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Loss of MOR-1 on different genetic backgrounds leads to increased BrdU labeling in the dentate gyrus only after repeated injection

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Abstract

The endogenous opioid system is involved in various physiological processes including neurogenesis in the dentate gyrus (DG) of the hippocampus. In the current study, we investigated the role of the mu opioid receptor (MOR-1) on DG neurogenesis and measured glucocorticoid levels following several injection paradigms to supplement the neurogenesis experiments. MOR-1 knockout (KO) mice on C57BL/6 and 129S6 backgrounds were injected with BrdU using either a single injection or two different repeated injection protocols and then sacrificed at different time points. The total number of BrdU and PCNA positive cells in the DG is significantly increased in MOR-1 KO mice compared to WT on both strains after repeated injection, but not after a single injection. Plasma corticosterone (CORT) levels increased similarly in MOR-1 KO and WT mice following both single and repeated injection, indicating that the stress response is activated following any injection protocol, but that the mechanism responsible for the increase in BrdU labeling in MOR-1 KO mice is CORT-level independent. Finally, WT 129S6 mice, independent of genotype, showed higher levels of plasma CORT compared to WT C57BL/6 mice in both non-injected controls and following injection at two separate time points; these levels were inversely correlated with low numbers of BrdU cells in the DG in 129S6 mice compared to C57BL/6 mice. In summary, these data demonstrate that loss of MOR-1 increases BrdU labeling in the DG

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independent of CORT levels, but only following a repeated injection, illustrating the capability of injection paradigms to influence cell proliferative responses in a genotype-dependent manner.

Keywords

dentate gyrus; hippocampus; opioid; neurogenesis; corticosterone; BrdU

A proliferative population of neuronal and glial precursors is maintained in the dentate gyrus (DG) throughout adulthood in many species (Kempermann et al., 1997; Cameron and McKay, 1998; Eriksson et al., 1998; Gould et al., 1999b). New neurons are produced from this progenitor population in the subgranular zone (SGZ) of the adult DG that, after maturation, are incorporated into the existing hippocampal network and are thought to be important for learning and memory processes (Gould et al., 1999a; Dobrossy et al., 2003; Drapeau et al., 2003; Leuner et al., 2004). Many factors impact neurogenesis in the DG including stress (Gould et al., 1998; Gould and Tanapat, 1999), exercise (van Praag et al., 1999b; van Praag et al., 1999a), mouse background strain (Kempermann et al., 1997; Hayes and Nowakowski, 2002), and drugs of abuse (Eisch et al., 2000; Yamaguchi et al., 2004; Yamaguchi et al., 2005; Crews et al., 2006; Noonan et al., 2008).

One class of drugs of abuse that can influence the population of neuronal precursors in the DG is the opiates. In vivo studies have shown that morphine, a mu opioid receptor (MOR-1) agonist, decreases neurogenesis, alters cell cycle dynamics, and leads to changes in the neurogenic microenvironment in the DG (Eisch et al., 2000; Mandyam et al., 2004; Kahn et al., 2005; Arguello et al., 2008; Arguello et al., 2009), while opioid antagonists like naltrexone and naltrindole instead increase neurogenesis in the DG (Persson et al., 2004). In addition, the survival rate of newborn cells in the DG is increased in MOR-1 knockout mice (Harburg et al., 2007).

While Bromodeoxyuridine (BrdU) labeling, utilizing several different injection protocols, has been used to assess levels of cell proliferation in the DG for many years (Kuhn et al., 1996; Wojtowicz and Kee, 2006; Kuhn and Cooper-Kuhn, 2007; Leuner et al., 2009), there has been little consideration of whether different injection paradigms might differentially influence the proliferative response in a genotype-dependent manner. Here, we used three separate BrdU injection paradigms to investigate the role of MOR-1 on cell proliferation in the DG and to determine if results vary when different injection paradigms are used. In addition, we utilized MOR-1 knockout mice on two distinct inbred strains of mice, 129S6 and C57BL/6, to determine if genetic background could influence MOR-1 effects on cell proliferation in the DG in any or all of these injection paradigms. Lastly, we measured plasma corticosterone levels in both strains basally and following injection to test whether BrdU labeling in WT and/or MOR-1 KO mice is correlated with basal or injection-stimulated corticosterone levels.

