Oligodendrocyte Responses to Buprenorphine Uncover Novel and Opposing Roles of μ-Opioid- and Nociceptin/Orphanin FQ Receptors in Cell Development: Implications for Drug Addiction Treatment During Pregnancy

ANDREW C. ESCHENROEDER,[1] ALLISON A. VESTAL-LABORDE,[1] EMILSE S. SANCHEZ,[1] SUSAN E. ROBINSON,[2,3] AND CARMEN SATO-BIGBEE[1]*

[1]*Department of Biochemistry and Molecular Biology, Virginia Commonwealth University School of Medicine, Richmond, Virginia*
[2]*Institute for Drug and Alcohol Studies, Virginia Commonwealth University School of Medicine, Richmond, Virginia*
[3]*Department of Pharmacology and Toxicology, Virginia Commonwealth University School of Medicine, Richmond, Virginia*

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ABSTRACT
Although the classical function of myelin is the facilitation of saltatory conduction, this membrane and the oligodendrocytes, the cells that make myelin in the central nervous system (CNS), are now recognized as important regulators of plasticity and remodeling in the developing brain. As such, oligodendrocyte maturation and myelination are among the most vulnerable processes along CNS development. We have shown previously that rat brain myelination is significantly altered by buprenorphine, an opioid analogue currently used in clinical trials for managing pregnant opioid addicts. Perinatal exposure to low levels of this drug induced accelerated and increased expression of myelin basic proteins (MBPs), cellular and myelin components that are markers of mature oligodendrocytes. In contrast, supra-therapeutic drug doses delayed MBP brain expression and resulted in a decreased number of myelinated axons. We have now found that this biphasic-dose response to buprenorphine can be attributed to the participation of both the μ-opioid receptor (MOR) and the nociceptin/orphanin FQ receptor (NOP receptor) in the oligodendrocytes. This is particularly intriguing because the NOP receptor/nociceptin system has been primarily linked to behavior and pain regulation, but a role in CNS development or myelination has not been described before. Our findings suggest that balance between signaling mediated by (a) MOR activation and (b) a novel, yet unidentified pathway that includes the NOP receptor, plays a crucial role in the timing of oligodendrocyte maturation and myelin synthesis. Moreover, exposure to opioids could disrupt the normal interplay between these two systems altering the developmental pattern of brain myelination.

INTRODUCTION

The use and abuse of opioids has significantly increased in the recent years (Compton and Volkow, 2006; Hall et al., 2008), an alarming trend representing a problem of particular importance during pregnancy (Broussard et al., 2011). We showed previously that rat brain myelination is significantly altered by perinatal exposure to buprenorphine (Sanchez et al., 2008), a synthetic opioid that functions as μ-opioid receptor (MOR) partial agonist and κ-opioid receptor (KOR) antagonist (Lewis, 1985). The Food and Drug Administration approved the use of buprenorphine for opioid dependence treatment (Amass et al., 2004; Jaffe and O'Keeffe, 2003) and furthermore, this drug is currently on clinical trials for the management of pregnant opioid addicts. Several clinical studies showed that buprenorphine effectively prevents the use of other opioids by pregnant addicts and reduces the incidence and severity of neonatal abstinence syndrome when compared with the approved treatment with methadone (Ebner and Wiedmann, 2006; Jones et al., 2005, 2010a; Unger et al., 2010). However, most studies on these treatments are limited to early neurodevelopment (Jansson et al., 2011; Jones et al., 2010b), so there is a lack of detailed systematic studies on the long-term effects of either buprenorphine or methadone on human brain development. In addition, buprenorphine and methadone are not only used in replacement therapies but abuse of both is also on the rise (Maxwell and McCance-Katz, 2010).

Potential effects on myelination and the myelin-forming oligodendrocytes are particularly important because the original perception of myelin as an inert structure, only responsible for axonal insulation and saltatory conduction, has radically changed to that of a complex membrane which is also implicated in the control of

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Emilse S. Sanchez is currently at Universidad Nacional de San Luis, San Luis, Argentina.

*Correspondence to: Carmen Sato-Bigbee, Department of Biochemistry and Molecular Biology, Virginia Commonwealth University School of Medicine, Richmond, VA 23298-0614, USA. E-mail: csatobig@vcu.edu

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axonal extension and radial growth (Yin et al., 1998), axolemmal integrity (Bruce et al., 2010) and the establishment of nodal and paranodal structures and ion channel localization at the nodes of Ranvier (Rasband and Trimmer, 2001). Furthermore, the oligodendrocytes appear to regulate axonal function by maintaining important bidirectional glial–neuronal interactions (Fields, 2008). These highly specialized cells originate from simple bipolar proliferative progenitors that undergo several steps of differentiation and morphological changes before becoming post mitotic, complex multipolar cells capable of myelination. Each of these developmental steps is accompanied by the expression of stage-specific markers of differentiation (Butt and Ransom, 1993; Campagnoni, 1988; Pringle et al., 1992; Sommer and Schachner, 1981), and this well-defined sequence of developmental changes implies the existence of highly controlled mechanisms that are still poorly understood. Interestingly, several lines of evidence support the idea that oligodendrocyte development may be modulated by the endogenous opioid system. Not only do these cells express opioid receptors (Knapp et al., 1998) but they are also capable of synthesizing endogenous opioids in a developmentally regulated manner (Knapp et al., 2001). Although cultured bipolar progenitors have been shown to produce dynorphin, the expression of this opioid is lost when the cells differentiate into mature oligodendrocytes. In contrast, proenkephalin-derived peptides were detected in both young and differentiated oligodendrocytes (Knapp et al., 2001). Activation of MOR in oligodendrocyte progenitor cultures was shown to result in elevated DNA synthesis, whereas inhibition of KOR was accompanied by increased membrane extensions (Knapp et al., 1998). Although the *in vivo* role of opioids in oligodendrocytes is not clear, these observations raise the possibility that interference with the endogenous opioid system could affect oligodendrocyte maturation and the process of myelination in the developing CNS.

As indicated above, previously we have found that perinatal exposure to buprenorphine alters myelination in the developing rat brain (Sanchez et al., 2008). Analysis of pups from mothers treated with buprenorphine indicated that exposure to therapeutic doses resulted in accelerated and increased brain expression of myelin basic proteins (MBPs), cellular and myelin components that are markers of mature oligodendrocytes. In contrast, supra-therapeutic doses of buprenorphine delayed MBP expression and resulted in a decreased number of myelinated axons. Importantly, a recent imaging study pointed to the development of white matter alterations in children prenatally exposed to drugs of abuse, in particular opioids (Walhovd et al., 2010). The importance of understanding the effects of opioids and drug abuse treatments on myelination is further underscored by the fact that, in agreement with a large number of animal studies, there is evidence correlating increased myelin formation with experience and enhanced function of the infant brain (Als et al., 2004; Bengtsson et al., 2005; Pujol et al., 2006). It is also important to consider that opioid effects may not be limited to early child development because there is also active brain myelination during adolescence (Berns et al., 2009; Qiu et al., 2008), a highly vulnerable period for drug abuse.

