

Evidence for increased nitric oxide production in the auditory brain stem of the aged dwarf hamster (*Phodopus sungorus*): an NADPH-diaphorase histochemical study

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Abstract

Age-related changes of the auditory system such as presbycusis are believed to be due, at least in part, to alterations of central structures. The superior olivary complex (SOC), a group of interrelated brain stem nuclei, projects to a variety of neuronal structures including the cochlea and the inferior colliculus (IC). The soluble gas nitric oxide (NO), believed to function as a neuroactive substance within the SOC and cochlea, is thought to be involved in ageing processes. Since it is unknown whether NO-production is altered in the ageing auditory system, the present study was conducted to investigate whether the number of NO-producing cells in the SOC is changed with increasing age. The histochemical detection of NADPH-diaphorase activity (NADPH-d), a marker for neurons containing NOS, was utilized to investigate the numbers of NO-producing cells in the SOC of adult and senile Djungarian dwarf hamsters (*Phodopus sungorus*). Our results demonstrate that the number of stained neurons was almost doubled in the SOC of senile hamsters. The most distinct changes were observed in the medial nucleus of the trapezoid body. In contrast, NO-producing preganglionic sympathetic neurons of the spinal intermediolateral nucleus, which was studied for comparison, did not exhibit significant differences between adult and senile animals. It is concluded that the increase of NO-production in the ageing auditory brain stem, as revealed by our data, may be related to hearing impairments with increasing age.

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1. Introduction

Brain ageing is thought to be based on many factors. There is evidence that ageing may be the result of free radicals which, upon generation by mitochondrial metabolism, may cause reduced cell functions or cell death. Many toxins within the environment may lead to free radicals, and recent research suggested that the excessive production of nitric oxide (NO) may be an important factor in ageing of the mammalian central nervous system (cf. Grozdanovic et al., 1994; McCann et al., 1998).

NO, a toxic gas with free-radical properties, serves as a neuroactive substance within the mammalian nervous system (cf. Grozdanovic et al., 1994). It is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS). Three NOS isoforms are known, one of which is neuronal NOS (nNOS). The enzyme is activated when glutamate, released from presynaptic terminals, acts on both postsynaptic *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors. This allows influx of calcium which in turn activates nNOS (cf. Garthwaite, 1991).

A sensory system that undergoes age-related changes is the auditory system in mammals. Well-known in humans and animals is hearing-loss, or presbycusis, which is accompanied by elevated thresholds, increased latencies and decreased amplitudes of brain stem auditory evoked potentials (Saitoh et al., 1994; Parham, 1997; Harada et al., 1999). These may be seen as signs of age-related dysfunctions of the auditory brain stem (Rosenhall et al., 1986). A major component of this system is the superior olivary complex (SOC) which consists of the lateral and medial superior olivary nuclei (LSO, MSO), the medial nucleus of the trapezoid body (MNTB) and the periolivary nuclei. SOC neurons provide the efferent innervation of cochlear hair cells via the olivocochlear bundle (OCB). The functions of the SOC include the detection of interaural sound intensity differences as the basis of spatial mapping (cf. Moore, 1994). It has been shown that this group of interrelated brain stem nuclei contains a relatively high number of nNOS cells (Reuss, 1998; Fessenden et al., 1999) some of which project to the cochlea (Riemann and Reuss, 1999a).

Although both NO-production and hearing are known to be altered with increasing age, it is unknown whether nNOS neurons of the auditory system undergo age-related changes. The present study, therefore, sought to investigate the auditory superior olivary complex with respect to the number of nNOS cells in adult and senile Djungarian dwarf hamsters (*Phodopus sungorus*). This rodent species was previously used as an animal model in ageing research (e.g. Hoffmann et al., 1985; Reuss and Bürger, 1994; Reuss and Rimoldi, 1998).

To compare the results with those of another nervous structure, the intermediolateral nucleus (IML) of the spinal cord was also investigated. It contains the preganglionic sympathetic neurons (PSN) most of which are NADPH-d-positive (Anderson, 1992). Although a number of changes were found in the ageing autonomic system (cf. Cowen, 1993), PSN numbers are unaffected by age (Santer, 1991).

Our results, obtained by histochemistry utilizing the NADPH-diaphorase (NADPH-d) activity of nNOS, demonstrate for the first time that NO is produced by neurons in the dwarf hamster auditory brain stem and provide evidence that the number of these neurons is augmented in senile hamsters.

