Published in final edited form as:

Neuroscience. 2012 September 27; 221: 203–213. doi:10.1016/j.neuroscience.2012.06.023.

Distribution of delta opioid receptor expressing neurons in the mouse hippocampus

ERBS Eric¹, FAGET Lauren¹, SCHERRER Gregory², KESSLER Pascal³, HENTSCH Didier³, VONESCH Jean-Luc³, MATIFAS Audrey¹, KIEFFER Brigitte L.¹, and MASSOTTE Dominique¹

¹Dpt of Human Genetics and Translational Medicine, Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), CNRS/INSERM/UdS, 1 Rue Laurent Fries, F-67404 Illkirch-Graffenstaden, France

²Dpt of Physiology and Cellular Biophysics, Columbia University, William Black Medical Res. Bld., 650 W. 168th St., New York, NY 10032, USA

³Imaging center IGBMC, CNRS/INSERM/UdS, 1 Rue Laurent Fries, F-67404 Illkirch-Graffenstaden, France

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. We examined the distribution of delta receptor-expressing cells in the hippocampus using fluorescent knock-in mice that express a functional delta receptor fused at its carboxyterminus with the green fluorescent protein in place of the native receptor. Colocalization with markers for different neuronal populations was performed by immunohistochemical detection. Fine mapping in the dorsal hippocampus confirmed that delta opioid receptors are mainly present in GABAergic neurons. Indeed, they are mostly expressed in parvalbumin-immunopositive neurons both in the Ammon's horn and dentate gyrus. These receptors, therefore, most likely participate to the dynamic regulation of hippocampal activity.

Keywords

G protein-coupled receptor; immunohistochemistry; GABAergic neurons; fluorescent knock-in mouse

Three opioid receptors mu, delta and kappa have been identified that belong to the superfamily of G protein-coupled receptors (GPCR). Together with the endogenous opioid peptides, they form a neuromodulatory system that plays a major role in the control of nociceptive pathways. The opioid system also modulates affective behavior, neuroendocrine physiology, and controls autonomic functions (Kieffer and Evans, 2009). Mu receptors are extensively studied since they are the molecular targets for exogenous opiate alkaloids such as heroin or morphine that constitute a major class of drugs of abuse. On the other hand,

© 2012 IBRO. Published by Elsevier Ltd. All rights reserved.

Corresponding author: Dominique MASSOTTE, Dept of Human Genetics and Translational Medicine, 1 rue Laurent Fries BP 10142, F67404 Illkirch cedex, Phone + 33 3 88 65 56 25, Fax +33 3 88 65 56 04, massotte@igbmc.fr.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

studies performed in rodents revealed that delta opioid receptors are involved in the control of emotional responses, including anxiety levels and depressive-like behaviors (Filliol et al., 2000) and are also involved in spatial memory (Robles et al., 2003). In addition, increasing evidence also emphasizes their implication in drug-context associations using pavlovian place conditioning (Shippenberg et al., 2009, Le Merrer et al., 2011) or context-induced reinstatement to drug seeking in rats trained to self-administer alcohol (Ciccocioppo et al., 2002, Marinelli et al., 2009). The role of the dorsal hippocampus in spatial memory has long been recognized (Burgess, 2008, Rudy, 2009) and the anatomical organization of the parahippocampal-hippocampal network well established (Witter et al., 2000).

The cellular architecture of the hippocampus has been extensively studied in rodents and its neuronal populations classified by combining several criteria relative to specific protein content, morphology, localization, orientation, as well as electrophysiological properties. In rats, immunohistochemical colocalization with several neuronal markers showed delta receptor distribution in interneurons positive for somatostatin or neuropeptide Y in the Ammon's horn CA1 and CA3 oriens layer and the hilus of the dentate gyrus (Commons and Milner, 1996, 1997, Williams et al., 2011). In addition, delta receptor mRNA expression was detected in GABAergic interneurons, mainly parvalbumin positive, in the pyramidal and oriens layers of the CA1 and CA3 or in the granular layer and hilus of the dentate gyrus (DG) (Stumm et al., 2004). In the oriens layer, delta mRNA was also present in somatostatin or pro-enkephalin positive neurons but not in calretinin ones (Stumm et al., 2004). In glutamatergic principal cells, very low expression was detected at the mRNA or protein levels, which may be hormonally controlled (Commons and Milner, 1997, Stumm et al., 2004, Williams et al., 2011). In mice, expression of the delta opioid receptor in the dorsal hippocampus is attested by ligand binding autoradiography ((Goody et al., 2002, Lesscher et al., 2003), for a recent review see (Le Merrer et al., 2009)). Delta receptors were also identified by dual immunolabeling within γ -aminobutyric acid (GABA) expressing interneurons including basket cells axons and terminals within the pyramidal layer. No delta receptors were detected in tyrosine hydroxylase positive processes (Bausch et al., 1995).