Thus, there is a true compelling need for deciphering the roles of the endogenous opioid system in oligodendrocyte differentiation and myelin formation, and understanding the mechanisms by which opioid abuse and related treatments may interfere with these processes in the developing CNS. We have now found that buprenorphine indeed has direct actions on the oligodendrocytes, inducing dose-specific effects on their differentiation. Interestingly, these buprenorphine actions uncovered novel and opposing functions of MOR and the nociceptin/orphanin FQ (NOP) receptor that underscore the complexity of mechanisms controlling oligodendrocyte development and myelination.

MATERIALS AND METHODS

Materials: Percoll, bovine pancreas DNAse, papain for cell isolation and cell culture medium components were from Sigma–Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12) (1:1) medium with high glucose and L-glutamine was obtained from Invitrogen (Grand Island, NY). Reduced-growth factor Matrigel™ was from Becton Dickinson (Franklin Lakes, NJ). Buprenorphine, methadone, and the MOR antagonist CTOP (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 [Disulfide Bridge: 2–7]) were purchased from Sigma–Aldrich (St. Louis, MO). The NOP receptor inhibitor J113397 (1-[(3*R**,4*R**)-1-(Cyclooctylmethyl)-3-(hydroxymethyl)-4-piperidinyl]-3-ethyl-1,3-dihydro-2*H*-benzimidazol-2-one) and nociceptin (Orphanin FQ) were from Tocris Bioscience (Ellisville, MO). All gel electrophoresis reagents and supplies were purchased from Bio-Rad Laboratories (Hercules, CA). Anti-β-actin and anti-MBP (82–87 region) antibodies were from Sigma–Aldrich and Millipore Corporation (Temecula, CA), respectively. The MOR and NOP receptor antibodies were from Neuromics (Edina, MN). The mouse O4 monoclonal antibody was kindly provided by Dr. Rashmi Bansal (University of Connecticut, Farmington, CT) and Dr. Babette Fuss (Virginia Commonwealth University, Richmond, VA). Super Signal West Dura chemiluminescence reagent was from Pierce (Rockford, IL). All appropriate secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Preparation of oligodendrocyte cultures: Sprague-Dawley rats were provided by Harlan Laboratories (Frederick, MD). Oligodendrocytes were directly isolated from rat brains as described previously (Colello and Sato-Bigbee, 2001) with minor modifications. For this, the animals were sacrificed by decapitation and the brains rapidly dissected out, transferred to ice, and the meninges and main blood vessels removed by rolling the tissue on sterile filter paper. After mincing into 1–2 mm pieces, the tissue was incubated for 25 min. at 37°C in the presence of 1 unit/mL papain and 0.01 mg/mL DNAse. After extensive washes and filtration through a

75 μm pore size nylon mesh, the resulting cell suspension was centrifuged for 15 min at 30,000×*g* in an isotonic self-generated Percoll gradient. The band enriched in oligodendrocytes was then subjected to differential adhesion on tissue culture-treated Petri dishes to eliminate microglial cells and residual astrocytes. The floating oligodendrocytes were then plated in 48-well plates (Falcon; for Western blot analysis), or on 10 mm cover slips in 24-well plates or 8-well slide Permanox chambers (for TUNEL assay and immunocytochemistry) all previously coated with 12.5 μL/well reduced-growth factor-Matrigel™ extracellular matrix. Before use, the cells were maintained overnight in chemically defined medium (CDM; DMEM/F-12 supplemented with 1 mg/mL fatty acid-free bovine serum albumin, 50 μg/mL transferrin, 5 μg/mL insulin, 30 nM sodium selenite, 0.11 mg/mL sodium pyruvate, 10 nM biotin, 20 nM progesterone, 100 μM putrescine, and 30 nM triiodothyronine). Astroglial contamination, as assessed by glial fibrillary acid protein staining, was less than 5%. When cells were exposed to buprenorphine, methadone, or receptor inhibitors, the CDM was supplemented with the appropriate drug concentrations, and the culture media changed every 48 h. Animal use and oligodendrocyte isolation were conducted in accordance with the guidelines from the National Institutes of Health and approved by the Virginia Commonwealth University Animal Care and Use Committee.

Western blot analysis: Oligodendrocyte cultures were lysed in 50–75 μL 60 mM Tris–HCl buffer (pH 6.8) containing 10% glycerol, 2% sodium dodecyl sulfate (SDS), and 5% 2-mercaptoethanol. Proteins in 10 μL samples were then separated by SDS-polyacrylamide gel electrophoresis in 15% acrylamide and electro-transferred to nitrocellulose. The membranes were then subjected to immunoblot analysis as previously reported (Saini et al., 2005), with minor modifications. Nonspecific antibody binding was blocked by incubation in 10 mM Na_2HPO_4, 2.7 mM KCl, and 137 mM NaCl, pH 7.4 [phosphate-buffered saline (PBS)], containing 3% nonfat-dry milk and 0.05% Tween-20 (blocking solution), for 1 h at room temperature. Blots were then incubated overnight with anti-MBP (dil. 1:100), anti-MOR (dil. 1:500), or anti-NOP receptor (dil.1:500) antibodies. β-Actin levels detected with anti-β-actin antibody (dil. 1:2,000) were used as loading controls. After extensive rinsing with PBS, blots were incubated for 30 min in blocking solution, followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibody for 2 h. All antibodies were diluted in blocking buffer. After extensive washing, the immunoreactive bands were detected by chemiluminescence with Super Signal West Dura reagent. The relative amount of immunoreactive protein in each band was determined by scanning densitometric analysis of the X-ray films using the NIH Image J program and divided by β-actin levels to correct for loading differences.

Detection of apoptotic cells: After fixation of the cells with 4% paraformaldehyde for 1 h at room temperature, the number of apoptotic oligodendrocytes was determined by detecting DNA fragmentation using a TUNEL assay (In Situ Cell Death Detection Kit; Roche Diagnostics, Indianapolis, IN), as reported previously (Saini et al., 2005). For each condition, at least 20 visual fields containing approximately 200 cells each were analyzed.