Experimental Procedures

Animals

All experiments were performed on 8-week-old male wild type (WT; +/+) and MOR-1 KO (-/-) mice (Schuller et al., 1999) maintained in either the isogenic 129S6 or the congenic C57BL/6 backgrounds. The 129S6 isogenic strain was generated by mating the original heterozygous male chimeras with 129S6 inbred females. The C57BL/6 congenic strain was obtained by backcrossing outbred MOR-1 KO mice to inbred C57BL/6 mice for at least fifteen generations (Wen et al., 2009). Genotypes for all mice were verified using PCR and/or Southern Blot analysis. Separate mating cages were kept for each genotype.

Experimental Design

Several BrdU injection paradigms were used in this study. In the initial experiment, mice were injected with BrdU (50 mg/kg) every four hours over a period of twelve hours (four BrdU injections total) and sacrificed twelve hours following the last injection. Since BrdU only labels cells in S-phase at a given time point, this schedule was selected in an attempt to label the entire proliferative population that exists in the SGZ, based on the estimate that the total cell cycle time in the DG is about twelve to fourteen hours with S-phase lasting for about four to six hours (Hayes and Nowakowski, 2002). Lower doses of BrdU do not label all of the cells in S-phase (Cameron and McKay, 2001; Mandyam et al., 2007); however, since the injection was repeated four times (totaling 200mg/kg) the 50mg/kg dose was chosen to reduce the possibility of BrdU induced cellular toxicity.

In order to replicate an earlier study on neurogenesis in MOR-1 KO mice (Harburg et al., 2007) and to assess baseline levels of cell proliferation, a second injection paradigm was used. In this paradigm, mice were injected once with BrdU (100 mg/kg) and then sacrificed either two hours or twenty-four hours later. Finally, a third BrdU injection paradigm was chosen in order to mimic the physiological and behavioral effects of the repeated intraperitoneal (IP) injection without labeling additional cells with BrdU. In this paradigm, mice were injected once with BrdU (50 mg/kg) and then injected with vehicle (sterile saline with .007M NaOH) every four hours following the BrdU injection (total of one BrdU and three vehicle injections over twelve hours). Mice were then sacrificed twelve hours after the last vehicle injection.

Preparation and Injection of BrdU

Solutions of BrdU were prepared in sterile saline with .007M NaOH at 5mg/ml for the 50mg/kg dose and 10mg/ml for the 100mg/kg dose. In order to ensure that all mice received the same dose and approximately the same injection volume, mice were dosed according to their body weight using the solution described above. The average injection volume was .30 ml.

Tissue Preparation and BrdU/PCNA Immunohistochemistry

At the time of sacrifice, animals were anesthetized and then perfused transcardially with 4% paraformaldehyde. Brains were dissected following perfusion and the left hemisphere was imbedded in paraffin. After imbedding, the brains (left hemisphere) were sectioned at 10um thickness on a microtome and every section through the DG was collected on slides. Every 16th section was stained for BrdU and Proliferating Cell Nuclear Antigen (PCNA) immunohistochemistry. First, sections were deparaffinized using xylene and an ethanol series. Then, for BrdU staining sections were pretreated with 0.1% trypsin in Tris buffer for antigen retrieval, 2N HCl to denature DNA, and 3% H₂O₂ in methanol to block endogenous peroxidase. For PCNA staining, sections were pretreated with citrate buffer (95°C) for antigen retrieval and 3% H₂O₂ in PBS to block endogenous peroxidase. Next, sections were blocked with 5% horse serum and then primary anti-BrdU antibody (BD Bioscience), 1:100, or primary anti-PCNA antibody (Santa Cruz), 1:500, was added. Following incubation with the primary antibody overnight at 4°C, sections were washed and then biotinylated anti-mouse secondary antibody (Vector) was added at a 1:300 dilution for 1 hour at room temperature. ABC (Vector) and DAB (Sigma) were added to visualize BrdU positive cells. Sections were then stained with methyl green to visualize the granule cell layer and all other cells that were not BrdU or PCNA positive.

BrdU/PCNA Quantification

The optical fractionator technique was used to determine the total number of BrdU/PCNA positive cells in the DG (West et al., 1991). Immunohistochemistry for BrdU/PCNA was performed on every 16th section through the entire DG. The number of BrdU/PCNA positive cells in the subgranular zone (SGZ) of the DG was counted under the 40X objective of a Leica (Leitz DMRB) microscope for each section with an average of 15 sections being counted for each animal. For each animal the number of BrdU/PCNA positive cells for each section (Q) was recorded and then a total number was determined by taking the sum of all the sections, ΣQ . In order to determine the total number (N) of BrdU/PCNA positive cells in the DG per animal the ΣQ was multiplied by 16. As an internal control, the total number of BrdU positive cells in the external capsule, a white matter track that projects between the cortex and sub-cortical structures, was determined as described above.

