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Impact of chronic morphine on delta opioid receptor expressing neurons in the mouse hippocampus

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Abstract

Delta opioid receptors participate to the control of chronic pain and emotional responses. Recent data also identified their implication in spatial memory and drug-context associations pointing to a critical role of hippocampal delta receptors. To better appreciate the impact of repeated drug exposure on their modulatory activity, we used fluorescent knock-in mice that express a functional delta receptor fused at its carboxy-terminus with the green fluorescent protein in place of the native receptor. We then tested the impact of chronic morphine treatment on the density and distribution of delta receptor-expressing cells in the hippocampus. A decrease in delta receptor positive cell density was observed in the CA1, CA3 and dentate gyrus without alteration of the distribution across the different GABAergic populations that mainly express delta receptors. This effect partly persisted after four weeks of morphine abstinence. In addition, we observed increased delta opioid receptor expression at the cell surface compared to saline treated animals. In the hippocampus, chronic morphine administration thus induces delta opioid receptor cellular redistribution and durably decreases delta receptor-expressing cell density. Such modifications are likely to alter hippocampal physiology, and to contribute to long-term cognitive deficits.

Keywords

G protein-coupled receptor; chronic morphine; delta opioid receptor; hippocampus; abstinence

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Delta opioid (DOP) receptors are known to play a critical role in the control of emotional responses, including anxiety-like levels and depressive-like behaviors (Filliol et al., 2000). Only recently, their implication in spatial memory (Robles et al., 2003), drug-context associations using pavlovian place conditioning (Shippenberg et al., 2009, Le Merrer et al., 2011), context-induced reinstatement of drug seeking in rats trained to self-administer alcohol (Ciccocioppo et al., 2002, Marinelli et al., 2009) or context induced withdrawal (Faget et al., 2012) has been recognized. Mu opioid (MOP) receptors on the other hand are extensively studied since they are the molecular targets of exogenous opiate alkaloids such as heroin or morphine that constitute a major class of drugs of abuse (Charbogne et al., 2014).

In previous studies, we mapped DOP receptors in the hippocampus using fluorescent knock-in mice expressing the DOP receptor in fusion with the enhanced green fluorescent protein (DOP-eGFP) (Erbs et al., 2012, Rezai et al., 2012). These studies revealed that DOP receptors are mainly expressed in GABAergic interneurons with no functional receptors present in pyramidal cells.

Because of a growing interest for DOP receptor implication in drug-context association, the question arises whether chronic opiate administration affects DOP receptor expression in the hippocampus and whether changes elicited at the protein level persist after protracted abstinence. Using the fluorescent knock-in DOP-eGFP mice, we therefore examined the impact of chronic morphine treatment on the density and distribution across GABAergic populations of DOP receptor expressing neurons in the dorsal hippocampus. We also examined the persistence of the changes following 4 weeks of abstinence, a time point where both behavioral and transcriptional long-term modifications are detectable (Goeldner et al., 2011, Le Merrer et al., 2012a).

Experimental procedures

Animals

DOP-eGFP knock-in mice expressing the DOP receptor fused to a green fluorescent protein were generated by homologous recombination. In these mice, the eGFP cDNA preceded by a five amino acid linker (G-S-I-A-T) was introduced into exon 3 of the DOP receptor gene, in frame and 5' from the stop codon as described previously (Scherrer et al., 2006). The genetic background of all mice was C57/BL6J;129svPas (50:50 %). Mice were housed in a temperature- and humidity-controlled animal facility (21±2°C, 45±5% humidity) on a 12 h dark-light cycle with food and water *ad libitum*. Male and female mice aged 8 to 12 weeks were used in all protocols. All experiments were performed in accordance with the European Communities Council Directive of 26 May 2010 and approved by the local ethical committee (Com'Eth 2010-003).

Drugs

Escalating doses of morphine (Francopia, Paris, France) ranging from 20 to 100 mg/kg were injected twice daily (i.p.) during 5 days. Control animals were injected twice daily with a saline solution.

Physical dependence to morphine was verified in a parallel group of mice (n=8 per group). For this purpose, withdrawal syndrome was precipitated by a naloxone (Sigma, St Louis, MO, USA) injection (1mg/kg, s.c.) 2 hours after the last morphine injection (100 mg/kg) on day 6. A global withdrawal score was calculated as previously described (Berrendero et al., 2003). Following chronic morphine administration, abstinent animals were housed for 4 weeks in their home cages.

