Journal of Chemical Neuroanatomy 36 (2008) 122–127

Contents lists available at ScienceDirect

Journal of Chemical Neuroanatomy

journal homepage: www.elsevier.com/locate/jchemneu

Delta opioid receptor mRNA expression is changed in the thalamus and brainstem of monoarthritic rats

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ARTICLE INFO

Article history:
Received 29 February 2008
Received in revised form 9 May 2008
Accepted 9 May 2008
Available online 16 May 2008

Keywords:
Reticular thalamic nucleus
Dorsal reticular nucleus
Parvocellular reticular nucleus
Chronic inflammatory pain
In situ hybridization

ABSTRACT

Changes in the mRNA expression of neurotransmitters receptors under chronic pain conditions have been described in various areas of the central nervous system (CNS). Delta opioid receptors (DORs) have been implicated in pain mechanisms but, although its mRNA expression has been studied in the rat CNS, there are no reports describing its distribution in specific thalamic and brainstem nuclei during chronic inflammatory pain. Here, *in situ* hybridization for DOR mRNA was performed in brain sections from control and monoarthritic (MA) rats with 2, 4, 7 and 14 days of inflammation. Grain densities were determined bilaterally in the ventrobasal complex (VB), posterior (Po), centromedial/centrolateral (CM/CL) and reticular (Rt) nuclei of the thalamus, and in the dorsal reticular (DRt), lateral reticular (LRt) and parvocellular reticular (PCRt) nuclei of the brainstem. Control animals exhibited weak mRNA expression in the VB, Po and CM/CL, as well as in PCRt, while moderate grain densities were observed in the Rt, DRt and LRt. During MA, DOR mRNA expression was significantly decreased (22%) in the Rt contralateral to the affected joint at both 7 and 14 days of inflammation, as compared to controls. A bilateral reduction (35%) was also observed in the DRt at 14 days of MA, while a contralateral increase was found in the PCRt at 7 days (+39%). No significant changes were observed in the other regions analyzed. Thus, data show changes in the DOR mRNA expression during the development of chronic inflammatory pain, in thalamic and brainstem nuclei implicated in pain processing mechanisms.

1. Introduction

Opioid receptors have been implicated in pain modulation (Przewlocki and Przewlocka, 2001; Fields, 2004), and drugs acting at these receptors have been widely used for a long time as the most effective therapy for treating chronic pain (King et al., 2005). In spite of this, there are still many questions regarding the role of these seven transmembrane G-protein coupled receptors in pain processing mechanisms at the various levels of the nociceptive system. Plastic changes of the opioidergic system in models of inflammatory pain have been documented especially at the primary afferents and spinal cord level (Besse et al., 1992; Maekawa et al., 1996; Goff et al., 1998; Cahill et al., 2003; Puehler et al., 2004; Shaqura et al., 2004; Puehler et al., 2006), and at lesser extent at supraspinal sites (Millan et al., 1986, 1987). Thus, Spetea et al. (2002) detected increased levels of opioid peptides in tissues of polyarthritic rats, while they did not find any significant differences in mu (MOR), delta (DOR) or kappa (KOR) opioid receptors binding affinities in several supraspinal regions. Other authors reported a significant time-dependent up-regulation of MOR binding and immunoreactivity in the dorsal root ganglions ipsilateral to a complete Freund's adjuvant- (CFA) induced inflammation of one hind paw, but no changes were observed within the spinal cord or hypothalamic neurons (Shaqura et al., 2004). In contrast, increased levels of MOR and KOR mRNA were observed in the ipsilateral spinal cord of rats with 11 days post-intradermal CFA injection in the hind limb in comparison to the contralateral side, while no changes were observed for DOR (Maekawa et al., 1996). Other studies, however, have shown increased bilateral expression for the DOR transcript in the spinal cord of inflamed arthritic rats, as compared to control animals (Cahill et al., 2003). At supraspinal sites, no changes were found in MOR- or DOR-mediated G-protein activity in many regions of the brain, including the caudate-putamen, medial thalamus and periaqueductal grey of polyarthritic rats (Cichewicz et al., 2004).

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doi:10.1016/j.jchemneu.2008.05.004

Similarly, no changes in the total binding density or binding affinity of diprenorphine, a non-subtype specific opioid ligand, were detected in the thalamus or midbrain of 3 weeks arthritic rats, but sequential blocking of the same ligand revealed a relative decrease in the proportion of KOR as compared to MOR in the thalamus, suggesting a relative loss of KOR (Millan et al., 1986), while, in the periaqueductal grey, a significant increase in binding to KOR sites was detected whereas MOR and DOR binding affinities were unaffected (Millan et al., 1987).