Immunocytochemistry: Cells were fixed in 4% paraformaldehyde in PBS, and immunocytochemistry was carried out as previously reported (Sato-Bigbee et al., 1999). Nonspecific antibody binding was blocked by incubation of the cultures for 1 h in PBS containing 5% nonfat dry milk, 0.05% Tween-20, and 0.5% normal goat serum (blocking solution). The cells were then incubated overnight with anti-MBP (dil, 1:20) or a mixture of O4 (dil.1:3) and MBP (dil. 1:20) antibodies in blocking solution. After several washes in PBS, the cells were incubated for 30 min in blocking solution and for 2 h with Texas Red-conjugated anti-rat IgG (dil. 1:150) to visualize MBP labeling or Alexa 488-conjugated anti-mouse IgM (dil. 1:250) and Texas Red-conjugated anti-rat IgG (dil. 1:150) for O4-MBP double immunocytochemistry. For detection of MOR and NOP receptor, cells were incubated overnight in a mixture of O4 and anti-MOR (1:100) or anti-NOP receptor (1:100) antibodies in PBS containing 0.1% Triton X-100 and 1% normal goat serum. After extensive washing and a second 30 min blocking step, cells were incubated for 2 h with a mixture of Alexa 488-conjugated anti-mouse IgM (dil. 1:250) for O4 labeling and Texas Red-conjugated anti-rabbit IgG (dil. 1:150) for MOR and NOP receptor visualization. The cultures were then analyzed using a Nikon Eclipse 800 M fluorescence microscope with ad hoc digital camera system.

Statistical analysis: Statistical analysis was performed by one-way analysis of variance and Bonferroni post test, using the GraphPad Prism program. Differences were considered statistically significant when P values were <0.05.

RESULTS

Our previous studies indicated that perinatal exposure to buprenorphine causes dose-specific and developmentally dependent effects on brain myelination. Although elevated MBP levels were observed at all studied ages in the brains of pups exposed to therapeutic drug doses, treatment with a supra-therapeutic buprenorphine dose was accompanied by delayed MBP expression and reduced number of myelinated axons (Sanchez et al., 2008). Because MBPs are considered to be markers of mature oligodendrocytes, these findings suggested that the differentiation of these cells is accelerated by therapeutic levels of buprenorphine but paradoxically delayed by higher drug doses. If this is the case, altered oligodendrocyte maturation could be the result of direct actions of buprenorphine or, in contrast, be mediated by drug effects on other cells, such as astrocytes or microglia, which could in turn influence the rate of oligodendrocyte development.

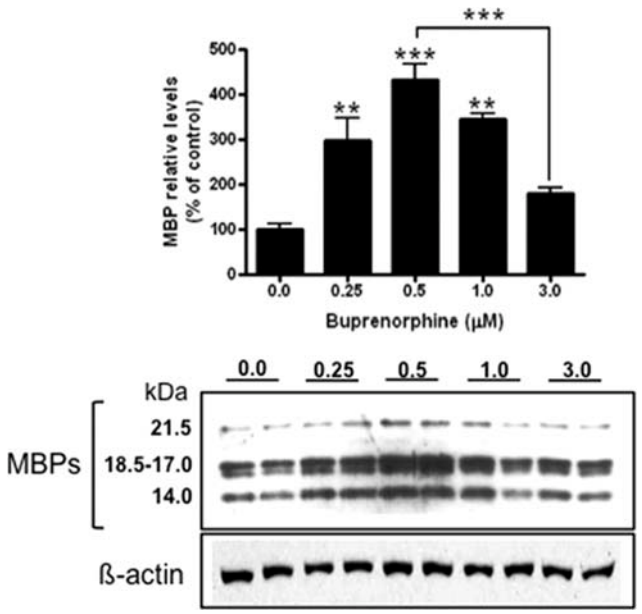

Fig. 1. Direct treatment of immature oligodendrocytes with buprenorphine alters MBP expression in a dose-specific manner. Cells isolated from 9-day-old rat brains were incubated for 4 days in CDM with or without 0.25, 0.5, 1.0, and 3.0 μM buprenorphine. MBP levels were determined by Western blotting using β-actin levels as loading controls. Figures correspond to representative experiments. Results in the bar graph are expressed as percentage of controls (0 μM buprenorphine) ± SEM from five experiments and correspond to the combined scanning of the four major MBP isoforms. $^{**}P < 0.005$ and $^{***}P < 0.0001$.

To begin to address these possibilities, we decided to study the potential direct effects of buprenorphine on the maturation of cultured oligodendrocytes. For this, we next investigated if treatment of the cells with buprenorphine could have any noticeable action on MBP expression. We began testing the response of cells directly isolated from 9-day-old rat brain. Cultures prepared from these cells are composed of postmitotic quiescent pre-oligodendrocytes, the majority of which already react with the O4 antibody (Sato-Bigbee et al., 1999). These cells represent a crucial and highly vulnerable developmental stage that immediately precedes the generation of mature oligodendrocytes capable of myelination.

As shown in Fig. 1, similar to the results previously observed in the *in vivo* studies, direct exposure of the cells to buprenorphine also resulted in a biphasic dose-specific effect on MBP expression. Low buprenorphine doses elicited a significant increase in MBP levels, with maximal stimulation observed between 0.25 μM and 0.5 μM drug concentrations. However, this stimulatory action was not detected when the cells were exposed to 3 μM buprenorphine.

Because morphine exposure was shown to increase apoptosis of cultured oligodendrocytes (Hauser et al., 2009), we next carried out an analysis of cell death by TUNEL assay. However, we failed to detect any differences between treatment conditions, indicating that the differences in MBP expression could not be attributed to potential effects of buprenorphine on apoptosis (data not shown). On the contrary, the results presented so far indicated that buprenorphine does exert direct dose-dependent effects on oligodendrocyte differentiation. In support of this possibility, immunostaining of the cell cultures with anti-MBP antibody demonstrated that buprenorphine actions on MBP expression levels are also accompanied by dose-specific effects on the morphology of the cells (Fig. 2). When treated with 0.5 μM buprenorphine which, as described above, induces maximal MBP stimulation, the cells showed significant increases in process outgrowth and membrane extension, a morphology that is indicative of highly mature oligodendrocytes. In contrast, in agreement with the lower MBP levels observed in the Western blots, both control cultures and those exposed to 3 μM buprenorphine exhibited MBP positive cells with shorter processes and simpler morphology characteristic of less mature cells.