Antibody characterization

Mouse monoclonal antibodies raised against calbindin D-28K (Cat. Nr 300, Swant, Bellinzona, Switzerland, dilution 1:1000), parvalbumin (Cat. Nr 235, Swant, Bellinzona, Switzerland, dilution 1:1000), rat monoclonal antibodies raised against somatostatin (Cat. Nr MAB 354, Millipore, Billerica, MA, USA, dilution 1:100), rabbit polyclonal antibodies raised against eGFP (Cat. Nr A-6455, Molecular Probes, Paisley, UK, dilution 1:1000) and GAD65/67 (Cat. Nr G5163, Sigma, St Louis, MO, USA, dilution 1:2000) were used.

The following AlexaFluor conjugated secondary antibodies (Molecular Probes, Paisley, UK) were used: goat anti rabbit AlexaFluor 488 conjugate (Cat. Nr A-11034, dilution 1:2000), goat anti mouse IgG AlexaFluor 594 conjugate (Cat. Nr A-11005, dilution 1:500), goat anti rabbit IgG AlexaFluor 594 conjugate (Cat. Nr A-11012, dilution 1:2000), goat anti rat IgG AlexaFluor 594 conjugate (Cat. Nr 1-11007, dilution 1:500). Absence of cross-reactivity (rabbit/mouse, rabbit/rat) was systematically checked in control experiments for each antibody. Immunohistochemistry was also performed without primary antibodies to verify absence of non-specific staining by the secondary antibody alone.

Tissue preparation and immunohistochemistry

Mice were anaesthetized with ketamine (Virbac, Carros, France)/xylazine (Rompun, Kiel, Germany) (100/10 mg/kg, i.p.) and perfused intracardially with 10 ml of 9.25% sucrose in PB 0.1M pH 7.4 (Sigma, St Louis, MO, USA) followed by 50 ml of 4% paraformaldehyde (Sigma, St Louis, MO, USA) (at 2-4°C) in PB 0.1M pH 7.4. Brains were post-fixed for 24 hours at 4°C in the 4% PFA solution, cryoprotected at 4°C in a 30% sucrose (Sigma, St Louis, MO, USA), PB 0.1M pH 7.4 solution and finally embedded in OCT (Optimal Cutting Temperature medium, Thermo Scientific) frozen and kept at -80°C. Brain sections (30 µm thick) were cut with a cryostat (CM3050, Leica) and kept floating in PB 0.1M pH 7.4.

Immunohistochemistry was performed according to standard protocols. Briefly, 30 µm thick sections were incubated in blocking solution (PB 0.1M pH 7.4, 0.5% Triton X100 (Sigma, St Louis, MO, USA), 5% normal goat serum (Invitrogen, Paisley, UK) for 1 hour at room temperature (RT). Sections were incubated overnight at 4°C in the blocking solution with appropriate primary antibodies. Sections were washed three times with PB 0.1M pH 7.4, 0.5% Triton ×100, incubated for 2 hours at RT with appropriate AlexaFluor conjugated secondary antibodies. Sections were washed three times and mounted on Superfrost™ glass (Menzel-Glaser) with Mowiol (Calbiochem, Darmstadt, Germany) and 4', 6-diamidino-2-phenylindole (DAPI) (Roche Diagnostic, Mannheim, Germany) (0.5 µg/ml). Double labeling was performed to co-localize DOR-eGFP with the chosen neuronal marker. For each neuronal marker, sections used for immunohistochemistry were distant by 150 µm.

DOR-eGFP fluorescence was enhanced by detection with an anti-GFP antibody and a secondary antibody coupled to the AlexaFluor 488. Antibodies specific for the neuronal markers were detected with a secondary antibody coupled to the AlexaFluor 594. For co-localization with GAD65/67, single immunofluorescence labeling was performed using a secondary antibody coupled to the AlexaFluor 594 with no amplification of the eGFP fluorescence.

Image acquisition and analysis

Image acquisition was performed with the slide scanner NanoZoomer 2 HT and fluorescence module L11600-21 (Hamamatsu Photonics, Japan). The light source LX2000 (Hamamatsu Photonics, Japan) consisted in an ultra high-pressure mercury lamp coupled to an optical fibre. Single RGB acquisition was made in the epifluorescence mode with the 3-chip TDI camera equipped with a filter-set optimized for DAPI, fluorescein and tetramethylrhodamine detection. The scanner was equipped with a time delay integration camera and performed line scanning that offered fast acquisition at high resolution of the fluorescent signal. The acquisition was performed using a dry 20× objective (NA: 0.75). The 40× resolution was achieved with a lens converter. The latter mode used the full capacity of the camera (resolution: 0.23 $\mu\text{m}/\text{pixel}$).

