Neuroscience 131 (2005) 293–302

DEEPLY LOCATED GRANULE CELLS AND MITRAL CELLS UNDERGO APOPTOSIS AFTER TRANSECTION OF THE CENTRAL CONNECTIONS OF THE MAIN OLFACTORY BULB IN THE ADULT RAT

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Abstract—The main olfactory bulb (MOB) is the first relay station of the olfactory system: it receives afferents from sensory neurons and sends efferents to the primary olfactory cortex. The MOB also receives many centrifugal afferents from various regions. Transection of peripheral afferents to the MOB has been reported to induce cell death in granule cells. However, little is known about the effect of transection of these central connections of the MOB in adult rats. Here, we used a unilateral olfactory peduncle transection model in the adult rat to examine neuronal degeneration in the MOB. In the MOB ipsilateral to the surgery, the granule cell layer (GCL) was smaller, and the number of mitral cells was decreased compared with the contralateral MOB at 7 days after surgery. Many degenerating cells were present in both the mitral cell layer (MCL) and GCL in the ipsilateral MOB at 3 days after surgery, although there were no obvious changes in the gross morphology. We also found terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick end labeling (TUNEL)-positive cells in the MCL and GCL in the ipsilateral MOB at 3 days after surgery. The majority of the degenerating and TUNEL-positive cells were located in the deep, rather than the superficial, GCL. Immunohistochemistry for activated caspase-9 further supported the occurrence of apoptotic cell death in the mitral and deeply located granule cells. These results indicate that not only axotomized mitral cells, but also deeply located granule cells that were not directly injured, underwent apoptosis after transection of the central connections, and suggest that sensitivities to transection of the central connections differ among granule cells according to their depth in the GCL. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: centrifugal afferent, axotomy, nuclear degeneration, TUNEL, caspase-9.

The mammalian main olfactory bulb (MOB) is a highly layered structure that receives afferent fibers from olfactory receptor neurons and sends efferent fibers toward higher structures (Shepherd, 1972; Scott, 1986; Kinoshita et al., 2002). The mammalian MOB is unusual in that neurogenesis is a normal feature of the adult MOB (Alvarez-Buylla and Lois, 1995; Seaberg and van der Kooy, 2002), and the addition of newly generated interneurons to the granule cell layer (GCL) continues throughout life (Altman, 1969; Lois and Alvarez-Buylla, 1994). There appears to be a high level of turnover of these newly generated granule cells (Biebl et al., 2000). A large proportion die within weeks after differentiation in the MOB (Petreanu and Alvarez-Buylla, 2002). Previous evidence has suggested that their survival is influenced by sensory inputs. Sensory deprivation by naris occlusion reduced the granule cell number in the developing MOB (Brunjes, 1994) through increased apoptosis (Najbauer and Leon, 1995). In the adult MOB, olfactory deprivation by naris closure (Henegar and Maruniak, 1991; Corotto et al., 1994) or by deafferentation (Mandairon et al., 2003) also induced granule cell death. Conversely, reopening of the naris following early occlusion (Cummings and Brunjes, 1997) or olfactory enrichment in adults (Rochefort et al., 2002) promoted granule cell survival.

In addition to receiving peripheral input fibers from olfactory receptor neurons, the MOB also receives a large number of centrifugal fibers from cells located in higher brain regions (De Olmos et al., 1978; Luskin and Price, 1983). It has been estimated that these fibers may equal or outnumber those of the olfactory receptors (Macrides and Davis, 1983). Although the significance of peripheral input fibers for neuronal survival in the MOB is relatively well understood, the significance of the central connections of the MOB has been poorly characterized to date, especially in adult animals. In developing animals, several studies have reported shrinkage of the MOB and loss of mitral cells at several weeks after transection of the lateral olfactory tract (LOT; Allison, 1953; Small and Leonard, 1983; Weiler and Farbman, 1999). In adult animals, mitral cell loss at a few months after LOT transection (Allison, 1953; Verhaagen et al., 1993) and granule cell loss at 6 weeks after olfactory peduncle transection were reported (Allison, 1953). However, the features of these degenerative changes at earlier time points after transection of the central connections were not investigated. In the present study, we used a unilateral olfactory peduncle transection model in the adult rat and examined the early phase of degenerative changes following the surgery using morphological, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin (DIG) nick end labeling (TUNEL) and immunohistochemical methods.

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Abbreviations: ANOVA, analysis of variance; DIG, digoxigenin; EPL, external plexiform layer; FITC, fluorescein 5′-isothiocyanate; GCL, granule cell layer; GL, glomerular layer; LOT, lateral olfactory tract; MCL, mitral cell layer; MOB, main olfactory bulb; NeuN, neuronal nuclei; PBS, phosphate-buffered saline; SEL, subependymal layer; TdT, terminal deoxynucleotidyl transferase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling.

