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DIFFERENTIAL EXPRESSION OF NK1 AND NK3 NEUROKININ RECEPTORS IN NEURONS OF THE NUCLEUS TRACTUS SOLITARIUS AND THE DORSAL VAGAL MOTOR NUCLEUS OF THE RAT AND MOUSE

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Abstract—Tachykinins (substance P, neurokinin A and neurokinin B) influence autonomic functions by modulating neuron activity in nucleus tractus solitarius (NTS) and dorsal motor nucleus of the vagus (DMV) through activation of neurokinin receptors NK1 and NK3. Our purpose was to identify and define by neurochemical markers, the subpopulations of NK1 and NK3 expressing neurons in NTS and DMV of rat and mouse.

Because the distribution of the NK1 and NK3 expressing neurons overlaps, co-expression for both receptors was tested. By double labeling, we show that NK1 and NK3 were not co-expressed in NTS neurons. In the DMV, most of neurons (87%) were immunoreactive for only one of the receptors and 34% of NK1 neurons, 7% of NK3 neurons and 12% of NK1–NK3 neurons were cholinergic neurons. None of the neurons immunoreactive for NK1 or NK3 were positive for tyrosine hydroxylase, suggesting that catecholaminergic cells of the NTS (A2 and C2 groups) did not express neurokinin receptors. The presence of NK1 and NK3 was examined in GABAergic interneurons of the NTS and DMV by using GAD65-EGFP transgenic mouse. Immunoreactivity for NK1 or NK3 was found in a subpopulation of GAD65-EGFP cells. A majority (60%) of NK3 cells, but only 11% of the NK1 cells, were GAD65-EGFP cells.

In conclusion, tachykinins, through differential expression of neurokinin receptors, may influence the central regulation of vital functions by acting on separate neuron subpopulations in NTS and DMV. Of particular interest, tachykinins may be involved in inhibitory mechanisms by acting directly on local GABAergic interneurons. Our results support a larger contribution of NK3 compared with NK1 in mediating inhibition in NTS and DMV.

Key words: tachykinin, GABA, interneuron, substance P, neurokinin A, neurokinin B.

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Abbreviations: ChAT, choline acetyl transferase; DMV, dorsal vagal motor nucleus; NK1−/−, mice knockout for NK1; NTS, nucleus tractus solitarius; PB, phosphate buffer; PBST, phosphate buffer containing 0.9% NaCl supplemented with 0.3% Triton X-100; TH, tyrosine hydroxylase.

In mammals, endogenous tachykinins, substance P, neurokinin A, and neurokinin B act as neuromodulators in peripheral and CNS (Severini et al., 2002). The biological activity of the tachykinins depends on their interaction with three G protein–coupled receptors (NK1, NK2, and NK3) which share considerable structural homology. Each of the tachykinins is considered to have a preferred receptor. Substance P has higher affinity for NK1, while neurokinin A and neurokinin B display higher affinity for NK2 and NK3, respectively. However, all three tachykinins can bind to each of the neurokinin receptors, if present at sufficient concentration (Nakanishi, 1991; Maggi, 1995). Therefore, they can exert physiological effects through both preferred and non-preferred receptors (Harlan et al., 1989; Nakanishi, 1991; Hastrup and Schwartz, 1996; Wijkhuisen et al., 1999; Colin et al., 2002).

Tachykinins and their receptors are involved in the central control of cardiovascular, digestive, gustatory and respiratory functions through brainstem neurons located in the nucleus tractus solitarius (NTS) and the dorsal vagal motor nucleus (DMV) (Improta and Broccardo, 1990; King et al., 1993; Otsuka and Yoshioka, 1993; Lawrence and Jarrott, 1996; Maubach and Jones, 1997; Liu et al., 1998; Mazzone and Geraghty, 2000; Severini et al., 2002; Potts et al., 2007). The NTS plays a key role in central regulation of autonomic functions as it receives primary afferents from gustatory, gastrointestinal, cardiovascular, and respiratory organs, particularly those supplied by the vagus nerve (Sawchenko et al., 1987; Berthoud and Neuhuber, 2000; Puizillout, 2005; Travagli et al., 2006). Apposed to the ventral face of the NTS, the DMV contains somata of the vagal efferent which innervate principally the digestive tract (Shapiro and Miselis, 1985; Blondeau et al., 2002) but also sub-diaphragmatic viscera such as the heart (Corbett et al., 2003) or lungs (Pérez Fontán and Velloff, 2001).

