A nitricergic projection from the superior olivary complex to the inferior colliculus of the rat

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Abstract

The present study was conducted to test whether the ascending auditory projection from the superior olivary complex (SOC) of the brainstem to the inferior colliculus (IC) may use nitric oxide (NO) as a neuroactive compound. We identified olivo-collicular projection neurons in subnuclei of the SOC by retrograde neuronal tracing with Fluoro-Gold (FG) injected into the central nucleus of the IC. Sections containing retrograde labelled neurons were subjected to immunohistochemical incubation in an antiserum directed against the enzyme responsible for NO production in nerve cells, neuronal NO synthase (nNOS). The analysis showed that FG-containing neurons as well as nNOS-immunoreactive neurons were present in the lateral superior olive (LSO), superior paraolivary nucleus (SPO), ventral nucleus of the trapezoid body (VNTB), medial superior olive (MSO) and in dorsal and ventral periolivary regions to different amounts. However, only in the LSO, SPO and VNTB double-labelled neurons were found. They made up to less than 10% of all nNOS neurons in the SOC. Considering that only about 5% of the nNOS cells in the SOC are olivocochlear neurons [Riemann and Reuss, 1999], it is still open whether the majority of nitricergic neurons of the SOC project to other sites or whether they rather have intrinsic actions in providing NO to the SOC.

Key words: Auditory brainstem; Retrograde tracing; Immunohistochemistry; Nitric oxide

1. Introduction

The superior olivary complex (SOC) is a group of interrelated nuclei bilaterally located in the basal brainstem. Its functions include the detection of interaural differences in sound intensity and time as the basis of spatial mapping, the feedback control of cochlear mechanisms and the processing of cochlear signals via the ascending auditory pathway.

The primary target of the ascending pathway originating in the SOC is the inferior colliculus (IC; cf. Helfert et al., 1991). The projection to the central nucleus of the IC (CNIC), consisting of excitatory and inhibitory inputs, uses substances such as glutamate and glycine, respectively (cf. Oliver, 2000). Another neuroactive substance produced in cell bodies of the SOC is nitric oxide (NO), since an isoform of the NO-producing enzyme, neuronal NO synthase (nNOS), was recently demonstrated in a subpopulation of SOC neurons (Reuss, 1998; Fessenden et al., 1999; Riemann and Reuss, 1999). In the auditory system, this substance was considered to regulate cochlear blood flow and, at the hair cell synapse, inhibit N-methyl-D-aspartate receptors or augment presynaptic transmitter release from inner hair cells (Reuss and Riemann, 2000).

However, the combination of nNOS immunodetection with retrograde tracing showed that less than 5% of the nNOS-immunoreactive (−IR) neurons in the SOC...
are olivocochlear neurons (Riemann and Reuss, 1999). It is as yet unknown whether the rest of these cells have local regulatory functions or whether they project to other sites. Despite the importance of the SOC–IC connection, the question whether this projection is (partly) nitrergic has not yet been addressed. We therefore sought to identify the SOC–IC projection neurons by retrograde neuronal tracing upon injection of the tracer Fluoro-Gold (FG) into the CNIC, combined with the immunofluorescent detection of nNOS and subsequent analysis of the sections with regard to double-labelling of the neurons.

2. Materials and methods

2.1. Animals and treatment

The procedures outlined in the following complied with German laws for the protection of animals and were approved by the local government office (Bezirks­regierung Rheinland-Pfalz, Az 177-07/961-30). Adult male Sprague–Dawley rats (200–220 g) were anesthetized with tribromoethanol (0.3 g/kg body weight, intraperitoneal) and received injections of 200 nl of a 5% FG solution (Fluorochrome, Englewood, CO, USA; dissolved in distilled water) into the IC. After 5 days, animals were anesthetized again and perfused transcardially with phosphate-bu­ffered 0.9% saline, to which 15 000 IU heparin/l were added, at room temper­ature, followed by an ice-cold periodate–lysine–paraformaldehyde solution as the fixative (McLean and Nakane, 1974). The right atrium was opened to enable venous outflow.

2.2. Tissue processing

The brains were removed, postfixed for 1 h, and stored overnight at 4°C in phosphate-buffered 30% sucrose. Tissue was sectioned serially at 40 μm thickness on a freezing microtome in the frontal plane and collected in phosphate buffer and, for immunohistochemistry, incubated free-floating in a polyclonal rabbit-raised antiserum directed against nNOS from rat cerebellum (1:1000 in PBS; Laboserv, Giessen, Germany), to which 1% normal swine serum and 0.1% Triton X-100 were added. This antibody is well-characterized (Alm et al., 1993) and has been used in our laboratory in many studies (Reuss, 1998; Riemann and Reuss, 1999; Reuss and Reuss, 2001). Various central nervous structures known to contain nNOS are labelled by this antibody. After three rinses in PBS, sections were incubated in Cy3-conjugated anti-rabbit IgG (1:100 in PBS; Amersham, Hannover, Germany). Sections were mounted onto gelatin-coated slides, briefly dehydrated in acetone, coverslipped with Merkoglas (Merck, Darmstadt, Germany) and analyzed by using a Leitz Orthoplan microscope with a Plomopak epifluorescence unit through filter sets D (FG) and N2 (Cy3).

