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Somatostatin and nociceptin inhibit neurons in the central nucleus of amygdala that project to the periaqueductal grey

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ABSTRACT

The central nucleus of amygdala (CeA) plays an important role in modulation of the descending anti-nociceptive pathways. Using whole-cell patch clamp recordings from brain slices, we found that CeA neurons responded to the endogenous ligands somatostatin (SST) and nociceptin/orphanin FQ (OFQ) via an increased K-conductance. Co-application with selective antagonists suggested that SST and OFQ act on SSTR2 and ORL1 receptors, respectively. Taking account of anatomical localisation of recorded neurons, the present study showed that many responsive neurons were located within the medial subdivision of CeA and all CeA projection neurons to the midbrain periaqueductal grey invariably responded to these peptides. Randomly selected agonist-responsive neurons in CeA predominantly classified physiologically as low-threshold spiking neurons. The similarity of SST, OFQ and, as previously reported, opioid responsiveness in a sub-population of CeA neurons suggests converging roles of these peptides to inhibit the activity of projections from CeA to vlPAG, and potentially similar antinociceptive actions in this pathway.

1. Introduction

The central nucleus of amygdala (CeA) plays an important role in modulation of responses to nociceptive stimuli and chronic pain states (Fields, 2000; Neugebauer et al., 2009) among a range of other somatic and autonomic functions including fear conditioning. Neurons in the CeA receive inputs from the lateral and basolateral amygdala (Cassell et al., 1999; LeDoux, 2007; Pitkanen et al., 1997), as well as key brainstem and midbrain regions that mediate nociception such as periaqueductal grey (PAG) and the lateral parabrachial nucleus (Gauriau and Bernard, 2002; Rizvi et al., 1991). Conversely, the medial subdivision of the CeA (CeM) provides direct projections to regions involved in both ascending and descending modulation of pain states, including the PAG (Cassell et al., 1999; Gauriau and Bernard, 2002; Rizvi et al., 1991).

The CeA expresses a range of neuropeptide systems that are known to be involved in modulation of nociception (Cassell et al., 1986; Neal et al., 1999b). Among these, activation of opioid-related-peptide receptors has been shown to produce antinociceptive actions in CeA (Manning and Mayer, 1995). We have previously reported that activation of mu and, in opioid tolerant animals, delta opioid receptors inhibit a sub-population of CeA neurons by increasing K-conductance (Chieng and Christie, 2009; Chieng et al., 2006). These actions are more pronounced in the CeM and occur primarily in one physiological class of neurons (Chieng and Christie, 2009; Chieng et al., 2006). We have also established that CeA neurons that project to major nociception modulating regions including PAG (Chieng and Christie, 2009; Chieng et al., 2006) are invariably inhibited by opioids. These neurons belong exclusively to a single class of low-threshold spiking cells (LTS) among the several physiological classes of CeA neurons (Chieng and Christie, 2009).

Receptors for the opioid-related peptide, nociceptin/orphanin FQ (OFQ), as well as somatostatin (SST) are closely related to mu and delta receptors and are expressed in CeA. The CeA also expresses the endogenous ligands somatostatin (SST) for SSTR2 receptors (Cassell et al., 1986; Dournaud et al., 1996) and OFQ for opioid-receptor-like type 1 (ORL1, aka NOPR, (Neal et al., 1999a, 1999b)). Intra-amygdalar injection of somatostatin reduces seizures, increases blood pressure and decreases heart rate (Brown and Gray, 1988; Mazarati and Telegdy, 1992) but its actions on nociception have not been studied and mechanisms of action on the physiology of single CeA neurons are unknown. OFQ is also likely to be involved in modulation of nociception in CeA. Microinjections of OFQ directly into CeA have been reported to produce analgesia and decrease signs of

Abbreviations: CeA, central nucleus of amygdala; PAG, periaqueductal grey; SST, somatostatin; OFQ, nociceptin/orphanin FQ; SSTR2, somatostatin receptor type 2; ORL1, opioid-receptor-like type 1; CeM, medial subdivision of CeA; CeI, intermediate subdivision of CeA; CeL, lateral subdivision of CeA; CeCd/Astr, dorsal capsular subdivision of CeA or amygdala–striatal transition zone; CeCv, ventral capsular subdivision of CeA; LTS, low-threshold spiking cell; LF, late-firing cell.

