Chapter 3

The Pontine Micturition Center Projects to Sacral Cord GABA Immunoreactive Neurons in the Cat

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ABSTRACT

Stimulation of the pontine micturition center (PMC) results in micturition, i.e. an immediate relaxation of the external urethral sphincter (EUS) and a contraction of the detrusor muscle of the bladder. Earlier studies have shown that the bladder contraction is brought about by a direct excitatory pathway from the PMC to the parasympathetic preganglionic bladder motoneurons in the sacral cord. How the PMC produces the inhibition of the EUS is not known.

The present study in two adult male cats demonstrates at the ultrastructural level a direct pathway from the PMC to the dorsal gray commissure of the sacral cord. More than half (55%) of these terminals made contact with $\gamma$-amino butyric acid (GABA) immunoreactive dendrites or somata, the others with non-GABA immunoreactive profiles. The PMC terminals contained many round vesicles, some dense cored vesicles and exclusively asymmetric synaptic clefts, which corresponds with an excitatory pathway. A concept is put forward in which this pathway produces the relaxation of the EUS during micturition.

INTRODUCTION

Micturition depends on a coordinated action of the smooth detrusor muscle of the bladder and the striated external urethral sphincter (EUS), which closes the bladder. During urine storage, the bladder is relaxed and the EUS is tonically contracted. When micturition takes place, this activation pattern is reversed, i.e. the bladder contracts and the EUS relaxes, resulting in elimination of urine.

The bladder and the bladder sphincter are innervated by respectively the sacral cord parasympathetic motoneurons (De Groat et al., 1981) and the motoneurons in the ventrolateral part of the nucleus of Onuf (Kuzuhara et al., 1980). The coordination between these two motoneuronal cell groups during micturition does not take place in the spinal cord, but in the caudal brainstem (Blaivas, 1982; Holstege et al., 1986; Blok et al., 1997).

Tracing studies in rat (Loewy et al., 1979) and cat (Holstege et al., 1979; Holstege et al., 1986; Blok and Holstege, 1997) have shown that a distinct cell group in the dorsal pontine tegmentum, the pontine micturition center (PMC), projects to the parasympathetic bladder motoneurons in the sacral intermediolateral cell group (IML), as well as to the dorsal gray commissure (DGC), but not to the nucleus of Onuf. Stimulation in the PMC results in an immediate and sharp decrease in the urethral pressure and pelvic floor electromyogram (EMG), followed in about 2 sec by a steep rise in the intravesical pressure, mimicking complete micturition (Holstege et al., 1986). The increased bladder pressure during this stimulation is probably caused by a direct projection from the PMC to bladder motoneurons, because the PMC terminals on bladder motoneuronal somata and dendrites contain round vesicles and have an asymmetric cleft, which corresponds with an excitatory func-
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Fig. 1. Schematic drawings of WGA-HRP injections in the dorsolateral pontine tegmentum in the two cats. The PMC is indicated by the dark cells (on the basis of Blok and Holstege, 1994).

tion (Blok and Holstege, 1997). However, this does not explain the decrease in the urethral pressure during micturition, because a direct PMC projection to the nucleus of Onuf does not exist (Holstege et al., 1979; Holstege et al., 1986).

The PMC, in order to inhibit the bladder sphincter, might use inhibitory interneurons as relay to the sphincter motoneurons in Onuf’s nucleus. In this respect the following observations are important: 1. Onuf’s nucleus receives a relatively large number of $\gamma$-amino butyric acid (GABA) immunoreactive terminals (Ramirez-Léon et al., 1994); 2. Almost all Onuf’s nucleus afferents from the lumbosacral cord originate from neurons in the DGC (Konishi et al., 1985; Nadelhaft and Vera, 1996), a few from the IML (Nadelhaft and Vera, 1996) but none from other areas in the lumbosacral cord (Holstege and Tan, 1987; Nadelhaft and Vera, 1996); 3. Many GABA immunoreactive neurons are located in the DGC and a few in the IML (Alvarez et al., 1996); and 4. The PMC projects directly to the DGC and IML bilaterally (Holstege et al., 1979; Loewy et al., 1979; Holstege et al., 1986).

In our concept the PMC uses GABA-ergic relay cells in the DGC, and probably also the few GABA-ergic cells in the IML, to inhibit the bladder sphincter motoneurons in Onuf’s nucleus. If that is true there must exist a direct PMC excitatory projection to GABA immunoreactive neurons in the DGC, the demonstration of which is the object of this study.

MATERIALS AND METHODS

Two adult male cats were used. The PMC was injected with the anterograde tracer wheat germ agglutinin horseradish peroxidase (WGA-HRP) to anterogradely label PMC terminals in the IMM. The surgery procedures, pre- and postoperative care, and handling and housing of the cats were in accordance with protocols approved by the Committee on Animal Experiments of the Faculty of Medicine of the University of
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Groningen. During surgery, heart rate and body temperature were monitored. The animals (weight 4.0 and 4.4 kg) were sedated with ketamin (30 mg/kg) intramuscular, followed by additional doses of 15 mg/kg as necessary.