Our laboratory has developed knock-in mice expressing the delta opioid receptor in fusion with the enhanced green fluorescent protein (DOR-eGFP) (Scherrer et al., 2006). Importantly, these mice express the fluorescent fusion under the control of the endogenous delta promoter at physiological level and eGFP fusion to the receptor did not produce detectable alteration in mouse behavior. Hence, these mice were successfully used to visualize receptor distribution under basal conditions and to evaluate receptor response to acute and chronic delta agonist administration (Scherrer et al., 2006, Pradhan et al., 2009, Pradhan et al., 2010). Recently, these genetically modified animals enabled us to establish the absence of functional delta receptors in pyramidal cells from the hippocampus by correlative light-electron microscopy (Rezai et al., 2012). Here, we used the fluorescent knock-in DOR-eGFP mice to examine the delta receptor distribution in the different strata of the mouse dorsal hippocampus. In addition, neuronal markers were used to map delta receptor distribution among GABAergic populations.

Experimental procedures

Animals

DOR-eGFP knock-in mice expressing the delta opioid 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 delta opioid receptor gene, in frame and 5' from the stop codon as described previously (Scherrer et al., 2006). The genetic background 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 (n=2) and female (n=3) mice aged 8 to 12 weeks were used. 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).

Antibody characterization

Mouse monoclonal antibodies raised against calbindin D-28K (Cat. Nr 300, Swant, Bellinzona, Switzerland, dilution 1:1000), calretinin (Cat. Nr MAB 1568, Millipore, Billerica, MA, USA, dilution 1:2000), parvalbumin (Cat. Nr 235, Swant, Bellinzona, Switzerland, dilution 1:1000), microtubule associated protein 2 (MAP2) (Cat. Nr M1406, Sigma, St Louis, MO, USA, dilution 1:2000), neuronal nuclei (NeuN) (Cat. Nr MAB 377, Millipore, Billerica, MA, USA, dilution 1:100), vesicular glutamate transporter type 1 (VGLUT1) (Cat. Nr 135511, Synaptic Systems, Göttingen, Germany, dilution 1:100), rat monoclonal antibodies raised against somatostatin (Cat. Nr MAB 354, Millipore, Billerica, MA, USA, dilution 1:1000), rabbit polyclonal antibodies raised against eGFP (Cat. Nr A-6455, Molecular Probes, Paisley, UK, dilution 1:1000), glutamate decarboxylase (GAD65/67) (Cat. Nr G5163, Sigma, St Louis, MO, USA, dilution 1:2000), glial fibrillary acidic protein (GFAP) (Cat. Nr 0334, Dako Cytomation, Glostrup, Denmark, 1:400), guinea pig polyclonal raised against vesicular glutamate transporter type 2 (VGLUT2) (Cat. Nr AB2251, Millipore, Billerica, MA, USA, dilution 1:2000), and goat polyclonal raised against choline acetyltransferase (CHAT) (Cat. Nr AB144, Millipore, Billerica, MA, USA, dilution 1:400), cholecystokinin (CCK) (Cat. Nr sc 21617, Santa Cruz Biotechnologies, Santa Cruz, CA, USA, dilution 1:50) were used.

The following AlexaFluor conjugated secondary antibodies (Molecular Probes, Paisley, UK) were used: goat anti rabbit AlexaFluor 488 conjugated (Cat. Nr A-11034, dilution 1:2000), donkey anti goat IgG AlexaFluor 594 conjugated (Cat. Nr A-11058, dilution 1:500), goat anti mouse IgG AlexaFluor 594 conjugated (Cat. Nr A-11005, dilution 1:500), goat anti guinea pig IgG AlexaFluor 594 conjugated (Cat. Nr A-11076, dilution 1:500), goat anti rabbit IgG AlexaFluor 594 conjugated (Cat. Nr A-11012, dilution 1:2000), goat anti rat IgG AlexaFluor 594 conjugated (Cat. Nr 1-11007, dilution 1:500), goat anti mouse IgG AlexaFluor 350 conjugated (Cat. Nr 1-21049, dilution 1:500). Absence of cross-reactivity (rabbit/mouse, rabbit/goat, rabbit/rat, mouse/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 or donkey serum (Invitrogen, Paisley, UK) (depending on the secondary antibody)) 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 X100, 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 colocalize 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 colocalization with GAD65/67, single immunofluorescence labeling was performed using a secondary antibody coupled to the AlexaFluor 594 with no amplification of the eGFP fluorescence. Somatostatin neurons expressing DOR-eGFP together with calbindin or parvalbumin were identified by triple labeling.

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 20x objective (NA: 0.75). The 40x resolution was achieved with a lens converter. The latter mode used the full capacity of the camera (resolution: 0.23 µm/pixel).

Neurons expressing a given fluorescent marker were counted manually and blindly 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 ($27.5 \pm 0.3 \text{ µm}$). The actual value of the latter was determined with a confocal microscope (SP2RS, Leica) using a 63x 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 DOR expression and the red or blue 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 1A, 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 40x (NA: 1.25) and 63x (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 double and triple colocalization. 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.

Results

DOR-eGFP was present in the three regions of the hippocampus (Figure 1A–D). Its distribution was not statistically different between male and female mice.

