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Mouse Delta Opioid Receptors are Located on Presynaptic Afferents to Hippocampal Pyramidal Cells

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Abstract

Delta opioid receptors participate in the control of chronic pain and emotional responses. Recent data have also identified their implication in drug-context associations pointing to a modulatory role on hippocampal activity. We used fluorescent knock-in mice that express a functional delta opioid receptor fused at its carboxy terminus with the green fluorescent protein in place of the native receptor to investigate the receptor neuroanatomical distribution in this structure. Fine mapping of the pyramidal layer was performed in hippocampal acute brain slices and organotypic cultures using fluorescence confocal imaging, co-localization with pre- and postsynaptic markers and correlative light-electron microscopy. The different approaches concurred to identify delta opioid receptors on presynaptic afferents to glutamatergic principal cells. In the latter, only scarce receptors were detected that were confined within the Golgi or vesicular intracellular

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compartments with no receptor present at the cell surface. In the mouse hippocampus, expression of functional delta opioid receptors is therefore mostly associated with interneurons emphasizing a presynaptic modulatory effect on the pyramidal cell firing rate.

Keywords

Delta opioid receptor; Mouse hippocampus; Pyramidal cells; G protein-coupled receptor; Correlative light-electron microscopy; Immunohistochemistry

Introduction

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). 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; Pradhan et al. 2011), 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 (Le Merrer et al. 2011; Shippenberg et al. 2009) or context-induced reinstatement to drug seeking in rats trained to self-administer alcohol (Ciccocioppo et al. 2002; Marinelli et al. 2009). Implication of the dorsal hippocampus in these behavioral aspects has long been recognized (Burgess 2008; Rudy 2009).

Delta receptor expression in hippocampal GABAergic interneurons is long established in rodents based on immunohistochemistry (Bausch et al. 1995; Commons and Milner 1996; Commons and Milner 1997), in situ hybridization (Stumm et al. 2004) and electrophysiological (Lupica 1995; Svoboda et al. 1999) data. In addition, delta receptor expression in the Ammon's horn principal cells has also been reported in rats. Indeed, low levels of mRNA transcript were detected in pyramidal cells identified by vGLUT1 labeling (Stumm et al. 2004), and delta receptor-positive immunoreactivity was detected in pyramidal dendritic processes (Commons and Milner 1997; Williams et al. 2011). In mice, however, immunoreactivity related to delta receptors was only found associated with GABA-positive cell bodies and with GABA-positive processes surrounding both GABA-positive and GABA-negative cell bodies, suggesting that they would not be present in principal cells (Bausch et al. 1995). Therefore, delta receptor expression in the pyramidal cells of the mouse hippocampus deserves further investigation to determine a possible impact on the hippocampal activity.

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). 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 (Pradhan et al. 2009, 2010; Scherrer et al. 2006). In addition, they represent a tool of choice to address the neuroanatomical distribution of delta opioid receptors. We previously examined delta opioid receptor distribution throughout the different strata of the dorsal hippocampus and identified the populations of interneurons that express the receptor (our own unpublished data). Here, we used hippocampal organotypic cultures and acute slices from DOR-eGFP mice in combination with fluorescence confocal imaging and correlative light-electron microscopy to more specifically address delta receptor expression in glutamatergic pyramidal cells.

Materials and Methods

Animals

Knock-in mice DOR-eGFP, expressing the delta opioid receptor coupled to a green fluorescent protein, were generated by homologous recombination. In these mice, the eGFP cDNA was introduced into exon 3 of the delta opioid receptor gene, in frame and 5' from the stop codon (Scherrer et al. 2006). Wild-type mice were used as control to verify the specificity of the anti-GFP antibody. The genetic background of all mice was C57/BL6 J: 129svPas (50:50%). Mice were housed in a temperature- and humidity-controlled animal facility ($21 \pm 2^\circ\text{C}$, $45 \pm 5\%$ humidity) on a 12-h dark-light cycle with food and water ad libitum. Both male and female mice were used. All experiments were performed in accordance with the European Communities Council Directive of May 26, 2010, and approved by the local ethical committee (Com'Eth 2010-003).

