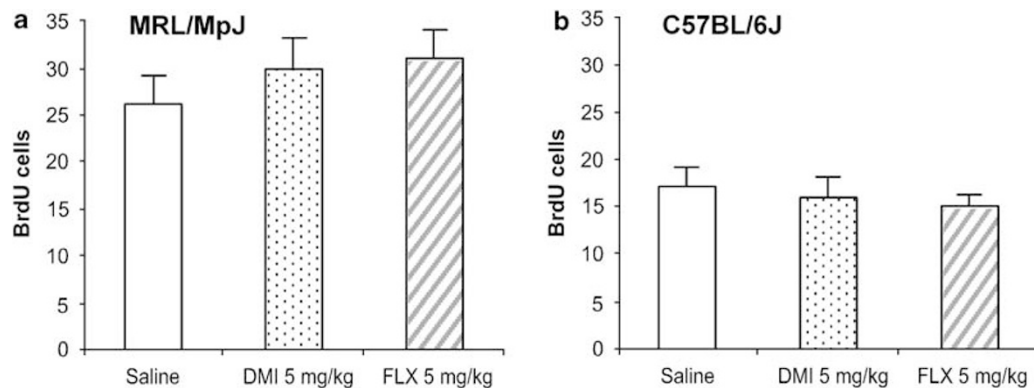
diminished reactions to novelty in the NIH test, whereas C57BL/6J mice did not show significant changes in these parameters after the same treatments. The background strain is critical for producing typical effects of antidepressants after acute (Lucki *et al*, 2001; Crowley *et al*, 2005) or chronic (Holick *et al*, 2008; Miller *et al*, 2008) treatments. The results of this study emphasize the importance of strain differences for measuring the effects of antidepressants requiring chronic drug administration. MRL/MpJ mice were studied because they are known distinctively for enhanced wound healing and tissue regeneration after injury (Clark *et al*, 1998; Leferovich *et al*, 2001; Heber-Katz *et al*, 2004). This study showed exaggerated neuroplastic responses in parallel with behavioral changes to chronic antidepressant drug treatments in these mice. Identification of genetic and neural substrates, responsible for these responses in MRL/MpJ mice, could provide important information concerning

the presently unknown mechanisms that regulate the chronic effects of antidepressants, and that may be associated more closely with their ultimate clinical therapeutic effects.

The existence of neurogenesis throughout the mammalian lifespan in particular brain regions has now been accepted. Adult hippocampal neurogenesis has been associated with a number of diseases, such as depression, schizophrenia, epilepsy, Parkinson's disease, and Alzheimer's disease (Balu and Lucki, 2008; Thompson *et al*, 2008; Zhao *et al*, 2008). As a result, there is much interest in studying the effects of various pharmacologic treatments on adult or developmental hippocampal neurogenesis. Hippocampal neurogenesis is regulated by a variety of neurotransmitters and hormones, and pharmacological regulation of neurogenesis can lead to the discovery of novel medications for these disorders. The most common method for measuring cellular



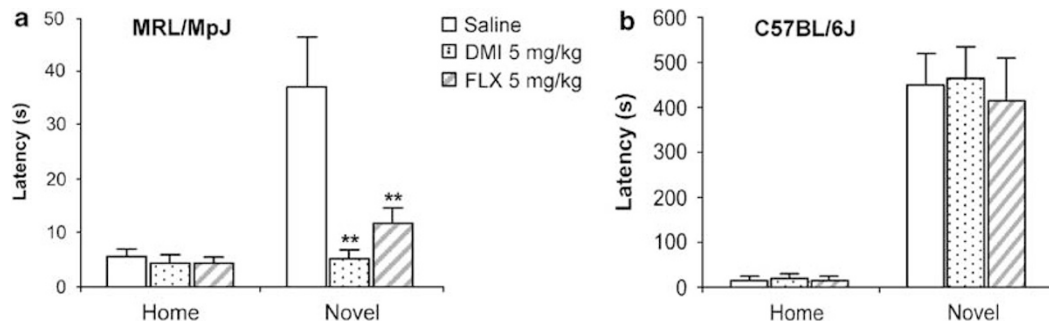
**Figure 3** Differential regulation of BDNF protein levels by chronic fluoxetine (FLX) and desipramine (DMI) treatments in MRL/MpJ mice and C57BL/6J mice. (a) MRL/MpJ and (c) C57BL/6J mice were administered saline (open bar,  $n = 10$ ) or FLX ( $n = 10$ /group) at 5 (striped bar) or 10 mg/kg b.i.d. for 21 days. In MRL/MpJ mice, FLX increased BDNF levels in the hippocampus ( $F(2,25) = 52.62$ ,  $P < 0.001$ ), frontal cortex ( $F(2,25) = 44.81$ ,  $P < 0.001$ ), and amygdala ( $F(2,25) = 155.08$ ,  $P < 0.001$ ). In C57BL/6J mice, FLX decreased BDNF levels significantly in the hippocampus ( $F(2,25) = 7.40$ ,  $P < 0.003$ ) and increased BDNF levels in the amygdala ( $F(2,25) = 14.04$ ,  $P < 0.001$ ). All other regional comparisons were not statistically significant. (b) MRL/MpJ and (d) C57BL/6J mice were administered saline (open bars,  $n = 10$ ) or DMI ( $n = 10$ /group) at 5 (dotted bars) or 10 (black bar) mg/kg b.i.d. for 21 days. In MRL/MpJ mice, DMI increased BDNF levels only in the frontal cortex ( $F(2,27) = 4.33$ ,  $P = 0.02$ ). In C57BL/6J mice, DMI decreased BDNF levels in the brain stem ( $F(2,27) = 11.36$ ,  $P < 0.001$ ). All other regional comparisons were not statistically significant. Values are expressed as the amount of BDNF protein per gram of tissue. Bars represent mean values  $\pm$  SEM. Asterisks indicate groups that differed significantly from saline treatment (\* $P < 0.05$ , \*\* $P < 0.005$ ) according to Dunnett's test.