Double-labeling/Immunofluorescence

To determine if cell fate is altered in MOR-1 KO mice following multiple injection and if BrdU positive cells are neuronal precursors, mice were injected with BrdU at a dose of 50 mg/kg every four hours over a period of twelve hours and then sacrificed four weeks following the last injection in order to allow newly born cells to fully mature. After sacrifice, brains were processed as described above and then stained for BrdU/NeuN (Neuronal Nuclei), to identify neurons, and BrdU/GFAP (Glial fibrillary acidic protein), to identify astrocytes, using fluorescent double-labeling as described below.

For immunofluorescent double-labeling of BrdU/NeuN and BrdU/GFAP in the DG every 24th section was used. After pretreatment (described above) and blocking step with 5% goat serum sections were incubated sequentially with BrdU primary antibody (Rat anti BrdU; Serotec; 1:100) and secondary antibody (Alexa Fluor 594 goat anti-rat; Invitrogen; 1:250) and then with either NeuN primary antibody (mouse anti-NeuN; Chemicon; 1:100) or GFAP primary antibody (rabbit anti-GFAP; Dako; 1:1000) and their respective secondary antibodies (Alexa Fluor 488 goat anti-mouse; Invitrogen; 1:250 and Alexa Fluor 488 goat anti-rabbit; Invitrogen; 1:1000). DAPI (Invitrogen) was used as a background stain. The number of BrdU positive cells in the granule cell layer (GCL) that co-localize with NeuN or GFAP was determined.

Corticosterone Measurement

In order to determine if changes in corticosterone (CORT) levels following single or repeated injection could explain any differences in BrdU labeling between genotypes, plasma CORT levels were measured in WT and MOR-1 KO mice on both strains. CORT levels were measured following four IP injections of vehicle every four hours over the course of twelve hours to mimic the repeated BrdU injection paradigm and following a single injection of vehicle to mimic the single BrdU injection paradigm.

Mice were sacrificed 15 minutes following the last IP injection and trunk blood was collected in tubes containing EDTA. Mice were sacrificed between 1000–1100 hours for the single injection and between 2000–2100 hours for the repeated injection. The blood was centrifuged for ten minutes at 3000 RPM. The plasma was separated and stored at -20°C until plasma corticosterone levels were determined using the ImmuChemTM Double Antibody Corticosterone ^{125}I Radioimmunoassay (RIA) Kit (MP Biomedicals, NY).

Statistical Analysis

Data are reported as mean $\pm$ SEM. ANOVA was used to perform statistical analysis and statistical significance was defined as $P < .05$, which is denoted by *, unless otherwise noted.

Results

Loss of MOR-1 on the C57BL/6 and the 129S6 strains leads to an increase in BrdU labeling following repeated BrdU injection

To determine if loss of MOR-1 leads to changes in cell proliferation in the SGZ of the DG, WT (+/+) and MOR-1 KO (-/-) mice on both the C57BL/6 and 129S6 backgrounds were injected with BrdU four times over a period of twelve hours (Hayes and Nowakowski, 2002). There was a 75% increase in the number of BrdU positive cells in the SGZ in C57BL/6 MOR-1 KO mice compared to WT mice ($F(1,18) = 6.745$, $*P < .05$) and a 60% increase in BrdU positive cells in the SGZ of 129S6 MOR-1 KO mice compared to WT mice ($F(1,11) = 7.174$, $*P < .05$) (Fig. 1, Table 1). This effect appears to be specific to the DG since no differences were observed in the number of BrdU labeled cells in the external capsule between genotypes on either strain. (C57BL/6: WT 12832 ± 671.5 , MOR KO 11053.7 ± 755.8 , $F(1,11) = 2.995$; 129S6: WT 10064 ± 1811.9 , MOR KO 8544 ± 1063.9 , $F(1,11) = .562$) Interestingly, the total number of BrdU positive cells in the DG is significantly lower in 129S6 mice compared to C57BL/6 mice ($F(1,13) = 60.631$, $\#P < .0001$, C57BL/6 WT vs. 129S6 WT) (Fig. 1, Table 1), though the magnitude of the increase in MOR-1 KO mice is similar between strains. These data indicate that MOR-1 is involved in the regulation of neurogenesis in the DG and that this effect is conserved between two separate genetic backgrounds.

