Cocaine- and amphetamine-regulated transcript (CART) peptide in the spinal neuronal networks involved in nociceptive information processing

Ph.D. thesis

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Introduction

Cocaine- and amphetamine-regulated transcript (CART) was discovered as a novel mRNA in the rat striatum, which is upregulated following acute administration of psychomotor stimulants. CART peptides of different sizes derived from the transcript have been identified, and the biologically active fragments have been implicated in the regulation of several physiological functions, including reward, food intake, and neuroendocrine functions.

Several lines of evidence suggest the involvement of CART peptide in spinal pain transmission: a dense plexus of CART-immunoreactive fibres has been described in the superficial laminae (I-II) of the spinal dorsal horn, intrathecal application of CART(55-102) caused hyperalgesia in acute pain states, attenuated hyperalgesia and allodynia in neuropathic but not inflammatory pain, enhanced the spinal analgesic actions of morphine and significantly enhanced NMDA- but not AMPA-induced nociceptive effects in vivo.

The spinal dorsal horn is the key area of sensory information processing and pain transmission. Aδ and C primary afferents, most of which are nociceptors, terminate in lamina I and II. C fibres can be subdivided into two overlapping populations: non-peptidergic C fibres that bind isolectin B4 from *Bandeiraea simplicifolia* (IB4) and peptidergic afferents, containing calcitonin gene-related peptide (CGRP) with substance P (SP) or somatostatin (SOM). Approximately the half of the peptidergic primary afferents express galanin (GAL), which is one of the most extensively studied peptides in pain transmission and chronic pain syndromes.

Lamina I neurons can be classified morphologically into fusiform, multipolar (flattened) and pyramidal cells; and based on responses to natural stimulation into nociceptive specific, polymodal nociceptive or innocuous thermoreceptive nerons. Correlation between morphology of a cell and its physiological response was reported in cat: all fusiform neurons were NS, multipolar cells were either NS or polymodal nociceptive and pyramidal cells were innocuous thermoreceptive.

The majority of neurons in lamina I–III are interneurons, and only a few cells in lamina I and III-V project to supraspinal areas, including the lateral parabrachial area (LPb), caudal ventrolateral medulla, periaqueductal gray and the thalamus. The majority of lamina I projection

neurons expresses neurokinin-1 receptors (NK1R), on which SP acts. These cells show internalization of the receptor or express c-fos after noxious stimulation. Selective ablation of lamina I NK1R-positive neurons by SP conjugated cytotoxin saporin results in attenuated responses to highly noxious stimuli and mechanical and thermal hyperalgesia. It has been also demonstrated that NK1R-expressing cells in superficial dorsal horn are essential for central sensitization, and for establishing and maintaining both inflammatory and neuropathic pain states.

One-third of interneurons in lamina I–III are inhibitory and immunoreactive for GABA and/or glycine, while the rest are thought to be glutamatergic and contain vesicular glutamate transporter 2 (VGLUT2), but further peptides such as the SP and SOM may also be present in their axon terminals. Serotoninergic and noradrenergic pathways descending from the brainstem also terminate in the dorsal horn, and can modulate sensory (including nociceptive) transmission.

Aims

It is well known that the segments of the spinal chord are functionally different. CART-immunoreactive fibres along the full length of the spinal cord have only been reported in mice, and such observations were limited to the cervical and thoracic segments in rats. For this reason CART-immunoreactivity was studied along the full length of the rat spinal cord first.

As the origin of CART-immunoreactive axons in the superficial laminae of the spinal cord was absolutely unknown, we wanted to determine if these fibres are primary afferents, axons of local interneurons or descend from the brainstem.

CART-ergic terminals were also investigated at electron microscopic level to determine their targets and the relationship between them.

Furthermore, we tested the hypothesis that CART-containing primary afferents selectively innervate particular classes of lamina I spinoparabrachial neurons, defined by morphology or NK1R expression.

Methods

All the experiments were carried out on male Wistar rats weighting 280-320 g.

Anti-CART primary antibody raised in mouse was used on transverse sections of the rat cervical, thoracic, lumbar and sacral segments for studying the distribution of CART-ergic fibres along the spinal cord.

Multiple immunofluorescent stainings were performed with CART antibody and markers for primary afferents, excitatory and inhibitory interneurons, and descending systems to determine the origin of the CART-immunoreactive axons in the superficial laminae of the rat spinal cord. CGRP was used as the specific marker of peptidergic C primary afferents, while non-peptidergic C fibres were identified by IB4-binding. Cholera toxin β-subunit (CTb) was used as a transganglionic tracer injected into the sciatic nerve for selective labelling of myelinated primary afferents (exclusively Aδ fibres in the superficial laminae). Excitatory interneurons express VGLUT2 and may also contain several peptides, such as the SP, SOM and neurotensin (NT). Inhibitory interneurons were identified according to their vesicular GABA transporter (VGAT) and/or glycin transporter 2 (GLYT2) immunoreactivity. Serotonin transporter (5-HTT) and dopamine-β-hydroxilase (DβH) were used as markers of the descending serotoninergic and noradrenergic pathways, respectively.

