Neuropeptides 46 (2012) 55–60

Contents lists available at SciVerse ScienceDirect

Neuropeptides

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

Neurochemical classification and projection targets of CART peptide immunoreactive neurons in sensory and parasympathetic ganglia of the head

Jason J. Ivanusic [a], Kate E. Goulding [a], Matthew M.K. Kwok [a], Ernest A. Jennings [a,b,*]

[a] Department of Anatomy & Cell Biology, University of Melbourne, Parkville 3010, Australia
[b] School of Medicine and Dentistry, James Cook University, Cairns 4870, Australia

ARTICLE INFO

Article history:
Received 14 June 2011
Accepted 22 September 2011
Available online 17 October 2011

Keywords:
CART
CGRP
Sphenopalatine ganglion
Otic ganglion
Trigeminal
Lacrimation

ABSTRACT

The aims of the present study were to determine if there is neuronal Cocaine and amphetamine regulated transcripts (CART) peptide expression (CART+) in parasympathetic (sphenopalatine (SPG); otic (OG)) and sensory (trigeminal (TG)) ganglia of the head and to examine the neurochemical phenotype (calcitonin gene-related peptide (CGRP), neurofilament 200 (NF200), isolectin B4 (IB4) binding, vasoactive intestinal peptide (VIP), neuropeptide Y (NPY) and enkephalin (ENK) immunoreactivity) and projection targets (lacrimal gland (LG), parotid gland (PG), nasal mucosa (NM), temporomandibular joint (TMJ), middle cerebral artery (MCA) and middle meningeal artery (MMA)) of CART expressing neurons in these ganglia. We found CART+ neurons in both the SPG (5.25 ± 0.07%) and OG (4.32 ± 0.66). A significant proportion of these CART+ neurons contained VIP, NPY or ENK (34%, 26% and 11%, respectively). SPG neurons retrogradely labelled from the lacrimal gland (29%) were CART+, but we were unable to demonstrate CART+ labelling in any of the SPG or OG neurons labelled from other targets. This supports a role for CART peptides in lacrimation or regulation of vascular tone in the lacrimal gland, but not in salivation or nasal congestion. CART+ neurons were also present in the trigeminal ganglion (1.26 ± 0.38%), where their size distribution was confined almost completely to neurons smaller than 800 μm^2 (mean = 410 μm^2; 98% < 800 μm^2), and were almost always CGRP+, but did not bind IB4. This is consistent with a role for CART peptides in trigeminal pain. However, there were few CART+ neurons amongst any of the trigeminal neurons retrogradely labelled from the targets we investigated and thus we cannot comment on the tissue type where such pain may have originated. Our study shows that some specialization of CART peptide expression (based on neurochemical phenotype and target projection) is evident in sensory and parasympathetic ganglia of the head.

1. Introduction

Cocaine and amphetamine regulated transcripts (CART) are mRNA that encode for the CART propeptide (proCART). ProCART has a long form and a short form, and each can undergo post-translational processing that results in the production of a number of biologically active CART peptides see (Rogge et al., 2008). Two of these CART peptides (CART 55–102 and CART 62–102) have been isolated in rat tissues and derive from the long form of proCART (Thim et al., 1999). In humans, only the short form of proCART is produced and the active peptides derived from this short form are called CART 42–89 and CART 49–89 (Rogge et al., 2008). The amino acid sequences of CART 55–102 and CART 62–102 are in fact identical to CART 42–89 and CART 49–89, respectively (the different amino acid numbering system is based on whether peptides are derived from the long or short form of proCART).