Analysis of the cell cultures by double immunocytochemistry with O4 and anti-MBP antibodies (Fig. 3) further supported the idea that the above observations reflect differences in the extent of cell differentiation. Although both immature pre-oligodendrocytes and mature cells are known to react with the O4 monoclonal antibody, only differentiated oligodendrocytes are expected to be labeled by both the O4 and anti-MBP antibodies. In the presence of 0.5 μM buprenorphine, the great majority of the cells are both highly O4 and MBP positive. In contrast, controls grown in the absence of buprenorphine or cultures treated with the 3.0 μM dose concentration exhibited a significant number of cells that were only O4 positive but just few that were highly stained by both the O4 and MBP antibodies. Quantitation of these results (Fig. 3, bar graph) indicated that in cultures exposed to 0.5 μM buprenorphine about 80% of the cells are labeled by both the O4 and MBP antibodies, whereas this number is only about 35% in the case of controls and cells treated with 3.0 μM buprenorphine.

Taken together, the above observations are consistent with the original hypothesis that buprenorphine exerts direct effects on oligodendrocyte maturation. In addition, the bell-shaped dose-dependent nature of the drug effects indicates the existence of a complex mechanism. Therefore, we next investigated the bases for these dose-specific effects.

Buprenorphine is a MOR partial agonist and KOR antagonist. However, as shown in Fig. 4, we found that the positive effect of 0.5 μM buprenorphine on MBP expression could be mimicked by methadone, an opioid analogue that functions as a MOR-specific agonist. Moreover, the stimulatory effects on MBP expression of methadone and 0.5 μM buprenorphine were both abolished by co-incubation with the highly specific MOR antagonist CTOP, supporting a novel role of this receptor as a positive regulator of oligodendrocyte maturation.

Although the results using methadone and CTOP pointed to 0.5 μM buprenorphine working through activation of MOR, these observations did not explain the differential effect of the higher 3 μM dose of buprenorphine. Similar to the dose-specific effects on MBP expression and oligodendrocyte development described

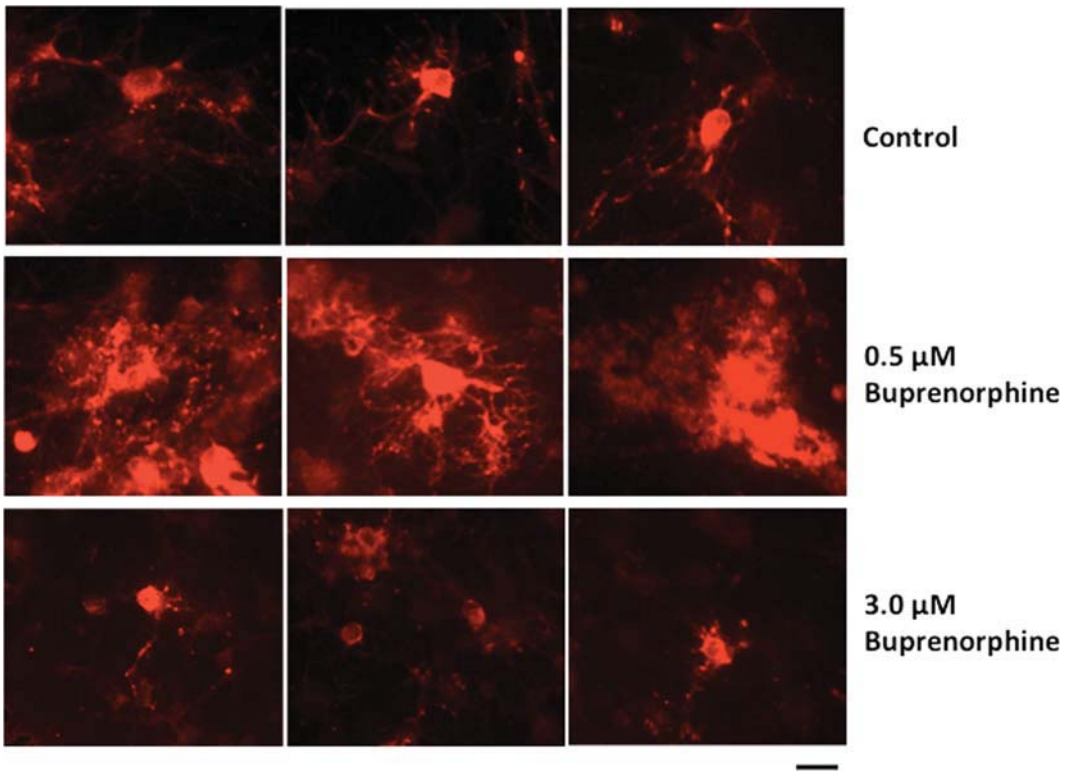

Fig. 2. Exposure of pre-oligodendrocytes to buprenorphine alters cell process extension and membrane outgrowth in a dose-specific manner. Developing oligodendrocytes isolated from 9-day-old rat brain were cultured for 4 days in CDM with or without buprenorphine (0.5 μM and 3.0 μM). Cells were then stained with anti-MBP antibody and visualized by fluorescent microscopy. The figure shows three representative panels corresponding to each of the treatment conditions. Note the extensive process and membrane extension in cells treated with 0.5 μM buprenorphine. Scale bar: 10 μm.

above, others have shown that buprenorphine antinociceptive effects also exhibit a bell-shaped dose-response. Low buprenorphine concentrations exert an analgesic effect but this antinociceptive action is significantly decreased by higher levels of the drug (Dum and Herz, 1981; Lizasoain et al., 1991). Later investigations also found that antinociception induced by buprenorphine is mediated by MOR but higher doses counteract this effect by concomitant activation of the NOP receptor (Lutfy et al., 2003), a molecule that has about 75% homology with the three different opioid receptors (Mogil and Pasternak, 2001).

The expression of the NOP receptor in oligodendrocytes has never been investigated before. However, Western immunobloting and immunocytochemistry revealed that pre-oligodendrocytes isolated from 9-day-old rat brain indeed express both MOR and the NOP receptor (Fig. 5). Moreover, the results depicted in Fig. 6 suggest that an antagonistic functional relationship between these two receptors also exists in the oligodendrocytes and underlies the dose-specific effects of buprenorphine on these cells. Analysis of MBP expression showed that the biphasic dose-dependent effect of buprenorphine is abolished by inhibition of the NOP receptor. As shown in Fig. 6A, cultures exposed to 3 μM buprenorphine in the presence of J-113397, a highly specific NOP receptor inhibitor, exhibited significantly higher MBP levels than controls as well as cells exposed to 3 μM buprenorphine alone. This is in contrast with the levels of MBP in cells treated with 3 μM buprenorphine and CTOP, in which MBP levels are even lower than in controls (Fig. 6B). Furthermore, treatment of the cells with the NOP receptor ligand NOP abrogates the stimulatory effect of 0.5 μM buprenorphine (Fig. 6C) which as described above (Fig. 4) implicates MOR activation.

