Distribution of signaling neurotransmitters and interaction in the sphenopalatine ganglion

Summary of Ph.D. Thesis

ANETT CSÁTI M.D.

Supervisor: János Tajti M.D., Ph.D.

Department of Neurology
Faculty of Medicine
Albert Szent-Györgyi Clinical Centre
University of Szeged

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LIST OF PAPERS

This doctoral thesis is based on the following publications:

I. **Csati A**, Tajti J, Kuris A, Tuka B, Edvinsson L, Warfvinge K. Distribution of vasoactive intestinal peptide, pituitary adenylate cyclase-activating peptide, nitric oxide synthase, and their receptors in human and rat sphenopalatine ganglion. Neuroscience. 2012. 202:158-68. IF: 3.122

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III. Tajti, J., Szok, D., Tuka, B., **Csáti, A.**, Kuris, A., Majláth, Zs., Lukács, M., Vécsei, L. Botulinum neurotoxin-A terápia migrénben. Ideggyógyászati Szemle. 2012. 65:77-82. IF: 0.348

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LIST OF ABBREVIATIONS

Ach	acetylcholine
cAMP	cyclic adenosine monophosphate
CGRP	calcitonin gene-related peptide
CLR	calcitonin receptor-like receptor
DAPI	4’,6-diamino-2-phenylindole
GFAP	glial fibrillary acidic protein
GPCRs	G protein-coupled receptors
GS	glutamine synthetase
NOS	nitric oxide synthase
PACAP	pituitary adenylate cyclase-activating peptide
RAMP1	receptor activity modifying protein 1
RCP	CGRP-receptor component protein
SGCs	satellite glial cells
SPG	sphenopalatine ganglion
SSN	superior salivatory nucleus
VIP	vasoactive intestinal peptide

1. INTRODUCTION

The head and neck regions and the intracranial circulation are innervated by parasympathetic nerve fibers from the sphenopalatine ganglion (SPG), otic and internal carotid ganglia (Suzuki et al., 1988). The central control emanates in the superior salivary nucleus (CN VII, the facial nerve) with cholinergic fibers that synapse in the SPG (Hara and Weir, 1988, Suzuki et al., 1988, Edvinsson et al., 1989, Hara et al., 1993, Edvinsson et al., 2001). The neuronal cell bodies in the human SPG contain vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating peptide (PACAP) and nitric oxide synthase (NOS) as the main parasympathetic signaling transmitters (Uddman et al., 1999). Traditionally the cell bodies have been considered to be cholinergic, but only a minor subpopulation of cells in the SPG have been reported to contain acetylcholine transferase (Lee et al., 1984). In addition, a large number of VIP and PACAP immunoreactive cell bodies have been shown to co-localize with NOS in rat (Uddman et al., 1999, Edvinsson et al., 2001).

VIP and PACAP belong to the secretin/glucagon/VIP superfamily of neuropeptides. VIP was first isolated from the ovine intestine and found to be a very potent neuropeptide (Said and Mutt, 1970) that consists of 28 amino acids. The peptide has various biological such as pain perception and inflammation (Harmar et al., 1998). PACAP, the newest member of this family of peptides was originally isolated from the rat hypothalamus (Miyata et al., 1989), and occurs in two forms: PACAP-27 and PACAP-38 (27 or 38 amino acids). Both forms of PACAP exert a large variety of biological effects including vasodilatation, immune modulation, stimulation of cell proliferation and differentiation, control of neurotransmitter release and pain transmission (Harmar et al., 1998, Vaudry and Laburthe, 2006). PACAP-38 predominates over PACAP-27 in most studied tissues (Sundler et al., 1996).

The actions of VIP and PACAP are mediated through the class B family of 7 transmembrane G protein-coupled receptors (GPCRs) (Vaudry and Laburthe, 2006, Dickson and Finlayson, 2009). VIP and PACAP act via VPAC1 and VPAC2 receptors with equally high affinity, while PACAP is more potent at the PAC1 receptor (Harmar et al., 1998).