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anxiety (Shane et al., 2003, 2001; Uchiyama et al., 2008). Despite expressing a low level of ORL1 mRNA and OFQ binding (Neal et al., 1999a, 1999b), pre- and post-synaptic inhibitory actions of OFQ have been reported in CeA in vitro (Chen et al., 2009; Meis and Pape, 1998; Roberto and Siggins, 2006). However, the types of neurons responsive to OFQ as well as their regional location in CeA are unknown.

In this study we identified the actions of OFQ and SST on specific classes of neuron in subdivisions of CeA, particularly in neurons with the identified projections to PAG that are likely to be involved in nociception. We found that SST and OFQ both increase K-conductance in CeA and CeA–PAG projection neurons. As with our previously reported finding for mu and delta opioids, these actions were observed overwhelmingly in low-threshold spiking (LTS) cells, especially in the CeM. These findings suggest that SST and OFQ can both act on the same population of neurons and in a manner similar to opioids to produce analgesia in CeA.

2. Materials and methods

2.1. Ethical approval

All animal experiments were approved by the University of Sydney and Royal North Shore Hospital/University of Technology Sydney Ethics Committees, which comply with National Health and Medical Research Council of Australia guidelines and NSW legislation. All efforts were made to minimise animal suffering and to reduce the number of animals used.

2.2. Injection of retrograde tracer

For studying CeA–PAG projection neurons, fluorescent latex nanospheres (30 nl, 0.04 μm, 565/580 nm, Invitrogen, Carlsbad, CA) were stereotaxically microinjected into the ventrolateral PAG (bregma −8.72 mm, 0.90 mm lateral, −6.00 mm depth) of rats under anaesthesia (ketamine 75 mg kg^{-1} i.m., xylazine 5 mg kg^{-1} i.m. and atropine 1 mg kg^{-1} i.p.) as previously described (Chieng and Christie, 2009; Chieng et al., 2006). Animals were used for experiment 5–7 days after surgery.

2.3. Brain slice preparation and electrophysiology

Male Sprague–Dawley rats (150–250 g) were overdosed with isoflurane, and brain slices (250 μm) were cut in ice-cold artificial cerebrospinal fluid (ACSF) using a vibrating slicer (Leica VT1000S, Germany). Slices were maintained at 34 °C in a submerged chamber containing ACSF equilibrated with 95% O_2 and 5% CO_2. The slices were then transferred to a recording chamber and superfused continuously (1.5 ml min^{-1}) with ACSF of composition (in mM): NaCl, 126; KCl, 2.5; NaH_2PO_4, 1.4; $MgCl_2$, 1.2; $CaCl_2$, 2.4; glucose, 11 and $NaHCO_3$, 25. CeA neurons were visualised on an upright microscope (Olympus BX50WI) using infra-red Normarski or Dodt tube optics. Whole-cell voltage-clamp recordings of membrane currents were made using patch electrodes (2–4 MΩ) containing (in mM): 115 KCH_3SO_4, 15 NaCl, 1 $MgCl_2$, 10 HEPES, 11 EGTA, 5 Mg–ATP, 0.33 Na–GTP and 0.2% biocytin, pH 7.3, osmolarity 285–290 mOsm l^{-1}. Series resistance (less than 20 MΩ) was monitored periodically during experiments with an Axopatch 200A amplifier (Molecular Devices, Sunnyvale, CA), connected to a Macintosh G4 computer and ITC-16 (Instrutech, Long Island, NY). Liquid junction potentials of −10 mV were corrected. Currents were sampled at 1 kHz and filtered online at 1 kHz, and again offline at 100 Hz for later analysis (Axograph 4.6, Molecular Devices, Sunnyvale, CA). Action potentials and current–voltage relationships were sampled at 5 kHz. Stock solutions of all drugs were diluted to working concentrations in the extracellular solution immediately before use and applied by continuous superfusion.

2.4. Immunohistochemistry

Immediately after physiological recording, brain slices containing biocytin-filled neurons were fixed overnight in 4% paraformaldehyde/0.16 M phosphate buffer solution then placed in 0.3% triton X-100/phosphate buffer for 4 days to permeabilise cells. Slices were then placed in 10% horse serum/phosphate buffer for 1 h before being incubated in primary sheep anti-tyrosine hydroxylase (1:1000, Chemicon, Temecula, CA) for 2 days at 4 °C to aid identification of subdivisions of the CeA (Chieng et al., 2006). The slices were rinsed in phosphate buffer and then in a one-step incubation containing both Cy3-conjugated donkey anti-sheep secondary antibody (1:500, Jackson Immuno Research Laboratories, West Grove, PA) and FITC-conjugated Streptavidin (1:500, Sigma–Aldrich, St Louis, MO) for 2 h. For slices containing retrogradely labelled nanospheres, they were incubated only in FITC-conjugated Streptavidin (1:500). Stained slices were placed briefly (5 min) in DAPI (1:200)/phosphate buffer solution before being rinsed, mounted onto glass slides, dried and coverslipped with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Sections of the PAG containing the injection site were placed in the same fixative solution and sectioned at 100 μm using a vibratome.