Most BrdU positive cells in the DG, independent of genotype or strain, express the neuronal marker, NeuN

It is possible that loss of MOR-1 could alter the phenotype of newly born cells. Therefore, cell fate was assessed four weeks following the repeated BrdU injection protocol by performing double labeling for BrdU and NeuN and for BrdU and GFAP to determine the percentage of BrdU labeled cells that express NeuN, a mature neuronal marker, or GFAP, a marker for astrocytes. The phenotype of newly born cells in the DG is not altered in MOR-1 KO mice compared to WT on both the C57BL/6 and the 129S6 strains (Fig. 2). BrdU and NeuN co-localize in approximately 85–90% of newly born cells in both C57BL/6 MOR-1 KO and WT mice (Fig. 2C) and in approximately 65–70% of newly born cells in both 129S6 MOR-1 KO and WT mice (Fig. 2D). These data indicate that loss of MOR-1 does not alter cell fate in the DG and that most of the newly born cells in the SGZ develop into neurons regardless of genotype.

BrdU labeling in the DG is unchanged in MOR-1 KO mice on the C57BL/6 and 129S6 strains following a single BrdU injection

Although the data described above indicate that MOR-1 KO mice, on two different genetic backgrounds, have an increased number of BrdU labeled cells in the SGZ of the DG, this finding was in contrast to a prior study in which no difference in the number of BrdU positive cells between MOR-1 KO and WT mice on a mixed background was seen two hours following a single BrdU injection (Harburg et al., 2007). Therefore, we next injected mice using a single injection paradigm in an attempt to replicate this prior study. Data from this experiment, as in the previously published study, showed no significant differences in the total number of BrdU positive cells in the SGZ between C57BL/6 or 129S6 WT and MOR-1 KO mice when sacrificed either two hours or twenty-four hours following one BrdU injection (Fig. 3, Table 1). These data suggest that the repeated injection is necessary to elicit the increase in BrdU labeling in MOR-1 KO mice compared to WT mice, as seen in the initial experiment.

MOR-1 KO mice on the C57BL/6 and 129S6 strains exhibit an increase in cell proliferation in the DG following a single BrdU injection and three vehicle injections

In order to determine if the increase in BrdU labeling in MOR-1 KO mice following multiple BrdU injections is elicited by a response to the repeated injection, we next injected mice once with BrdU followed by three vehicle injections over twelve hours. This paradigm was thus identical to that used in the initial experiment except that vehicle was substituted for BrdU in the last three injections. The results again showed a significant increase in the total number of BrdU positive cells in MOR-1 KO mice on both the C57BL/6 ($F(1,8) = 8.763$, $*P < .05$) and the 129S6 ($F(1,5) = 11.724$, $*P < .05$) strains when the initial BrdU injection was followed by three vehicle injections (Fig. 4, Table 1). This result was in contrast to the lack of change in BrdU labeling in MOR-1 KO mice twenty-four hours following a single BrdU injection (Fig. 3), even though the total dose of BrdU was higher in the single injection experiment (100mg/kg vs. 50mg/kg). In addition, these results were confirmed using PCNA; there is a significant increase in the total number of PCNA positive cells in the SGZ in C57BL/6 and 129S6 MOR-1 KO mice compared to WT (C57BL/6: $F(1,8) = 14.127$, $n=5$, $*P < .01$; 129S6: $F(1,8) = 7.202$, $n=5$, $*P < .05$) (Fig. 5). Together these data indicate that the repeated injection paradigm, even if comprised of primarily vehicle injections, leads to a genotype-dependent increase in cell proliferation in the DG on two independent mouse strains.

Plasma CORT levels are similar in MOR-1 KO mice compared to WT on the C57BL/6 and 129S6 strains following both single and repeated vehicle injection

Since increased levels of CORT have been shown to decrease neurogenesis in the DG (Gould and Tanapat, 1999), we next wanted to test the hypothesis that loss of MOR-1 decreases the rise in plasma CORT expected following multiple injections, which in turn could lead to a relative increase in BrdU labeling in the DG of MOR-1 KO mice compared to WT. In order to accomplish this, we measured plasma CORT levels in WT and MOR-1 KO mice following both the single and repeated injection paradigms used for BrdU labeling. CORT levels increased significantly in mice of both genotypes and both strains either injected once or injected repeatedly compared to non-injected controls; no differences were detected between genotypes (Fig. 6) (Single Injection; C57BL/6 injected vs. control, $F(1,14) = 43.619$, $*P < .001$, $n=4$ WT, $n=5$ MOR-1KO; 129S6 injected vs. control, $F(1,14) = 16.401$, $*P < .01$, $n=5$ WT, $n=4$ MOR-1KO) (Repeated Injection; C57BL/6 injected vs. control, $F(1,21) = 17.853$, $*P < .001$, $n=6$ WT and MOR-1 KO control, $n=5$ WT injected, $n=8$ MOR-1 KO injected; 129S6 injected vs. control, $F(1,22) = 8.247$, $*P < .01$, $n=7$ WT and MOR-1 KO injected, $n=6$ WT and MOR-1 KO control). These results demonstrate that both the single and repeated injection lead to increased CORT levels and thus activate this aspect of the stress response to the same magnitude in both genotypes on both strains. Thus, the data indicate that a genotype-dependent attenuated CORT response following repeated injection may not explain the increased number of BrdU positive cells in the DG in MOR-1 KO mice.