In the CA1 and CA3, a similar profile was observed with the highest DOR-eGFP density in the pyramidal layer then, only half of it in the oriens layer and, far less abundance in the stratum radiatum and stratum lacunosum-moleculare (Figure 1B–C and Table 1). In the DG, DOR-eGFP was mainly expressed in the hilus and, to a lesser extent in the granular and molecular layers (Figure 1D and Table 1). Colocalization studies using anti-GFAP, a glial marker (Figure 2A, E), anti-NeuN, a marker specific to neuronal nuclei (Figure 2I), and anti-MAP2, a marker specific to the neuronal somatodendritic compartment (Kieffer and Evans, 2009), revealed that DOR-eGFP was only detectable in neurons. In addition, colocalization was observed with GAD65/67 (Figure 2J) but not with CHAT (Figure 2B, F), VGLUT1 (Figure 2C, G) or VGLUT2 (Figure 2D, H) markers indicating that DOR-eGFP is mainly expressed in GABAergic neurons.

Global distribution of the different GABAergic neuronal populations through the dorsal hippocampus

Using GAD 65/67 immunolabeling, the numerical density of GABAergic neurons was estimated to 2779 ± 205 cells/ mm^3 in the dorsal hippocampus (Figure 3A). Parvalbumin, calbindin, somatostatin, and calretinin immunoreactivities were used to further characterize and identify the different GABAergic populations (Figures 3B–E). Parvalbumin positive neurons (1261 ± 77 cells/ mm^3) were the most abundant corresponding to 46 ± 3 % of GAD 65/67 labeled neurons (Figure 4). Calbindin (594 ± 54 cells/ mm^3), somatostatin (632 ± 49 cells/ mm^3) and calretinin (326 ± 39 cells/ mm^3) respectively represented 20 ± 2 %, 23 ± 6 %, and 11 ± 2 % of the GABAergic population in the whole hippocampus (Figure 4).

Most parvalbumin positive neurons were located in the pyramidal layer of the CA1 and CA3 regions. In the DG, these neurons were mainly present in the granular layer and at the boundary to the hilus. A large proportion was also present in the stratum oriens of the CA1 and CA3 areas whereas only few were expressed in the radiatum layer of the CA1 and CA3 areas (Figure 3B).

Calbindin positive cells were most abundant in the CA1, CA3 and DG principal layers where they were previously identified as glutamatergic principal cells (Klausberger and Somogyi, 2008). In the other layers of the CA1 and CA3 regions, calbindin immunoreactive

cells were GABAergic neurons. Calbindin intense staining was also observed in the stratum lucidum of the CA3 where the mossy fibers project (Figure 3C).

Somatostatin positive cells were mostly present in the stratum oriens of the CA1 and hilus of the DG. In the CA1, somatostatin either co-expressed with calbindin or with parvalbumin. Some immunoreactivity was also detected in the stratum radiatum of the CA3 and occasionally in the principal cell layer of the Ammon's horn (Figure 3D).

Calretinin positive neurons were scattered through all layers (Figure 3E). They were previously identified as a GABAergic population that showed negligible overlaps with calbindin or parvalbumin positive neurons (Miettinen et al., 1992).

Only few cholecystokinin positive neurons were detected. They were mainly located in the stratum radiatum and stratum pyramidale of the CA1 and were very sparse in the oriens layer (not shown). These neurons were described either as basket cells or, for those with cell bodies located at the border between the stratum radiatum and the stratum moleculare-lacunosum, as several populations whose functions are not well known (Somogyi and Klausberger, 2005, Klausberger and Somogyi, 2008). Since only occasional colocalization with DOR-eGFP was observed, this population was not further analyzed.

Altogether, numerical densities, distribution and relative proportions of the different GABAergic populations are in good agreement with a previous study performed in adult male C57/BL/6J mice using a stereological approach (Jinno and Kosaka, 2006).

Global DOR-eGFP distribution in the hippocampus

A similar distribution was observed within the three areas of the hippocampus where DOR-eGFP mainly colocalized with parvalbumin ($69 \pm 7\%$) and to a lesser extent with calbindin ($14 \pm 2\%$) and somatostatin ($16 \pm 2\%$) immunoreactive cells (Figure 5A–C, E). In terms of GABAergic neurons, parvalbumin and somatostatin positive cells co-expressed DOR-eGFP to a similar extent ($35 \pm 4\%$ and $40 \pm 3\%$ respectively) whereas calbindin positive neurons co-expressing DOR-eGFP were twice less abundant ($16 \pm 2\%$) (Figure 5F). No colocalization with calretinin was ever detected in agreement with a previous study based on DOR mRNA expression in rat (Stumm et al., 2004) (Figure 5D).

DOR-eGFP distribution in the CA1 region

In the CA1, $36 \pm 5\%$ of the parvalbumin positive neurons expressed DOR-eGFP (Figure 5F). They were mainly located in the oriens and pyramidal layers where respectively $38 \pm 5\%$ and $35 \pm 5\%$ of the parvalbumin positive neurons also expressed DOR-eGFP. Some were also detected in the stratum radiatum immediately close to the pyramidal layer (Figure 6). Interestingly, the very few parvalbumin immunoreactive neurons detected in the stratum lacunosum-moleculare all expressed DOR-eGFP. We also identified DOR-eGFP expression in a few cells co-expressing both parvalbumin and somatostatin within the stratum pyramidale. They were likely of the bistratified type that also mainly projects on pyramidal cells (Klausberger, 2009).

