Oxytocin innervation of spinal preganglionic neurons projecting to the superior cervical ganglion in the rat

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Abstract. The paraventricular nucleus of the hypothalamus is a major integrative nucleus for relaying information from the suprachiasmatic nucleus to the autonomic system. The precise pathway by which this information can influence autonomic functions, such as melatonin synthesis in the pineal gland, is not clear. In the present study, we used a retrograde tracer injected in the superior cervical ganglion to identify spinal preganglionic neurons. One of the main neurotransmitters present in descending projections of the paraventricular nucleus of the hypothalamus, oxytocin, was detected with immunocytochemistry to visualise possible contacts with the neurons located in the intermediolateral column of the spinal cord and projecting to the superior cervical ganglion. Although many appositions could be seen at the light-microscopic level, this abundance could not be confirmed at the electron-microscopic level. The implications of these observations for the overall timing message received by the spinal preganglionic neurons are discussed.

Key words: Oxytocin – Immunocytochemistry – Paraventricular nucleus – Superior cervical ganglion – Spinal cord – Sympathetic nervous system – Retrograde tracing – Rat

Introduction

Photoperiodic information is conveyed to the organism through the rhythmic release of the pineal hormone, melatonin (Mel). In mammals, light information is received by the retina and directly relayed via the retinohypotha-

lamic tract, to the suprachiasmatic nucleus (SCN), which contains the biological circadian clock. Subsequently, this information is transmitted from the SCN to the pineal gland via a polysynaptic pathway comprising the dorsal hypothalamus, the spinal cord and the superior cervical ganglion (SCG). The important role of the paraventricular nucleus of the hypothalamus (PVN) as a relay station for SCN output has been illustrated by a number of lesion experiments (Klein et al. 1983; Lehman et al. 1984; Bartness et al. 1985; Hastings and Herbert 1986; Brown et al. 1988). Among the neurotransmitters identified in the PVN, oxytocin (OXT) has been localized in 11–16% (Sawchenko and Swanson 1982) and 20–25% (colchicine treatment; (Cechetto and Saper 1988) of spinal projecting neurons. Moreover, lesions of the PVN resulted in a severe decrease of OXT content in the spinal cord (Lang et al. 1983; Hawthorn et al. 1985). OXT is therefore a good marker for PVN efferents to the spinal cord. It is assumed that hypothalamic information may reach the sympathetic input neurons of the pineal gland located in the SCG through descending projections of the PVN to the sympathetic preganglionic neurons (SPNs; Saper et al. 1976; Badura et al. 1989; Smale et al. 1989) located mainly in the intermediolateral cell column (IML) in lower cervical and upper thoracic segments of the spinal cord. Hosoya et al. (1991) showed in the rat, by means of double tracing, that there is a clear coincidence of the location in the spinal cord of PVN efferent fibres identified by anterograde tracing and preganglionic neurons in the spinal cord identified by retrograde tracing from the superior cervical ganglion. For the present study, we attempted to identify one of the PVN transmitters that might be involved in this pathway by means of OXT staining to establish synaptic contacts at the electron-microscopic level.

Materials and methods

Fifteen adult male rats were used for this study. They were anaesthetised with Hypnorm. The left SCG was approached ventrally from a mid-line incision in the neck. For electron microscopy,

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Fig. 1. Horizontal section of the spinal cord showing cholera-toxin b-subunit (Ctb)-labelled sympathetic preganglionic neurons (SPNs; *brown*) and oxytocin OXT-containing nerve fibres (*black*).

Labelled SPNs receive numerous appositions from OXT-containing fibres (*arrows*). Bar: 80 µm

0.1–0.5 µl of horseradish peroxidase (HRP; Sigma type IV) 50% in phosphate buffer 0.1 M, pH 7.4, was slowly pressure-injected into the SCG with a glass pipette. The glass pipette was left in place for 5 min to prevent leakage. For light microscopy, 1 µl of cholera toxin b subunit 1% (Ctb; Sigma) in phosphate buffer 0.1 M, pH 7.4, was injected iontophoretically (positive current of 7 µA for 15 min, on/off for 7 s) into the SCG. To control for the possibility of leakage of the tracer, 0.1–0.5 µl of Ctb or HRP was delivered (1) onto the ganglion without the glass pipette entering, or into the ganglion (2) after the cervical sympathetic nerve was cut or (3) with the cervical sympathetic nerve cut immediately after the injection.