Although, for some time, scientific research on the field has privileged MOR and established this subtype as the main responsible for mediating opioid analgesic effects (Fields, 2004), recent data suggests that delivery and trafficking of DORs are crucial mechanisms in the modulation of opioid analgesia (Zhang et al., 2006). Thus, Morinville et al. (2004) have shown an increase in spinal cord's neuronal plasma membrane density of DOR in CFA-injected mice while the same was not observed when analyzing CFA-inflamed MOR knock-out mice, suggesting that changes in DOR density were due to an increased trafficking elicited by stimulation of MOR by endogenous opioids released in response to chronic inflammatory pain. Behavioral analysis of supraspinal administration of DOR ligands also point to a key role for these opioid receptors subtype in mediating analgesia. Thus, for example, supraspinal injection of DOR agonists reversed hyperalgesia associated with CFA-induced peripheral inflammation and attenuated the nociceptive response to acute thermal stimuli in both normal and CFA-inflamed animals (Fraser et al., 2000; Hurley and Hammond, 2000). Indeed, recent data has provided evidence for the existence of modulatory interactions between MOR and DOR, and, in fact, an enhancement of the analgesic potency and efficacy has been verified when MOR ligands are concomitantly administered with DOR ligands (review in Ananthan, 2006).

After the cloning of DOR (Evans et al., 1992; Kieffer et al., 1992; Fukuda et al., 1993), its mRNA expression in the rat central nervous system (CNS) has been mapped (Mansour et al., 1994). Yet, there are no studies analyzing DOR mRNA expression during chronic inflammatory pain conditions in supraspinal nuclei specifically involved in pain processing mechanisms. Thus, in the present study, the expression of DOR mRNA was evaluated by using *in situ* hybridization techniques, in some regions of the thalamus and brainstem of monoarthritic rats, a well established experimental model of chronic inflammatory pain which is induced by intraarticular injection of CFA (Butler et al., 1992), at specific time points of evolution of the disease when the changes of neuronal metabolic activity (Neto et al., 1999) that were found exhibited a non-linear profile in respect to the time of progression of the inflammatory painful disease.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (Institute of Molecular and Cellular Biology, Porto, Portugal) weighing between 250 and 300 g were housed in pairs in cages with water and food *ad libitum*. The animal room was maintained at a constant temperature of 22 °C with controlled lightning (12 h light/dark cycles). All experiments were conducted following the regulations of local authorities in handling laboratory animals, the ethical guidelines for the study of experimental pain in conscious animals (Zimmermann, 1983) and the European Communities Council Directive of 24 November 1986 (86/609/EEC). Moreover all efforts were made to minimize animal suffering or discomfort and to reduce the number of animals used.

2.2. Chronic inflammation

To induce MA, rats were injected intraarticularly with 50 μL of complete Freund's adjuvant (CFA) into the left tibiotarsal joint according to Butler et al. (1992), under brief isofluorane anaesthesia (4% for induction and 2–2.5% for maintenance). CFA was prepared by mixing 60 mg of killed and desiccated *Mycobacterium butyricum* (Difco laboratories, USA) to paraffin oil (6 ml), saline (4 ml) and Tween 80 (1 ml), and this suspension was then autoclaved at 120 °C for 20 min. The inflammatory reaction was assessed daily by using a subjective scoring (Castro-Lopes et al., 1992) ranging from 0 to 4, where 0 corresponds to no inflammatory signs and 4 to severe inflammatory symptoms affecting the animal's motor activity. Monoarthritic animals were sacrificed by decapitation 2, 4, 7 and 14 days after injection ($n = 6$ per group). The control group ($n = 6$) was injected similarly with saline and sacrificed 2 days after injection. Animals displaying any symptom of polyarthritis were discarded from the experiments.

2.3. Riboprobes preparation

A plasmid containing cDNA for the rat DOR was kindly provided by Professor Huda Akil from the Mental Health Research Institute, University of Michigan. Briefly, a polymerase chain reaction (PCR) fragment of 985 bp (Fukuda et al., 1993; 402–1386 pb, Genbank Acession # D16348) was cloned into a plasmid vector containing a T7 RNA polimerase promotor (pCR2.1) and used to generate a [α-^{35}S]-UTP (1200 Ci/mmol; PerkinElmer) cRNA probe against the rat DOR by *in vitro* transcription (kit T7, Promega).