129S6 mice have elevated plasma CORT levels compared to C57BL/6 mice

The plasma CORT measurements described above also showed that 129S6 mice have elevated plasma CORT levels compared to C57BL/6 mice (Fig. 7) independent of genotype both in the morning (between 1000–1100 hours) when circulating levels of CORT are normally low and in the evening (between 2000–2100 hours) when circulating levels of plasma CORT are higher due to circadian regulation (Buijs and Kalsbeek, 2001). Plasma CORT levels were significantly higher in the morning in 129S6 mice compared to C57BL/6 mice both in non-injected controls and following a single vehicle injection (Fig. 7) (Control: 129S6 vs. C57BL/6, $F(1,16) = 13.430$, $*P < .01$, $n=9$; Injected: 129S6 vs. C57BL/6, $F(1,16) = 20.670$, $*P < .001$, $n=9$). In addition, plasma CORT levels were significantly higher in

129S6 mice compared to C57BL/6 mice in the evening both in non-injected controls and following repeated injection (Fig. 7). (Control: 129S6 vs. C57BL/6, $F(1,22) = 4.390$, $*P < .05$, $n=12$; Injected: 129S6 vs. C57BL/6, $F(1,25) = 20.932$, $*P < .01$, $n=13$ C57BL/6 and $n=14$ 129S6). Since increased CORT has been shown to decrease neurogenesis, it is of note that WT 129S6 mice with higher levels of plasma CORT have lower levels of BrdU labeled cells in the DG compared to WT C57BL/6 mice.

Discussion

Our data indicate that BrdU labeling is increased in the DG of MOR-1 KO mice compared to WT after both repeated injection of BrdU (4X) and after one BrdU injection followed by three vehicle injections, but not following only a single BrdU injection. Increases in CORT levels elicited by any injection paradigm are unchanged between genotypes, though 129S6 mice have higher levels of plasma CORT compared to C57BL/6 mice independent of genotype. These data demonstrate that MOR-1 can regulate neurogenesis in the DG under specific conditions and suggest that the increase in BrdU labeling in MOR-1 KOs results from a CORT-level independent physiological or behavioral response elicited by the action of the repeated injection. The current study also shows that different injection paradigms can alter proliferative responses in a genotype-dependent manner, illustrating the importance of considering the BrdU injection paradigm when designing experiments and interpreting results assessing cell proliferation in genetic knockout models.

MOR-1 effects on cell proliferation

The changes in BrdU labeling in MOR-1 KOs in the repeated injection experiment do not appear to reflect a genotype-dependent increase in basal levels of cell proliferation. If the increase in BrdU labeled cells in MOR-1 KOs shown in the four BrdU injection experiment were due to changes in basal cell proliferation, we would also expect to see a significant increase in the number of BrdU positive cells in MOR-1 KOs when BrdU is injected once and mice are sacrificed two hours later. However, BrdU labeling is not increased in MOR-1 KOs in this paradigm, though increases are seen when a single, lower-dose BrdU injection is followed by three vehicle injections. Thus, the increase in BrdU labeling in MOR-1 KO mice is paradigm-specific and is even evident when lower levels of BrdU are injected. The absence of an increase in BrdU labeling in MOR-1 KO mice following a single injection agrees with a prior study in which MOR-1 KOs on an outbred background also did not show alterations in DG cell proliferation after a single BrdU injection (Harburg et al., 2007). If changes in basal cell proliferation do not account for the increase in BrdU labeling in the DG of MOR-1 KOs, it is likely that loss of MOR-1 influences another factor introduced by the repeated injection paradigm, for instance, activation of some aspect of the stress response, which then indirectly affects neurogenesis in the DG.

