The thalamic paraventricular nucleus relays information from the suprachiasmatic nucleus to the amygdala: A combined anterograde and retrograde tracing study in the rat at the light and electron microscopic levels

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Abstract

The relationship between efferents of the hypothalamic suprachiasmatic nucleus (SCN) and neurons of the thalamic paraventricular nucleus (PVT) projecting to the amygdala was investigated in the rat using tract tracing in light and electron microscopy. Biotinylated dextran amine was used to label anterogradely SCN efferents. These fibers were found to reach the thalamic midline, terminating in PVT, through three pathways: anterodorsally through the preoptic region, dorsally through the periventricular hypothalamus, and through the contralateral medial hypothalamic and preoptic areas after crossing the midline in the optic chiasm. Preterminal and terminal-like elements labeled from the SCN were distributed throughout the rostrocaudal extent of PVT, with an anteroposterior gradient of density. Labeled terminal elements were densest in the dorsal portion of PVT beneath the ependymal lining and some of them entered the ependyma. Anterograde tracing of SCN fibers was combined with injections of retrograde tracers in the amygdala. Numerous retrogradely labeled cell bodies were seen throughout PVT, with a prevalence in its anterodorsal portion. Overlap was detected between puncta labeled from the SCN and retrogradely labeled neurons, especially in the anterodorsal sector of PVT, where numerous puncta were in close apposition to thalamo-amygdaloid cells. Electron microscopy revealed that boutons labeled from the SCN established synaptic contacts with dendritic profiles of PVT neurons labeled from the amygdala. The findings demonstrate that information processed in the biological clock is conveyed to the amygdala through PVT, indicating that this nucleus plays a role in the transfer of circadian timing information to the limbic system.

Introduction

The thalamic paraventricular nucleus (PVT) is the most dorsal component of the thalamic midline group of nuclei, and extends through most of the anteroposterior extent of the thalamus, lining the dorsal third ventricle. It has been suggested that PVT is a highly conserved structure through phylogeny, representing the homologue to the dorsomedial thalamic nucleus of reptiles (Hereida et al., 2002), and PVT is a stable thalamic structures through mammalian evolution (see for review Bentivoglio et al., 1993). Together with the other thalamic midline nuclei, PVT has been traditionally considered part of the so-called thalamic “nonspecific” system, characterized, among other features, by widely divergent cortical projections (Bentivoglio et al., 1991). However, PVT is now known to be composed by a mosaic of cell groups whose main targets are represented by limbic and limbic-related cortical and subcortical regions, and in particular the amygdala, nucleus accumbens and prefrontal cortex (see for review Bentivoglio et al., 1993). It has been reported that PVT is a crucial structure in the representation of motivational drives (see for review Sowards & Sowards, 2003). PVT projections converge with dopamine fibers on neurons of the nucleus
accumbens (Pinto et al., 2003). It has also been shown (Moga et al., 1995; Krout et al., 2002) that PVT neurons project to the hypothalamic suprachiasmatic nucleus (SCN), which plays a role of circadian pacemaker in the mammalian brain.

Besides the corticothalamic input, PVT receives multiple subcortical afferents, deriving mainly from sources in the brainstem and diencephalon (reviewed by Bentivoglio et al., 1991). Among these, increasing evidence indicates that PVT receives information from the centers of the circadian timing system involved in the entrainment of endogenous biological rhythms, including the SCN and the intergeniculate leaflet (Moga et al., 1995; see also Moore, 1996 for review). In particular, PVT, and especially the anterior portion of the nucleus (aPVT), is the major extrahypothalamic target of the efferent projections of the SCN (Watts et al., 1987; Kalsbeek et al., 1993). PVT is therefore considered one of the effectors of the circadian timing system (Leak & Moore, 2001). However, the targets of cell populations of the thalamic midline recipient of SCN input have not been hitherto determined.

The present study was designed to verify direct interconnections between SCN efferents and the PVT neurons projecting to the amygdala. To this purpose, we used a combined anterograde-retrograde tracing strategy in light microscopy (LM) and electron microscopy (EM). Biotinylated dextran amine (BDA) was used to label PVT neurons projecting to the amygdala. Two different retrograde tracers, namely wheat germ agglutinin conjugated with enzymatically inactivated horseradish peroxidase and with colloidal gold (Au) and the fluorescent tracer Fluoro-Gold (FG), were used for pilot experiments, a single BDA injection was made in the SCN to test the sensitivity of different protocols for the visualization of BDA reaction products. Combined injections of BDA in the SCN and FG (Watts et al., 1992), whereas an immunohistochemical protocol (Chang et al., 1990) was exploited to visualize FG labeling in PVT neurons.