2. Materials and methods

The procedures concerning animals reported in this study complied with German legislation for the protection of animals and were approved by the county-government office (Bezirksregierung Rheinhessen-Pfalz, Az 177-07/961-30). Djungarian hamsters (*Phodopus sungorus*) of either sex with body weights of 46–54 g were used in this study. The animals stem from our own breeding colony and were held under constant conditions (light:dark 16:8 h, food and water ad libitum, room temperature $21 \pm 1^\circ\text{C}$). The average life span of hamsters in our colony is approximately 20 months, with less than 50% of the population remaining at an age of 2 years.

Two groups of animals were studied. Group one consisted of five adult hamsters (3–7 months old), group two of five senile animals (25–28 months old). The animals were anesthetized and perfused transcardially with phosphate-buffered saline (PBS) to which 15 000 IU/l heparin were added at room temperature, followed by an ice-cold periodate-lysine-paraformaldehyde solution (McLean and Nakane, 1974). The right atrium was opened to enable venous outflow. The brains and spinal segments C8-Th2 were removed, postfixed for 4 h and stored overnight at 4°C in phosphate-buffered 30% sucrose. Tissue was sectioned serially at $40 \mu\text{m}$ on a freezing microtome in the frontal plane and collected in PBS.

The histochemical reaction for NADPH-diaphorase activity consisted of incubating the free-floating sections for 15 min at room temperature in 50 mM Tris-HCl, pH 8.0 and then for 60 min at 37°C in the same solution containing 1 mM β -NADPH (Sigma, München, Germany), 8.0 mM malic acid, 0.8 mM nitroblue tetrazolin (NBT; Sigma) and 0.8% Triton X-100. Histochemical reactions in the absence of β -NADPH or NBT, respectively, served as controls in which no staining was observed. Care was taken that sections from hamsters of either group were treated simultaneously under identical conditions.

The sections were then mounted onto gelatin-coated slides, dehydrated, cover-slipped with Merckoglas (Merck, Darmstadt, Germany) and analyzed. Neurons labeled by histochemistry were quantified from 7–9 sections from each hamster with regard to their location within the SOC, in a way that the investigator was unaware from which group of animals the material was taken. The total number per animal was calculated for each nucleus. Brain stem regions were identified

according to the stereotaxic atlas of the rat brain (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. The region 100 μm dorsally to the superior paraolivary nucleus (SPO) and lateral superior olivary nucleus (LSO) was defined as dorsal periolivary nucleus (DPO) and the region 100 μm ventrally to the medial nucleus of the trapezoid body (MNTB), the medial superior olivary nucleus (MSO) and the LSO was defined as ventral periolivary nucleus (VPO).

NADPH-d-stained neurons in the IML of the spinal segments C8-TH2 were counted from five sections per segment and animal.

The numbers obtained were tested for statistical significant differences between groups by the Mann–Whitney *U*-test.

3. Results

Examinations of frontal sections of the dwarf hamster brain showed that the nuclei of the SOC were labelled by the NADPH-diaphorase method. Scattered perikarya exhibiting dark reaction product were detected in the VPO and DPO and the lateral part of the SPO (Fig. 1). Some faintly labeled cell bodies were also found in the LSO and MSO. The largest numbers of stained cell bodies was found in the MNTB, where most neurons contained reaction product (Fig. 2). Staining was observed in particular in the MNTB's anterior part. The distribution of stained cell bodies was similar to that in rats and guinea pigs (Reuss, 1998; Fessenden et al., 1999; Riemann and Reuss, 1999a) and did not reveal any differences between female and male animals of either group.

The quantification of histochemically stained neurons (Table 1) showed a difference between the animal groups. Senile hamsters exhibited approximately twice as many stained neurons in the MNTB when compared to the adult hamsters ($P < 0.01$). The other SOC nuclei displayed only small differences in number between adult and senile animals (not statistically significant). The numbers of sections containing the SOC were similar in both groups, i.e. there was no indication that the nuclei investigated were different in size between groups.

In cross sections of the spinal cord, stained neurons were seen in several laminae, in particular in the dorsal horn and in the sympathetic regions (Fig. 3). The quantification, conducted from the intermediolateral nucleus, showed that the average number of stained neurons did not vary significantly between the groups (adult animals: C8: 9.0 ± 2.0 , Th1: 13.5 ± 1.1 , Th2: 13.5 ± 1.4 ; senile animals: C8: 10.1 ± 2.9 , Th1: 11.3 ± 1.5 , Th2: 11.1 ± 1.9 ; mean/animal \pm SD, $n = 5$ per group).