The animals were allowed to recover for 2–4 days. They then received an overdose of sodium pentobarbital (Nembutal) and were perfused intracardially with 0.9% NaCl, followed by 1% paraformaldehyde and 3% glutaraldehyde in phosphate-buffered saline, 0.1 M, pH 7.4. The spinal cord was exposed by laminectomy, cut into segments using the midpoint between the entry of two adjacent ventral roots as rostral and caudal limits and postfixed for 1 h in the same fixative. To enhance the fixation, microwave treatment was used as described by Buijs et al. (1993). After removing the dura from the surface, 50-µm-thick transversal sections were cut on a Vibratome and saved in TRIS-HCL buffered saline (TBS) 0.05 M, pH 7.6.

Sections from animals injected with Ctb were incubated overnight at 4° C with rabbit anti-Ctb (List Biological) 1:10000 in TBS with 0.5% Triton. They were then successively incubated with biotinylated sheep anti-rabbit (Vector) 1:500 and ABC complex (Vector) 1:1000 in TBS with 0.5% Triton for 1 h. In between each incubation the sections were rinsed 3×10 min with TBS. The sections were finally reacted with 0.025% DAB with 0.01% H₂O₂ in TBS with 0.5% ammonium nickel sulfate.

Sections from animals injected with HRP were prepared for electron microscopy as follows: The sections were incubated for 30 min in 0.025% DAB with 0.01% H₂O₂ in TBS. DAB staining was intensified with silver substituted with gold according to the following protocol: (1) 3×10 min sodium acetate 2%; (2) incubation in a solution consisting of 150 ml 3% methenamine, 20 ml 5% silver nitrate and 20 ml 1% sodium tetraborate for 5 min at 60° C, mixed thoroughly until disappearance of white precipitate just before utilization; (3) 3×10 min sodium acetate 2%; (4) 5 min 0.1% gold chloride; (5) 3×10 min sodium acetate 2%; (6) 5 min sodium thiosulphate 3%; (7) 3×10 min sodium acetate 2%; (8) rinse in TBS. After several rinses, the sections were incubated in 1% NaBH₄ for 10 min. After thorough rinsing they were incubated in 50% ethanol.

All sections were incubated overnight in rabbit anti-OXT (O2T, Netherlands Institute for Brain Research) 1:2000 in TBS with 0.25–0.5% Triton, at 4° C. Specificity controls have been de-

Fig. 2A–C. Electron micrographs through the intermediolateral cell column. **A** HRP-labelled cell body and dendrites. An HRP-containing dendrite (*asterisk*) in an apposition to an HRP-containing soma. HRP is contained in large endocytotic vesicles (*arrows*). **B** Enlargement of the HRP-containing dendrite shown by asterisk in **A** in contact with an HRP-containing cell body. An HRP-containing granule is indicated by the *arrow*. Arrowheads indicate the membrane specialization. **C** OXT-containing profile showing diffuse DAB staining (*open arrow*) in close proximity to a dendrite containing HRP vesicles which are stained with DAB intensified with silver (*full arrow*). Bars: **A** 2 µm, **B** 0.6 µm, **C** 0.7 µm

scribed elsewhere (Buijs et al. 1990). The sections were then rinsed and incubated with biotinylated sheep anti-rabbit (Vector) for 1 h, followed by ABC complex for 1 h. They were finally incubated with 0.025% DAB with 0.01% H_2O_2 in TBS for 30 min. Representative sections were mounted on gelatin-coated slides, dehydrated in a graded series of ethanol, cleared in xylene, coverslipped in Eukitt and viewed with a light microscope. The rest of the sections were postfixed for 30 min in 1% osmium tetroxide and 1.5% potassium ferrocyanide in 0.1 M cacodylate buffer, dehydrated in ethanol and embedded flat in Epon resin between two glass slides. The IML was cut out and glued on an empty block. A minimum of one Vibratome section per spinal segment was used. Before sectioning, the block was further trimmed to make sure of the location within the IML. Ultrathin sections (silver) were made by use of a microtome and viewed with an electron microscope (CM10, Philips).

