Neuroscience 278 (2014) 11–19

LOSS OF THE MU OPIOID RECEPTOR INDUCES STRAIN-SPECIFIC ALTERATIONS IN HIPPOCAMPAL NEUROGENESIS AND SPATIAL LEARNING

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Abstract—Alterations in hippocampal neurogenesis affect spatial learning, though, the relative contributions of cell proliferation and cell survival on this process are poorly understood. The current study utilized mu opioid receptor (MOR-1) knockout (KO) mice on two background strains, C57BL/6 and 129S6, to assess cell survival as well as determine the impact on spatial learning using the Morris water maze. These experiments were designed to extend prior work showing that both C57BL/6 and 129S6 MOR-1 KO mice have an increased number of proliferating cells in the dentate gyrus (DG) when compared to wild-type (WT) mice. The current study indicates that newly born neurons in the DG of C57BL/6 MOR-1 KO mice exhibit enhanced survival when compared to WT mice, while new neurons in the DG of 129S6 MOR-1 KO mice do not. In addition, C57BL/6 MOR-1 KO mice have a lower number of apoptotic cells in the DG compared to WT mice while, in contrast, 129S6 MOR-1 KO mice have a higher number of apoptotic cells in this region. These alterations collectively contribute to an increase in the granule cell number in the DG of C57BL/6 MOR-1 KO mice, while the total number of granule cells in 129S6 MOR-1 KO mice is unchanged. Thus, although C57BL/6 and 129S6 MOR-1 KO mice both exhibit increased cell proliferation in the DG, the impact of the MOR-1 mutation on cell survival differs between strains. Furthermore, the decrease in DG cell survival displayed by 129S6 MOR-1 KO mice is correlated with functional deficits in spatial learning, suggesting that MOR-1-dependent alterations in the survival of new neurons in the DG, and not MOR-1-dependent changes in proliferation of progenitor cells in the DG, is important for spatial learning. **© 2014 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: neurogenesis, spatial learning, mu opioid receptor, dentate gyrus, cell survival, hippocampus.

INTRODUCTION

Neurogenesis, the production of new neurons, occurs throughout adulthood in the granule cell layer (GCL) of the dentate gyrus (DG) in essentially all species examined thus far (Kempermann et al., 1997; Cameron and McKay, 1998; Eriksson et al., 1998; Gould et al., 1999b). Although DG neurogenesis is a widely studied process, its role in hippocampal-dependent learning is not entirely clear. Hippocampal-dependent learning tasks including the Morris water maze, a spatial learning task, and trace eyeblink conditioning, a hippocampal-dependent associative learning task increase the proliferation of neuronal precursors and the survival of newly born cells in the DG (Gould et al., 1999a; Dobrossy et al., 2003; Drapeau et al., 2003; Leuner et al., 2004; Sisti et al., 2007). In addition, decreasing DG neurogenesis leads to performance deficits in contextual fear conditioning and trace eyeblink conditioning (Dupret et al., 2008; Nokia et al., 2012). In contrast, however, other studies suggest that DG neurogenesis is not required for spatial learning (Wojtowicz et al., 2008; Hernandez-Rabaza et al., 2009; Groves et al., 2013). Thus, while there is evidence suggesting that neurogenesis is involved in spatial learning, there is additional evidence suggesting the contrary, indicating the need for further investigation.

The mu opioid receptor (MOR-1) is one specific receptor that has been linked to hippocampal-dependent learning, though results have varied depending on strain background. C57BL/6 MOR-1 knockout (KO) mice exhibit impaired performance in learning tasks such as the Morris water maze (Jamot et al., 2003; Jang et al., 2003) while MOR-1 KO mice on mixed strain backgrounds do not have deficits in spatial learning (Lubbers et al., 2007). Since DG neurogenesis is involved in hippocampal-dependent learning, one explanation for these overall effects is that alterations in hippocampal-dependent learning in MOR-1 KO mice could result from alterations in DG neurogenesis, perhaps in a background strain-dependent manner.