2.4. Tissue preparation and in situ hybridization

The brains were quickly removed, frozen on dry ice and stored at −80 °C. Serial coronal brain sections 14 μm thick were cut on a cryostat (−20 °C) and thaw-mounted on poly-L-lysine coated glass slides. Tissue sections were fixed for 5 min in 4% paraformaldehyde (PFA) at 4 °C, washed in 1× phosphate buffer saline (PBS), dehydrated and stored in 96% ethanol at 4 °C (Wisden and Morris, 1994; Neto et al., 2000). Radioactive *in situ* hybridization was performed by adapting the method described by Mansour et al. (1994). In the day of the *in situ* hybridization, tissue was quickly hydrated in 96, 70 and 30% ethanol and then fixed for 1 h in 4% PFA. Sections were then washed three times for 5 min in 2× SSC (saline sodium citrate, pH 7.0) and afterwards treated with proteinase K (1 μg/mL in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0) for 15 min at 37 °C. Subsequently slides were rinsed in water followed by 0.1 M triethanolamine, pH 8.0 and next treated with a solution containing 0.1 M triethanolamine pH 8.0 and acetic anhydride (400:1, v/v) for 10 min. The slides were then rinsed in water, dehydrated through graded ethanol and air dried. Riboprobes were heated to 65 °C for 3 min and then diluted in hybridization buffer (75% formamide, 10% dextran sulphate, 3× SSC, 50 mM Na_2PO_4, pH 7.4, 1× Denhardt's, 0,1 mg/mL yeast tRNA, 10 mM dithiothreitol) to a final concentration of 10^6 cpm/100 μL. Coronal brain sections from control, 2, 4, 7 and 14 days MA rats were hybridized (100 μL probe/slide) overnight at 55 °C in sealed and humidified chambers (50% formamide, 4× SSC). To test the hybridization signal's specificity, a few sections were incubated with a 100-fold excess of unlabeled probe with the corresponding ^{35}S-labeled probe. The next day the slides were washed in 2× SSC at RT for 5 min and in 0.1× SSC at 65 °C for 60 min. After being rinsed in water, dehydrated and air dried, slides were dipped in NTB Kodak photographic emulsion diluted in 0.05% glycerol in distilled water for 15 days. Subsequently, sections were developed (D-19 developer from Kodak), fixed and washed in water and then counterstained with thionin for analysis under bright and dark field optic microscopy.

2.5. Image acquisition and data analysis

Image acquisition and DOR mRNA expression quantitative analysis was performed by a "blind" experimenter in thalamic and brainstem nuclei known to play an important role in nociceptive transmission and/or modulation (facilitatory or inhibitory). For each animal, four thalamic regions, the ventrobasal complex (VB), the posterior (Po), centromedial/centrolateral (CM/CL) and reticular (Rt) nuclei, and three brainstem nuclei, the dorsal reticular (DRt), lateral reticular (LRt) and the parvocellular reticular (PCRt) nuclei, were sampled separately, at three different rostro-caudal levels in every nuclei that extended throughout all of these levels, as previously described (Neto et al., 2000). Delimitation of the nuclei was done according to the rat brain atlas of Paxinos and Watson (1998) in serial coronal sections. Quantification of grain density values (silver grains area/neuronal somata area) in each nuclei were obtained from images acquired in brain sides ipsi- and contralateral to the saline- or CFA-injected paw using a computer-assisted image analyzer (Optimas-Bioscan) equipped with a Leica Axioplan and a Sony Hyper HAD Digital camera, as described (Neto et al., 2000). Values obtained from each analyzed image of a specific brain region, at different rostro-caudal levels, were pooled and averaged separately for ipsi- and contralateral sides in each animal, then divided by background levels and expressed as "times background". Background levels were determined randomly in the images analyzed, in areas where no cell bodies were observed, and therefore no specific labeling occurred, as previously (Neto et al., 2000). As no statistically significant differences of grain density values between the ipsi- and contralateral sides were found (Student's *t*-test) in any of the nuclei and experimental groups analyzed, values from both sides were pooled for each animal and each region. Mean group values of mRNA expression for each region were then obtained by pooling and averaging the values of each animal ($n = 6$ per experimental group). These final values are presented as