In the stratum oriens, about half of the somatostatin immunoreactive neurons co-expressed DOR-eGFP ($44\% \pm 4\%$). They were located close to the alveus border and exhibited horizontal orientation. Refined colocalization studies using confocal microscopy identified them as either somatostatin/parvalbumin or somatostatin/calbindin populations (Figure 7). In this layer, about 13% of DOR-eGFP expressing cells colocalized with calbindin and somatostatin, whereas 56% colocalized with parvalbumin and somatostatin.

In the CA1, DOR-eGFP was expressed in 13 ± 3 % of the calbindin positive population (Figure 5F). In the oriens layer, around 17 ± 7 % of the calbindin positive neurons were identified as DOR-eGFP neurons. These neurons were all somatostatin positive (see above). In the pyramidal layer, calbindin immunoreactivity was mostly associated with principal cells, a large portion of which were tightly packed on the stratum radiatum side (Ng and Iacopino, 1995, Somogyi and Klausberger, 2005, Klausberger and Somogyi, 2008). Colocalization with DOR-eGFP (less than 1% of the total DOR-eGFP expressing GABAergic neurons in the CA1) was only occasionally observed in this layer. The morphology of these neurons suggested that they could correspond to a recently described population of unknown function (Klausberger and Somogyi, 2008). Scarce calbindin positive cells located at the border of the stratum radiatum and stratum lacunosum-moleculare also expressed DOR-eGFP (less than 3% of the total calbindin interneurons that expressed DOR-eGFP in the CA1). They were attributed to the Schaffer collateral associated cell type (Klausberger and Somogyi, 2008).

DOR-eGFP distribution in the CA3 region

DOR-eGFP expression was detected in 38 ± 4 % of the parvalbumin positive neurons and equally distributed across the oriens, pyramidal and radiatum layers (Figure 5F).

Similarly to the CA1, about half of the somatostatin immunoreactive cells (56 ± 13 %) expressed DOR-eGFP (Figure 5F). Colocalization was mostly present in the oriens layer where they represented about 50% of the somatostatin positive cells.

One fifth of the calbindin positive neurons (21 ± 2 %) expressed DOR-eGFP with a colocalization essentially restricted to the stratum oriens (39 ± 7 %) (Figure 5F).

DOR-eGFP distribution in the dentate gyrus

In our study, DOR-eGFP was mainly detected in the hilus with few scattered neurons in the granular and molecular layers. DOR-eGFP was expressed in 29 ± 7 % of the parvalbumin population (Figure 5F) equally distributed in the hilus and granular layer. No colocalization was detected in the stratum moleculare.

All somatostatin positive neurons (25 ± 5 %) that expressed DOR-eGFP were detected in the hilus (Figure 5C, F). In addition, no colocalization with calbindin immunoreactive cells was identified (Figure 5E, F).

Discussion

In this study, we documented delta receptor expression in the dorsal hippocampus.

Methodological considerations

Identification of the different neuronal types in the hippocampus was essentially conducted in rats for which detailed analysis of the GABAergic populations was performed by combined electrophysiological and immunohistological characterizations (Miettinen et al., 1992, Somogyi and Klausberger, 2005, Jinno et al., 2007, Klausberger and Somogyi, 2008). The cellular architecture of the mouse hippocampus (C57BL/6J strain) assorted with a quantitative survey of the GABAergic neurons and characterization of several neuronal populations was also published (Jinno and Kosaka, 2000, 2002, 2004, 2006, 2010). In addition, a comparative study performed on four different mouse strains (including C57BL/6 and 129SvJ) concluded that there were no qualitative differences in the features among the four strains for any of the tested markers. The authors also confirmed that the morphofunctional classification of interneurons established in the rat was largely

transposable to mouse (Matyas et al., 2004). The genetic background of the DOR-eGFP knock-in mice being C57/BL6J-129svPas (50/50 %), we directly referred to the literature to assign neuronal populations according to their immunoreactive profile, morphology and layer distribution. In addition, neuronal markers that would cover the entire spectrum of hippocampal GABAergic neurons were selected according to published data. As expected, the distribution of the different GABAergic populations that we identified in mice matched previously published descriptions and the different populations were present in similar proportions to those reported in the C57BL/6J mouse line (Jinno and Kosaka, 2006) or in rat (Potier et al., 2006, Hu et al., 2010). Only few cholecystokinin positive neurons were detected under our conditions (not shown). Though it may in part reflect a poor detection by the anti-cck antibody, cck expressing neurons seem indeed of low abundance in mouse (around 5% of the GABAergic population) (Jinno and Kosaka, 2006). Altogether, densities of the different GABAergic populations are in good agreement with a previous stereological study in mice (Jinno and Kosaka, 2006).