**Figure 4** Chronic treatments with fluoxetine (FLX) or desipramine (DMI) did not affect the survival of newly born hippocampal progenitor cells. Cells were labeled by administration of BrdU (100 mg/kg, i.p. for 4 days) prior to the initiation of chronic treatment. (a) MRL/MpJ mice or (b) C57BL/6J mice ( $n = 10$  mice/group) were administered saline (open bar), FLX (5 mg/kg, b.i.d.; striped bar), or DMI (5 mg/kg, b.i.d.; dotted bar) for 21 days. Values for ANOVA were not statistically significant ( $P > 0.05$ ). Values are expressed as the number of BrdU-positive cells per 10 000 7-AAD events. Bars represent mean values  $\pm$  1 SEM.

genesis in brain tissues involves marking DNA synthesis by administering the thymidine analog BrdU and then counting the number of BrdU-labeled cells using immunohistochemistry. However, the intensive labor and time required

to perform these studies has made hippocampal neurogenesis an impractical target for screening novel compounds in animals. This study used flow cytometry to quantify BrdU incorporation into hippocampal cells after administering



**Figure 5** Chronic treatments with fluoxetine (FLX) and desipramine (DMI) reduced reactivity to novelty in the NIH test in MRL/MpJ mice but not in C57BL/6J mice. (a) MRL/MpJ mice ( $n = 7-9$ ) or (b) C57BL/6J mice ( $n = 7-9$ ) were administered saline (open bar), DMI (5 mg/kg, b.i.d.; dotted bar), or FLX (5 mg/kg; striped bar) for 21 days. The chronic antidepressant treatments reduced the latency to initiate feeding in a novel environment for MRL/MpJ mice ( $F(2,25) = 8.13$ ,  $P = 0.002$ ) but not for C57BL/6J mice ( $F(2,20) = 0.10$ ,  $P = 0.90$ ). The antidepressant treatments did not alter home cage feeding for both strains. Values are expressed as the latency (s) to consume peanut butter chips in either the home cage (Home) or a novel environment (Novel). Bars represent mean values + SEM. Asterisks (\*\*) indicate groups that differed significantly from saline ( $P < 0.005$ ) according to Dunnett's test.

chronic antidepressant drug treatments and showed significant drug and strain differences in cell proliferation (see also (Balu *et al*, 2008a; Bilsland *et al*, 2006; Shankaran *et al*, 2006). This technique circumvents many technical limitations associated with immunohistochemistry, and provides the speed and automated analysis necessary to facilitate drug discovery and understand the mechanisms underlying drug effects on neurogenesis. The enhanced analytical power of flow cytometry was associated with slightly inferior precision, when compared with immunohistochemistry, which may reflect the ability to topographically restrict counting labeled cells to the dentate gyrus. Future advances of cell phenotyping using flow cytometry, double-labeling BrdU-positive cells with antibodies for neural, glial, and developmental markers, will enable more specific morphogenic changes produced by chronic antidepressant drug treatments to be identified rapidly. Nevertheless, rapid screening can still identify those conditions requiring more detailed anatomical analysis.

The value of a rapid quantitative screening approach for measuring hippocampal cell proliferation was shown in this study. Both FLX and DMI showed an inverted U-shaped dose-response curve for increased proliferative activity in MRL/MpJ mice after chronic treatment. The inverted U-shaped curve for these antidepressants could be caused by their affinity for receptors unrelated to their primary effect of selectively blocking serotonin and norepinephrine transporters, respectively (Richelson, 2003). In contrast, C57BL/6J mice did not show an increase in cell proliferation. There have been mixed reports on the ability of antidepressants to regulate hippocampal neurogenesis in C57BL/6J mice. Some studies have also found that FLX administered for 10 days (10 mg/kg, i.p.) (Beauquis *et al*, 2006) or for 24 days (10, 16, or 25 mg/kg in drinking water) (Navailles *et al*, 2008) failed to increase hippocampal cell proliferation. However, other studies reported that FLX (10 mg/kg, i.p.) administered for 21 days (Lagace *et al*, 2007) or amitriptyline (in drinking water) given for 28 days (Caldarone *et al*, 2004) resulted in a 25% increase in hippocampal cell proliferation in C57BL/6J mice. It is unclear whether procedural differences in drug administration, BrdU loading protocols, or differences in levels of endogenous stress contributed to different findings between laboratories. Differences in the response to chronic FLX

have been reported for other strains (Santarelli *et al*, 2003; Holick *et al*, 2008; Miller *et al*, 2008), but no strain showed a response as large as shown by the MRL/MpJ mice. Most of the cells in the adult hippocampus generated from amplifying progenitor cells become neurons (Encinas *et al*, 2006; Wang *et al*, 2008).