During the investigation on primary headache pathophysiology a close correlation between the calcitonin gene-related peptide (CGRP) and the head pain was observed in acute attacks of migraine (Ho et al., 2010). In some cases of migraine and in all cluster headache cases, additional facial symptoms (reddening of sclera, rhinorrhea, nasal congestion, etc.) were associated with co-release of the sensory neuropeptide CGRP from the trigeminal system and VIP from the cranial parasympathetic system into the cranial venous outflow (Goadsby and

Edvinsson, 1994a). CGRP consists of 37 amino acids and plays an important role in vasodilatation and pain transmission in craniocervical structures (Ho et al., 2010). Sumatriptan, acting on 5-hydroxytryptamine type 1B/1D subtypes of receptors, aborts not only the CGRP release and the head pain, but also the VIP release and the parasympathetic symptoms (Goadsby and Edvinsson, 1994a). The mechanisms involved are not clear, however, experimental studies have provided some support of a link between the two systems (Goadsby and Edvinsson, 1994b).

The receptor for CGRP belongs to the family of G-protein-coupled receptor of the B-subtype (Hay et al., 2008). The functional CGRP receptor consists of three proteins: (i) the calcitonin receptor-like receptor (CLR) which forms the ligand binding site with (ii) receptor activity modifying protein 1 (RAMP1), and together they determine the specificity of the receptor (McLatchie et al., 1998, Heroux et al., 2007). (iii) The CGRP-receptor component protein (RCP) is essential in coupling the receptor to intracellular signal-transduction pathways and, in particular, to adenylyl cyclase (Evans et al., 2000).

2. AIMS

(i) Reveal the presence and distribution of VIP/PACAP receptors in human and rat SPG using indirect immunofluorescence and Western blot techniques.

(ii) Examine the SGCs and their relation to neurons both in human and rat SPG.

(iii) Study CGRP and CGRP receptor elements (CLR and RAMP1) in human and rat SPG neurons, nerve fibers and SGCs as an indication of putative local function.

(iv) Compare the distribution of examined neurotransmitters between human and rat SPG, using immunofluorescence technique.

3. EXPERIMENTAL PROCEDURES

3.1. Tissue material

3.1.1. Human

Human SPG were obtained at autopsy from 5 adult subjects with an average age of 72.6 years (range 60-81 years). The collection of tissue samples was done in accordance with the University of Szeged, Faculty of Medicine guidelines for ethics in human tissue experiments (6/1996 – 13/12/2010).

Human SPG were post-fixed overnight. After fixation, specimens were rinsed repeatedly in sucrose-enriched Tyrode solution, snap frozen, embedded and stored at -80ºC.

3.1.2. Rat

SPG were collected from eight adult male Sprague-Dawley rats (weighing 300-400 g). The animals were raised and maintained under standard laboratory conditions. The study followed the guidelines of the European Communities Council (86/609/ECC) and was approved by the Ethics Committee of The Faculty of Medicine, University of Szeged.

The rats were deeply anesthetized and perfused and fixed transcardially. The ganglia were removed and post-fixed overnight. After fixation rat specimens were rinsed repeatedly in sucrose-enriched Tyrode solution, frozen on dry ice and stored at -80ºC.

Both human and rat specimens were embedded in gelatin medium, cryosectioned at 10 µm, mounted on Superfrost Plus coated slides (Menzel GmbH Co KG, Braunschweig, Germany) and stored at -20ºC until use.

3.2. Regular tissue staining

3.2.1. Hematoxylin-Eosin

For orientation and examination of the tissue condition, human and rat sections were stained with Hematoxylin-Eosin (Htx-Eosin) using a standard protocol.

3.3. Immunohistochemistry

Indirect immunofluorescence technique was used for immunohistochemical demonstration of VIP, PACAP, NOS, glutamine synthetase (GS), glial fibrillary acidic protein (GFAP), VIP and PACAP common receptors (VPAC1, VPAC2), PACAP receptor (PAC1), CGRP, the CGRP receptor components as CLR and RAMP1 in human and rat SPG. In addition, double labeling was carried out to reveal the co-localization of neurotransmitters.

3.4. Image analysis

Cryostat sections were examined and images were obtained using a light- and epifluorescence microscope coupled to a camera to visualize co-labeling by superimposing the digital images.