Neurons expressing a given fluorescent marker were counted manually and blindly (3-4 sections par animal) on screen using the NDP viewer system with an integrated high-resolution zoom and equipped with a counter to simultaneously number two different objects. The NDP viewer also enables separation of the different fluorescent components. Neurons were considered as immunopositive for a given neuronal marker when the red fluorescence was filling objects with a mean diameter of 12 μm that showed DAPI labeled nucleus. No threshold was applied to fluorescence detection. The counting three-dimensional box was delineated by the surface of the hippocampus ($2.035 \pm 0.025 \text{ mm}^2$) and the thickness of the slice ($27.5 \pm 0.3 \mu\text{m}$). The actual value of the latter was determined with a confocal microscope (SP2RS, Leica) using a 63× oil objective (NA: 1.4) on nine randomly chosen sections with three independent measurements per section. Identification of each neuron according to its labeling (AlexaFluor 488 or AlexaFluor 594) was performed with the NDP counter which both prevented overcounting and overlooking. Colocalization between the green fluorescence associated with DOP expression and the red fluorescence associated with expression of the neuronal markers was determined manually for each stratum.

Counting was performed in the three well-described areas of the dorsal hippocampus (Bregma: -1.58 mm to -1.94mm): the dentate gyrus (DG), the Ammon's horn 3 (CA3) and the Ammon's horn 1 (CA1) regions using the mouse Paxinos atlas as anatomical reference (Paxinos & Franklin, 2004, 2nd edition). Boundaries between internal hippocampal layers, as annotated in figure 2A, were manually defined with the NDP viewer accordingly to (Lister et al., 2005). Briefly, the hilus of the dentate gyrus was defined as the entire polymorphic cellular layer enclosed between the two densely packed layers of dentate granule cells, but excluded the dense CA3 pyramidal cells that often extend into the hilus. Because of the small size of the CA2 subfield, it was grouped with the CA3 pyramidal layer. The border

between CA3 and CA1 areas was identified where the large dense neurons of the CA3 give way to the smaller, more densely packed neurons of the CA1 pyramidal layer. Also, due to its small size, the prosubicular transition zone at the distal end of the CA1 pyramidal cells was included as part of the CA1 area. Surface areas of the different regions were systematically measured with the NDP viewer. Cell density values correspond to the total number of immunoreactive cells counted in the region of interest divided by the volume of the analyzed region.

Some samples were also observed with a confocal microscope (SP2RS, Leica) using 40× (NA: 1.25) and 63× (NA: 1.4) oil objectives and images were acquired with the LCS (Leica) software. Confocal acquisitions in the sequential mode (single excitation beams: 405, 488 and 568 nm) to avoid potential crosstalk between the different fluorescence emissions were also used to validate colocalizations. In addition, we checked for the penetration of each antibody by confocal microscopy. For each marker, two sections were randomly selected and stacks of 20 serial optical sections (1.5 µm apart) were acquired. We did not detect any significant variation in the number of labeled cells with depth.

DOP-eGFP subcellular distribution

DOP-eGFP subcellular distribution was expressed as a ratio of membrane associated versus cytoplasmic fluorescence densities determined as described in (Scherrer et al., 2006). Briefly, quantification of internalization was performed using the IMAGE J software on 8-bit raw confocal images from neurons randomly sampled in the CA1 and CA3 areas. Nuclear fluorescence was used to define the background level (no threshold was applied). Cytosolic fluorescence intensity was subtracted from whole cell fluorescence intensity to obtain surface fluorescence intensity. Fluorescence intensity values were divided per surface unit (pixel) to obtain densities. Ratio of surface (Df surf) versus cytoplasmic (Df cyto) fluorescence densities was calculated to normalize data across neurons examined. A value of 1.0 results from equal densities of DOP-EGFP at the cell surface and in the cytoplasm.

Statistical analysis

Statistical analysis was performed with Graph-Pad Prism v4 (GraphPad, San Diego, CA) or Statistica v10 (StatSoft, France). Two-way ANOVA (treatment × region) analysis was performed to compare the impact of chronic morphine treatment and morphine abstinence across regions or layers followed by one-way ANOVA with multiple comparisons using Tukey's test for post-hoc analysis to compare changes between saline, chronic morphine and morphine abstinent animals in each area or layer independently. Student's t test was used for behavioral analysis and for comparison of DOP-eGFP subcellular distribution between saline and chronic morphine conditions.

Results

Chronic morphine administration induces physical dependence

Chronic administration of escalating doses of morphine is a robust treatment that induces drug dependence in mice (Matthes et al., 1996). We first verified that chronic morphine treatment indeed elicited physical dependence under our conditions. A group of mice

chronically treated with escalating doses was subjected to naloxone-precipitated withdrawal. Somatic and vegetative signs (horizontal activity, paw and body tremors, head shakes and wet dog shakes, sniffing, jumps, ptosis, teeth chattering, piloerection and diarrhea) were scored. As expected, morphine-treated mice exhibited higher global withdrawal score compared to saline treated animals (Figure 1). Chronic morphine-treated animals therefore exhibited physical dependence.