The affinity of buprenorphine for the NOP receptor is lower than its affinity for the MOR (Toll et al., 1998), and thus, our findings are consistent with a model in which buprenorphine exerts direct effects in the oligodendrocytes by binding to two different receptors with opposing roles on cell development (Fig. 7). Because the affinity of buprenorphine for the NOP receptor is lower than its affinity for the MOR (Toll et al., 1998), it is logical to suppose that low drug levels activate the high affinity MOR, whereas high buprenorphine concentrations are required to bind to both MOR and the low affinity NOP receptor. MOR activation results in stimulation of oligodendrocyte maturation, whereas this effect is counteracted by an inhibitory effect induced by concomitant signaling through the NOP receptor. Therefore, it is tempting to hypothesize that balance between these two pathways may play a crucial role in "timing" oligodendrocyte differentiation and the beginning of myelin synthesis. Buprenorphine, methadone, and other opioid

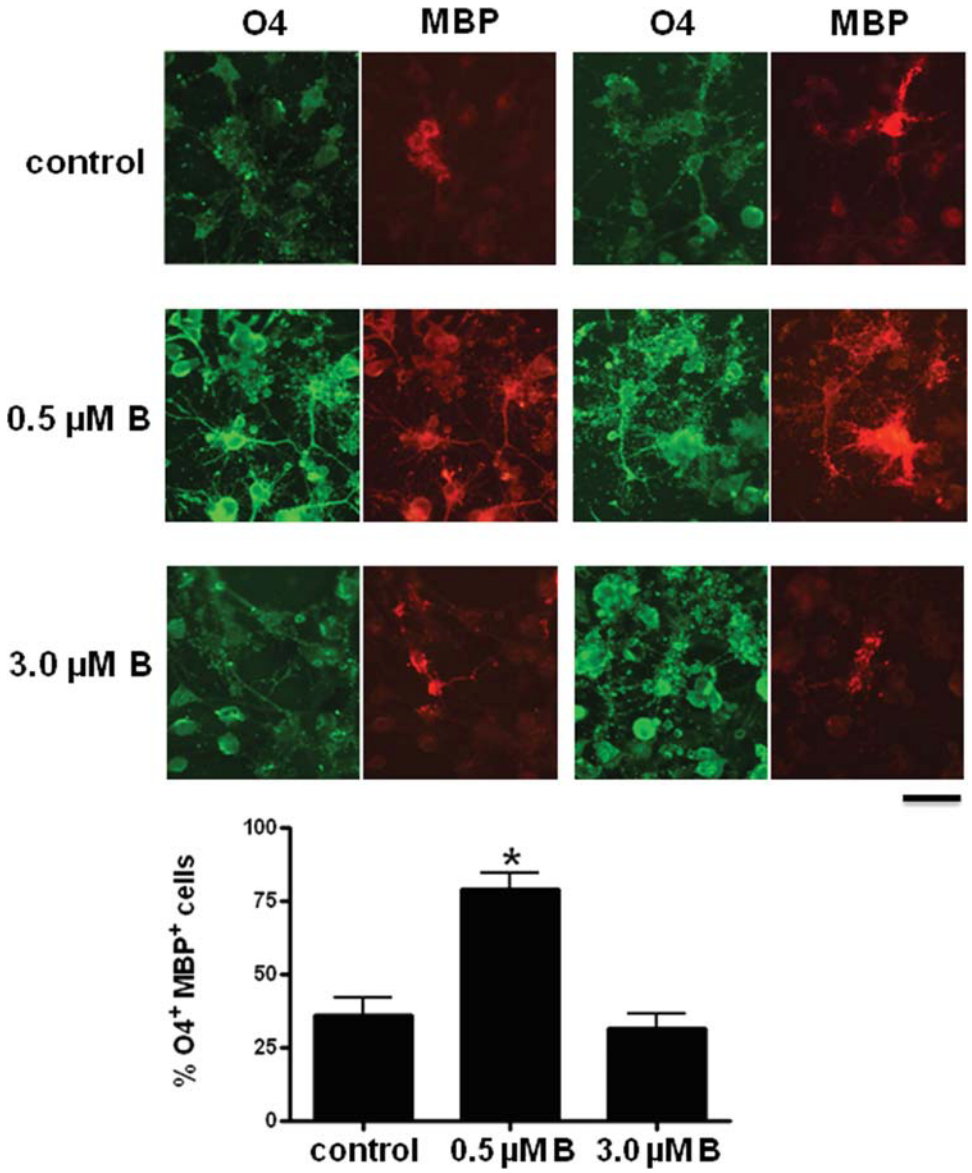

Fig. 3. Treatment of O4 positive pre-oligodendrocytes with buprenorphine results in dose-specific differences in the capacity of the cells to transition into the mature MBP-expressing developmental stage. Pre-oligodendrocytes isolated from 9-day-old rat brain were cultured for 4 days in CDM alone (control) or supplemented with 0.5 μM buprenorphine (0.5 μM B) or 3.0 μM buprenorphine (3.0 μM B). After 4 days of treatment, cells were analyzed by double immunocytochemistry with O4 (green) and anti-MBP (red) antibodies. Notice that cells which are both O4 and highly MBP positive are particularly increased in the cultures exposed to 0.5 μM buprenorphine. Scale bar: 20 μm. The bar graph indicates the percentage of O4 positive cells that are also MBP positive under each experimental condition. The results represent the average ± SEM from 5 different fields and at least 100 cells per condition. *$P < 0.001$.

drugs could alter this interplay affecting myelination in the maturing brain.

DISCUSSION

Our previous studies showed that perinatal exposure to buprenorphine affects myelination in the developing rat brain and suggested that these actions may in part reflect alterations in the timing of oligodendrocyte development (Sanchez et al., 2008). We have now found that buprenorphine indeed exerts direct actions on the oligodendrocytes. These effects are highly dose-specific and furthermore, they reveal novel functions of the MOR and the NOP receptor as regulators of oligodendrocyte development. The present results demonstrate that exposure of the cells to low doses of buprenorphine not only results in elevation of MBP levels but is also accompanied by increased morphological complexity and elevated proportion of O4-positive pre-oligodendrocytes that are able to progress into the mature MBP-expressing developmental stage. Thus, although potential effects of buprenorphine on the expression of other myelin components remain to be investigated, these