From each section, labelled cells were counted with regard to their location within the nuclei of the SOC. Single- and double-labelled neurons were counted separately and the respective percentages were calculated for each nucleus. Brainstem regions were identified using a stereotaxic atlas (Paxinos and Watson, 1986). The nomenclature of these authors, and of Schwartz (1992) and Webster (1995), was adopted to characterize the components of the SOC. According to the latter, we defined the ventral portion of the trapezoid nucleus as ventral nucleus of the trapezoid body (VNTB) while the remaining ventral periolivary regions were referred to as the ventral periolivary nucleus (VPO). The average total cell number of each SOC region was taken from a previous study (Riemann and Reuss, 1999) in which numbers showed relatively little variation between animals when counted from five rats of the same origin, strain, sex and age as in the present study.

Table 1

Distribution of retrogradely labelled and/or nNOS-IR neurons in the SOC

<table>
<thead>
<tr>
<th>(1) Total cell number SOC</th>
<th>(2) FG cell number (in % of total cell number)</th>
<th>(3) nNOS cell number (in % of total cell number)</th>
<th>(4) FG and nNOS cell number (in % of FG cell number)</th>
<th>(5) FG and nNOS cell number (in % of nNOS cell number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSO 7800</td>
<td>1349 ± 124 (17.3 ± 1.6)</td>
<td>1248 ± 218 (16.1 ± 2.8)</td>
<td>171 ± 37 (12.7 ± 2.8)</td>
<td>171 ± 37 (13.7 ± 2.9)</td>
</tr>
<tr>
<td>SPO 2000</td>
<td>298 ± 28 (14.9 ± 1.4)</td>
<td>358 ± 68 (17.9 ± 3.4)</td>
<td>118 ± 6.9 (39.6 ± 2.3)</td>
<td>118 ± 6.9 (32.9 ± 1.9)</td>
</tr>
<tr>
<td>VNTB 1100</td>
<td>266 ± 214 (24.2 ± 20.6)</td>
<td>415 ± 87 (37.7 ± 3.3)</td>
<td>164 ± 6.3 (61.6 ± 3.2)</td>
<td>164 ± 6.3 (39.5 ± 2.1)</td>
</tr>
<tr>
<td>MNTB 6300</td>
<td>0</td>
<td>3019 ± 52 (47.8 ± 0.7)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MSO 1700</td>
<td>26 ± 1.8 (1.5 ± 1.1)</td>
<td>155 ± 30 (9.1 ± 1.8)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DPO 950</td>
<td>11 ± 8 (1.2 ± 0.9)</td>
<td>49 ± 35 (5.2 ± 3.7)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>VPO 1000</td>
<td>71 ± 8 (7.1 ± 0.8)</td>
<td>99 ± 46 (9.9 ± 4.6)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total 20850</td>
<td>2021 ± 482 (9.7 ± 2.3)</td>
<td>5343 ± 502 (25.7 ± 2.4)</td>
<td>453 ± 24 (22.7 ± 1.2)</td>
<td>453 ± 24 (8.5 ± 0.5)</td>
</tr>
</tbody>
</table>

The numbers given are the means of five animals ± standard deviations, or the percentages with regard to total cell number or to numbers of FG or nNOS cells, respectively.
3. Results

Following unilateral injection of FG into the CNIC, the tracer was found bilaterally (mainly ipsilateral) in neuronal cell bodies and processes of the SOC (Fig. 1A and column 2 of Table 1). The largest amounts were found in the lateral superior olivary nucleus (LSO), superior periolivary nucleus (SPO) and VNTB, while in the other nuclei labelled neurons were either not found (medial nucleus of the trapezoid body, MNTB) or amounted to a few percent only. The data presented here stem from five animals with successful injections centered in the CNIC. Although the placement of the pipette tip varied slightly in the anteroposterior axis, all injections included the central nucleus. The injections of the present study are part of a larger series from which a typical injection site was shown in a previous paper dealing with some other aspects of the ascending auditory projection (Reuss et al., 1999).

Neurons exhibiting nNOS immunoreactivity (Fig. 1B and column 3 of Table 1) were observed in different amounts in the subnuclei of the SOC. In the LSO and SPO, less than 20% were nNOS-IR. Less than 10% of the respective neurons were labelled in the medial superior olivary nucleus (MSO) and in the dorsal and ventral periolivary nuclei (DPO, VPO), while nearly half of the cells in the MNTB were labelled by the antibody. The VNTB, also known as medial preolivary cell group (Schwartz, 1992) or medioventral periolivary nucleus (Warr and Beck, 1996), exhibited IR neurons to nearly 40%.