3.5. Western blot

Western blot technique was used to demonstrate the existence of VIP/PACAP receptors and CGRP receptor components in rat SPG. Adult male Sprague-Dawley rats (500-600g; n=8) were used. Omission of primary antibodies were used as negative controls.

4. RESULTS

4.1. Regular tissue staining

4.1.1. Hematoxylin-Eosin

Most of the human material displayed qualitatively adequate morphology as visualized with Htx-Eosin staining. The SPG were found as well-defined ganglia with neurons intermingled within the sphenopalatine branches of the maxillary nerve. Ganglia consisted of neurons of various size enveloped by SGCs. Minor cell shrinkage was observed.

Rat SPG showed similar morphology with neurons of various size surrounded by SGCs.

4.2. Immunohistochemistry

4.2.1. Human SPG

Due to the subject's old age, many neurons in the human material contained lipofuscin granulae in their cytoplasm.

VIP immunoreactive neurons as well as fibers were frequently found in human SPG. The immunoreactivity was granular-like in both neurons and fibers. Many NOS (homogenously stained) immunoreactive neurons were found, but no positive fibers. In addition, PACAP immunoreactivity was found in some of the neurons and in fibers. As for VIP, the PACAP staining displayed granular-like immunoreactivity. The neuronal staining was often localized to, or close to, the cell surface. In order to reveal if the peptide only was localized to the neurons, and not the SGCs, double-staining with different glial cell specific antibodies were performed. Double staining with PACAP/Vimentin revealed that PACAP staining was not localized in the SGCs. Double stainings of human SPG – VIP/NOS, PACAP/NOS and VIP/PACAP – were carried out. In our hands, co-localization was found between VIP/NOS, and PACAP/NOS. We were not able to establish, with the methods used, if the peptides were present in different neuronal subpopulations.

Pearl-like CGRP immunoreactive fibers were frequently found in human SPG. In order to scrutinize the CGRP immunoreactivity, we used different primary and secondary antibodies. More CGRP positive fibers were visible with the use of CGRP anti-mouse primary and DyLight 549 secondary antibodies relative to CGRP anti-rabbit and FITC anti-rabbit. Texas Red secondary antibody revealed the same staining patterns as DyLight 549. CGRP immunoreactivity was not observed in human SPG neurons or SGCs using either of the combination of antibodies.

Many CLR immunoreactive SGCs and fibers were found. For the demonstration of CLR, FITC or DyLight 488 was used as secondary antibody. Both antibodies visualized immunoreactive SGCs, but only FITC revealed immunoreactive fibers in the SPG. No CLR immunoreactive neurons were found.

For the visualization of RAMP1, the use of Cy2 secondary antibodies revealed RAMP1 homogenously stained neurons. Some large and medium-sized neurons were positive for RAMP1. Furthermore, RAMP1 positive SGCs were detected with Alexa 488 secondary antibodies. There was no RAMP1 immunoreactivity in the fibers.

In order to examine co-localization between CGRP, CLR and RAMP1, double stainings were performed. No co-localization was found.

4.2.2. Rat SPG

In the rat material, VIP, NOS and PACAP immunoreactivity were found in many neurons and fibers. PACAP immunoreactivity was often localized close to the cell membrane, whereas VIP and NOS stainings were more evenly visualized within the cell soma, although somewhat granular-like for VIP. We were also able to observe co-localization of PACAP and NOS, but not between VIP/NOS or PACAP/VIP. PACAP and GS double staining revealed that the PACAP immunoreactivity was localized in or close to the cell membrane, but not in the SGCs.

In rat SPG CGRP immunoreactive fibers were frequently found. CGRP anti-rabbit antibody disclosed many homogenously stained neurons, while CGRP anti-mouse antibody revealed only few CGRP positive neurons. CLR immunoreactive fibers and SGCs were found using FITC anti-rabbit, but not noted in neurons. Both Cy2 and Alexa 488 secondary antibodies revealed RAMP1 positive fibers and some homogenously stained neurons. Double staining with RAMP1 and CLR revealed co-localization of the immunoreactive fibers.