4. Discussion

The present results are the first to demonstrate NADPH-d-stained neurons in the superior olivary complex of the dwarf hamster auditory brain stem. Our data further show that these neurons are numerically augmented in senile animals. In

contrast, no age-related differences were observed in preganglionic sympathetic neurons of the same animals.

The SOC is the source of the olivocochlear bundle (OCB). This efferent innervation of cochlear hair cells is thought to regulate biomechanical parameters of the cochlea, and to be involved in the modulation of otoacoustic emissions, the enhancement of signal detection and the protection of the cochlea from noise damage (cf. Guinan, 1996). SOC neurons have been shown to contain nNOS (Reuss, 1998; Fessenden et al., 1999) and are among the neuronal sites that project to the cochlea (Riemann and Reuss, 1998; Reuss et al., 1999; Riemann and Reuss, 1999a,b). It is therefore likely that the OCB provides nNOS, and thus nitric oxide, to the cochlea. Our present results reveal that, in aged animals, a higher amount of NO is produced and released within the auditory brain stem and in the cochlea.

Some discrepancies were previously observed with regard to age-related changes of NOS-immunoreactivity. For example, while Uttenthal et al. (1998) described the

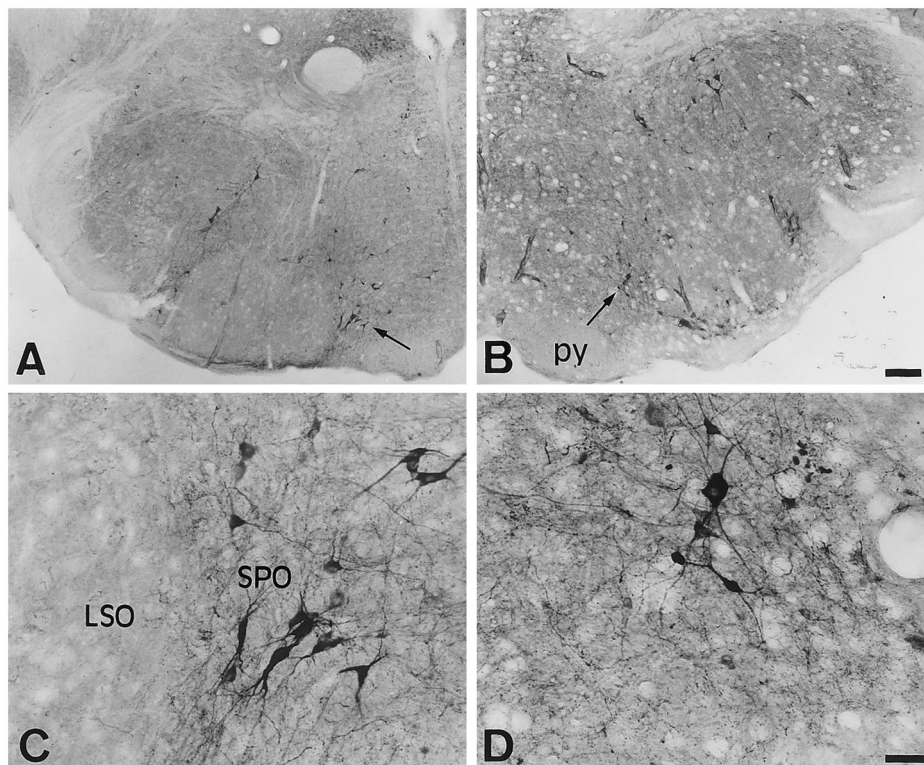


Fig. 1. Histochemical staining of NADPH-diaphorase activity in the dwarf hamster auditory brain stem. A: was taken from an adult animal; and B: from a senile hamster. Groups of stained neurons that are depicted by arrows in A, B are shown in higher magnification in C and D. Magnification bars: 200 μ m (A, B; same magnification), 50 μ m (C, D; same magnification). Abbreviations: LSO, lateral superior olivary nucleus; SPO, superior paraolivary nucleus; and py, pyramidal tract.