### Materials and methods

#### ANIMALS AND SURGERY

This study was based on a total of 24 adult male Wistar rats, selected from a larger series of experiments on the basis of successfully targeted tracer injections. The rats, weighing 200–350 g, were kept in basal conditions (12 h/12 h light-dark cycle; food and water freely available). The experiments were performed following the European Community Council Directive (86/609/EEC), with protocols approved by the Italian Ministry of Health and NIH (Animal Welfare Assurance A5155-01). For injections of tracers, the animals were anesthetized with barbiturate (pentobarbital, 50 mg/kg, i.p.) and placed on a stereotaxic instrument. Holes were opened in the skull by a dental drill at the desired coordinates using the atlases of Paxinos and Watson (1986) and Swanson (1992). Special care was taken to avoid tearing of the sagittal sinus, which may result in fatal bleeding and tissue disruption while opening the bone along the midline.

The tracers were injected using pipettes with a tip diameter of 30 µm. BDA (Molecular Probes Inc., Eugene, OR, USA) was dissolved at a 10% concentration in 0.01 M phosphate-buffered saline (PBS), pH 7.2. An aqueous solution of FG (Fluorochrome, Inc., Englewood, CO, USA; 2% in distilled water) was used. For the preparation of Au, wheat germ agglutinin conjugated with enzymatically inactive horseradish peroxidase was purchased from Sigma (St. Louis, MO, USA) and was conjugated with colloidal gold following the protocol of Basbaum and Ménetéry (1987).

Injections of Au, whose diffusion at the injection site is very limited, were performed by pressure in the amygdala at a rate of 0.05 µl per min, up to a total volume of 0.4 µl. In order to obtain restricted injections of the other tracers, iontophoretic administration of BDA in the SCN and of FG in the basal amygdaloid nucleus was made using 5 µA positive current, 7 sec on/7 sec off, for 20 min. In four animals (rats 1–4, Table 1), used for pilot experiments, a single BDA injection was made in the SCN to test the sensitivity of different protocols for the visualization of BDA reaction products. Combined injections of BDA in the SCN and FG (n = 4) or Au (n = 16) in the amygdala were performed in the other animals (Table 1). After surgery, the rats were returned to the animal room and allowed to survive for 10 to 24 days.

### Table 1. Experimental parameters.

<table>
<thead>
<tr>
<th>Animals: total number (code)</th>
<th>Fixative*</th>
<th>BDA ant. labeling from SCN: Protocol**</th>
<th>Retr. labeling from amygdala</th>
<th>Tracer</th>
<th>Protocol</th>
<th>EM</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 (1–4)</td>
<td>4% para</td>
<td>A, B, C</td>
<td>Au</td>
<td>Silver enhancement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 (5–15)</td>
<td>4% para</td>
<td>C</td>
<td>Au</td>
<td>Silver enhancement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 (26,27,30,32,35,36)</td>
<td>4% para + 0.5% glut</td>
<td>C for LM, B for EM</td>
<td>Au</td>
<td>Silver enhancement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>41 (43,46,48)</td>
<td>4% para + 1% acrolein</td>
<td>B</td>
<td>FG</td>
<td>Immunostaining (A)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Diluted in 0.1 M phosphate buffer, pH 7.4
** Protocol A: standard 3,3’-diaminobenzidine (DAB) reaction; Protocol B: nickel-intensified DAB reaction; Protocol C: tetramethylbenzidine-sodium tungstate + nickel-intensified DAB reaction (see text).

Abbreviations: ant, anterograde; Au, wheat germ agglutinin conjugated with enzymatically inactivated horseradish peroxidase and with colloidal gold; BDA, biotinylated dextran amine; EM, electron microscopy; LM, light microscopy; FG, Fluoro-Gold; glut, glutaraldehyde; para, paraformaldehyde; retr, retrograde; SCN, suprachiasmatic nucleus.