Material

Rabbit polyclonal primary antibodies directed against the green fluorescent protein (Molecular Probes A6455) or against the AMPA glutamate receptor subunit 1 (GluR1) (Abcam 31232) were used at a 1:1,000 dilution, and rabbit polyclonal primary antibodies directed against synaptophysin (Synaptic Systems 101002) were used at a 1:100 dilution. Mouse monoclonal primary antibodies directed against synaptotagmin (clone 1D12 (Martin-Moutot et al. 1993)) or against the postsynaptic density protein 95 (PSD95) (Millipore MAB1596) were used at a 1:1,000 and 1:100 dilution, respectively. The anti-calbindin D-28 K (Swant 300) was used at a 1:1,000 dilution. Anti-rabbit AlexaFluor 488- or 594-conjugated secondary antibodies (Molecular Probes A-11034 and A-11012, respectively) were used at a 1:2,000 dilution, and anti-mouse AlexaFluor 594-conjugated secondary antibodies (Molecular Probes A-11005) were used at 1:500 dilution. All reagents were from Sigma.

Organotypic Culture and Acute Hippocampal Slice Preparation

DOR-eGFP knock-in pups (aged 4–9 days) were used for organotypic hippocampal slice cultures that were prepared as previously described (Gogolla et al. 2006a). Slices were then fixed for 30 min at 37°C in paraformaldehyde (PFA) 4% in phosphate buffer (PB) 0.1 M pH 7.4 containing 0.5% Triton X100.

Mice (aged 4 weeks) were anaesthetized with ketamine/xylazine (100/10 mg/kg, i.p.) and perfused intracardially with 10 mL PB 0.1 M pH 7.4 followed by 50 mL of 4%

paraformaldehyde (PFA) (at 2–4°C) in PB 0.1 M or PBS 1× (Dulbecco's Phosphate Buffer Saline, Sigma Aldrich), pH 7.4. Brains were post-fixed for 1 h at 4°C in 4% PFA solution. 60-μM-thick acute brain sections were cut with a vibratome (Leica VT1000S).

Immunohistochemistry and Image Acquisition

After fixation, 60-μM coronal sections or organotypic slices were washed three times with PB pH 7.4 then incubated in a blocking solution (PB 0.1 M pH 7.4, 5% normal goat serum (NGS), 0.5% Triton X100) for 2 h at room temperature (RT). Incubation with the primary antibodies was performed overnight under shaking at 4°C in the blocking solution. Following three washes with PB 0.1 M pH 7.4, 0.5% Triton X100, sections were incubated with Alexa-Fluor-conjugated secondary antibodies for 2 h at RT. Samples were washed three times with PB 0.1 M pH 7.4 and mounted with Mowiol and 4',6-diamidino-2-phenylindole (DAPI) (0.5 μg/mL). Samples were observed with a confocal microscope (SP2RS, Leica) using 40× (NA: 1.25) and 63× (NA: 1.4) objectives with the LCS (Leica) software for image acquisition. Confocal acquisitions were performed in the sequential mode (single excitation beams: 405, 488 and 568 nm) to avoid potential cross talk between the different fluorescence emissions.

DOR-eGFP Intracellular Localization by Correlative Light-Electron Microscopy

We used a correlative light-electron microscopy approach to identify neurons of interest by GFP fluorescence in confocal microscopy and then used immunogold pre-embedding technique for electron microscopy to label DOR-eGFP in 100-μM-thick brain sections (Schikorski 2010). Mice (aged 8–12 weeks) were anaesthetized with ketamine/xylazine (100/10 mg/kg, i.p.) and perfused intracardially with 100 mL of 4% PFA at 2–4°C in PB 0.1 M, pH 7.4 with 0.1% glutaraldehyde at a fast flow rate of 20 mL/min. Brains were post-fixed for 2 h at 4°C in 4% PFA solution. 100-μM-thick brain sections were cut with a vibratome (Leica VT1000S). Sections were manually dissected into thin lamellae that were approximately 100 μm thick, 500 μm wide and 2 mm long and washed in glycine 150 mM PB 0.1 M pH 7.4 solution, and neurons of interest were localized with confocal microscope. Lamellae were washed 3 times 5 min in HBS 1× pH 7.4 (Hepes buffer saline: 154 mM NaCl, 0.2 mM CaCl₂ and 20 mM Hepes-NaOH Sigma in H₂O mQ) and permeabilized for 30 min in a 10% BSA, 0.025% Triton X100 and HBS 1× pH 7.4 solution. Samples were incubated overnight at 4°C with anti-GFP antibodies (1/1,000, Invitrogen) in 1% BSA, 0.0025% Triton X100 and HBS 1× pH 7.4 solution. Samples were washed 3 times for 5 min in 0.05% BSA, HBS 1× pH 7.4, incubated overnight at 4°C with anti-rabbit secondary antibodies coupled to ultra-small immunogold particles 0.8 nm (1/200, Aurion) diluted in 2% FSG, 1% BSA-c, 0.0025% Triton X100 and HBS 1× pH 7.4 solution. Samples were then washed 3 times for 5 min in HBS 1× pH 7.4 and post-fixed in 1% glutaraldehyde HBS 1× pH 7.4 solution for 10 min. Lamellae were then washed 2 times for 5 min in HBS 1× pH 7.4 and then 3 times 5 min in H₂O mQ before silver enhancement (gold particle size raised to 10–15 nm) for transmission electron microscope (TEM) observation. Samples were incubated with silver enhancement reagents (R-Gent SE-EM, Aurion) for 40 min in a light-protected place and washed at least 5 times for 5–10 min with H₂O mQ to eliminate unfixed silver reagents. Samples were then post-fixed in 0.5% osmium (OsO₄) in H₂O for 10 min to fix and contrast membranes. Lamellae were washed 3 times for 5 min in H₂O, then