In addition to variations due to species specificities, the use of different methodologies may also impact on the estimate of delta receptor expression and, hence, its extent of colocalization with neuronal markers. In our study, we visualized the actual delta receptor protein content. Compared to a detection based on mRNA transcript, estimation of DOR-eGFP positive neurons by fluorescence should therefore more accurately reflect the level of delta receptor expression.

Delta receptor expression in interneurons

DOR-eGFP was mostly present in the oriens and pyramidal cell layers of the CA1 and CA3 regions as well as the hilus and granular layer of the dentate gyrus. Expression was mostly detected in parvalbumin positive neurons and was also detected in calbindin and somatostatin positive cells but not in calretinin positive interneurons. Our results qualitatively matched those reported in rats for delta receptor expression based on *in situ* hybridization (Stumm et al., 2004). However, some differences were noted in the extent of colocalization compared to previous immunohistochemical detection in which delta receptors were mainly identified in somatostatin positive neurons using double labeling colocalization (Commons and Milner, 1996; 1997). The reason for this discrepancy is unclear but likely reflects both species and methodological differences.

DOR-eGFP colocalized with parvalbumin, somatostatin or calbindin but not calretinin immunoreactivities. Delta receptors are thus distributed among neurons that modulate principal cells activity and, hence, hippocampal firing. Indeed, parvalbumin positive neurons were previously described as basket or chandelier cells (Somogyi and Klausberger, 2005, Jinno and Kosaka, 2006, Klausberger and Somogyi, 2008). These neurons play a similar role in the three regions of the hippocampus where they innervate principal glutamatergic cells and exert an inhibiting action on their activity (Figure 8). Basket cells contact the soma and proximal dendrite parts of the principal cells whereas chandelier, also called axo-axonic cells, exclusively contact the axon initial segment therefore differentially regulating postsynaptic firing (Klausberger and Somogyi, 2008) (Figure 8). In the CA1 oriens layer, DOR-eGFP was present in two other populations that were previously identified as oriens-lacunosum moleculare (O-LM) cells (somatostatin/parvalbumin positive) or hippocampo-septal cells (somatostatin/calbindin positive) (Figure 8). O-LM cells innervate the apical tuft of the pyramidal cells. These cells receive glutamatergic input from local CA pyramidal cells and therefore provide GABAergic feedback inhibition (Matyas et al., 2004, Klausberger and Somogyi, 2008). Hippocampo-septal cells, on the contrary, are long-range projecting neurons that target both the septum and the subiculum (Jinno and Kosaka, 2002, Klausberger, 2009). In the stratum oriens, a limited amount of DOR-eGFP expression was detected in these cells, which represented less than 10 % of the total DOR-eGFP population

in the CA1 (Figure 8). Delta receptors therefore appeared to essentially contribute to the local regulation of hippocampal activity by influencing local hippocampal connectivity and glutamatergic firing rate. Indeed, local GABAergic interneurons modulate the spike timing of principal cells and synchronize their activity. They also largely contribute to the different types of network oscillations of the hippocampal formation. Theta oscillations occur during spatial navigation, learning and memory formation whereas ripple oscillations take place during resting and consummatory behaviors as well as memory consolidation (Jinno and Kosaka, 2002, Klausberger and Somogyi, 2008, Klausberger, 2009). Basket, chandelier and O-LM cells are firing with different temporal patterns during these two rhythmic activities (Klausberger and Somogyi, 2008, Klausberger, 2009).

In conclusion, we have shown that delta receptors are localized in GABAergic neuronal populations that modulate principal cell firing rate. These receptors, therefore, most likely participate to the dynamic regulation of hippocampal activity. Future functional investigations will be needed to determine the precise role of hippocampal delta receptors in memory processes in particular when involved in drug-context association or drug seeking behavior.

Acknowledgments

We thank E. Lenys and S. Boudon for excellent technical assistance, Drs K. Befort and C. Gaveriaux-Ruff for critical reading. We are grateful to Hamamatsu Photonics for allowing access to the NanoZoomer 2HT acquisition and NDP viewer systems. This work was supported by NIDA Center for Opioid Receptors and Drugs of Abuse (#DA 005010), ANR, CNRS, INSERM and University Strasbourg. LF was a recipient of a region Alsace PhD fellowship.