Stress dependent effects of MOR-1 on neurogenesis

The repeated injections can be considered a stressor since they lead to a genotype-independent increase in plasma CORT-levels compared to non-injected controls. Although both single and repeated injections lead to an increase in plasma CORT-levels in both WT and MOR-1 KO mice, only the repeated injection paradigm appears to produce a sufficient stress response to evoke a genotype-specific response in BrdU labeling. The data show, however, that differential regulation of the primary stress effector CORT is not altered in the two injection paradigms over the time course examined.

Chronic stress is known to decrease neurogenesis in the DG, most likely due to elevated levels of glucocorticoids (Gould et al., 1992; Gould et al., 1998; Gould and Tanapat, 1999; Mirescu and Gould, 2006). Therefore, one possibility is that MOR-1 KO mice may respond

differently compared to WT to injection stress, which in turn alters neurogenesis in our more acute treatments. Though our data indicate that this effect is independent of CORT-levels over the time period examined it remains possible that MOR-1 KO mice may develop an attenuated CORT response at a later time point following multiple injections. Therefore, to completely rule out attenuated plasma-CORT levels following injection in MOR-1 KO mice as a possible mechanism for increased BrdU labeling in the DG, additional time points following injection would need to be analyzed.

It is also possible that other components mediating the stress response are altered in MOR-1 KO mice and could contribute to the increased BrdU labeling following repeated injection. For instance, glucocorticoid receptors are present in the hippocampus in high abundance and are important in regulating negative feedback mechanisms of CORT (McEwen et al., 1968; Feldman and Weidenfeld, 1999). Previous studies have suggested that the opioid system can regulate both glucocorticoid receptors (GR, low affinity CORT receptors) and mineralocorticoid receptors (MR, high affinity CORT receptors). There is evidence that chronic morphine decreases GR density, but not MR density in the hippocampus (Budziszewska et al., 1995), while prenatal morphine exposure prevents the stress-induced up-regulation of GR and MR binding in the hippocampus (Rimanoczy et al., 2006). Given the data from these studies, changes in MR and GR density in the hippocampus of MOR-1 KO mice could explain the differences in BrdU labeling between MOR-1 KO and WT mice induced by the repeated injection paradigm. Finally, studies have shown that neurosteroids are not only increased following stress, but that neurogenesis in the DG is enhanced following neurosteroid treatment (Mayo et al., 2005; Reddy, 2010). Thus, changes in neurosteroid levels between MOR-1 KO and WT mice could potentially contribute to the differences seen in our study as well.

MOR-1 dependent changes in the proliferative population

It is possible that MOR-1 KO mice have a larger pool of quiescent nestin-expressing stem cells in the SGZ and upon induction by a stressful stimulus this larger pool of stem cells would create a larger number of BrdU positive cells. In this case, consistent with our data, baseline levels cell proliferation would not differ between MOR-1 KO and WT mice. Given the complexity of the process of neurogenesis, it is evident that further studies, such as with nestin reporter mice, are necessary to completely understand the mechanism by which loss of MOR-1 increases the number of proliferating cells in the DG.

Strain effects on Neurogenesis

In the current study, the increase in BrdU labeling in MOR-1 KO mice following repeated injection was detected on both the C57BL/6 and the 129S6 strains, even though 129S6 mice have reduced levels of proliferating cells compared to C57BL/6 (Fig. 1). Previous work has shown that genetic strain can impact cell proliferation in the DG (Kempermann et al., 1997; Hayes and Nowakowski, 2002; Kempermann and Gage, 2002). In these studies, similar to the current study, C57BL/6 mice were shown to have increased numbers of BrdU labeled cells in the DG compared to several other strains including BALB/c, CD1, and 129/SvJ, A/J, C3H/HeJ, and DBA/2J. A more recent study showed that genotype specific proliferative differences in NK1 receptor knockout mice are also dependent on strain; in this study increased BrdU labeling is only evident in NK1 receptor knockout mice on the mixed C57BL/6 X 129/Sv strain and not in NK1 receptor knockout mice on the C57BL/6 strain (McCutcheon et al., 2008).

Interestingly, the reduced number of BrdU positive cells seen in 129S6 mice correlates with significantly higher levels of plasma CORT (Fig. 5), while low CORT-levels in C57BL/6 mice are associated with higher cell proliferation. The association between high CORT and

lower neurogenesis provides additional support to previous work showing the CORT suppresses neurogenesis in the DG (Gould et al., 1992).