transferred in 2-mL eppendorf tubes for dehydration and embedded in epoxy resin for ultra-microtome cutting (Leica Ultracut UCT).

Sections were observed with a Philips CM12 transmission electron microscope operated at 80 kV. Images were acquired with an Orius 1000 CCD camera (Gatan). Observation was restricted to neurons located in the first 2–5 μm of the hippocampal lamellae for optimal antibody labeling. The presence of labeled profiles was verified by examining serial tissue sections. Two to three animals were used for each condition.

Image Analysis

Profiles were considered to be immunogold-labeled when they contained one or more gold particles. 89 profiles were counted. The soma of principal cells was distinguished by the presence of a large nucleus and narrow cytoplasm. Dendritic projections from interneurons located within the pyramidal layer were recognized by their size (mean width: 0.9 μm) and by correlation with confocal images. Axon terminals were also identified by size (at least 0.2 μm diameter) and the presence of synaptic vesicles and mitochondria. Synapses were defined as either symmetric or asymmetric, according to the presence of either thin or thick postsynaptic specializations, respectively.

Results

In the pyramidal layer from acute brain slices (Fig. 1a) as well as organotypic cultures (Fig. 1b), DOR-eGFP expressing neurons always presented a morphology that was clearly distinct from pyramidal cells, and DOR-eGFP fluorescence was only detected on the periphery of pyramidal cells. In addition, no co-localization was observed with calbindin-positive pyramidal cells (Fig. 1c). Extensive co-localization between DOR-eGFP and the presynaptic markers synaptotagmin and synaptophysin was observed in organotypic cultures (Fig. 1d, e) as well as acute slices (Fig. 1g, h), but no co-localization between DOR-eGFP and the postsynaptic density protein 95 (PSD 95) and GluR1 subunit of AMPA receptors was observed in organotypic cultures (Fig. 1f) nor in brain slices (Fig. 1i).

We then investigated subcellular delta opioid receptor distribution using correlative light-electron microscopy (Fig. 2). In the pyramidal layer, gold particles were distributed among three distinct profiles: dendritic processes from interneurons (Fig. 3a), axonic presynaptic boutons (Fig. 3b, c) and pyramidal cells (Fig. 3d, e). No gold labeling was visible in wild-type mice confirming the specificity of our GFP detection (Fig. 3f). DOR-eGFP was mostly located in presynaptic terminals contacting pyramidal cells (77%) (Fig. 3g) with some proteins also in dendrites from interneurons (22%) (Fig. 3g). Only few DOR-eGFP proteins (7%) were detected in the soma of pyramidal cells (Fig. 3g) that were all located in the Golgi apparatus or vesicular compartments that could be related to Golgi export (Fig. 3d, e).