References

- Bausch SB, Patterson TA, Appleyard SM, Chavkin C. Immunocytochemical localization of delta opioid receptors in mouse brain. *J Chem Neuroanat.* 1995; 8:175–189. [PubMed: 7598816]
- Burgess N. Spatial cognition and the brain. *Ann N Y Acad Sci.* 2008; 1124:77–97. [PubMed: 18400925]
- Ciccocioppo R, Martin-Fardon R, Weiss F. Effect of selective blockade of mu(1) or delta opioid receptors on reinstatement of alcohol-seeking behavior by drug-associated stimuli in rats. *Neuropsychopharmacology.* 2002; 27:391–399. [PubMed: 12225696]
- Commons KG, Milner TA. Cellular and subcellular localization of delta opioid receptor immunoreactivity in the rat dentate gyrus. *Brain Res.* 1996; 738:181–195. [PubMed: 8955512]
- Commons KG, Milner TA. Localization of delta opioid receptor immunoreactivity in interneurons and pyramidal cells in the rat hippocampus. *J Comp Neurol.* 1997; 381:373–387. [PubMed: 9133574]
- Filliol D, Ghazizadeh S, Chluba J, Martin M, Matthes HW, Simonin F, Befort K, Gaveriaux-Ruff C, Dierich A, LeMeur M, Valverde O, Maldonado R, Kieffer BL. Mice deficient for delta- and mu-opioid receptors exhibit opposing alterations of emotional responses. *Nat Genet.* 2000; 25:195–200. [PubMed: 10835636]
- Goody RJ, Oakley SM, Filliol D, Kieffer BL, Kitchen I. Quantitative autoradiographic mapping of opioid receptors in the brain of delta-opioid receptor gene knockout mice. *Brain Res.* 2002; 945:9–19. [PubMed: 12113946]
- Hu W, Zhang M, Czeh B, Flugge G, Zhang W. Stress impairs GABAergic network function in the hippocampus by activating nongenomic glucocorticoid receptors and affecting the integrity of the parvalbumin-expressing neuronal network. *Neuropsychopharmacology.* 2010; 35:1693–1707. [PubMed: 20357756]
- Jinno S, Klausberger T, Marton LF, Dalezios Y, Roberts JD, Fuentealba P, Bushong EA, Henze D, Buzsaki G, Somogyi P. Neuronal diversity in GABAergic long-range projections from the hippocampus. *J Neurosci.* 2007; 27:8790–8804. [PubMed: 17699661]

- Jinno S, Kosaka T. Colocalization of parvalbumin and somatostatin-like immunoreactivity in the mouse hippocampus: quantitative analysis with optical disector. *J Comp Neurol*. 2000; 428:377–388. [PubMed: 11074441]
- Jinno S, Kosaka T. Immunocytochemical characterization of hippocamposeptal projecting GABAergic nonprincipal neurons in the mouse brain: a retrograde labeling study. *Brain Res*. 2002; 945:219–231. [PubMed: 12126884]
- Jinno S, Kosaka T. Patterns of colocalization of neuronal nitric oxide synthase and somatostatin-like immunoreactivity in the mouse hippocampus: quantitative analysis with optical disector. *Neuroscience*. 2004; 124:797–808. [PubMed: 15026120]
- Jinno S, Kosaka T. Cellular architecture of the mouse hippocampus: a quantitative aspect of chemically defined GABAergic neurons with stereology. *Neurosci Res*. 2006; 56:229–245. [PubMed: 16930755]
- Jinno S, Kosaka T. Stereological estimation of numerical densities of glutamatergic principal neurons in the mouse hippocampus. *Hippocampus*. 2010; 20:829–840. [PubMed: 19655319]
- Kieffer BL, Evans CJ. Opioid receptors: from binding sites to visible molecules in vivo. *Neuropharmacology*. 2009; 56(Suppl 1):205–212. [PubMed: 18718480]
- Klausberger T. GABAergic interneurons targeting dendrites of pyramidal cells in the CA1 area of the hippocampus. *Eur J Neurosci*. 2009; 30:947–957. [PubMed: 19735288]
- Klausberger T, Somogyi P. Neuronal diversity and temporal dynamics: the unity of hippocampal circuit operations. *Science*. 2008; 321:53–57. [PubMed: 18599766]
- Le Merrer J, Becker JA, Befort K, Kieffer BL. Reward processing by the opioid system in the brain. *Physiol Rev*. 2009; 89:1379–1412. [PubMed: 19789384]
- Le Merrer J, Plaza-Zabala A, Del Boca C, Matifas A, Maldonado R, Kieffer BL. Deletion of the delta opioid receptor gene impairs place conditioning but preserves morphine reinforcement. *Biol Psychiatry*. 2011; 69:700–703. [PubMed: 21168121]
- Lesscher HM, Bailey A, Burbach JP, Van Ree JM, Kitchen I, Gerrits MA. Receptor-selective changes in mu-, delta- and kappa-opioid receptors after chronic naltrexone treatment in mice. *Eur J Neurosci*. 2003; 17:1006–1012. [PubMed: 12653976]
- Lister JP, Blatt GJ, DeBassio WA, Kemper TL, Tonkiss J, Galler JR, Rosene DL. Effect of prenatal protein malnutrition on numbers of neurons in the principal cell layers of the adult rat hippocampal formation. *Hippocampus*. 2005; 15:393–403. [PubMed: 15669101]
- Marinelli PW, Funk D, Harding S, Li Z, Juzysch W, Le AD. Roles of opioid receptor subtypes in mediating alcohol-seeking induced by discrete cues and context. *Eur J Neurosci*. 2009; 30:671–678. [PubMed: 19686472]
- Matyas F, Freund TF, Gulyas AI. Immunocytochemically defined interneuron populations in the hippocampus of mouse strains used in transgenic technology. *Hippocampus*. 2004; 14:460–481. [PubMed: 15224983]
- Miettinen R, Gulyas AI, Baimbridge KG, Jacobowitz DM, Freund TF. Calretinin is present in non-pyramidal cells of the rat hippocampus--II. Co-existence with other calcium binding proteins and GABA. *Neuroscience*. 1992; 48:29–43. [PubMed: 1584423]
- Ng MC, Iacopino AM. Quantitative measurement of neuronal calbindin-D28k by radioimmunocytochemistry. *Brain Res Mol Brain Res*. 1995; 32:82–86. [PubMed: 7494466]
- Potier B, Jouvenceau A, Epelbaum J, Dutar P. Age-related alterations of GABAergic input to CA1 pyramidal neurons and its control by nicotinic acetylcholine receptors in rat hippocampus. *Neuroscience*. 2006; 142:187–201. [PubMed: 16890374]
- Pradhan AA, Becker JA, Scherrer G, Tryoen-Toth P, Filliol D, Matifas A, Massotte D, Gaveriaux-Ruff C, Kieffer BL. In vivo delta opioid receptor internalization controls behavioral effects of agonists. *PLoS ONE*. 2009; 4:e5425. [PubMed: 19412545]
- Pradhan AA, Walwyn W, Nozaki C, Filliol D, Erbs E, Matifas A, Evans C, Kieffer BL. Ligand-directed trafficking of the delta-opioid receptor in vivo: two paths toward analgesic tolerance. *J Neurosci*. 2010; 30:16459–16468. [PubMed: 21147985]
- Rezaï X, Faget L, Bednarek E, Schwab Y, Kieffer BL, Massotte D. Mouse delta opioid receptors are located on presynaptic afferents to hippocampal pyramidal cells. *Cell Mol Neurobiol*. 2012; 32:509–516. [PubMed: 22252784]