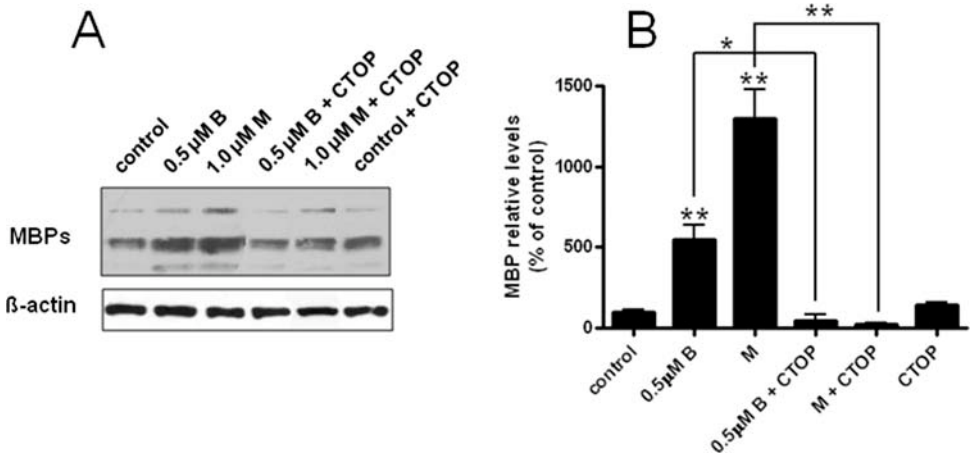

Fig. 4. The stimulatory effect of buprenorphine is mimicked by methadone and both drug effects are abolished by the MOR antagonist CTOP. Pre-oligodendrocytes from 9-day-old rat brain were cultured for 4 days in the presence or absence of 1 μM methadone (1 μM M) or 0.5 μM buprenorphine (0.5 μM B) with or without 1 μM CTOP. MBP levels were determined by Western blot analysis. (**A**) The picture shows a representative experiment. (**B**) The results in the bar graph correspond to the combined scanning of the four major MBP isoforms from at least three experiments and are expressed as percentage of controls (0 μM buprenorphine) ± SEM. $^{*}P < 0.02$ and $^{**}P < 0.0001$.

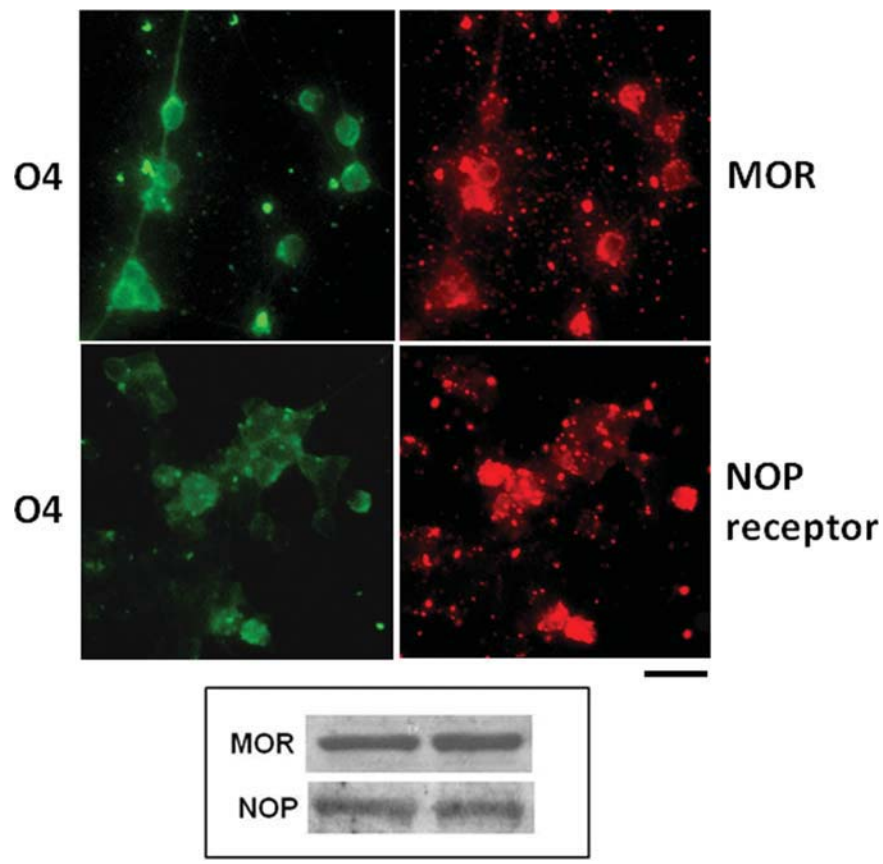

Fig. 5. Pre-oligodendrocytes express both MOR and the NOP receptor. Cells isolated from 9-day-old rat brain were allowed to fully attach on the culture plates by overnight incubation and stained by double immunocytochemistry with O4 (green) together with anti-MOR or anti-NOP receptor antibodies (red). Scale bar: 20 μm. The Western blot shows MOR and NOP receptor expression in two different samples of developing oligodendrocytes directly isolated from 9-day-old rat brains.

observations suggest that the increased levels of MBP do not result from a specific up-regulation of this protein but are instead a reflection of low buprenorphine doses stimulating oligodendrocyte differentiation. Interestingly, exposure to high buprenorphine doses exerts an opposing effect on oligodendrocyte development.