Double-labelled neurons were observed only in the LSO, SPO and VNTB but not in MNTB, MSO, DPO or VPO (Fig. 2 and columns 4 and 5 of Table 1). In the LSO and SPO, both neuronal populations overlapped by approximately 13 and nearly 40%, respectively. Sixty percent of the retrogradely labelled neurons of the VNTB exhibited nNOS immunoreactivity (column 4 of Table 1). Forty percent of these nNOS-IR perikarya were labelled by the tracer FG (column 5 of Table 1).

4. Discussion

The present study, conducted to investigate whether the SOC neurons providing ascending projections to the IC are nitrergic, demonstrates for the first time that approximately one-fifth of these ascending neurons contain the enzyme responsible for NO production in neurons, nNOS. This projection may be the origin of NOS-IR neuropil in the IC detected previously (Druga and Syka, 1993; Endoh et al., 1994).

The presence of this enzyme in neurons of the SOC was first mentioned by Vincent and Kimura (1992) and Southam and Garthwaite (1993) but was analyzed in detail recently by means of histochemistry, immunofluorescence and in situ hybridization in rat, hamster and guinea pig (Reuss, 1998; Fessenden et al., 1999; Riemann and Reuss, 1999; Reuss and Riemann, 2000; Reuss et al., 2000). In a study combining nNOS immunofluorescence with the identification of olivocochlear projection neurons by retrograde tracing (Riemann and Reuss, 1999), it was found that only a relatively small portion of nitrergic neurons in the SOC belong to this neuronal subpopulation. While approximately one-fourth of all SOC neurons were nNOS-IR (a finding confirmed in the present study), less than 5% of the olivocochlear neurons are nitrergic. This finding thus yielded the question as to where the other NO-
producing neurons may project? A major candidate site is the IC as an important relay station in the ascending auditory pathway. Our data now demonstrate that a subpopulation of these ascending projection neurons may produce NO.

We do not know presently whether these neurons built a separate group of ascending neurons or whether they belong to the excitatory (glutamatergic) or inhibitory (glycinergic, gamma-aminobutyric acid (GABA)-ergic) subpopulations of the SOC that project to the IC (cf. Oliver, 2000). In general, as part of a built-in negative feedback system, NO may dampen responses that are mediated by the activation of glutamate or of GABA receptors (cf. Ahern et al., 2002). In the IC, NO may be involved in the processing and transmission of the acoustic input to the auditory cortex since treatment of rats with an NOS inhibitor reduced the amplitude of the auditory middle latency responses (Grassi et al., 1995) and of sound-evoked electrocortical desynchronization (Iannone et al., 1996).

Fig. 2. Higher magnifications from Fig. 1 showing regions of the LSO, VNTB and SPO, respectively, which exhibit large amounts of double-labelled neurons. A,C,E: Cell bodies labelled by unilateral injection of the retrograde tracer FG into the CNIC. B,D,F: Neurons exhibiting nNOS-like immunoreactivity. Examples of neurons of the ascending projection that are not nNOS-IR are marked by asterisks. Some nNOS-IR neurons not retrogradely labelled by FG are indicated by arrows. Magnification bars = 30 μm.
To identify neurons of the ascending projection, we injected FG unilaterally into the CNIC. Following retrograde axonal transport, the tracer was found in a distinct neuronal population distributed throughout the SOC. This is in general accordance with previous studies localizing olivo-collicular projection neurons (Schwartz, 1992; Gonzalez-Hernandez et al., 1996; Warr and Beck, 1996; Kelly et al., 1998). We also conducted nNOS immunohistochemistry and observed immunofluorescent neurons in a similar amount and distribution as in a previous study (Riemann and Reuss, 1999).

The largest portion of FG-labelled somata were observed in the LSO, SPO and VNTB. This projection pattern was found previously in rats (cf. Schwartz, 1992; Kelly et al., 1998). In these regions, we observed nNOS-IR perikarya as well as double-labelled neurons in considerable amounts.

Retrogradely labelled neurons were also observed in the MSO and in periolivary nuclei, in accordance with previous studies in rats and guinea pigs (Schofield and Cant, 1992; Gonzalez-Hernandez et al., 1996; Kelly et al., 1998). Although nNOS neurons were also found in these regions, we did not detect any double-labelled neurons in either the MSO, DPO or VPO.

Our initial question about where nNOS-containing neurons of the SOC project can still not be answered completely. It was previously demonstrated that less than 5% of nNOS-IR neurons project to the cochlea as part of the descending auditory pathway in rats (Riemann and Reuss, 1999). The present study shows that less than 10% of these neurons innervate the IC.

We conclude that the projection target of 85-90% of the nNOS-IR neurons of the SOC is still unknown. The nuclei of the lateral lemniscus and the cochlear nuclei should be considered as other well-known major targets of ascending SOC projections, and we will test these pathways in future studies. Alternatively, or additionally, nNOS-IR neurons of the SOC may have local actions. This view is supported by the strong immunostaining for the NO receptor, guanylate cyclase, and for its product, cyclic guanosine monophosphate in neuronal somata and processes of the SOC (Southam and Garthwaite, 1993; Fessenden et al., 1999).

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