Neuropeptide content of the primary afferents and the coexistence of CART and GAL was analyzed in sections undergoing quadruple immunofluorescent staining with CART, SP, CGRP and GAL antibodies.

CTb injected into the LPb was used as a retrograde tracer for labelling lamina I projection neurons. Quadruple immunofluorescent staining was carried out on horizontal lumbar segments with antibodies raised against CART, CGRP, NK1R and CTb. Following the 3D reconstruction of the retrogradely labelled lamina I projection neurons the contact density of CART+ and CART+/CGRP+ terminals was quantified and compared between the NK1R-expressing and non-expressing neurochemical subclasses and among the NK1R+ fusiform, pyramidal and multipolar morphological types.

All the confocal images were captured with a Nicon Eclipse E800 microscope attached to a Bio-Rad Radiance 2100 Rainbow confocal laser scanning system, and the *Neurolucida for Confocal 4.34* (MicroBrightField,

USA) software pack was used for the colocalization analysis and for the 3D reconstruction of the lamina I projection cells.

CART-containing fibres for the ultrastructural and synaptological studies were labelled with immunoperoxidase reaction where diaminobensidine was used as a chromogen, or the *silver-intensified gold* technique was used.

Results

As reported earlier, CART staining was present from cervical to sacral segments, and CART-stained fibres and terminals were found in each lamina and in the dorsolateral fasciculus including the lateral spinal nucleus. The densest network of CART-positive fibres was observed in the superficial laminae, especially in lamina I, and the CART-positive axon density dramatically decreased away from the superficial laminae. CART-immunoreactive cell bodies were found around the central canal, and a few pale neurons appeared in the dorsal horn. Strongly stained neurons and fibres were also located in the intermediolateral cell column of the thoracic cord.

CGRP was present in 73% and 35% of CART-immunoreactive axons in lamina I and II, respectively. The majority of these fibres also contained SP, while a few were SOM-positive. The other subpopulation of CART-immunoreactive boutons in lamina I and II also expressed SP and/or SOM without CGRP, but contained vesicular glutamate transporter 2, which is present mainly in excitatory interneuronal terminals (Table 1). Our data demonstrate that the majority of CART-immunoreactive axons in the spinal dorsal horn originate from peptidergic nociceptive primary afferents, while the rest arise from excitatory interneurons that contain SP or SOM. This strongly suggests that CART peptide can affect glutamatergic neurotransmission as well as the release and effects of SP and SOM in nociception and other sensory processes.

Almost all randomly selected CGRP-immunoreactive terminals in the lamina I were SP-positive and CART was detected in approximately half of them. Most of the CGRP/SP-ergic boutons were GAL-positive and approximately half of these contained CART. Many of the CART-ergic boutons which expressed CGRP were also immunoreactive for GAL (Table 2). These results demonstrate a substantial overlap between CART and GAL in peptidergic nociceptive afferents, and this raises the possibility of

pre- and/or postsynaptic interaction between the two peptides in acute and chronic pain states.

Table 1. Colocalization of CART peptide with various markers for primary afferents, interneurons and descending fibres in the lamina I-II. All values are given as percentage (%), mean ±standard deviance.

	Markers	Lamina I	Lamina II
Primary afferents			
Peptidergic afferents			
substance P-containing	CGRP+, SP+	62.7±0.9	33.9±1.3
somatostatin-containing	CGRP+, SOM+	9.9±1.2	0.9±0.3
Non-peptidergic C afferents	CGRP-, IB4+	0	0
Aδ nociceptive afferents	CTb+	0	0
Glutamatergic primary afferent and interneuronal endings	VGLUT2+	57.2±4.3	48.8±8.4
Interneuronal terminals			
substance P-containing	CGRP-, SP+	17.1±0.1	33.9±2.7
somatostatin-containing	CGRP-, SOM+	15.3±1.8	25.5±1.4
neurotensin-containing	NT+	3.2±0.5	3.4±0.2
GABA-ergic	VGAT+	2.4±0.4	6.5±1.7
glycinergic	GLYT2+	0	0
Descending axons			
serotoninergic	5-HTT+	0	0
noradrenergic	DβH+	0	0

Table 2. CART peptide and GAL in the peptidergic primary afferents and their colocalization in the lamina I terminals.

Peptidergic (CGRP-expressing) primary afferent terminals in the lamina I	**Mean, %**	**SEM**
CGRP+	2.10	0.49
CGRP+ / SP+	11.40	0.89
CGRP+ / SP+ / CART+	7.04	1.45
CGRP+ / SP+ / GAL+	39.08	1.56
CGRP+ / SP+ / CART+ / GAL+	41.54	0.56
CART-ergic terminals in the lamina I	**Mean, %**	**SEM**
CART+ / CGRP+ / GAL+	58.08	4.04
CART+ / CGRP+ / GAL-	21.61	7.41
CART+ / CGRP- / GAL+	8.55	1.93
CART+ / CGRP- / GAL-	11.76	3.93

Electron microscopy showed that most of the CART terminals formed asymmetrical synapses mainly with dendrites. When the silver-intensified gold technique was used, large dense core vesicles were observed in the labelled terminals, which are characteristic for primary afferents.