The NTS and DMV are enriched with axon terminals containing substance P, neurokinin A and neurokinin B (Kalia et al., 1984; Baude et al., 1989; Lucas et al., 1992; Marksteiner et al., 1992; Saha et al., 1995). All of them are putatively able to activate neurokinin receptors in NTS and DMV (King et al., 1993; Maubach and Jones, 1997; Liu et al., 1998; Colin et al., 2002). Autoradiographic studies have described high specific labelings for NK1 and NK3 binding sites in NTS and DMV, while none was detected for NK2 (Saffroy et al., 2003). mRNAs coding for NK1 and NK3 receptors have been detected (Nakaya et al., 1994; Ding et al., 1996), and NK1 and NK3 receptor proteins are present in NTS and DMV neurons (Carpentier et al., 1996; Ding et al., 1996; Baude and Shigemoto, 1998). In addi-

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tion, pharmacological studies using specific agonists and antagonists have demonstrated that NK1 and NK3 receptors within the NTS and DMV were involved in the central control of autonomic functions (Maubach and Jones, 1997; Mazzone and Geraghty, 2000; Pickering et al., 2003). Because the distribution of NK1 positive cells overlaps with the distribution of NK3 positive cells in both NTS and DMV, we have determined first whether tachykinins act on the same or different neuronal populations or on different populations by examining the co-localization of NK1 and NK3. Then, the expression of NK1 or NK3 was examined in chemically identified neurons (i.e. in cholinergic, catecholaminergic and GABAergic neurons). Characterizing the neuronal subpopulations on which tachykinins act in NTS and DMV will provide information concerning their role in the central control of vital functions.

EXPERIMENTAL PROCEDURES

Animals and tissue preparation

All experimental procedures were in accordance with the European Communities Council directive (86/609/EEC) for the care and use of laboratory animals.

Experiments were performed on adult Wistar rats of either sex ($n=6$) and adult mice of either sex ($n=3$) expressing EGFP under the control of the GAD65 promoter (De Marchis et al., 2007; Lopez-Bendito et al., 2004). For controls, some experiments were performed on wild type mice (C57/Bl6) and mice knockout for NK1 (NK1−/−; De Felipe et al., 1998). Animals were anesthetized with a ketamine (50 mg/ml) and xylazine (7.5 mg/ml) solution at a dose of 2 ml/kg (i.m.), then perfused through the aorta with a 0.9% NaCl solution followed by a fixative solution containing 4% paraformaldehyde in phosphate buffer (PB, 0.1 M, pH 7.4). The medulla was removed and cut in the coronal plane with a vibratome (section thickness 50 μm).

Immunohistochemistry

Free-floating sections were incubated for 1 h in 10% normal goat serum (NGS; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in phosphate buffer containing 0.9% NaCl supplemented with 0.3% Triton X-100 (PBST). Sections were sequentially incubated with primary antibodies (Table 1) diluted in PBST overnight, washed in PBST, incubated in species-specific secondary antibodies conjugated with either Al 488 (1/500; Molecular Probes™, Invitrogen Corporation, Carlsbad, CA, USA), Cy3 or Cy5 (1/500; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for 1 h, and washed in PBST. Two combinations of labeling were used on rat sections: triple labeling of NK1, NK3 and choline acetyl transferase (ChAT); double labeling for tyrosine hydroxylase (TH) and NK1 or NK3. Single labeling for NK1 or NK3 was used on sections from GAD65-EGFP expressing mice. All the incubations were performed at room temperature on a rotating platform. Following washes, sections were coverslipped using glycerol-PB 0.2 M (50:50) as mounting medium.