Strain effects on plasma CORT levels

In addition to strain differences in BrdU labeling in the DG, we found that, independent of genotype, 129S6 mice have higher levels of plasma CORT compared to C57BL/6 mice at two separate time points. To our knowledge, this study is the first to compare CORT levels in 129S6 and C57BL/6 mice, though previous studies have compared CORT levels in other mouse strains. For example, mixed C57BL/6 X 129/Sv mice have elevated levels of stress-induced CORT compared to C57BL/6 (McCutcheon et al., 2008), while outbred ICR and Sabra strains and inbred C57BL/6J and Balb/c strains show no differences in CORT (Nesher et al., 2011). Genetic background has a major influence on various behavioral phenotypes (Crawley et al., 1997; Abramov et al., 2008). Thus, the alterations in CORT levels between the 129S6 and the C57BL/6 strains may contribute to strain-specific differences in physiology and behaviors that are regulated by the hypothalamic-pituitary adrenal axis. This possibility may be particularly important to consider in genetic studies since these strains are extensively used to maintain genetically modified mice.

Conclusions

The current study shows that DG BrdU labeling is increased in a paradigm-specific manner in MOR-1 KO mice on both the C57BL/6 and 129S6 strains. Though future work is necessary to understand fully how loss of MOR-1 leads to an increase in BrdU labeling in the DG, we provide some initial insight by showing that this effect, while related to the injection paradigm and thus likely involving stress, is CORT-level independent. This study also demonstrates that 129S6 mice have higher levels of plasma CORT compared to C57BL/6 mice and that these CORT levels are inversely correlated with DG neurogenesis. Since mice and other organisms can encounter different patterns of stress throughout life, the increase in neurogenesis in MOR-1 KO mice following a specific, repeated stress paradigm, may exemplify how genetic factors in concert with stressful stimuli could lead to significant changes in dentate gyrus structure and function.

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Highlights

- BrdU labeling in the dentate gyrus is increased in C57BL/6 and 129S6 MOR-1 KO mice
- Increased BrdU labeling in MOR-1 KO mice is only seen following repeated injection
- Increased BrdU labeling in MOR-1 KO mice is independent of corticosterone levels
- WT 129S6 mice have increased corticosterone levels compared to WT C57BL/6

Figure 1. Loss of MOR-1 on the C57BL/6 and the 129S6 strains leads to an increase in BrdU labeling following repeated BrdU injection

A) Experimental timeline for repeated BrdU injection protocol **B)** Dentate gyrus of a (+/+) 129S6 mouse, scale bar = 50 μ m; inset scale bar = 20 μ m; arrows mark BrdU positive cells **C)** Dentate gyrus of a MOR-1 (-/-) mouse, scale bar = 50 μ m; inset scale bar = 20 μ m; arrows mark BrdU positive cells **D)** Total number of BrdU positive cells in the subgranular zone (SGZ) of the DG in (+/+) and MOR-1 (-/-) C57BL/6 mice 12 hours following repeated BrdU injection (BrdU injected four times over twelve hours). * $P < .02$, WT vs. MOR-1 KO; # $P < .0001$, WT C57BL/6 vs. WT 129S6 **E)** Total number of BrdU positive cells in the SGZ of the DG in (+/+) and MOR-1 (-/-) 129S6 mice 12 hours following repeated BrdU injection. * $P < .05$, WT vs. MOR-1 KO

Figure 2. Most BrdU positive cells in the DG, independent of genotype or strain, express the neuronal marker, NeuN

A) Fluorescent immunohistochemistry for BrdU (red) and NeuN (green). Merged image shows co-localization of BrdU and NeuN; scale bar = 50 μ m **B)** Fluorescent immunohistochemistry for BrdU (red) and GFAP (green). Dapi (blue) was used as a background nuclear stain in order to visualize the GCL of the DG. Merged image shows that BrdU and GFAP do not co-localize; scale bar = 50 μ m **C)** Percent of BrdU positive cells that co-localize with NeuN or GFAP in (+/+) and MOR-1 (-/-) C57BL/6 mice. **D)** Percent of BrdU positive cells that co-localize with NeuN or GFAP in (+/+) and MOR-1 (-/-) 129S6 mice. (n=4–5 per group)

Figure 3. BrdU labeling in the DG is unchanged in MOR-1 KO on the C57BL/6 and 129S6 strains following a single BrdU injection

A) Experimental timeline for single BrdU injection protocol **B)** Total number of BrdU positive cells in the SGZ of (+/+) and MOR-1 (-/-) C57BL/6 mice 2 hours and 24 hours following a single BrdU injection. **C)** Total number of BrdU positive cells in the SGZ of (+/+) and MOR-1 (-/-) 129S6 mice 2 hours and 24 hours following a single BrdU injection. * $P < .01$, significantly larger number of BrdU positive cells at 24 hours compared to 2 hours in both groups. There are no significant differences between genotypes.