Chronic morphine administration durably decreases the density of DOP-eGFP receptor expressing neurons in the dorsal hippocampus

To address the impact of chronic morphine administration on DOP receptor expression and distribution in the hippocampus, we explored changes in the density of DOP-eGFP expressing neurons that took place in the different layers of the dorsal hippocampus after treatment with escalating doses of morphine (Figure 2). DOP-eGFP was detected in the three regions of the hippocampus with no statistical difference between male and female mice. We first estimated the impact of chronic morphine and the persistence of the effect in the CA1, CA3 and DG. We observed significant impact of the treatment (two-way ANOVA effect of treatment $F_{(4,78)}=8.92$, $p<0.001$, effect of region $F_{(2,78)}=145.51$, $p<0.0001$; interaction between treatment and region $F_{(4,78)}=1.53$, $p=0.2$). Statistically significant decrease in the density of DOP-eGFP expressing neurons was identified in the CA1, CA3 and dentate gyrus areas compared to saline treated animals (Table 1).

We then focused on the persistence of the changes induced by escalating doses of morphine after 4 weeks of protracted abstinence in DOP-eGFP mice (Figure 2). This time frame was chosen since previous work indicated major modifications in emotional-like responses after such a period of abstinence, whereas signs of physical dependence are attenuated during protracted abstinence (Goeldner et al., 2011). Statistically significant decrease in DOP-eGFP positive neurons persisted after 4 weeks of abstinence in the CA1 and CA3 areas as well as in the whole dorsal hippocampus (Table 1).

We also analyzed in more detail changes in DOP-eGFP expression across layers in the different hippocampal areas (Table 1). Chronic morphine decreased the density of DOP-eGFP expressing neurons in the oriens and pyramidal layers of the CA1 area, the pyramidal layer of the CA3 area and in the hilus and molecular layer of the DG. After four weeks abstinence, changes persisted in the CA1 pyramidal layer and in the hilus and stratum moleculare of the DG (Table 1).

On the other hand, the density in GABAergic neurons, measured by GAD65/67 positive immunoreactivity, remained unaffected by chronic morphine treatment or morphine abstinence (two-way ANOVA, effect of treatment $F_{(2,72)}=0.85$, $p=.43$; effect of region $F_{(2,72)}=22.83$, $p<0.0001$; interaction treatment $\times$ region $F_{(2,72)}=1.34$, $p=0.265$) (Figure 3). No statistical significant changes were observed in the CA1 (2880 ± 231 , 2442 ± 309 and 2678 ± 283 ND/mm³ for saline, chronic morphine and morphine abstinent animals respectively, $F_{(2,24)}=0.72$, $p=0.499$), the CA3 (3698 ± 269 , 3243 ± 394 and 3542 ± 363 ND/mm³ for saline, chronic morphine and morphine abstinent animals respectively, $F_{(2,24)}=0.46$, $p=0.639$), the DG (1660 ± 181 , 1791 ± 203 and 1791 ± 203 ND/mm³ for saline, chronic morphine and morphine abstinent animals respectively, $F_{(2,24)}=0.17$,

$p=0.842$) and globally in the hippocampus (2733 ± 205 , 2460 ± 263 and 2635 ± 240 ND/mm³ for saline, chronic morphine and morphine abstinent animals respectively, $F_{(2,24)}=0.36$, $p=0.699$) which suggests a specific loss in DOP receptor expressing neurons.

Altogether, data indicate that chronic morphine administration durably decreases DOP-eGFP expression throughout the dorsal hippocampus.

Chronic morphine administration does not modify the distribution of DOP-eGFP receptor expressing neurons across GABAergic populations in the dorsal hippocampus

We then investigated whether chronic morphine specifically decreased the density of DOP-eGFP expressing neurons among the different GABAergic populations previously identified in saline animals (Erbs et al., 2012). We compared the extent of co-localization between DOP-eGFP and parvalbumin, DOP-eGFP and calbindin or DOP-eGFP and somatostatin.

A global analysis showed no difference in the extent of co-localization with parvalbumin positive neurons across hippocampal regions (effect of treatment $F_{(2,72)}=1.52$, $p=0.226$, effect of region $F_{(2,72)}=2.26$, $p=0.112$). In the CA1, CA3 and DG of saline, chronic morphine and abstinent morphine mice, the percentage of DOP-eGFP positive neurons expressing parvalbumin did not statistically differ (Figure 4 A; Table 2).

Similarly, no difference was observed in the extent of co-localization with calbindin positive neurons across hippocampal regions (effect of treatment $F_{(2,60)}=0.369$, $p=0.693$, effect of region $F_{(2,60)}=17.97$, $p<0.001$). In the CA1 and CA3 of saline, chronic morphine and abstinent morphine mice, the percentage of DOP-eGFP positive neurons expressing calbindin did not statistically differ whereas only rare colocalization between DOP-eGFP and calbindin was detected in the DG (Figure 4 B; Table 2).