Results

Retrogradely labelled neurons after a Ctb injection in the SCG were found in the spinal segments T1 to T4. They occurred mainly in the IML, the lateral funiculus (LF; Fig. 1), the intercalated nucleus (IC) and the central autonomic area (CA). Many thin varicose OXT-containing fibres were found in the IML stretching to the LF (Fig. 1). Fibres could be seen encircling the central canal and in the intermediate grey linking the CA and the IML. They were observed in apposition to cell bodies and dendrites of retrogradely labelled neurons in the IML and LF (Fig. 1). Ultrastructural observation of retrograde labelling was restricted to the IML after HRP injection into the SCG. The location of HRP-labeled SPN in the spinal cord was similar to that observed after Ctb injection, showing that the extent of uptake of HRP by en-passant fibres in the SCG was very limited. A great number of ultrathin sections obtained from Vibratome sections from different animals were found to contain HRP in large, probably endocytotic, vesicles in the cell bodies and dendrites (Fig. 2A, B). Silver staining enhancement was restricted to these vesicles. HRP-containing profiles could be seen close to HRP-containing cell bodies (Fig. 2A), and in some cases specialised contacts could be observed (Fig. 2B). Axonal profiles containing OXT staining appeared faint, and the DAB precipitate was diffuse (Fig. 2C) and could be easily distinguished from HRP labelling; however, only in the range of the first few micrometers of the Vibratome section. Therefore, we examined only the first few ultrathin sections from each spinal cord section. For the same reason, no semithin sectioning was done. OXT-containing profiles could be seen in the IML close to HRP-labelled dendrites (Fig. 2C) and somata. Although many sections were observed, a count of labelled profiles in the IML was done on only three consecutive ultrathin sections from one Vibratome section from each of two different animals to get a rough estimate. Among 17 OXT profiles, 12 could be seen to have a synaptic contact on an unidentified dendrite, 2 were close to HRP-containing dendrites and on only one occasion could we see a synaptic specialisation between an HRP soma and an OXT-containing profile (not shown). Four HRP-labelled somata (two per animal) were observed and 21 HRP-containing profiles, among

which 10 were seen to have a synaptic contact with an unidentified fibre, 4 did not have any membrane specialisation and 7 were myelinated.

Discussion

An intact PVN-spinal cord pathway is necessary for the generation of a rhythm of Mel synthesis (Kneisley et al. 1978; Badura et al. 1989; Smale et al. 1989). Several studies have shown the existence of a direct PVN-spinal cord projection employing electrophysiological techniques (Yamashita et al. 1984; Caverson et al. 1984), immunocytochemistry (Swanson 1977; Buijs 1978) or neural tracing (Hosoya and Matsushita 1979; Swanson and Kuypers 1980; Cechetto and Saper 1988; Pretel and Piekut 1989). Electrical stimulation of the PVN inhibits Mel synthesis of rats during the night (Reuss et al. 1985; Olcese et al. 1987). Total lesions of the PVN abolish the rhythmic pattern of Mel synthesis (Klein et al. 1983). Gilbey et al. (1982) showed that the effect of an infusion of OXT on identified SPN is the same as an electrical stimulation of the PVN. The fact that lesions of the PVN induce a dramatic decrease in the vasopressin and OXT content in the spinal cord (Hawthorn et al. 1985; Lang et al. 1983) clearly indicates that OXT is a component of this descending pathway. OXT-containing fibres in the IML of the rat and of the cat are found in restricted areas (rat: Buijs 1978; Swanson and McKellar 1979; cat: Krukoff 1987). This does not hold true for other peptides that can be found in different amounts along the full extent of the thoracolumbar spinal cord. This fact favours the notion of a specific pathway, containing OXT and extending from the PVN to a discrete subset of SPNs. The presence of localised OXT binding sites in the thoracic spinal cord (Reiter et al. 1994) further indicates that one of the transmitters from the PVN to the spinal cord could well be OXT.