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Abbreviations: BrdU, bromodeoxyuridine; DG, dentate gyrus; GCL, granule cell layer; KO, knockout; MOR-1, mu opioid receptor; SGZ, subgranular zone; WT, wild-type; TUNEL, Terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling; TQ, target quadrant.

http://dx.doi.org/10.1016/j.neuroscience.2014.07.039
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Pharmacologic and genetic alterations of MOR-1 activity *in vivo* also alter DG neurogenesis. Thus morphine, a MOR-1 agonist, decreases DG neurogenesis, alters cell cycle dynamics, and leads to changes in the neurogenic microenvironment (Eisch et al., 2000; Mandyam et al., 2004; Kahn et al., 2005; Arguello et al., 2008, 2009), while opioid antagonists like naltrexone and naltrindole increase neurogenesis in the DG (Persson et al., 2004). Genetic KO of MOR-1 in outbred mice increases the survival rate of newborn cells in the DG (Harburg et al., 2007), while MOR-1 KO mice, on two separate genetic backgrounds, show increased levels of cell proliferation in the DG as measured by bromodeoxyuridine (BrdU) and proliferating cell nuclear antigen (PCNA) immunohistochemistry (Cominski et al., 2012).

As mentioned above, we have previously shown that loss of MOR-1, on two separate strains of mice, increases cell proliferation in the DG (Cominski et al., 2012). In the current study, the increase in DG cell proliferation in MOR-1 KO mice on both the C57BL/6 and the 129S6 strains was further characterized by analyzing additional parameters related to neurogenesis in the DG, including cell survival in the subgranular zone (SGZ), total granule cell number in the GCL, and cell death. The impact of these changes on hippocampal-dependent learning was also examined using the Morris water maze.

EXPERIMENTAL PROCEDURES

Animals

All experiments were performed on 8-week-old male wild-type ((+/+), WT) and MOR-1 knockout ((−/−), 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. All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the IACUC of Rutger's University, Piscataway, New Jersey. All efforts were made to minimize the number of animals used and their suffering.

BrdU injections

129S6 and C57BL/6 MOR-1 KO and WT mice were injected with BrdU at a dose of 50-mg/kg every 4 h over a period of 12 h (four BrdU injections total). Animals were sacrificed 4 weeks following the last injection, in order to allow a sufficient amount of time for maturation of the newly born cells. Four repeated BrdU injections were administered in an attempt to label the entire proliferative population that exists in the SGZ, based on the idea that the total cell cycle time in the DG is approximately 12 h with S-phase lasting for about 4 h (Hayes and Nowakowski, 2002).

Preparation and injection of BrdU

Solutions of BrdU were prepared in sterile saline with .007 M NaOH at a concentration of 5-mg/ml. 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 immunohistochemistry

At the time of sacrifice, mice 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 10-μm thickness on a microtome and every section through the DG was collected on slides. Every 16th section was stained for BrdU immunohistochemistry. First, sections were deparaffinized using xylene and an ethanol series. Then, sections were pretreated with 0.1% trypsin in Tris buffer for antigen retrieval, 2 N HCl to denature DNA, and 3% H_2O_2 in methanol to block endogenous peroxidase. Next, sections were blocked with 5% horse serum and then primary anti-BrdU antibody (BD Bioscience, San Jose, CA, USA) was added at a 1:100 dilution. Following incubation with the primary antibody overnight at 4 °C, sections were washed and then biotinylated anti-mouse secondary antibody (Vector, Burlingame, CA, USA) was added at a 1:300 dilution for 1 h at room temperature. ABC (Vector) and DAB (Sigma, St. Louis, MO, USA) were added to visualize BrdU-positive cells. Sections were then stained with methyl green to visualize the GCL and all other cells that were not BrdU-positive.

BrdU quantification

The optical fractionator technique was used to determine the total number of BrdU-positive cells in the DG (West et al., 1991). Immunohistochemistry for BrdU was performed on every 16th section through the entire DG. The number of BrdU-positive cells in the SGZ of the DG was counted for each section with an average of 15 sections being counted for each animal. For each animal the number of BrdU-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-positive cells in the DG per animal the ΣQ was multiplied by16.

Terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL)

Tissue was collected and sectioned as described above. For cell death analysis in the DG, every 32nd section was processed using the TUNEL ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit (EMD Millipore, Billerica, MA, USA). The number of TUNEL-positive cells was counted throughout the entire DG for each tissue section and then quantified in the same manner as for BrdU cells, except that the ΣQ was multiplied by 32.

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