2.5. Data analysis

Only neurons with "healthy" appearance in slices examined using infra-red Normarski or Dodt tube optics were studied. All neurons included in data analyses had overshooting action potential amplitudes of at least 60 mV from a threshold of approximately −45 mV. These cells had a resting membrane potential −66 ± 2 mV ($n = 34$), similar to those previously reported in CeA (Chieng and Christie, 2009; Chieng et al., 2006; Dumont et al., 2002). All pooled values are expressed as mean ± S.E.M. Statistical tests between treatment groups were made using Student's unpaired *t*-tests and comparisons within group used a paired *t*-test. Significance was accepted at $p < 0.05$. *N*-values refers to numbers of neurons recorded from throughout.

2.6. Chemicals used

Nociceptin/orphanin FQ, somatostatin, biocytin, DAPI and CYN 154806 were from Sigma–Aldrich (St Louis, MO), and J113397 from Tocris (UK).

3. Results

3.1. SST and OFQ increase K-conductance in CeA-random and CeA–PAG projection neurons

In CeA neurons randomly sampled from untreated rats, superfusion of near-maximum concentrations of SST (0.3 or 1 μM, (Connor et al., 1997)) and OFQ (0.3 or 1 μM, (Chiou and Fan, 2002; Connor et al., 1996)) induced an outward current when membrane potential was set at −70 mV (19/19 cells for SST; 18/22, 82% cells for OFQ, Fig. 1, Table 1). The outward K-current amplitudes of SST and OFQ were not significantly different from neurons of untreated rats (26 ± 3 pA, $n = 19$ for SST versus 28 ± 4 pA, $n = 18$ for OFQ (unpaired *t*-test, $p > 0.05$)). When both SST and OFQ were sequentially applied to a single neuron, all cells that responded to one agonist also responded to the other but the mean amplitude of SST was significantly smaller than that of OFQ (23 ± 5 pA for SST versus 33 ± 5 pA for OFQ, $n = 11$, paired *t*-test, $p < 0.05$, Fig. 1A). Current–voltage relationship analyses showed that SST- and OFQ-induced currents reversed polarity close to predicted K-equilibrium potential (predicted −101 mV; −94 ± 5 mV, $n = 4$ and −94 ± 3 mV, $n = 6$ for SST and OFQ, respectively, Fig. 1B). SST- and OFQ-induced currents were specifically mediated by SSTR2 and ORL1 receptors because the increases in K-conductance was blocked by co-application of the respective SSTR2 (CYN 154806 1 μM, $n = 5$, (Mori et al., 2010)) and ORL1 (J113397 1 μM, $n = 4$, (Berger et al., 2006; Chiou and Fan, 2002) antagonists (Fig. 1C and D)).

In retrogradely labelled CeA–PAG projection neurons, all cells responded with an increased K-conductance during either SST (9/9 cells) or OFQ (6/6 cells) superfusion (Fig. 2B and D). The outward K-current amplitudes of SST and OFQ were not significantly different in CeA–PAG projection neurons (25 ± 4 pA for SST versus 27 ± 6 pA for OFQ, (unpaired *t*-test, $p > 0.05$)). In CeA–PAG projection neurons that were tested sequentially with both SST and OFQ, all neurons responded to both agonists and the mean amplitude of SST was not significantly different to that of OFQ (20 ± 6 pA for SST versus 24 ± 6 pA for OFQ, $n = 5$, paired *t*-test, $p > 0.05$).

3.2. All CeM-PAG projection neurons express SSTR2 and ORL1 responses

The main focus of this study is the CeA–PAG projection neurons that are predominantly localised in the medial (CeM) subdivision of CeA (Fig. 2; Rizvi et al., 1991). In randomly selected CeA neurons from untreated rats, CeM neurons were all responsive to SST (18/18 cells) and OFQ (15/15 cells, Fig. 2C, Table 1). From post hoc histological confirmation, one CeA neuron that responded to SST and a further three neurons out of seven that responded to OFQ were localised