Table 1. Primary antibodies

Antigen	Species	Dilution	Source	Code no.
NK1	Guinea pig	1/500	Chemicon[a]	AB5800
NK3	Rabbit	1/500	Novus Biologicals[a]	NB300-102
TH	Mouse	1/10,000	Chemicon	MAB5280
ChAT	Sheep	1/5000	Chemicon	AB1582

[a] Chemicon International, Inc., Temecula, CA, USA; Novus Biologicals, Inc., Littleton, CO, USA.

Controls

NK1 antiserum was generated in guinea pig against a synthetic peptide corresponding to amino acids 393–407 of the C-terminus region of rat NK1. Specificity of the anti-NK1 guinea-pig antibody was tested on brain sections of NK1−/− mice. No immunoreactivity was visible on brainstem sections, while strong labeling was present on tissue from wild-type mice brains providing evidence that this antibody recognized NK1 (Fig. 1). NK3 antiserum was generated in rabbit against a synthetic peptide corresponding to amino acids 438–452 of the C-terminus region of rat NK3. Specificity was tested by Western blot and the antibody recognizes a 52 kDa band in rat brain as reported earlier (Ding et al., 1996; Fig. 1); the antibody has been successfully used in mouse tissues (Feigenspan et al., 2004). TH monoclonal antibody was obtained in mouse against purified TH from rat pheochromocytoma. Specificity was tested by Western blot and the antibody recognized a 56–60 kDa band from rat adrenal medulla peptide (manufacturer's technical information). ChAT antiserum was generated in sheep against a synthetic peptide corresponding to amino acids 168–189 of porcine ChAT. This antibody was quality control tested using standard immunohistochemical methods (manufacturer's technical information).

No staining corresponding to the specific labeling was observed when the primary antisera were omitted. For multiple labeling, the omission of one of the primary antisera in the presence of the fluorescent secondary antisera resulted in only the appropriate labeling; this demonstrated that no cross-reaction occurred during immunocytochemical processing.

Image acquisition

Confocal image acquisition was performed on a Leica TCS SP2 laser scanning microscope (Leica Microsystems, Heidelberg, Germany) using the 488 nm band of an Ar laser for excitation of Alexa Fluor 488 (spectral detection: 500–531 nm), the 543 nm band of an He–Ne laser for excitation of Cy3 (spectral detection: 565–635 nm) and the 633 nm band of an He–Ne laser for excitation of Cy5 (spectral detection: 650–750 nm). Image editing was performed using Adobe Photoshop (Adobe Systems France, Paris, France). Brightness and contrast were adjusted for a whole frame and no part of an image was modified separately.

Semi-quantitative analysis

We have differentiated two parts along the caudal–rostral extent of the NTS and DMV using the rostral border of the area postrema as the boundary between the caudal and rostral parts. In addition, we used the solitary tract as landmark to distinguish between the lateral and the medial parts of the NTS.

We have estimated the number of immunopositive cells throughout the caudal–rostral extent of NTS and DMV on nonadjacent sections of rat or mouse brainstem. Slightly overlapping optical sections were taken on confocal microscope from each 50 μm thick section in order to survey the mediolateral extent of the NTS and DMV, bilaterally. Counting was performed on single optical section of 800 nm theoretical thickness. Co-expression of NK1 and NK3 was analyzed in a total of six brainstem sections (three for caudal level and three for rostral level) per rat ($n=3$). NK1 or NK3 immunoreactive cell profiles were scored and tested for NK3 or NK1, respectively, as well as for ChAT immunoreactivity using a 40× objective (numerical aperture 1.2). Expression of NK1 and NK3 in TH positive cells was analyzed in a total of four sections (two for caudal and rostral level, respectively) per rat ($n=3$). NK3 or NK1 receptors immunoreactive cell profiles were scored and tested for TH immunoreactivity. The presence of NK1