4.2.3. Human and rat SPG

Distribution of the receptors PAC1, VPAC1 and VPAC2 was investigated in both human and rat. PAC1 and VPAC1 immunoreactivity was found in the SGCs. VPAC1 immunoreactivity was also observed in few fibers in both the human and rat SPG. In addition, we observed VPAC2 immunoreactive fibers in both human and rat specimens. However, the staining was not as distinct as for PAC1 and VPAC1. No co-localization between the peptides and the receptors were found.

Overview of CGRP, CLR and RAMP1 immunoreactivity in human and rat SPG is shown in Table 1.

Table 1. Summary of PACAP, VIP, NOS, PAC1, VPAC1, VPAC2, CGRP, CLR and RAMP1 results in human and rat SPG

	Neurons		Satellite glial cells		Nerve fibers	
	Human	Rat	Human	Rat	Human	Rat
PACAP	+	+	-	-	+	+
VIP	+	+	-	-	+	+
NOS	+	+	-	-	-	+
PAC1	-	-	+	+	-	-
VPAC1	-	-	+	+	+	+
VPAC2	-	-	-	-	+	+
CGRP	-	+	-	-	+	+
CLR	-	-	+	+	+	+
RAMP1	+	+	+	-	-	+

4.2.4. Negative controls

Negative controls (omission of primary antibodies) displayed no immunoreactivity, except for autofluorescent lipofuscin.

4.3. Western blot

Western blot revealed protein expression of PAC1, VPAC1, VPAC2, RAMP1 and CLR in rat SPG. PAC1 receptor gave a 60 kDa band, VPAC1 receptor a 58 kDa band and VPAC2 receptor a 65 kDa band. RAMP1 was visualized as a 30 kDa band, while CLR as a 60 kDa band. Bands were identified by protein molecular weight marker. No bands were visualized after omission of primary antibodies.

5. DISCUSSION

In recent years there has been considerable interest in the pathomechanism of migraine (Tajti et al., 2011a, Tajti et al., 2011b, Tajti et al., 2012, Vecsei et al., 2013). Clinical observations and experimental studies have suggested a possible role for the SPG in the pathophysiology of migraine (Barbanti et al., 2002, Tepper et al., 2009). Interestingly, systemic administration of PACAP but not VIP has been found to induce “migraine-like” headache in migraine patients, although both peptides elicited similar changes in the vessel tone (Rahmann et al., 2008, Schytz et al., 2009). The present results do not, however, suggest a morphological reason for this differential response. In addition, vascular studies have revealed that VIP is by far a stronger and more potent vasodilator than PACAP of human and

rat cerebral and meningeal arteries (Jansen-Olesen et al., 2004, Boni et al., 2009, Chan et al., 2011). *In vivo* studies in man also showed relaxation of cranial vessels by VIP (Hansen et al., 2006, Rahmann et al., 2008) and PACAP (Birk et al., 2007, Schytz et al., 2009). It was argued that dilatation alone could not be the direct cause of the migraine-like attacks after the PACAP-38 infusion but perhaps this response could involve neurons or other cells that contain VIP/PACAP receptors such as cranial ganglia (Schytz et al., 2009). It has been revealed that PACAP-38 is expressed in the trigeminal ganglion (Tajti et al., 1999) and in the caudal trigeminal nucleus (Tajti et al., 2001). It has been demonstrated that blood plasma PACAP-38-like immunoreactivity is increased following the electrical stimulation of the trigeminal ganglion in rat (Tuka et al., 2012). Furthermore, it has been suggested that PACAP is one of the mediators of light aversion, because it elicited photophobia in wild-type mice, while it did not in PACAP-gene deficient mice (Markovics et al., 2012). Recent data showed that the concentration of PACAP-38 together with CGRP was elevated during the migraine attack period versus to the attack-free periods (Tuka et al., 2013). It has been postulated that elevation of cellular cyclic adenosine monophosphate (cAMP) plays a role in the development of delayed headache via sensitization of trigeminal neurons after CGRP or cGMP (Ingram and Williams, 1996, Lassen et al., 2002, Birk et al., 2004, Birk et al., 2006). PACAP activates VPAC1-2 and PAC1 receptors, which induces cAMP level elevation (Dickson et al., 2006), which more resembles the mode of action of CGRP (Walker et al., 2010).