- Robles Y, Vivas-Mejia PE, Ortiz-Zuazaga HG, Felix J, Ramos X, Pena de Ortiz S. Hippocampal gene expression profiling in spatial discrimination learning. *Neurobiol Learn Mem.* 2003; 80:80–95. [PubMed: 12737936]
- Rudy JW. Context representations, context functions, and the parahippocampal-hippocampal system. *Learn Mem.* 2009; 16:573–585. [PubMed: 19794181]
- Scherrer G, Tryoen-Toth P, Filliol D, Matifas A, Laustriat D, Cao YQ, Basbaum AI, Dierich A, Vonesh JL, Gaveriaux-Ruff C, Kieffer BL. Knockin mice expressing fluorescent delta-opioid receptors uncover G protein-coupled receptor dynamics in vivo. *Proc Natl Acad Sci U S A.* 2006; 103:9691–9696. [PubMed: 16766653]
- Shippenberg TS, Chefer VI, Thompson AC. Delta-opioid receptor antagonists prevent sensitization to the conditioned rewarding effects of morphine. *Biol Psychiatry.* 2009; 65:169–174. [PubMed: 18950747]
- Somogyi P, Klausberger T. Defined types of cortical interneurone structure space and spike timing in the hippocampus. *J Physiol.* 2005; 562:9–26. [PubMed: 15539390]
- Stumm RK, Zhou C, Schulz S, Holtt V. Neuronal types expressing mu- and delta-opioid receptor mRNA in the rat hippocampal formation. *J Comp Neurol.* 2004; 469:107–118. [PubMed: 14689476]
- Williams TJ, Torres-Reveron A, Chapleau JD, Milner TA. Hormonal regulation of delta opioid receptor immunoreactivity in interneurons and pyramidal cells in the rat hippocampus. *Neurobiol Learn Mem.* 2011; 95:206–220. [PubMed: 21224009]
- Witter MP, Wouterlood FG, Naber PA, Van Haeften T. Anatomical organization of the parahippocampal-hippocampal network. *Ann N Y Acad Sci.* 2000; 911:1–24. [PubMed: 10911864]

- fluorescent knock-in mice were used to map delta opioid receptor distribution
- delta opioid receptors are present in all hippocampal areas
- hippocampal delta opioid receptors are primarily expressed in GABAergic neurons
- delta opioid receptors are mostly identified in parvalbumin-positive neurons

Figure 1. Expression of DOR-eGFP across the different layers of the dorsal hippocampus
 A. General view of the dorsal hippocampus. DOR-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 (hf), dentate gyrus (DG), hilus (h), stratum granulosum (g), stratum moleculare (m). Scale bar 500 μ m.
 B–D. Enlargement in the CA1 (B), CA3 (C) and DG (D) regions showing DOR-eGFP expression. Scale bar 100 μ m.

Figure 2. DOR-eGFP is mainly expressed in GABAergic neurons

Representative confocal fluorescence photomicrographs in 30 μm thick brain sections showing DOR-eGFP detected with an anti-GFP antibody revealed with an AlexaFluor 488 coupled secondary antibody and specific markers immunoreactivities revealed by an AlexaFluor 594 conjugated secondary antibody. No colocalization was observed with the GFAP (A, E), CHAT (B, F), VGLUT1 (C, G) or VGLUT2 (D, H) markers whereas DOR-eGFP colocalized with NeuN (I) and GAD 65/67 positive neurons (J) (arrow) revealing delta receptor expression in GABAergic neurons. Nuclei were stained with DAPI. Scale bars 25 μm (A–D) and 10 μm (E–J).

