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Distribution of neuropeptide W in the rat brain

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ABSTRACT

Neuropeptide W (NPW), which was recently isolated from the porcine hypothalamus, has been identified as the endogenous ligand of the orphan G protein-coupled receptors GPR7 (NPBWR1) and GPR8 (NPBWR2). Infusion of NPW increases food intake in the light phase, whereas in the dark phase, it has the opposite effect. In this study, we used RT-PCR analysis to examine the gene expression of NPW mRNA in the rat brain, and performed a detailed analysis of the distribution of NPW-positive neurons by use of immunohistochemistry at both the light and electron microscopic levels. NPW mRNA expression was demonstrated in the hypothalamic paraventricular nucleus (PVN), arcuate nucleus (ARC), ventromedial nucleus (VMH) and lateral hypothalamus (LH). At the light microscopic level, NPW-like immunoreactive (NPW-LI) cell bodies were found in the preoptic area (POA), PVN, ARC, VMH, LH, PMD (dorsal premammillary nucleus), periaqueductal gray (PAG), lateral parabrachial nucleus (LPB), and prepositus nucleus (Pr). NPW-LI axon terminals were shown in the POA, bed nucleus of the stria terminalis (BST), amygdala, PVN, ARC, VMH, LH, and PAG, LPB. In addition, at the electron microscopic level, NPW-LI cell bodies and dendritic processes were often seen to receive inputs from other unknown neurons in the ARC, PVN, VMH and amygdala. Our observations indicate that NPW-LI neurons widely distributed in the rat brain region. These finding suggest that NPW may have important roles in feeding behavior, energy homeostasis, emotional response and regulation of saliva secretion.

1. Introduction

O'Dowd et al. (1995) cloned two novel genes, GPR7 (NPBWR1) and GPR8 (NPWBR2), that encode opioid- and somatostatin-like orphan G-protein-coupled receptors (GPCRs) in the brain. NPBWR1 and NPBWR2 share 70% nucleotide and 64% amino acid identities with each other, with NPBWR1 being found in both humans and rodents whereas NPBWR2 is apparently expressed only in humans

Abbreviations: ac, Aterior commissure; aca, Aterior commissure, anterior part; acp, Aterior commissure, posterior part acp; AP, Area postrema; Aq, Aqueduct; Arc, Arcuate hypothalamic nucleus; ArcL, Arcuate nucleus, lateral part ArcL; BSTLP, Bed nucleus of the stria terminalis, lateral division, posterior part; BSTMA, Bed nucleus of the stria terminalis, medial division, anterior part; BSTMV, Bed nucleus of the stria terminalis, medial division, ventral part; BSTMPL, Bed nucleus of the stria terminalis, medial division, posterolateral part; Ce, Central amygdaloid nucleus; CeC, Central amygdaloid nucleus, capsular part; CeL, Central amygdaloid nucleus, lateral division; CeM, Central amygdaloid nucleus, medial division; cp, Cerebral peduncle, basal part; DLPAG, Ddorsolateral periaqueductal gray; DTM, Dorsal tuberomammillary nucleus; DRC, Dorsal raphe nucleus, caudal part; ec, External capsule; EW, Edinger–Westphal nucleus; f, Fornix; HDB, Nucleus of the horizontal limb of the diagonal band; ic, Internal capsule; ICj, Islands of Calleja; IPR, Interpeduncular nucleus, rostral subnucleus; LA, Lateroanterior hypothalamic nucleus; LGP, Lateral globus pallidus; LH, Lateral hypothalamic area; LPAG, Ventrolateral periaqueductal gray; LSV, Lateral septal nucleus, ventral part; Ml, Medial lemniscus; LDTg, Laterodorsal tegmental nucleus; MnR, Median raphe nucleus; MPA, Medial preoptic area; MPO, Medial preoptic nucleus; MPOC, Medial preoptic nucleus, central part; MRe, Mammillary recess of the 3rd ventricle; ox, Optic chiasm; opt, Optic tract; OPT, Olivary pretectal nucleus; PaAP, Paraventricular hypothalamic nucleus, anterior parvicellular part; PaMP, Paraventricular hypothalamic nucleus, medial parvicellular part; PaPo, Paraventricular hypothalamic nucleus, posterior part; PaV, Paraventricular hypothalamic nucleus, ventral part; Pe, Periventricular hypothalamic nucleus; PMD, Premammillary nucleus, dorsal part; RMg, Raphe magnus nucleus; Py, Pyramidal tract; pr, Prepositus nucleus; scp, Superior cerebellar peduncle; Sch, Suprachiasmatic nucleus; SO, Supraoptic nucleus; sox, Supraoptic decussation; st, Stria terminalis; VLPAG, Ventrolateral periaqueductal gray; VMPO, Ventromedial preoptic nucleus; VMHA, Ventromedial hypothalamic nucleus, anterior part; VTA, Ventral tegmental area; VP, Ventral pallidum; 7, Accessory abducens/facial nucleus; 12, Hypoglossal nucleus.