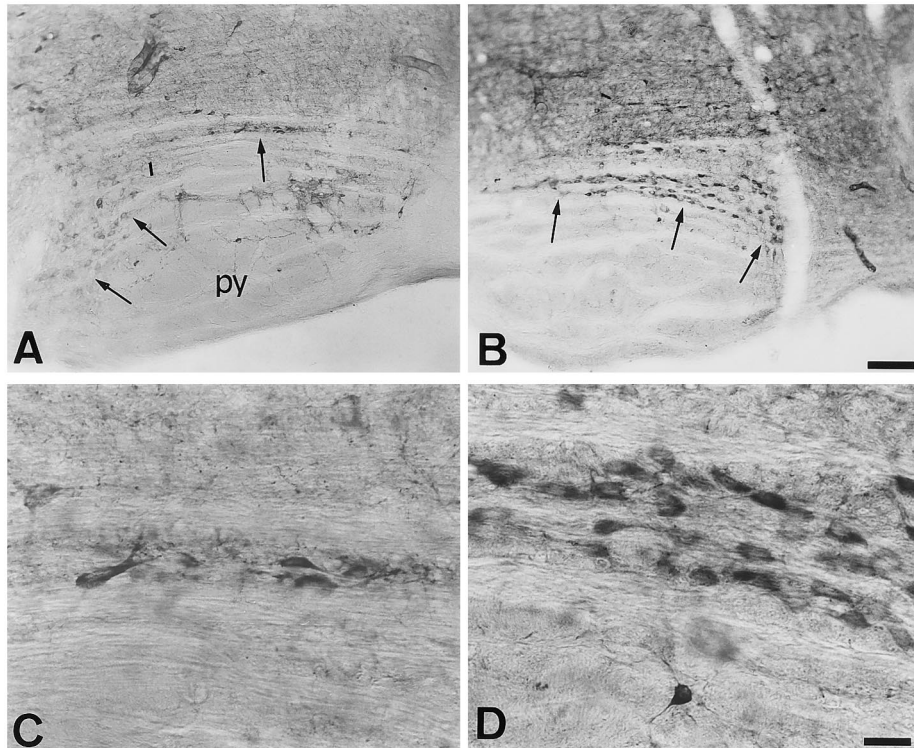


Fig. 2. Histochemical staining of NADPH-d activity in the medial nucleus of the trapezoid body (MNTB), the ventral borders of which are depicted by arrows. A: adult animal; and B: senile animal. C and D are higher magnifications of A and B, respectively. A numerical increase of stained neurons is seen in the senile animal. Magnification bars: 100 μm (A, B; same magnification), 20 μm (C, D; same magnification). Abbreviation: py, pyramidal tract.

Table 1

Numbers of NADPH-diaphorase-stained neurons in the nuclei of the superior olivary complex (SOC) of the auditory brain stem of adult and senile hamsters (mean/animal \pm SD; $n = 5$ per group)^a

SOC nucleus	Adult animals	Senile animals
LSO	20.7 \pm 6.0	23.3 \pm 7.8
MSO	4.8 \pm 4.4	12.4 \pm 8.8
MNTB	136.1 \pm 27.3	285.2 \pm 41.2
SPO	6.2 \pm 4.8	5.4 \pm 1.2
DPO	13.0 \pm 3.8	17.7 \pm 4.8
VPO	9.9 \pm 4.7	13.3 \pm 2.5
Total of means	190.7 (100%)	357.2 (187.3%)

^a Abbreviations: DPO, dorsal periolivary nucleus; LSO, lateral superior olivary nucleus; MNTB, medial nucleus of the trapezoid body; MSO, medial superior olivary nucleus; SPO, superior paraolivary nucleus; VPO, ventral periolivary nucleus.

numerical increase of nNOS and iNOS-immunoreactive neurons in the rat cerebral cortex, no age-related changes were found in the striatum (Kawamata et al., 1990), and declines in NO-production were also found in the mammalian brain (cf. Yamada and Nabeshima, 1998). A possible explanation for these differing findings may be that age-related changes in NO-production are regionally specific. This hypothesis may also explain our present observation that there was apparently no change in the quantity of NADPH-d-stained cells of the intermediolateral cell column of the spinal cord. The IML was studied because there is evidence for age-related changes of sympathetic system function in mammals (cf. Cowen, 1993).