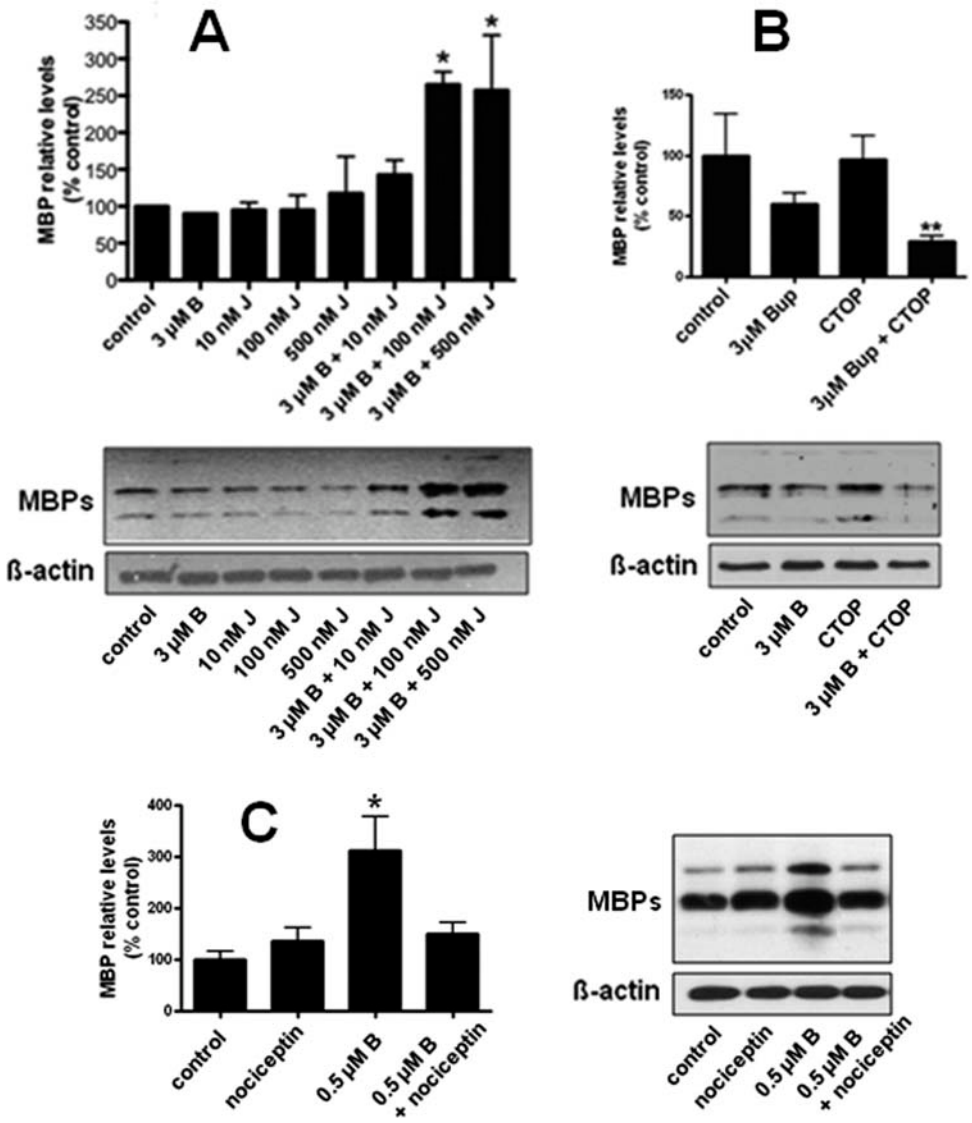

Fig. 6. The NOP receptor/nociceptin system downregulates MBP expression. (**A**) Cells isolated from 9-day-old rat brain were cultured for 4 days in CDM in the presence or absence of 3 μM buprenorphine (3 μM B) and different concentrations of the NOP receptor inhibitor J-113397 (J). (**B**) Cells were cultured for 4 days in CDM in the presence or absence of 3 μM buprenorphine with or without the MOR inhibitor CTOP. For both A and B, results in the bar graphs are expressed as percentage of controls (0 μM buprenorphine) ± SEM from at least 5 experiments and correspond to the combined scanning of the four major MBP isoforms. $^{*}P < 0.02$ and $^{**}P < 0.005$. (**C**) Cells were cultured in the presence or absence of 0.5 μM buprenorphine (0.5 μM B) with or without 1 μM nociceptin. Results are expressed as percentage of controls (0 μM buprenorphine) ± SEM from four experiments and correspond to the combined scanning of the four major MBP isoforms. $^{*}P < 0.05$. MBP levels were determined by Western blotting using β-actin levels as loading controls.

Understanding of the molecular mechanisms underlying buprenorphine effects in the oligodendrocytes is complicated by the complex pharmacology of this drug. Buprenorphine is generally regarded as a partial MOR agonist (Yu et al., 1997) and KOR antagonist (Leander, 1987) that could also exert both agonist and antagonist actions on the δ-opioid receptor (DOR) (Huang et al., 2001; Sadee et al., 1982). Initial studies have shown that the MOR is expressed very early in the oligodendroglial lineage, whereas the other two opioid receptors appear at later stages of development (Knapp et al., 1998; Tryoen-Toth et al., 2000). However, more recent analysis has indicated the presence of the three opioid receptors at all stages along the oligodendroglial lineage (Hauser et al., 2009). The present results support the idea that the observed stimulatory actions of low buprenorphine doses on the pre-oligodendrocytes are mediated by agonist effect on the MOR. Early studies implicated this receptor as a stimulator of proliferation for both neonatal oligodendrocyte progenitors (Knapp and Hauser, 1996) and adult neuroprogenitors (Persson et al., 2003). The supposition of an *in vivo* role of the endogenous opioid system in controlling cell proliferation during CNS development is strengthened by the observation that synthesis of pro-opiomelanocortin and its processing into the MOR and DOR agonist β-endorphin

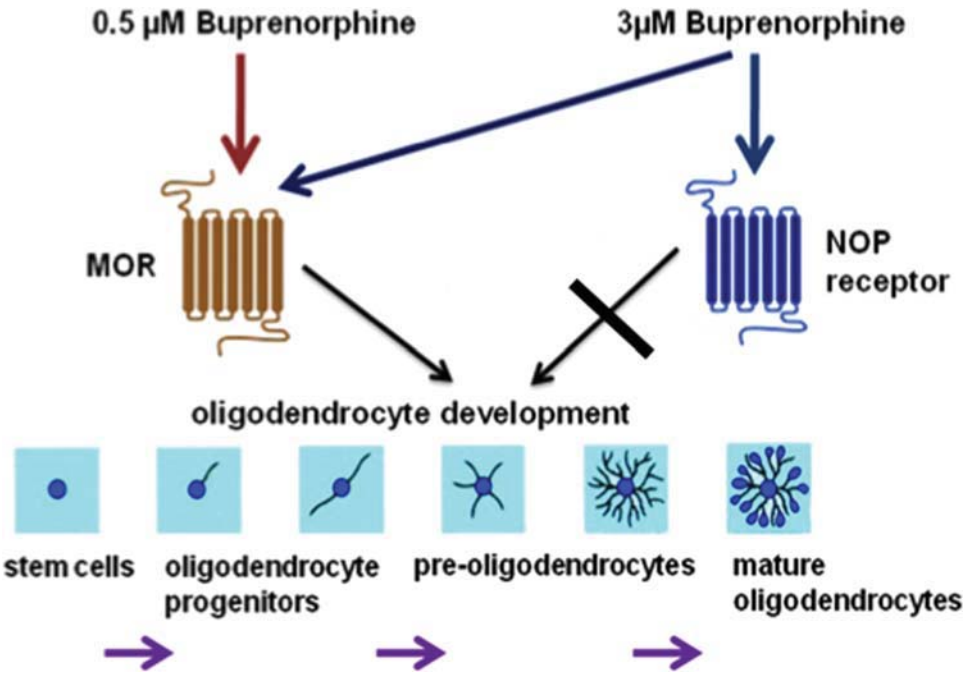

Fig. 7. Buprenorphine effects and proposed involvement of MOR and the NOP receptor in oligodendrocyte maturation. Low buprenorphine doses activate the high affinity MOR, whereas at high concentrations the drug is able to bind to both MOR and the low affinity NOP receptor. MOR activation results in stimulation of oligodendrocyte maturation, whereas this effect is counteracted by an inhibitory effect induced by signaling through the NOP receptor. Balance between both receptor activities could modulate the timing of oligodendrocyte maturation.