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(Lee et al., 1999). NPBWR1 and NPBWR2 mRNA has been detected at high levels in tissues of the human central nervous system (CNS) using RT-PCR analysis (Fujii et al., 2002). In particular, high levels of NPBWR1 mRNA were found in the hippocampus and amygdala (Brezillon et al., 2003) whereas, in the rat, strong NPBWR1 mRNA expression was detected in the hypothalamus and amygdala (Fujii et al., 2002). Similarly, *in situ* hybridization studies have revealed that NPBWR1 mRNA is present in the rat hypothalamus, including the arcuate nucleus (ARC), ventromedial nucleus (VMH), paraventricular nucleus (PVN), dorsomedial nucleus (DMH) and supraoptic nucleus (SON) (Lee et al., 1999), which are well known for their role in feeding regulation and energy homeostasis. Recently, several studies have reported the identification of two endogenous ligands for NPBWR1 and NPBWR2, neuropeptides W and B (NPW and NPB, respectively) (Fujii et al., 2002; Brezillon et al., 2003; Tanaka et al., 2003), which were isolated from the porcine hypothalamus. In the case of NPW, this has led to the characterization of two endogenous peptides consisting of 23- and 30-amino acid residues, termed neuropeptide W-23 (NPW23) and neuropeptide W-30 (NPW30), respectively. Synthetic NPW23 and NPW30 have been shown to be similarly effective in binding and activating both NPBWR1 and NPBWR2 (Shimomura et al., 2002; Tanaka et al., 2003). In *in vitro* experiments, human NPW23 and NPW30 were shown to have a similar potency in terms of activating human NPBWR1. In rat, NPBWR1 is expressed in the hypothalamus, including its feeding centers, and administration of NPW to NPBWR1 knockout mice results in hyperphagic and decreased energy expenditure effects, suggesting a modulatory role for NPW in the control of feeding regulation. Recently, a physiological study reported that the intracerebroventricular (icv) injection of NPW induced acute food intake for the first 2 h in the light phase (Shimomura et al., 2002). However, Mondal et al. (Mondal et al. (2003)) reported that icv infusion of NPW significantly reduced dark-phase feeding 4 h after administration, an effect that was maintained for 48 h at higher doses, concomitant with increased body temperature and heat production. Administration of NPW30 has also been shown to increase arterial blood pressure heart rate and plasma catecholamine levels in rats (Yu et al., 2007), with Baker et al. demonstrating that administration of NPW23 elevates prolactin, corticosterone (Baker et al., 2003), and growth hormone levels. In addition, Niimi and Murao (Niimi and Murao (2005)) have reported that icv administration of NPW results in significant Fos expression in the PVN, suggesting that NPW is involved in stress-responsive signal transduction, and may be a modulator of the hypothalamus–pituitary–adrenal axis. On the other hand, the presence of NPBWR1 in the PVN is suggestive of a role in the modulation of neuroendocrine functions. Finally, Yamamoto et al. (Yamamoto et al. (2005)) have demonstrated that intrathecal administration of NPW23 or NPB in rats also suppresses inflammatory pain. Overall, these various studies reveal that NPW is a multifunctional peptide that mediates a range of physiological outcomes.