A global analysis also showed no difference in the extent of co-localization with somatostatin positive neurons across hippocampal regions (effect of treatment $F_{(2,72)}=0.19$, $p=0.822$, effect of region $F_{(2,72)}=1.21$, $p=0.306$). In the CA1, the percentage of DOP-eGFP positive neurons expressing somatostatin decreased upon chronic morphine administration but without reaching statistical significance. No statistically significant change was observed in the CA3, and the DG (Figure 4 C; Table 2).

Detailed analysis of DOP-eGFP distribution in the CA1 and CA3 oriens and pyramidal layers or hilus of the DG revealed no statistically significant change after chronic morphine administration or in morphine abstinent mice in the percentage of DOP-eGFP positive neurons co-localized with parvalbumin (Figure 5A; Table 2), calbindin (Figure 5 B; Table 2) or somatostatin positive neurons (Figure 5 C; Table 2). Noteworthy, a decrease in the extent of co-colocalization with somatostatin was observed in the CA1 pyramidal layer after chronic morphine though not reaching statistical significance (Figure 5 C; Table 2).

Altogether, data indicate no change in DOP-eGFP distribution across the different GABAergic populations in chronic morphine and morphine abstinent groups compared to saline control.

Chronic morphine induces DOP-eGFP subcellular redistribution

We also investigated whether chronic morphine administration induced subcellular redistribution of DOP-eGFP by quantifying the fluorescence respectively associated with the membrane and cytoplasm (Figure 6). The ratio of fluorescence associated with the cell surface compared to the fluorescence associated with the intracellular compartments was significantly increased after chronic morphine treatment (1.32 ± 0.04 versus 1.17 ± 0.03 in saline animals, Student's *t* test $p=0.0033$) (Figure 6 C), which strongly suggests DOP-eGFP recruitment at the plasma membrane following chronic morphine administration compared to basal conditions.

Discussion

In this study, we used DOP-eGFP knock-in mice to evaluate the impact of chronic morphine administration on DOP receptor distribution in the dorsal hippocampus as well as the persistence of changes after four weeks of morphine abstinence.

Impact of chronic morphine treatment

Chronic morphine significantly decreased DOP-eGFP expression in all areas of the dorsal hippocampus. On the other hand, we observed no statistically significant decrease in the density of GABAergic neurons. Since DOP-GFP neurons represent at most one third of the GABAergic population (Erbs et al., 2012), this result is consistent with chronic morphine specifically affecting DOP-eGFP expressing neurons rather than a general pro-apoptotic effect induced by chronic exposure to the drug (Atici et al., 2004, Liu et al., 2013). Interestingly, similar results were observed whether animals were injected daily with escalating doses (20-100 mg/kg) or at 30mg/kg s.c. (data not shown), another protocol known to induce physical dependence (Faget et al., 2012). This is in agreement with a previous report in which similar impairment of the hippocampal function, in particular long-term potentiation and spatial memory, was observed after administration of escalating doses (20-100mg/kg) or equal daily doses (20mg/kg) of morphine that both induced physical dependence (Lu et al., 2010).

Importantly, the distribution of DOP-eGFP neurons across the different GABAergic populations remained unchanged after chronic morphine suggesting that the treatment does not target any particular population of DOP expressing neurons. In the CA1, DOP receptors are expressed in parvalbumin-, calbindin, and/or somatostatin-positive neurons that correspond to basket, chandelier and O-LM cells (Erbs et al., 2012). These neurons modulate and synchronize the firing frequency of principal cells. They are involved in the modulation of theta oscillations and scale excitatory input when pyramidal cells are most active (Somogyi and Klausberger, 2005, Klausberger, 2009). A decrease in the density of these neuronal populations would therefore result in reduced inhibition of the firing activity of the hippocampus. This is in line with the strong increase in theta rhythm observed in mice chronically treated with morphine (Liu et al., 2010). Theta oscillations occur during spatial navigation, learning and memory formation (Jinno and Kosaka, 2002, Klausberger and Somogyi, 2008, Klausberger, 2009, Somogyi and Klausberger, 2005) and previous reports indicated spatial memory impairment in heroin abusers (Ornstein et al., 2000) as well as in

morphine-dependent rats (Zhang et al., 2005, Marinelli et al., 2007, Hu et al., 2010, Taubenfeld et al., 2010) and mice (Lu et al., 2010). It also supports a role for DOP receptors in drug-context associations (Ciccocioppo et al., 2002, Marinelli et al., 2009, Le Merrer et al., 2011, Faget et al., 2012, Le Merrer et al., 2012b).