Although the regulation of hippocampal cell proliferation by chronic antidepressant treatments is established, their ability to enhance the long-term survival of newly born neurons is less well defined. Treatment with DMI or FLX for 21 days did not enhance the survival of newly born progenitors in either the MRL/MpJ or C57BL/6J strain in this study, when BrdU was administered before the antidepressant treatments. An earlier study showed that hippocampal cell survival in rats was not altered by chronic treatment with FLX (5 mg/kg) for 14 days (Malberg *et al*, 2000). However, more recent studies showed that FLX given chronically to rats (5 mg/kg, i.p.) or 129SvEv mice (18 mg/kg, in drinking water) for 28 days was effective in prolonging cell survival (Nakagawa *et al*, 2002; Wang *et al*, 2008). Treatments with other doses, longer durations, or BrdU-labeling protocols could show increased cell survival, and this remains to be determined. However, increased survival of newborn hippocampal cells was not produced under chronic treatment conditions that increased cell proliferation in MRL/MpJ mice.

Another important marker of chronic antidepressant treatments is their ability to increase levels of BDNF protein or mRNA, particularly in the hippocampus and frontal cortex (Duman and Monteggia, 2006). Although similar findings have been reported in the mouse (Conti *et al*, 2002; Song *et al*, 2006; Tsankova *et al*, 2006), BDNF was measured only at the mRNA level. In MRL/MpJ mice, chronic administration of FLX increased BDNF protein levels in the hippocampus, frontal cortex, and amygdala, whereas DMI elevated BDNF levels specifically in the frontal cortex, which is similar to effects reported recently in rats (Balu *et al*, 2008b). In contrast, the chronic antidepressant treatments did not increase BDNF levels in the frontal cortex or hippocampus in C57BL/6J mice. FLX (10 mg/kg) increased BDNF in the amygdala and decreased BDNF in the hippocampus, whereas DMI dose-dependently and selectively, reduced BDNF protein levels in the brain stem. Mobilization of neurotrophins after chronic antidepressant



drug treatments has been considered to play a key role in promoting neuroplasticity and behavioral change (Duman and Monteggia, 2006; Sairanen *et al*, 2005). In this study, the increased BDNF levels in MRL/MpJ mice was associated with increased hippocampal cytochrome and changes in behavior, and is a candidate for driving these changes. In addition, the augmentation of other forms of synaptic plasticity after chronic antidepressant treatments, such as enhanced LTP (Wang *et al*, 2008), circuit level activity (Airan *et al*, 2007) in the dentate gyrus of the hippocampus, and increased dendritic arborization of adult-born hippocampal neurons (Wang *et al*, 2008), could be mediated, in part, by BDNF mobilization (Elmariah *et al*, 2005; Mamounas *et al*, 1995).

The differential effects of FLX and DMI in neuroplasticity and behavior between the two mouse strains could result from differences in pharmacokinetics. However, as these effects were produced by two pharmacologically distinct antidepressants, this explanation is unlikely.

Reduction of hyponeophagia is one of the few behavioral responses produced by chronic antidepressant drug treatments (Merali *et al*, 2003; Dulawa and Hen, 2005), and was used to compare behavioral effects between these mouse strains. Chronic administration of FLX or DMI to MRL/MpJ mice, but not C57BL/6J mice, reduced their latency to consume food in the NIH paradigm. The divergent behavioral responses of the mouse strains paralleled their response to chronic antidepressant treatments on hippocampal cell proliferation and cortico-limbic BDNF levels, although the link between these factors is unclear (Dranovsky and Hen, 2006). Hippocampal neurogenesis appeared to be required for the behavioral effects of chronic antidepressant treatments in the novelty suppression of feeding paradigm in 129SvEvTac mice because cessation of neurogenesis by X-irradiation prevented chronic antidepressant behaviors (Santarelli *et al*, 2003). However, hippocampal neurogenesis did not correlate with the ability of chronic FLX to mediate NIH behavior in unperturbed BALB/cJ mice (Holick *et al*, 2008). Nevertheless, a causal link may still exist when changes in hippocampal neurogenesis occur with the moderation of NIH behavior after chronic antidepressant drug treatments, and future studies are needed to assess this relationship in the MRL/MpJ strain. Taken together with hippocampal neurogenesis and BDNF mobilization, these results using markers of chronic, rather than acute, antidepressant drug activity highlight the potential utility of the MRL/MpJ mouse to test novel compounds for potential antidepressant activity.

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## DISCLOSURE/CONFLICT OF INTEREST

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