Figure 3. Immunohistochemical labeling of GABAergic populations

Examples of immunohistochemical labeling with primary antibodies specific for different GABAergic populations as revealed by an anti-AlexaFluor 594 conjugated secondary antibody: anti-GAD65/67 (A), anti-parvalbumin (B), anti-calbindin (C), anti-somatostatin (D) or anti-calretinin (E) labeling. Scale bar 50 μ m.

Figure 4. Distribution of the different GABAergic populations within the hippocampus
 Relative expression of parvalbumin, calbindin, somatostatin or calretinin immunoreactive neurons in the CA1, CA3, DG regions as well as in the whole hippocampus expressed as a percentage of the GABAergic population defined as GAD 65/67 immunoreactive neurons (n=10 hippocampal sections for each GABAergic marker). Data are represented as means $\pm$ SEM.

Figure 5. Distribution of DOR-eGFP expression within GABAergic populations

A–D. General view of the dorsal hippocampus showing immunohistochemical labeling with primary antibodies specific for different GABAergic populations as revealed by an anti-AlexaFluor 594 conjugated secondary antibody and DOR-eGFP fluorescence amplified by immunohistochemistry using an anti-eGFP antibody revealed by a secondary AlexaFluor 488 conjugated antibody. (A) Parvalbumin-positive immunoreactivity, (B) calbindin-positive immunoreactivity, (C) somatostatin-positive immunoreactivity, (D) calretinin-positive immunoreactivity. Scale bar 500 μ m.

E. Percentage of DOR-eGFP expressing cells in the CA1, CA3, DG regions as well as in the whole hippocampus that also expressed parvalbumin, calbindin or somatostatin as GABAergic markers. No colocalization with calretinin was detected. (n=10 hippocampal sections for each GABAergic marker). Data are presented as mean $\pm$ SEM.

F. Percentage of parvalbumin, calbindin or somatostatin immunoreactive neurons in the CA1, CA3, DG regions as well as in the whole hippocampus that also expressed DOR-eGFP. No colocalization with calretinin was detected. (n=10 hippocampal sections for each GABAergic marker). Data are presented as mean $\pm$ SEM.

Figure 6. DOR-eGFP mainly colocalizes with parvalbumin immunoreactivity in the oriens and pyramidal layers of the Ammon's horn

Representative confocal fluorescence photomicrographs in 30 μm thick brain sections showing DOR-eGFP detected with an anti-GFP antibody revealed with an AlexaFluor 488 coupled secondary antibody (A, D) and parvalbumin immunoreactivity revealed by an AlexaFluor 594 conjugated secondary antibody (B, E). Merge images (C, F). (A–C) Scale bar 100 μm. (D–F) Scale bar 10 μm.

Figure 7. DOR-eGFP is expressed in two populations of horizontal somatostatin positive neurons
Representative confocal fluorescence photomicrographs in 30 μm thick brain sections showing DOR-eGFP detected with an anti-GFP antibody revealed with an AlexaFluor 488 coupled secondary antibody (A, D), somatostatin immunoreactivity revealed by an AlexaFluor 594 conjugated secondary antibody (B, E), and immunoreactivity revealed by an AlexaFluor 350 conjugated secondary antibody for parvalbumin (C) or calbindin (F). Scale bar 10 μm .

Figure 8. Schematic diagram presenting the principal GABAergic populations expressing DOR-eGFP in the Ammon's horn

DOR-eGFP colocalized with parvalbumin immunoreactive cells (parva) in the oriens and pyramidal layers that correspond to basket and chandelier cells and innervate principal cells. DOR-eGFP was also identified in somatostatin immunoreactive horizontal neurons located in the oriens. These neurons belong to two different categories: parvalbumin immunoreactive cells corresponding to oriens-lacunosum moleculare (O-LM) neurons or calbindin immunoreactive cells corresponding to hippocampo-septal neurons. With the exception of the latter category that projects to the septum and the subiculum, DOR-eGFP neurons make local contacts at different levels of the principal glutamatergic cells and may therefore temporally control the inhibition of postsynaptic firing. Percentages refer for each GABAergic type to the proportion that also expresses DOR-eGFP.

Table 1

Density of DOR-eGFP expressing neurons across the different hippocampal layers.

Area	ND/mm ³
Hippocampus	1207 ± 37
CA1	1443 ± 48
S. Oriens	2357 ± 95
S. Pyramidale	4653 ± 181
S. Radiatum	310 ± 24
S. Moleculare	266 ± 49
CA3	1862 ± 100
S. Oriens	1773 ± 154
S. Pyramidale	3182 ± 201
S. Radiatum	1008 ± 78
DG	468 ± 25
Hilus	1675 ± 107
S. Granulare	525 ± 56
S. Moleculare	110 ± 27

Data are presented as means ± SEM (n= 36 hippocampal sections from 5 animals)