Chronic morphine treatment also increased DOP-eGFP expression at the neuronal surface. This result is in agreement with previous reports showing that chronic but not short-term morphine treatment induces translocation of DOP receptors from intracellular compartments to the plasma membrane in cortical neurons or *in vivo* in the spinal cord, basal ganglia and dorsal root ganglia (Cahill et al., 2001, Morinville et al., 2003, Lucido et al., 2005, Gendron et al., 2006), also recently reviewed in (Gendron et al., 2014)). Importantly, we observed increased receptor expression at the plasma membrane in neurons with significant DOP-eGFP expression under basal conditions indicating that the presence of the fused C-terminal fluorescent protein does not significantly impact on DOP receptor trafficking as already suggested by previous studies on DOP-eGFP internalization (Scherrer et al., 2006, Pradhan et al., 2009). Increased expression of DOP receptors at the surface of the neuron following chronic morphine treatment has been associated with increased receptor function. Electrophysiological recordings revealed that DOP receptors inhibited synaptic GABA release in neurons of the periaqueducal gray matter of mice chronically treated with morphine, an effect that was not detected in naive animals (Hack et al., 2005). In the brainstem, DOP receptor stimulation induced thermal analgesia in rats chronically treated with morphine that was not present in saline treated animals (Ma et al., 2006). In the hippocampus, increased DOP receptor localization at the surface of the GABAergic interneurons would contribute to decrease the inhibitory control on the pyramidal cell firing leading to increased power of theta rhythm (Liu et al., 2010). Next to the decrease in density of DOP-eGFP positive interneurons, this represents an additional mechanism to modulate/inhibit the activity of principal glutamatergic cells.

Increased export of DOP receptors to the neuronal surface following chronic morphine administration is mediated by MOP receptors (Morinville et al., 2004), also recently reviewed in (Gendron et al., 2014)), the primary molecular target of morphine as established using MOP receptor deficient mice (recently reviewed in (Charbogne et al., 2014)). Interestingly, DOP and MOP receptors co-localize in parvalbumin, calbindin and somatostatin positive neurons where they can associate to form heteromers under basal conditions, as established by co-immunoprecipitation experiments (Erbs et al., 2014). MOP-DOP heteromerization has already been proposed as a possible mechanism to explain DOP receptor recruitment at the neuronal surface (Xie et al., 2009). Using antibodies specific for MOP-DOP heteromers, L.A. Devi's group showed that chronic morphine treatment increased MOP-DOP heteromers in the hippocampus (Gupta et al., 2010). Abundant co-expression is also detected in double fluorescent knock-in mice for DOP and MOP receptors after chronic morphine treatment (our own unpublished observations). Since increased DOP receptor activity may also contribute to the development of morphine tolerance, possible involvement of MOP-DOP heteromerization in the regulation of DOP receptor subcellular distribution now deserves in-depth investigation.

Persistence of changes after protracted abstinence

Neuronal adaptations in response to chronic morphine administration were evidenced at the behavioral level but also in gene expression (Ribeiro Do Couto et al., 2005, McClung, 2006, Befort et al., 2008, McClung and Nestler, 2008). However, long-term consequences of chronic morphine treatment remain poorly investigated (Goeldner et al., 2011, Le Merrer et al., 2012a). Modifications in dendrites and dendritic spine density induced by chronic opiates were reported in different brain regions, including the hippocampal formation, and persisted for at least one month in rats (Robinson et al., 2002, Robinson and Kolb, 2004).

Here, we provide evidence that the impact of chronic morphine administration on DOP receptor expression persists after four weeks of abstinence. Indeed, changes in DOP-eGFP expression were maintained in the CA3 and CA1 areas, in particular in the CA1 pyramidal layer as well as in the hilus and molecular layer of the DG. The long-lasting decrease in the CA1 principal layer indicates persistent alteration in the modulatory control exerted on glutamatergic cell activity in abstinent animals that likely contribute to long-term perturbations of memory processes and needs to be further explored.

In conclusion, we have shown that chronic morphine treatment decreases the density of DOP expressing neurons in the dorsal hippocampus and that this effect persists after four weeks of abstinence. Chronic morphine treatment also promotes DOP receptor recruitment at the plasma membrane. Both alterations likely alter the rhythmic activity of the hippocampus and, hence, may underlie long-standing alterations of specific cognitive functions in abstinent individuals.