CART peptide immunoreactivity has been reported in dense core vesicles within nerve terminals (Dun et al., 2002; Smith et al., 1997), suggesting CART peptides are secreted from nerve terminals and might act as neurotransmitters or neuromodulators. CART mRNA and peptides have a wide distribution throughout the mammalian central nervous system. They have been reported in the nucleus accumbens, amygdala, olfactory bulbs, hypothalamus, somatosensory cortical barrels, thalamus, brain stem and cerebellum, and supraspinal and spinal cord territories associated with pain processing (Douglass et al., 1995; Koylu et al., 1998, 1997). Given this wide distribution, it is not surprising that CART peptides have been implicated in a variety of physiological processes, including reward and reinforcement, feeding/apetite, stress, anxiety, autonomic regulation, sensory–motor processing and pain (Dominguez et al., 2004; Hunter et al., 2004; Koylu et al., 2006, 1998, 1997; Stanek, 2006; Vicentic and Jones, 2007).

* Corresponding author at: School of Medicine and Dentistry, James Cook University, PO Box 6811, D1-213, Cairns 4870, Australia. Tel.: +61 7 4042 1911; fax: +61 7 4042 1920.
E-mail address: ernest.jennings@jcu.edu.au (E.A. Jennings).

0143-4179/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.npep.2011.09.002

In the peripheral nervous system, CART peptides have been reported in small diameter sensory neurons in the dorsal root ganglia (DRG) (Kozsurek et al., 2007), vagal afferent neurons in the nodose ganglia (de Lartigue et al., 2007, 2010), autonomic efferent neuronal somata in the cardiac ganglia (parasympathetic) and fibres in the stellate ganglia (sympathetic) (Gonsalvez et al., 2010; Richardson et al., 2006). These findings are consistent with a role for CART peptides in pain processing and autonomic regulation of the vasculature. However, there is only a single report of CART peptide expression in peripheral ganglia of the head. Superior cervical ganglion contains CART immunoreactive fibres that originate in preganglionic sympathetic neurons of the upper thoracic spinal cord (Gonsalvez et al., 2010). The aim of the present study was to determine if there is neuronal CART peptide expression in parasympathetic (sphenopalatine (SPG); otic (OG)) and sensory (trigeminal (TG)) ganglia of the head and examine the characteristics (neurochemical phenotype and projection targets) of CART expressing neurons in these ganglia.

2. Materials and methods

At total of 47 Male Sprague Dawley rats, each weighing between 200 and 250 g, were used in this study. Each observation we describe was made in experiments on at least three animals. All experiments conformed to the *Australian National Health and Medical Research Council* code of practice for the use of animals in research, and were approved by the University of Melbourne *Animal Experimentation Ethics Committee*.

2.1. Tissue preparation and Immunohistochemistry

Each animal was given an overdose of sodium pentobarbitone (Lethobarb; 150 mg/kg; i.p.), and was perfused via the ascending aorta with 250 ml of heparinized 0.01 M phosphate buffered saline (PBS) followed by 250 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). SPG, OG and TG were dissected, post-fixed in the above fixative for 1 hr, and left overnight in 30% sucrose in 0.01 M PBS. Multiple series through the entire SPG and OG (1 in three sections; 20 μm per section), and TG (1 in 10 or 15 sections; 12 μm per section), were cut onto gelatinized glass slides (1% gelatin) using a cryostat (−17 °C). Sections were air dried, washed three times in 0.01 M PBS, incubated for one hour in a solution containing 10% normal horse serum and 0.5% Triton X-100 in 0.01 M PBS and then incubated for two nights (at 4 °C) in primary antisera. Primary antisera were chosen to reveal immunoreactivity for the CART peptide, calcitonin-gene related peptide (CGRP; a peptide found in a subpopulation of sensory neurons), vaso-active intestinal peptide (VIP; a peptide found in a subpopulation of parasympathetic postganglionic neurons), neuropeptide Y (NPY; another peptide found in a subpopulation of parasympathetic postganglionic neurons), neurofilament 200 (NF200; a marker of myelinated sensory neurons), enkephalin (ENK; an opioid peptide found in some sensory and autonomic neurons) and isolectin-B4 binding (IB4; a marker for a subpopulation of small diameter non-peptidergic sensory neurons) (see Table 1 for antibody source, specificity and characterization). All primary antisera were diluted in 0.01 M PBS containing 0.5% Triton X-100 and 0.1% sodium azide. Following three further washes in 0.01 M PBS, sections were incubated in secondary antibody for 2 h, and washed again three times in 0.01 M PBS. Secondary antisera (donkey anti-rabbit 594, donkey anti-mouse 488 or donkey anti-goat 488; AlexaFluor; Molecular Probes) were diluted to 1:200 in 0.01 M PBS. The sections were cover-slipped using Dako (Carpinteria, CA) fluorescence mounting medium. In the cases that double labelling of the CART peptide antibody with other antisera was performed, both antisera were diluted in the same incubation solution. Primary omission controls were performed for each antibody to exclude non-specific staining from secondary antibodies and resulted in no labelling.