In the present study, the simultaneous application of a retrograde tracer and OXT immunocytochemistry showed at the light-microscopic level that SPNs projecting to the SCG were in apposition with OXT-containing fibres. These appositions were verified under the electron microscope. However, their apparent abundance could not be confirmed at the electron-microscopic level. A reason for this low number of labelled synaptic contacts may be the limited penetration of the OXT antibody in the section. The number of OXT-containing fibres is not the limiting factor, since we could see many OXT-containing fibres in apposition with SPNs. Moreover, Appel and Elde (1988), by use of fluorogold applied to the cut end of the cervical sympathetic trunk innervation the SCG, showed that, at the light-microscopic level, OXT-containing fibres form appositions on approximately 25% of labelled SPNs. Furthermore, our findings agree with data recently published by Hosoya et al. (1995), who also failed to see synaptic contacts between retrogradely labelled SPNs and OXT fibres inside the IML. Hosoya et al. (1995) showed that synaptic contacts between OXT-containing fibres and SPNs occur frequently outside the IML, within the transverse den-

dritic bundles and in the CA. The pattern of the innervation of SPNs seems to depend on the transmitter studied. The innervation by serotonin- (Jensen et al. 1995) and substance P (Bacon and Smith 1988); containing elements is somatic and dendritic, whereas catecholaminergic innervation is almost exclusively dendritic (Milner et al. 1988). Most of these neurotransmitters were found to provide a restricted innervation to SPNs compared with the total number of synapses. Scarce innervation and specific targetting of the SPNs may indicate that several transmitters, such as OXT, substance P or serotonin, are mainly modulatory. Nevertheless, their message can be overridden by strong inhibition from, for example, GABA, which is present in one-third of the synapses (Bacon and Smith 1988), or excitation from glutamate, present in two-thirds of the synapses or appositions on SPNs (Llewellyn-Smith et al. 1992). This would be in accord with the idea that timing information conveyed by OXT innervation does not trigger autonomic functions but modulates them, according to the „day“ or „night“ information OXT neurons receive via the SCN. In accordance with this, in SCN-lesioned animals Mel is still synthesised at a high basal level but not in a rhythmic way (Kalsbeek et al. 1996). Therefore, OXT, although present in a high number of spinal cord projecting neurons (Sawchenko and Swanson 1982; Chechitto and Saper 1988), might not be the major transmitter for conveying photoperiodic information to the pineal gland. Hosoya et al. (1991) showed that as a whole, only a small number of dendrites were studded by varicosities or terminals of PVN fibres, whereas others displayed hardly any appositions. Serotonin-containing afferents to SPNs projecting to the SCG appear to innervate some neurons very densely, and others hardly at all (Jensen et al. 1995), indicating the high level of target specificity. Contradictory electrophysiological results show that vasopressin and OXT have either an excitatory (Backman and Henry 1984), or an inhibitory effect (Gilbey et al. 1982) on IML neurons. These results suggest that these peptides may also affect interneurons that contact the SPNs. Indeed, the existence of a network of interneurons in connection with SPNs projecting to the SGC has been shown (Cabot et al. 1994). These can be inhibitory glycine-containing (Campistron et al. 1986; Cabot et al. 1992) or excitatory glutamate-containing (Llewellyn-Smith et al. 1992) interneurons. Such microcircuits, combined with region-specific targetting, would allow greater flexibility in the way the central nervous system can influence SPNs. Reciprocal connections between SPNs as shown in our results and previous studies (Vera et al. 1986) add to the complexity of the regulation of the activity of SPNs.

Another effect probably mediated by the SCN-pineal pathway, as described previously, is more acute. A light pulse during the night immediately inhibits the synthesis of Mel in the pineal gland (Reiter et al. 1986). A direct SCN GABA-containing projection to the dorsal hypothalamus has been described (Buijs et al. 1994), and microdialysis studies suggest that this projection transmits a fast signal that inhibits the Mel signal (Kalsbeek et al. 1996). It is possible that GABA acts directly on spinal-

projecting PVN neurons. The effect of acute light pulses during the night would then be further processed in the PVN, shutting down the whole pathway. However, the neurotransmitter content of the SCN input on spinal-projecting PVN neurons needs to be demonstrated in further detail to support this hypothesis.

In conclusion, OXT afferents to SPNs are located primarily on dendrites and may involve, in addition, inhibitory and excitatory interneurons. The control by the SCN of autonomic functions can thus be translated into finely tuned SPN activity according to the time of day, resulting in a rise or fall in Mel synthesis in the pineal gland.

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