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- chronic morphine decreases the density of delta receptor expressing neurons
- chronic morphine equally affects all delta opioid receptor expressing populations
- chronic morphine increases cell surface expression of delta opioid receptors
- mu opioid receptors are required to observe chronic morphine effects
- most changes induced by chronic morphine persist after four weeks of abstinence

Figure 1. Chronic morphine treatment induces a drug dependent state in mice

Global score of pharmacological withdrawal precipitated by naloxone (1 mg/kg, s.c.) in mice treated with escalating doses of morphine (20, 40, 60, 80, 100 mg/kg) or in saline-treated controls (n=8 per group). ***: $p < 0.001$, Student's t test

Figure 2. Expression of DOP-eGFP neurons in naive, morphine dependent and abstinent mice
 A. General view of the dorsal hippocampus. DOP-eGFP fluorescence is amplified by immunohistochemistry using an anti-eGFP antibody revealed by a secondary AlexaFluor 488 conjugated antibody. Ammon's horn regions (CA1, CA3), alveus (a), stratum oriens (o), stratum pyramidale (p), stratum radiatum (r), stratum lacunosum moleculare (l) and hippocampal fissure (h.f), dentate gyrus (DG), hilus (h), stratum granulosum (g), stratum moleculare (m). Scale bar 250 μ m. B-J Enlargements in the CA1 (B, E, H), CA3 (C, F, I) and DG (D, G, J) of naïve (B-D), morphine dependent (E-G) and morphine abstinent (H-J) mice. Scale bar 100 μ m.

Figure 3. Chronic morphine treatment does not affect the density of GAD 65/67 expressing neurons

The density of GAD 65/67 expressing neurons was similar in saline treated mice (n=13), mice chronically treated with morphine (n=8) and mice after 4 weeks of morphine abstinence (n=6) in the different regions of the hippocampus. Data are presented as means $\pm$ SEM.

Figure 4.

Distribution of DOP-eGFP expressing neurons in GABAergic populations in saline, morphine dependent and morphine abstinent mice. No change was observed in the extent of co-localization of DOP-eGFP expressing neurons with parvalbumin (A), calbindin (B) or somatostatin (C) positive populations in the different regions of the hippocampus. Co-localization with parvalbumin or somatostatin (n=13 saline, n=8 chronic morphine, n=6 morphine abstinent). Co-localization with calbindin (n=12 saline, n=7 chronic morphine, n=5 morphine abstinent). Data are presented as means $\pm$ SEM.

Figure 5.

Distribution of DOP-eGFP expressing neurons in GABAergic populations across layers in saline, morphine dependent and morphine abstinent mice. No change was observed in the extent of co-localization of DOP-eGFP expressing neurons with parvalbumin (A), calbindin (B) or somatostatin (C) positive populations in the oriens and pyramidal layers of the CA1 and CA3 areas or in hilus of the dentate gyrus from saline (n=13), morphine dependent (n=8) and morphine abstinent (n=6) mice. Data are presented as means $\pm$ SEM.

Figure 6. Chronic morphine administration induces DOP-eGFP subcellular redistribution

Representative fluorescence micrographs of a DOP-eGFP expressing neuron in saline (A) or chronic morphine treated (B) animal. Scale bar 10 μ m. C. DOP-eGFP subcellular redistribution expressed as a ratio of membrane-associated versus intracellular fluorescence densities is increased after chronic morphine administration. Data are presented as mean $\pm$ SEM (n=25). **p < 0.01 compared to saline.

Table 1
DOR-eGFP distribution in saline, chronic morphine treated and abstinent mice

DOR-eGFP expressing neurons (ND/mm ³)	Saline	Chronic morphine	Abstinent	Statistical analysis
Hippocampus	1554 ± 108	1167 ± 58 [*]	1202 ± 69 [*]	F _{2,26} =5.74, p=0.0086
CA1	1862 ± 126	1279 ± 61 ^{**}	1394 ± 66 [*]	F _{2,26} =9.23, p=0.0009
S. Oriens	2493 ± 142	1998 ± 115 [*]	2676 ± 132	F _{2,26} =5.64, p=0.0092
S. Pyramidale	5034 ± 195	4101 ± 217 [*]	3845 ± 358 ^{**}	F _{2,26} =7.15, p=0.0033
S. Radiatum	401 ± 52	330 ± 42	486 ± 53	F _{2,26} =1.86, p=0.1766
S. Lacunosum Moleculare	230 ± 44	290 ± 72	393 ± 71	F _{2,26} =1.67, p=0.2079
CA3	2490 ± 176	1925 ± 117 [*]	1910 ± 92 [*]	F _{2,26} =5.14, p=0.0135
S. Oriens	1976 ± 135	2422 ± 246	2488 ± 345	F _{2,26} =1.75, p=0.1945
S. Pyramidale	2897 ± 134	2325 ± 120 [*]	2522 ± 133	F _{2,26} =5.27, p=0.0120
S. Radiatum	995 ± 90	1137 ± 72	1103 ± 97	F _{2,26} =0.77, p=0.4735
DG	468 ± 38	346 ± 26 [*]	376 ± 35	F _{2,26} =3.63, p=0.0412
Hilus	1619 ± 98	1192 ± 101 [*]	1101 ± 106 ^{**}	F _{2,26} =7.75, p=0.0024
S. Granulare	511 ± 68	396 ± 47	462 ± 123	F _{2,26} =0.64, p=0.5356
S. Moleculare	147 ± 33	46 ± 18 [*]	38 ± 15 [*]	F _{2,26} =5.05, p=0.0140

Data are presented as means ± SEM from 13 (saline), 9 (chronic morphine) and 7 (abstinent) animals respectively. One way ANOVA was performed on each region or layer with Tukey's posthoc analysis.