We found that all spinoparabrachial projection neurons that were analysed received contacts from CART-immunoreactive nociceptive afferents. Statistical analysis showed that neither the CART nor the CART/CGRP contact densities were significantly different between either the NK1R/non-NK1R cell groups or the different morphological cell types of NK1R-positive projection neurons. In contrast, the contact density of CGRP axons not containing CART is significantly higher on projection neurons which express NK1R than on those lacking the receptor, but there is no difference among the morphological subtypes of NK1R-positive projection neurons (Table 3).

Table 3. Contact density of CART-ergic (CART+/CGRP+ and CART+/CGRP- together), CART/CGRP-containing (CART+/CGRP+) and CGRP-expressing (CART-/CGRP+) terminals on various neurochemical and morphological subtypes of lamina I projection neurons.

	CART+	**CART+/CGRP+**	**CART-**
	contact density (mean ±SEM; min.-max.; bouton/1000 μm²)		
NK1R-positive neurons	20.95 ±1.52 5.25 – 69.64	13.36 ±1.93 1.74 – 60.06	23.55 ±1.39 1.58 – 43.19
fusiform	20.05 ±2.42 5.25 – 53.68	12.10 ±3.41 3.00 – 45.61	23.25 ±2.22 11.65 – 39.15
pyramidal	24.66 ±3.06 6.74 – 69.64	18.58 ±3.98 1.74 – 60.06	23.18 ±2.63 1.58 – 42.78
multipolar	17.93 ±2.20 6.37 – 58.82	9.89 ±2.53 3.05 – 48.63	24.03 ±2.32 7.19 – 43.19
NK1R-negative neurons	21.66 ±3.04 8.05 – 48.58	11.82 ±2.35 4.25 – 26.66	12.79 ±1.58 5.62 – 20.98

Our work demonstrates that the subpopulation of CGRP/SP primary afferents which contains CART does not make similar distinction

between the two cell groups. This suggests a diffuse, non-selective innervation of lamina I projection neurons from a population of nociceptive primary afferents that contain CART peptide and a selective input from those afferents not containing the peptide. These data also suggest that the different neurochemical subtypes of the peptidergic afferents containing SP and CGRP may be functionally different (Table 3).

Conclusions

Our results provide anatomical bases for involvement of CART peptide in spinal pain transmission:

(1) Dense CART-immunoreactive fibres are present in the superficial laminae - especially in the lamina I - along the full length of the rat spinal cord and the superficial laminae are considered as the key areas of nociceptive information processing;

(2) CART peptide is present in the half of the CGRP/SP-containing C primary afferent terminals in the lamina I, which fibres are all thought to be nociceptive;

(3) There is a strong colocalization between the CART peptide and the GAL, the latter is known as one of the most important peptides in development and maintenance of chronic pain;

(4) CART-ergic fibres directly terminate on lamina I projection neurons, most of which are nociceptive specific or polymodal nociceptive, thus, forward painful stimuli toward the supraspinal areas.

It is known, that CART peptide potentiates NMDA-glutamatergic neurotransmission. Further experiments are necessary to clarify if CART peptide can affect the long term potentiation, which is mainly based on the altered NMDA-glutamatergic signalling and explains several types of chronic pain syndromes. It is still not obvious and should be investigated how CART peptide interacts with opioids, which are the most powerful anaesthetics in clinical practice. Invention and cloning the CART receptor or receptors would also help to understand how CART peptide is involved in nociceptive mechanisms. Later, various agonists or antagonist acting on these receptors alone or in combination with the anaesthetics used nowadays could contribute to a more effective therapy of pain.

Research articles related to the thesis

Kozsurek M, Lukácsi E, Fekete Cs, Wittmann G, Réthelyi M, Puskár Z. (2007) Cocaine- and amphetamine-regulated transcript peptide (CART) is present in peptidergic C primary afferents and axons of excitatory interneurons with a possible role in nociception in the superficial laminae of the rat spinal cord. *Eur J Neurosci*, 26:1624-1631.
IF: 3,673

Kozsurek M, Lukácsi E, Fekete Cs, Puskár Z. (2009) Non-selective innervation of lamina I projection neurons by cocaine- and amphetamine-regulated transcript peptide (CART) containing fibres in the rat spinal dorsal horn. *Eur J Neurosci* (accepted for publication).
IF: 3,673 (2007)

Research articles not related to the thesis

Hajdú J, Marton T, Kozsurek M, Pete B, Csapó Z, Beke A, Papp Z. (2008) Prenatal diagnosis of abnormal course of umbilical vein and absent ductus venosus--report of three cases. *Fetal Diagn Ther*, 23:136-139.
IF: 0,844

Meeting abstracts

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The Ph.D. thesis is available online in full length at:
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