In order to properly place the needle in the PMC, it was identified by means of electrical stimulation prior to the WGA-HRP injection (for details see reference 4). At the optimal stimulation site 40 nl 2.5% WGA-HRP was injected. Three days after the WGA-HRP injection, the animals were deeply anaesthetized with 60 mg/kg sodium pentobarbital, perfused intracardially with 1.5 l heparinized saline (pH 7.4) at room temperature, followed by 1.5 l fixative containing 1% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4, room temperature). The spinal cords were removed and placed in the same fixative for 2 hours at 4°C. The brainstems were dehydrated overnight in 25% sucrose in 0.1 M phosphate buffer (pH 7.4), and the next day the tissue was frozen to -55°C in a isopentane bath and cut in 40 µm sections on a cryostat. One out of four sections were incubated according to the diaminobenzidine (DAB; Sigma, USA) method to determine the extent of the injection site. Ultrathin sections were collected on formvar-coated nickel grids and processed for the ultrastructural localization of GABA (De Zeeuw et al., 1988). The ultrathin sections were etched in 1% periodic acid and 1% sodium periodate and incubated for 2 hours at room temperature with 1:1000 rabbit GABA antiserum (Sigma, USA). After rinsing, the sections were incubated for 1 hour with 1:30 goat anti-rabbit IgG coupled to colloidal gold particles (15 nm; Aurion, The Netherlands). Method specificity was controlled by omitting the primary antibody. After contrasting with uranyl acetate for 2 minutes and lead citrate for 1 minute, the sections were studied with an electron microscope.

RESULTS

The injection sites of the cases 2350 and 2366 were placed in the left dorsolateral pontine tegmentum, including the PMC (Fig. 1). At the light microscopic level, a large number of anterogradely labeled fibers and presumptive terminal labeling was observed bilaterally in the sacral IML and DGC (Fig. 2). At the ultrastructural level numerous anterogradely labeled terminals with WGA-HRP crystals were found bilaterally in the sacral DGC. These terminals were filled with many round and occasionally dense core vesicles and were often in close apposition with dendrites or somata. When a synaptic cleft was present it was always asymmetric, and no labeled profiles with a symmetric cleft were found. In both cases WGA-HRP labeled terminals were found contacting dendrites or somata immunoreactive for GABA. The GABA immunoreactive somata contained many immunoreactive particles (Fig. 3 left), but their dendrites contained only a few (5-10; Fig. 3 right), which is normal for dendrites of

![Fig. 2. Polarized light photomicrograph of a section of the second sacral segment of case 2366. Anterogradely labeled fibers are visible in the IML and the DGC. Bar represents 300 µm.](image)
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Table 1 Number of WGA-HRP labeled terminals on GABA immunoreactive profiles

<table>
<thead>
<tr>
<th>WGA-HRP labeled terminals</th>
<th>Case 2350</th>
<th>Case 2366</th>
<th>Total</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA immunoreactive dendrites</td>
<td>29</td>
<td>24</td>
<td>53</td>
<td>49</td>
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<td>GABA immunoreactive somata</td>
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<td>6</td>
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<tr>
<td>GABA negative profiles</td>
<td>21</td>
<td>28</td>
<td>49</td>
<td>45</td>
</tr>
<tr>
<td>Total</td>
<td>54</td>
<td>55</td>
<td>109</td>
<td>100</td>
</tr>
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GABA immunoreactive neurons (De Biasi et al., 1997). Since the background labeling in our preparations was extremely low, it was not necessary to make a statistical analysis of the distribution of gold particles to judge a profile as labeled or not (see also De Biasi et al., 1997). A selected area of the DGC was screened on the presence of WGA-HRP positive terminals with a synaptic cleft, and their postsynaptic contacts were characterized (Table 1). In each maze (=10,000 µm²), 2 to 3 WGA-HRP positive terminals were found to contact GABA immunoreactive profiles. A total of 109 labeled terminals were found, of which more than half (60 of 109 =55%) contacted GABA immunoreactive profiles.

Fig. 3. Left: Electron microscopic photograph of a WGA-HRP labeled terminal (asterisk) making contact with a GABA immunoreactive dendrite (PSD = postsynaptic dendrite) in the second sacral segment of case 2366. Arrowheads indicate anti-GABA immunogold particles. Right: Electron microscopic photograph of an anterogradely labeled terminal (asterisk) making contact with a GABA immunoreactive soma in the second sacral segment of case 2350. S = GABA immunoreactive soma. Bar represents 400 nm.
DISCUSSION
The results are in agreement with the concept that the PMC produces micturition via a direct excitatory connection with the bladder detrusor muscle motoneurons and with the GABA immunoreactive inhibitory interneurons in the DGC (Fig. 4). Although not yet demonstrated, it seems likely that the PMC fibers to the DGC GABA immunoreactive neurons are collaterals of PMC fibers to the IML (Nadelhaft and Vera, 1996). Theoretically, it is possible that the GABA immunoreactive DGC cells, which receive afferents from the PMC do not project to Onuf’s nucleus. However, this seems extremely unlikely, because almost all premotor interneurons projecting to Onuf’s nucleus are located in the DGC, and a few in the IML. Other sources of GABA immunoreactive innervation of the Onuf’s nucleus do not exist, because it is not affected by spinal cord transection rostral to the sacral cord or dorsal rhizotomy (Ramirez-Léon et al., 1994).
Thus, although GABA immunoreactive neurons are also located in the dorsal horn, the GABA immunoreactive premotor interneu-

Fig. 4. Schematic overview of the pathways involved in the control of the bladder and bladder sphincter motoneurons during micturition. Pathways are indicated on one side only.
rons projecting to Onuf’s nucleus, must be located in the DGC and to a very limited extend in the IML. Both regions receive PMC projections. This leads to the concept that the PMC excites the bladder motoneurons via a direct pathway to their parasympathetic motoneurons, and inhibits the bladder sphincter motoneurons via an excitatory projection to the GABA-ergic premotor interneurons of Onuf’s nucleus.