Table 1
Primary antibody source and characterization.

Primary antibody	Source	Specificity and characterization
Rabbit α CART (55–102)	Phoenix	Dun et al. (2000) and Gonsalvez et al. (2010)
1:2000	#H-003-62	Manufacturers information
Goat α CGRP	Biogenesis	Yasuhara et al. (2008)
1:1000	#1720-9007	Manufacturers information
Mouse α ENK	Chemicon	Yeo et al. (2010)
1:100	#MAB350	Manufacturers information
Mouse α NF200	Sigma	Fukuoka et al. (2008)
1:2000	#N0142	Manufacturers information
Sheep α NPY	Chemicon	Furness et al. (1985) and Zhu et al. (1997)
1:500	#AB1583	Manufacturers information
Sheep α VIP	Chemicon	Liu et al. (2008)
1:100	#AB1581	Manufacturers information
Biotinylated IB4	Sigma	Manufacturers information
1:100	#L2140	

2.2. Retrograde tracing

In some experiments, retrograde tracing was used to identify sensory neurons in the TG, and postganglionic parasympathetic neurons originating in the SPG and OG, that innervate the lacrimal gland, nasal mucosa, parotid gland, temporomandibular joint and the intracranial vasculature. Animals were anaesthetized with ketamine (100 mg/kg; i.p.) and xylazine (10 mg/kg; i.p.). For lacrimal and parotid gland injections, a skin incision was made over the lateral aspect of jaw and inferior to the ear to reveal the two glands. Two injections of 0.5 μl of the retrograde tracer Fast Blue ((4′-Amino-2′,5′-diethoxybenzanilide); 2% in distilled water; FB) were placed directly into either the lacrimal or parotid gland with a Hamilton syringe. The injection site was washed extensively with 0.1 M PBS and inspected for tracer leakage using a hand-held UV illumination device. No animals showed evidence of tracer leakage to surrounding tissues. Skin incisions were closed with stainless steel autoclips. Injections into the temporomandibular joint (1 μl FB) were made with a Hamilton syringe that was introduced through the skin, and into the joint cavity between the mandibular condyle and posterior aspect of the zygomatic arch. Injections into the nasal mucosa (1 μl FB) were also made with a Hamilton syringe, through the nostril and into the mucosa on the lower aspect of the lateral wall of the nasal cavity. For retrograde labelling from the intracranial vasculature, we targeted the middle meningeal artery in the dura (MMA) and the middle cerebral artery (MCA). In the case of the middle meningeal artery, a skin incision was made over the calvarium and a small square piece of bone removed from the skull posterior to the orbital rim with a dental drill. A small piece of gel foam soaked in FB was placed directly onto the dura over the middle meningeal artery. In the case of the middle cerebral artery, a skin incision was made between the eye and ear, the temporalis muscle retracted, and then a small square piece of bone removed from the temporal bone to expose the underlying middle cerebral artery. The dura and arachnoid were cut and retracted from the middle cerebral artery and a small piece of gel foam soaked in FB was placed directly onto the artery. The square pieces of bone were placed back over the holes created to expose the intracranial vessels and were covered with parafilm to prevent leakage of the tracer to surrounding tissues. Skin incisions were closed with stainless steel autoclips. All animals used for retrograde tracing were allowed to survive for 5 days to allow for transport of the tracer to soma in the TG, SPG and/or OG. Subsequently, the protocol described above