in the rat brain are elevated at embryonic days and postnatal ages that coincide with periods of crucial proliferative activity of neuronal and glial progenitors (Angelogianni et al., 2000). Moreover, several lines of evidence support the idea that opioid signaling may not only be important during development but also play a role in maintaining adequate numbers of different cell populations in the adult brain. Treatment of cultured adult hippocampal progenitors with MOR and DOR antagonists decreases proliferation and neurogenesis (Persson et al., 2003). Additional work also has shown that incubation with β-endorphin preferentially stimulates oligodendrogenesis in a population of cultured rat adult hippocampal progenitors (Persson et al., 2006; Raynor et al., 1994). More recently, MOR and KOR were shown to modulate oligodendrogenesis from stem cell-derived neural progenitors via MAP kinases (Hahn et al., 2010). However, to our knowledge, this study is the first to directly implicate the MOR in the last stages of oligodendrocyte development.

Particularly interesting is the observation that pre-oligodendrocytes exhibit increased differentiation when exposed to low concentrations of buprenorphine but not in response to elevated doses of the drug. A potential explanation to the observed bell-shaped dose-dependent effect of buprenorphine on oligodendrocyte maturation and MBP expression could be receptor desensitization by high drug concentrations. However, our present observations indicated that buprenorphine dose-specific effects in the oligodendrocytes implicate a delicate balancing act between MOR and the NOP receptor. This finding is particularly intriguing because the NOP/nociceptin system has been primarily linked to behavior and pain regulation but a role in CNS development or myelination has not been described before.

Similar to the biphasic bell-shaped curve observed in our present studies, different lines of evidence have demonstrated that while low doses of buprenorphine exert an analgesic effect, this antinociceptive action is decreased by higher drug concentrations (Dum and Herz, 1981; Lizasoain et al., 1991). Later studies found that while buprenorphine antinociceptive effects are mediated by the MOR, this action is counteracted by the concomitant activation of NOP receptors (Lutfy et al., 2003). In those studies, the co-administration of J-113397, the specific NOP receptor inhibitor used in our present experiments, not only enhanced the antinociceptive effect of buprenorphine but also eliminated the characteristic bell-shaped response. Moreover, the biphasic response was not observed in NOP receptor knockout mice. The results of this study suggest for the first time that an interaction between the MOR and NOP receptor-mediated signaling plays a crucial role timing oligodendrocyte maturation.

It remains to be determined the extent to which this type of dose-specific receptor activation is responsible for the biphasic effects on myelination that we previously observed in the brains of rats perinatally exposed to buprenorphine (Sanchez et al., 2008). It is important to consider that the *in vivo* effects may not be entirely explained by the direct effects of buprenorphine on the oligodendrocytes. The ubiquity of opioid receptors in the

CNS and the importance of neuron–glial interactions in the developmental coordination of myelination raise the possibility of combined drug effects in different cell populations. In this regard, previous observations found that perinatal exposure of rodents to buprenorphine delays the generation of cholinergic neurons and reduces the expression of nerve growth factor in the striatum (Robinson, 2002; Wu et al., 2001). In support of additional effects on neuron–glial interactions, we also found before that regardless of the dose, pups perinatally exposed to buprenorphine exhibited a significant increase in the caliber of the myelinated axons (Sanchez et al., 2008). Surprisingly, these axons were characterized by having a disproportionately thinner myelin sheath. These changes were also accompanied by increased levels of the myelin-associated glycoprotein (MAG), a molecule that has been implicated in glial–axonal communication (Yin et al., 1998). Exposure to therapeutic low buprenorphine doses also exhibited increased MAG glycosylation and interaction with the Src-family tyrosine kinase Fyn. Interestingly, others have implicated both MAG and Fyn as key molecules potentially mediating bidirectional signals between neurons and oligodendrocytes (Biffiger et al., 2000). On the basis of these observations, we hypothesized that opioid signaling may be part of the molecular mechanisms that coordinate axonal radial growth with myelination (Sanchez et al., 2008). Altogether, our previous and present findings suggest that interference with the endogenous opioid system during development may have significant consequences on oligodendrocyte maturation and myelination.

Although perinatal opioid exposure is the most immediate clinical correlate to this study, drug effects on oligodendrocytes and myelin formation may not be limited to early brain maturation. It is now known that human cognitive development and memory, as well as learning experience beyond early infancy, are positively correlated with late white matter deposition in functionally related areas of the brain (Bengtsson et al., 2005; Deutsch et al., 2005; Niogi and McCandliss, 2006; Rimrodt et al., 2010; Takeuchi et al., 2010; Uhlhaas et al., 2010; Whitaker et al., 2008). A major concern in this regard is the existence of active myelination during adolescence (Pfefferbaum et al., 1994; Qiu et al., 2008; Whitaker et al., 2008), a highly vulnerable period for drug abuse. This is also a critical developmental window for circuit refining and changes in the brain reward system and the magnitude of these structural and functional changes is evident both physically and behaviorally (Paus et al., 2008). The importance of myelin in the later phases of brain maturation is further supported by a growing number of neurological diseases and psychotic disorders being associated to white matter pathology during adolescence, including, among others, schizophrenia (Chambers and Perrone-Bizzozero, 2004; Kerns et al., 2010; Kubicki et al., 2005; Walterfang et al., 2005; Whitford et al., 2010), bipolar disorder (Brambilla et al., 2009; Chambers and Perrone-Bizzozero, 2004; Mahon et al., 2010), and autism (O'Hearn et al., 2008). It would therefore be important to consider that opioid addiction at adolescence may critically affect myelination resulting in long-lasting behavioral and cognitive problems.

In conclusion, the present results indicate that both buprenorphine and methadone exert direct specific effects on the oligodendrocytes. Together with the previous observations, the present findings further support an important role for opioid signaling in regulating brain maturation and, in particular, oligodendrocyte development and myelination. The novel finding of opposing actions of the MOR and NOP receptor further emphasize the intricate mechanisms underlying opioid function in glial biology. Future studies investigating the molecular mechanisms by which buprenorphine and methadone affect myelination and neuron-glial interactions should provide deeper understanding into these developmental processes and new and better strategies for the managing of both pregnant addicts and drug addiction in adolescence.

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