Discussion

We previously investigated delta opioid receptor distribution throughout the hippocampus using DOR-eGFP knock-in mice. Our results evidenced that DOR-eGFP fluorescence was mainly associated with GABAergic interneurons, mostly of the somatostatin- or

parvalbumin-positive types (Erbs et al. unpublished). Here, we examined DOR-eGFP distribution in the pyramidal layer in more detail to determine its possible expression in glutamatergic principal cells. We analyzed DOR-eGFP subcellular localization by fluorescence and correlative light-electron microscopy. We also compared data from acute brain slices that retain neuronal connections similar to the in vivo situation as well as organotypic cultures. In the latter, the general hippocampal architecture is preserved, and plasticity of neuronal circuits can be evaluated for several weeks in vitro (Del Turco and Deller 2007; Gogolla et al. 2006b; Lossi et al. 2009; Noraberg et al. 2005).

In the pyramidal layer, fluorescence confocal imaging clearly indicated DOR-eGFP distribution in neurons presenting a morphology distinct from principal cells. The fluorescent signal associated with DOR-eGFP was only detected on the periphery of principal cells. These data are in agreement with a previous immunohistochemical study where mouse delta opioid receptors were only detected in GABAergic interneurons and GABA-positive processes surrounding both GABA-positive and GABA-negative cell bodies, suggesting that they are not expressed in principal cells (Bausch et al. 1995). Similarly, Cahill et al. (Cahill et al. 2001) reported few intensely labeled neurons distributed within the rat pyramidal layer based on immunohistochemical data using two antibodies directed against the N-terminus or the C-terminus of delta opioid receptors.

Immunohistochemical co-localization of DOR-eGFP with specific markers was then performed to further assess pre- and postsynaptic distributions of delta opioid receptors in the pyramidal layer. Synaptotagmin and synaptophysin are part of the SNARE complex involved in presynaptic neurotransmitter release (Bonanomi et al. 2006). Extensive co-localization with DOR-eGFP was observed in organotypic cultures as well as acute slices clearly pointing to its localization on presynaptic terminals contacting pyramidal cells. The postsynaptic density protein 95 (PSD 95) and the GluR1 subunit of AMPA receptors are, on the other hand, prototypical proteins from the postsynaptic compartments (Shiraishi et al. 2003). No co-localization with DOR-eGFP was observed by confocal imaging in organotypic cultures or in brain slices. Altogether, data strongly suggest that delta opioid receptors in the pyramidal layer are mainly expressed in presynaptic afferents corresponding to GABAergic projections on glutamatergic principal cells. This distribution is in agreement with electrophysiological studies performed in rats that also identified delta opioid receptors in interneurons (Lupica 1995; Lupica et al. 1992; Svoboda et al. 1999). Local GABAergic interneurons are known to modulate the spike timing of principal cells and synchronize their activity, and delta receptors expressed in these neurons therefore modify GABAergic inhibition and, hence, dynamically influence local hippocampal connectivity and glutamatergic neuron firing rate (Klausberger 2009).

We then investigated subcellular delta opioid receptor distribution by electron microscopy. We took advantage of the fluorescent DOR-eGFP construction to perform correlative light-electron microscopy. eGFP fluorescence and surrounding non-fluorescent landmarks were identified restricting further processing to neurons of interest. This approach has already been successfully used to locate DOR-eGFP receptors in GABAergic interneurons (Faget et al. unpublished). Within the pyramidal layer, gold particles were distributed among three distinct profiles: dendritic processes from interneurons, axonic presynaptic boutons and

pyramidal cells. A substantial amount of DOR-eGFP proteins was observed in presynaptic terminals contacting pyramidal cells, and the remaining gold particles were mostly identified in dendrites from interneurons. Data are in agreement with a detection of the DOR-eGFP fluorescence restricted to interneurons in the pyramidal layer.

Only few DOR-eGFP proteins were detected in the soma of pyramidal cells that were all located in the Golgi apparatus or vesicular compartments that could be related to Golgi export. A previous report also mentioned receptor cytoplasmic localization in rat glutamatergic cells (Commons and Milner 1997), suggesting that, in both species, delta opioid receptors located in principal cell bodies are not functional. This hypothesis is supported by electrophysiological data collected in rats reporting no direct opioid effect on principal cell membrane properties (Madamba et al. 1999) though weak mRNA content was detected by in situ hybridization (Stumm et al. 2004). However, the latter does not necessarily reflect the actual protein content nor the receptor subcellular distribution. Interestingly, a recent report pointed to a hormonal regulation of both receptor expression and cellular trafficking in rat pyramidal cells, which may impact the hippocampal function (Williams et al. 2011).