Sensory and neural parts of the auditory system, however, may be a region in which NO-production is not reduced but rather augmented with advancing age. Interestingly, Kimura et al. (1998) reported an increase of inducible NOS (iNOS) in the cochlear tissue of senile mice. This enzyme is involved in the immunological process of macrophages, and has also been considered to contribute to cell death and degeneration (Xie and Nathan, 1994).

In the present study, the most distinct change was observed in the MNTB where nNOS neurons represent more than fifty percent of all MNTB cells in rat and

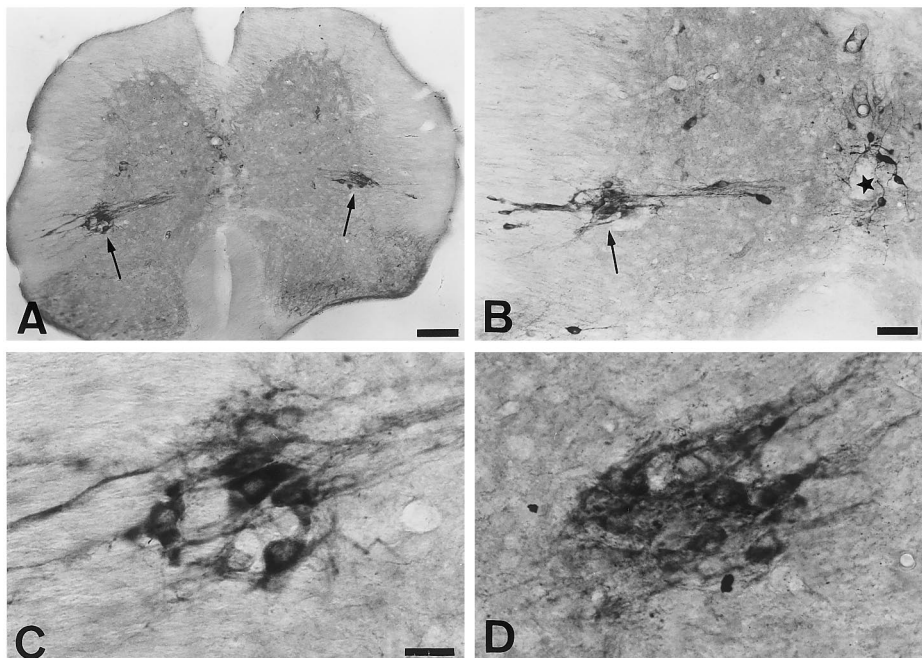


Fig. 3. A: cross section of the spinal segment Th2 taken from an adult hamster, stained by the histochemical NADPH-diaphorase method. The NO-producing neurons in the intermediolateral nucleus (IML) are depicted by arrows. B: higher magnification showing stained neurons in the IML (arrow) and surrounding the central canal (asterisk) in an adult hamster. The comparison of C (adult animal) and D (senile hamster) demonstrates the similarity in the number of stained neurons in the IML. Magnification bars: 100 μ m (A), 50 μ m (B), 20 μ m (C, D; same magnification).

guinea pig (Riemann and Reuss, 1999a). The MNTB projects to the cochlea as well as to other SOC regions such as the LSO, MSO and periolivary nuclei (cf. Helfert et al., 1991) and plays a critical role in sound localization. Interestingly, age-related impairments in directional hearing and speech perception in ‘cocktail party’ environments have been described (Harrison, 1981; Duquesnoy, 1983).

So the question arises, what are the effects of augmented NO-levels in the MNTB and cochlea? NO is known to inhibit the activity of NMDA receptors (Manzoni et al., 1992) which are expressed by principal MNTB cells. It is thus suggested that, in this subdivision of the SOC, NO diminishes the effects of glutamate which would result in altered neural transmission. On the other hand, excess glutamate can be neurotoxic (cf. McCann et al., 1998), and the increased NO-production may damp these effects.

It is also conceivable that the presently observed increase in NO-producing neurons of the SOC is related to impairments of the ageing auditory system such as presbycusis. Since the loss of inner hair cells (IHC) is minimal in the aged guinea pig cochlea (Ingham et al., 1999), age-related changes may occur rather in functional parameters of sensory cells. It is possible that nNOS-immunoreactive terminals, found predominantly at the base of IHC (Riemann and Reuss, 1999a), provide more NO to sensory cells in the ageing inner ear which also would result in altered function. However, the mechanisms underlying the augmentation of NADPH-d activity in the senile auditory system and its functional implications require elucidation.

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