It has been demonstrated that postganglionic parasympathetic fibers from the SPG mediate meningeal blood flow elevations and meningeal vasodilatation (Bolay et al., 2002) and, in addition, neurogenic inflammation which in turn may sensitize meningeal nociceptors (Burstein and Jakubowski, 2005). The preganglionic fibers to the SPG originate from the superior salivatory nucleus (SSN) and synapse in the SPG. The SSN can be activated/modified by trigeminal sensory nerve fibers. This is a trigeminal-autonomic reflex which may be active in migraine attacks (Zagami et al., 1990).

It seems that the fundament for a trigeminal action is at place by the presence of CGRP receptor components. The role of the SGCs in the SPG is largely unknown. However, increasing glial cell research suggests a ganglion function at many levels (Hanani, 2010), especially the characterized SGCs and neurons forming a morphological unite in the SPG. The available data suggest that SGCs are involved in synaptic maintenance and remodeling. Our study is the first that examines SGCs in cranial parasympathetic ganglion in man. Future may provide more insight on how SGCs may influence synaptic transmission, and information processing in autonomic and sensory ganglia.

There are some functional data which suggest an interaction between the trigeminal and sphenopalatine ganglia. Cluster headache is associated with activation of both ganglia since there is co-release of CGRP and VIP (Goadsby and Edvinsson, 1994a). Treatment with sumatriptan aborts both symptoms of parasympathetic activation and neuropeptide release, presumably by the triptan acting as inhibitor on the sensory nervous system via a presynaptic mechanism or the formation of CGRP (Durham and Russo, 1999). Further, experimental activation of the superior sagittal sinus results in co-release of VIP and CGRP (Zagami et al., 1990). Cutting of the trigeminal nerve abolished not only the CGRP release but also that of VIP which supports an interaction between the two systems. It is tempting to speculate that the present finding reveals a direct link between the trigeminal ganglion CGRP-containing fibers and the SPG. The nature of this is unclear but available data would imply that intense activation of the trigeminal ganglion can result in parasympathetic symptoms (cluster headache, red eye, rhinorrhea, conjunctival injection, and tearing) associated with VIP release (Goadsby and Edvinsson, 1994a).

5.1. Conclusion

The present work has revealed the presence of VIP, PACAP and NOS in nerve fibers within the ganglion, in addition, VIP/NOS and PACAP/NOS show co-localization in the human SPG neurons. Western blot verified the presence of VPAC1, VPAC2 and PAC1 receptors in rat SPG. Immunohistochemistry showed that PAC1 and VPAC1 are localized in the SGCs, while VPAC1 and VPAC2 in the nerve fibers in both human and rat SPG. These results suggest that the peptides may be involved in intraganglionic activity.

Moreover, the present finding demonstrates that CGRP-positive fibers, probably originating from the trigeminal ganglion as C-fibers, are present in both human and rat SPG. In rat CGRP positive neurons are found. Since both components of the CGRP receptor, CLR and RAMP1, are present in the ganglion (Western blot) and these are localized to SGCs (human) and fibers (rat), an interaction between parasympathetic and sensory ganglia is plausible.

The immunohistochemical differences between human and rat SPG suggest that in human CGRP is produced by the trigeminal neurons, transmitted to the SPG through CGRP positive nerve fibers and acts on the SGCs in the SPG, since both of the CGRP receptor components are present. In rat CGRP can be produced or stored in SPG neurons and act through the nerve fibers, where both of the receptor components are present.

6. SUMMARY OF NEW FINDINGS

(i) Our work has revealed VIP and PACAP immunoreactivity in nerve fibers besides the neurons but not in satellite glial cells both in human and rat SPG.

(ii) We disclose that the SPG contains the VPAC1, VPAC2 and PAC1 subtypes of receptor proteins. We found PAC1 and VPAC1 immunoreactivity in the satellite glial cells and VPAC1 and VPAC2 immunoreactive nerve fibers of both human and rat.

(iii) We demonstrate that CGRP-positive fibers are present in human and rat SPG and CGRP immunoreactive neurons in rat.

(iv) CGRP receptor components (CLR and RAMP1) are localized to SGCs in human and to fibers in rat SPG.

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