Figure 4. MOR-1 KO mice on the C57BL/6 and 129S6 strains exhibit an increase in BrdU labeling in the DG following a single BrdU injection and three vehicle injections

A) Experimental timeline for BrdU/vehicle injection protocol **B)** Total number of BrdU positive cells in the SGZ of (+/+) and MOR-1 (-/-) C57BL/6 mice 12 hours following a single BrdU injection and 3 vehicle injections. * $P < .02$ **C)** Total number of BrdU positive cells in the SGZ of (+/+) and MOR-1 (-/-) 129S6 mice 12 hours following a single BrdU injection and 3 vehicle injections. * $P < .02$

Figure 5. MOR-1 KO mice on the C57BL/6 and 129S6 strains exhibit an increase in the number of PCNA positive cells in the DG following a single BrdU injection and three vehicle injections
A) Experimental timeline for BrdU/vehicle injection protocol **B)** Total number of PCNA positive cells in the SGZ of wild-type (+/+) and MOR-1 (-/-) C57BL/6 mice 12 hours following a single BrdU injection and 3 vehicle injections. * $P < .01$ **C)** Total number of PCNA positive cells in the SGZ of (+/+) and MOR-1 (-/-) 129S6 mice 12 hours following a single BrdU injection and 3 vehicle injections. * $P < .05$

Figure 6. Plasma CORT levels are similar in MOR-1 KO mice compared to WT on the C57BL/6 and 129S6 strains following both single and repeated vehicle injection

A) Experimental timeline for single injection **B)** Plasma corticosterone levels in (+/+) and MOR-1 (-/-) C57BL/6 mice following a single vehicle injection (injected) or in mice that did not receive injection (control). * $P < .05$, control vs. injected. Blood was taken between 1000–1100 hours. **C)** Plasma corticosterone levels in (+/+) and MOR-1 (-/-) 129S6 mice following a single vehicle injection (injected) or in mice that did not receive injection (control). * $P < .05$, control vs. injected. Blood was taken between 1000–1100 hours. **D)** Experimental timeline for repeated injection **E)** Plasma corticosterone levels in (+/+) and MOR-1 (-/-) C57BL/6 mice following four vehicle injections (injected) or in mice that did not receive injections (control). * $P < .001$, control vs. injected. Blood was taken between 2000–2100 hours. **F)** Plasma corticosterone levels in (+/+) and MOR-1 (-/-) 129S6 mice following four vehicle injections (injected) or in mice that did not receive injections (control). * $P < .01$, control vs. injected. Blood was taken between 2000–2100 hours.

Figure 7. 129S6 mice have elevated plasma CORT levels compared to C57BL/6 mice

A) Plasma corticosterone levels in C57BL/6 and 129S6 mice following a single vehicle injection (injected) or in mice that did not receive injection (control). * $P < .01$, C57BL/6 vs. 129S6. Blood was taken between 1000–1100 hours. **B)** Plasma corticosterone levels in C57BL/6 and 129S6 mice following four vehicle injections (injected) or in mice that did not receive injections (control). * $P < .05$, C57BL/6 vs. 129S6. Blood was taken between 2000–2100 hours.

Table 1

Total number of BrdU positive cells in the SGZ of the DG for all experiments

Experiment	Strain and Genotype	<i>n</i>	# of BrdU+ cells in the SGZ ± SEM
4X BrdU	WT C57BL/6	10	3964± 216 [#]
	MOR-1 KO C57BL/6	10	6963± 1134 [*]
	WT 129S6	6	1380± 178
	MOR-1 KO 129S6	7	2286± 272 [*]
1X BrdU - 2 hr	WT C57BL/6	5	1731± 169
	MOR-1 KO C57BL/6	5	1673± 143
	WT 129S6	6	440± 29
	MOR-1 KO 129S6	5	496± 50
1X BrdU - 24 hr	WT C57BL/6	6	2520± 215
	MOR-1 KO C57BL/6	6	2651± 207
	WT 129S6	4	836± 70
	MOR-1 KO 129S6	5	934± 56
1X BrdU/3X Vehicle	WT C57BL/6	5	1776± 108
	MOR-1 KO C57BL/6	5	2336± 155 [*]
	WT 129S6	4	740± 56
	MOR-1 KO 129S6	3	1152± 120 [*]

^{*} MOR-1 KO (−/−) significantly different than WT (+/+),

^{*} P<.05;

[#] P< .0001, WT C57BL/6 vs. WT 129S6.