* p<0.05,

** p<0.01, significantly different from saline mice

Table 2
Distribution of DOP-eGFP expressing neurons across GABAergic populations

Extent of co-localization with	Saline	Chronic morphine	Abstinent	Statistical analysis
Parvalbumin-positive neurons	%	%	%	
Hippocampus	66 ± 4	55 ± 7	60 ± 5	$F_{(2,24)}=0.99$, $p=0.385$
CA1	61 ± 5	46 ± 7	54 ± 4	$F_{(2,24)}=2.27$, $p=0.125$
s. oriens	55 ± 7	48 ± 11	56 ± 6	$F_{(2,24)}=0.26$, $p=0.776$
s. pyramidale	94 ± 8	91 ± 17	89 ± 12	$F_{(2,24)}=0.041$, $p=0.960$
CA3	67 ± 6	73 ± 8	72 ± 10	$F_{(2,24)}=0.18$, $p=0.836$
s. oriens	44 ± 5	57 ± 10	48 ± 11	$F_{(2,24)}=0.85$, $p=0.438$
s. pyramidale	94 ± 8	97 ± 15	97 ± 21	$F_{(2,24)}=0.018$, $p=0.982$
DG	68 ± 11	55 ± 13	58 ± 12	$F_{(2,24)}=0.34$, $p=0.715$
hilus	79 ± 13	77 ± 14	73 ± 23	$F_{(2,24)}=0.037$, $p=0.964$
Calbindin-positive neurons				
hippocampus	14 ± 2	11 ± 2	9 ± 3	$F_{(2,20)}=0.97$, $p=0.396$
CA1	11 ± 2	8 ± 2	8 ± 3	$F_{(2,20)}=0.79$, $p=0.466$
s. oriens	11 ± 2	12 ± 3	7 ± 3	$F_{(2,24)}=0.70$, $p=0.507$
s. pyramidale	1.5 ± 0.6	2.7 ± 1.4	5.7 ± 2.6	$F_{(2,24)}=2.35$, $p=0.120$
CA3	18 ± 3	17 ± 3	15 ± 4	$F_{(2,20)}=0.214$, $p=0.810$
s. oriens	18 ± 4	22 ± 6	26 ± 5	$F_{(2,24)}=0.70$, $p=0.507$
s. pyramidale	3.6 ± 1.9	1.7 ± 1.7	0 ± 0	$F_{(2,24)}=0.84$, $p=0.448$
DG	nd	nd	nd	
Somatostatin-positive neurons				
hippocampus	14 ± 1	10 ± 2	14 ± 3	$F_{(2,24)}=1.15$, $p=0.333$
CA1	16 ± 2	9 ± 2	15 ± 3	$F_{(2,24)}=3.12$, $p=0.064$
s. oriens	31 ± 4	20 ± 4	33 ± 8	$F_{(2,24)}=1.50$, $p=0.243$
s. pyramidale	5.3 ± 1.8	0 ± 0	3.0 ± 1.4	$F_{(2,24)}=2.98$, $p=0.069$
CA3	9 ± 2	12 ± 4	12 ± 3	$F_{(2,24)}=0.50$, $p=0.616$
s. oriens	27 ± 5	22 ± 4	21 ± 7	$F_{(2,24)}=0.49$, $p=0.620$
s. pyramidale	6.7 ± 2.8	1.5 ± 1.5	3.5 ± 2.2	$F_{(2,24)}=1.17$, $p=0.329$
DG	12 ± 4	8 ± 3	8 ± 3	$F_{(2,24)}=0.38$, $p=0.688$
hilus	24 ± 7	14 ± 6	22 ± 9	$F_{(2,24)}=0.44$, $p=0.649$

Data are presented as means ± SEM from 13 (saline), 8 (chronic morphine) and 6 (abstinent) animals respectively for colocalization with parvalbumin or somatostatin and from 12 (saline), 7 (chronic morphine) and 5 (abstinent) animals respectively for colocalization with calbindin. Oneway ANOVA was performed on each region or layer with Tukey's posthoc analysis.