In conclusion, our data clearly substantiate that, in mouse, delta opioid receptors present in the pyramidal layer are mostly expressed in interneurons. In addition, we evidence low delta opioid receptor expression in pyramidal cells similarly to the distribution reported in rats. Importantly, receptor intracellular localization in glutamatergic principal cells suggests that, under basal conditions, only presynaptic receptors present in interneurons innervating pyramidal cells are functional. Since GABAergic neuronal populations modulate principal cell firing rate, the participation of delta opioid receptors in the dynamic regulation of hippocampal activity is emphasized.

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Fig. 1.

Presynaptic DOR-eGFP expression in the pyramidal layer of hippocampal organotypic culture or acute slice. Immunohistochemical detection of DOR-eGFP confirms that the general hippocampal architecture observed in acute slices (**a**) is retained in organotypic cultures (**b**). DOR-eGFP expressing neurons do not co-localize with calbindin-positive principal cells labeled with AlexaFluor 594-conjugated secondary antibodies and exhibit a distinct morphology (**c**). Representative confocal fluorescence photomicrographs in organotypic cultures (**d–f**) and acute brain slices (**g–i**). DOR-eGFP co-localizes (*white arrows*) with the presynaptic markers synaptophysin (**d, g**) or synaptotagmin (**e, h**) but not with the postsynaptic markers PSD95 (**f**) or GluR1 (**i**). Pre- and post-synaptic markers are labeled with AlexaFluor 594-conjugated secondary antibodies. Nuclei are stained with DAPI. *Scale bars* 100 μm (**a, b**), 20 μm (**c**) and 10 μm (**d–i**)

Fig. 2. Correlative lightelectron microscopy (CLEM) methodology. Fixed brain sections (100 µm) are dissected in thin lamellae for optimal mapping and neuron identification by confocal microscopy. eGFP labeling is then performed using immunogold pre-embedding technique. Principal cells of the pyramidal layer (*arrows*) together with surrounding nonfluorescent landmarks (*arrow heads*) can be identified throughout the procedure restricting electron microscope processing to neurons of interest

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DOR-eGFP subcellular distribution in the pyramidal layer			
Profile type	% immunogold particles (Nb versus total)	Subcellular compartment	% immunogold particles (Nb versus profile)
Axonic terminals	71% (113/160)	Plasmamembrane	30% (34/113)
		Intracellular vesicles	70% (79/113)
		Golgi	0% (0/113)
GABAergic dendrites	22% (36/160)	Plasmamembrane	44% (16/36)
		Intracellular vesicles	44% (16/36)
		Golgi	11% (4/36)
Pyramidal cells	7% (11/160)	Plasmamembrane	0% (0/11)
		Intracellular vesicles	45% (5/11)
		Golgi	55% (6/11)

Fig. 3.

DOR-eGFP subcellular distribution in the pyramidal layer. **a** Dendritic projections from GABAergic neurons in the pyramidal layer (*white arrows*) are identified by confocal fluorescence (1) and electron microscopy (2). DOR-eGFP immunogold labeling is visible at the plasmamembrane and in intracellular vesicles (*black arrows*) (3). Gold particles are also present in a presynaptic terminal connecting the dendrite (*black arrow head*). **b, c** Axonic terminals recognizable by the presence of dense vesicles and mitochondria show DOR-eGFP immunogold labeling at the synapse and in intracellular vesicles (*black arrow heads*). **d, e** In pyramidal cells, DOR-eGFP immunogold labeling is present in the Golgi apparatus and vesicular compartments close to it (*white arrows*). DOR-eGFP immunogold labeling is also visible at the level of synaptic contacts (*black arrow heads*). **f** Representative electron

micrograph showing the absence of gold particles in a hippocampal ultra-thin section of a wildtype animal. *Scale bars* 500 nm (**a, c, e, f**) and 200 nm (**b, d**). **g** Immunogold particles were counted in different profiles ($n = 89$) corresponding to axonic terminals, pyramidal cells and GABAergic dendrites. In each profile, immunogold particles were observed in three different subcellular compartments corresponding to plasmamembrane, intracellular vesicles and Golgi apparatus. Distribution among the three different profiles is expressed as a percentage of the total number of gold particles and distribution among the subcellular compartments is expressed as a percentage of the number of gold particles in each profile. Values in parentheses represent the number of counted particles relative to the total number of particles ($n = 160$) or relative to the number of particles present in the profile