Based on immunohistochemical analysis, NPW mRNA expression in the human CNS has been shown to be strongest in the substantia nigra, amygdala and hippocampus (Fujii et al., 2002; Singh et al., 2004). We (Takenoya et al., 2008), together with Dun et al. (Dun et al. (2003)) have also reported that NPW-like immunoreactive (NPW-LI) cell bodies are found in various regions of the rat brain, including the preoptic area (POA), PVN, SON, ARC, and dorsal and lateral hypothalamic areas, as well as the anterior and posterior pituitary gland. In contrast, Kitamura et al. (Kitamura et al. (2006)) reported expression in the Edinger–Westphal nucleus (EW), periaqueductal gray (PAG), lateral parabrachial nucleus (LPB), and medial parabrachial nucleus (MPB), but saw no NPW-LI cell bodies in the PVN. Given these somewhat conflicting results, the present study was designed to provide a detailed analysis of NPW expression in the rat brain. This was achieved by first determining NPW gene expression by reverse-transcription polymerase chain reaction (RT-PCR), followed by immunohistochemical localization of NPW-LI neurons and the neuronal network between NPW-containing neurons and other hypothalamic neurons at the light and electron microscopic levels.

2. Materials and methods

2.1. Animals

Male Wistar and Sprague–Dawley (SD) rats (Saitama Experimental Animal Supply, Saitama, Japan) weighing approximately 300 g were used. The animals were maintained on a 12/12 h light/dark cycle and supplied with standard laboratory chow and tap water *ad libitum*. All protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Showa University. Rats were placed under deep Nembutal anesthesia (40 mg/kg, i.p.) and colchicine (200 μg/25 μl saline) injected into the third ventricle of the brain. After 48 h, the rats were perfused through the ascending aorta with 50 ml of saline (37 °C), followed by 250–300 ml of 2% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) fixative for 20 min. The brains were enucleated, trimmed, and immersed in the same fixative for 12 h at 48 °C. After washing, the fixed brains were transferred to a solution containing 20% sucrose in 0.1 M PB for 2 days at 4 °C.

2.2. RT-PCR

The hypothalamic LH, ARC, VMH and PVN were punched out of the brain slices of the SD rats. The stomach was also taken from the SD rat. Total RNA was prepared from these tissues using TRIzol reagent (Invitrogen Corp), according to the manufacturer's protocol. Total RNA from the stomach was used as a positive control for NPW (Mondal et al., 2003, 2006, 2008). Total RNA was treated with DNase (Roche, Basel, Switzerland). RNA was quantified spectrophotometrically and confirmed by ethidium bromide staining of 18S and 28S ribosomal RNA after electrophoresis on 1.0% agarose/formaldehyde gel. Four micrograms of total RNA were converted into cDNA using oligo $d(T)_{12-18}$ primer (Invitrogen Corp.) and SuperScript III (Invitrogen Corp.) in a RT-reaction mixture (20 μl). PCR was performed using 0.8 μl of the RT-reaction mixture, 0.5 μM of each primer and AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster, CA, USA) in a total reaction volume of 25 μl. The primers used for rat NPW were 5′-GAGCTGTGGGAGGTA CGAAG-3′ and 5′-CTGACAGGATCGGCAAAGAT-3′ (GeneBank Accession No. AB084278). The PCR products were visualized by ethidium bromide staining under UV light following electrophoresis on a 1.5% agarose gel. After TA-cloning of PCR products, the nucleotide sequence of each inserted cDNA in the plasmid was confirmed by an automatic sequencing analyzer (ABI377, Applied Biosystems).

2.3. Preparation of sections

Rats were anesthetized with pentobarbital (50 mg/kg, Dainippon Pharmaceutical, Saitama, Japan) then perfused with saline, followed by 250–300 ml of 2% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). The brain was transferred to 2% PFA/PB overnight at 4 °C, after which it was transferred first to 0.1 M PB solution containing 20% sucrose at 4 °C and then to 0.1 M PB solution containing 30% sucrose at 4 °C. The fixed brain was finally embedded in O.C.T compound in liquid nitrogen-cooled isopentane and stored at −80 °C. Sections 7 μm-thick were subsequently cut by cryostat (MICROM HM 500; MICROM, Heidelberg, Germany), and used for immunohistochemistry.