0306-4522/05$30.00+0.00 © 2005 Published by Elsevier Ltd on behalf of IBRO.
doi:10.1016/j.neuroscience.2004.10.040

EXPERIMENTAL PROCEDURES

Animals and surgical procedure

Eight-week-old male Wistar rats (250–280 g) were used in this study. All animal experiments were performed in accordance with the European Community Council Directive of 24 November, 1986 (86/609/EEC), and the regulations of the University of Tokyo School of Medicine. All efforts were made to minimize the number of animals used and their suffering. Rats (n=12) were anesthetized intraperitoneally with pentobarbital (65 mg/kg) or ketamine/xylazine (74 mg/kg and 9 mg/kg, respectively), and positioned in a stereotaxic apparatus. The posterior end of the right MOB was exposed by drilling a small groove in the dorsal skull surface. The scalpel blade was lowered into the right MOB, slightly anterior to the sinus on the tip of the frontal cortex and lateral to the superior olfactory sinus. After touching the base of the skull, the scalpel blade was moved laterally, cutting the olfactory peduncle at the posterior end of the right MOB. Care was taken to avoid damage to the superior olfactory sinus and rostral confluence of sinuses. The contralateral MOB was left intact and served as a control. Histological examination (see below) verified that at least the lateral two-thirds of the right olfactory peduncle was transected completely at the level of the anterior tip of the anterior olfactory nucleus, and that the contralateral MOB was intact.

Tissue preparation

At 3 days (n=6), 7 days (n=3) and 21 days (n=3) after surgery, rats were anesthetized intraperitoneally with a lethal dose of sodium pentobarbital (260 mg/kg), and perfused intracardially with saline at 4 °C to clear the vessels of blood. This was followed by perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4 °C as a fixative. After removal from the skull, the brains were postfixed for 24 h in the same fixative at 4 °C, cryoprotected in 20% sucrose in phosphate-buffered saline (PBS), pH 7.4, at 4 °C until they sank, and then frozen in M-1 embedding matrix (Thermo Shandon, Pittsburgh, PA, USA). The MOBs from nine of the rats, perfused at 3 days (n=3), 7 days (n=3) and 21 days (n=3) after surgery, were used for histological analysis. The same three MOBs at 3 days after surgery were also used for TUNEL assay and double-labeling. The MOBs from the other three rats, perfused at 3 days after surgery, were used for immunohistochemistry.

Histological analysis

The MOBs were cut coronally into 12-μm cryostat sections, thaw-mounted onto slides, stained with Cresyl Violet, and coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ, USA). Images were obtained using a Leica DC500 digital camera (Leica Microsystems, Wetzlar, Germany) fitted to a Nikon Optiphot-2 microscope (Nikon Corporation, Tokyo, Japan).

For quantification, we used three equally spaced coronal sections along the rostrocaudal axis from each animal. These sections were positioned approximately 1.2, 1.6 and 2.0 mm caudal to the anterior tip of the MOB. All sections used for quantification were at least 2.0 mm anterior to the transection site.

The numbers of degenerating cells in the GCL, mitral cell layer (MCL) and the subependymal layer (SEL) were counted on images captured through a ×20 objective. A cell was identified as degenerating if nuclear condensation or fragmentation was observed (Perry et al., 1983; Rabacchi et al., 1994). We next characterized the distribution of degenerating cells throughout the depth of the GCL. The GCL was shaped like a slightly distorted ellipse in the coronal plane of the MOB. We divided the GCL into six concentric ring-shaped subdivisions of equal depth, and then counted the number of degenerating cells within each subdivision. The density of degenerating cells was calculated as the number of cells per mm^2 of each subdivision.

The areas of the GCL/SEL, external plexiform layer (EPL) and glomerular layer (GL) were measured using the Scion Image Beta 4.0.4 software (Scion Corporation, Frederick, MD, USA). The total number of mitral cells in each section was counted along the MCL on images captured through a ×20 objective. A cell was counted as a mitral cell if it had a large (>15 μm) and clearly stained cell body with a visible nucleus, and was located within the MCL (McCollum et al., 1997).

TUNEL assay

To detect DNA fragmentation *in situ*, TUNEL was performed using an ApopTag Apoptosis Detection Kit (Intergen, Purchase, NY, USA) following the manufacturer's instructions with slight modifications for fixed frozen sections. The 12-μm sections were washed twice in PBS, permeabilized with ethanol/acetic acid (2:1) for 5 min at −20 °C, washed twice in PBS, and then treated with 10 μg/ml proteinase K (Roche Diagnostics, Basel, Switzerland) for 10 min at room temperature. After washing twice in PBS, the sections were incubated for at least 10 s with the equilibration buffer. Subsequently, the sections were incubated for 1 h with DIG-conjugated dUTP and TdT enzyme at 37 °C, and then for 10 min in the stop buffer at room temperature, and finally washed three times in PBS. The sections were then incubated with a fluorescein 5′-isothiocyanate (FITC)-conjugated anti-DIG antibody for 30 min at room temperature, and washed three times in PBS. After couterstaining with 0.3 μM bisbenzimide (Hoechst 33258; Molecular Probes Inc., Eugene, OR, USA) for 5 min, the sections were washed in PBS and coverslipped with PermFluor (Thermo Shandon).

Fluorescence images were captured using an Olympus DP50 digital camera (Olympus Corporation, Tokyo, Japan) fitted to an Olympus BX51 fluorescent microscope (Olympus Corporation). For quantification, we captured images of TUNEL and bisbenzimide counterstaining through a ×5 objective, created overlaid images using image-editing software (Photoshop; Adobe Systems, San Jose, CA, USA), and counted the number of TUNEL-positive cells in each overlaid image. The distribution of TUNEL-positive cells in the GCL was analyzed as described under Histological analysis.

Immunohistochemistry for caspase-9

To investigate the activation of caspase signaling, we used a rabbit anti-cleaved-caspase-9 polyclonal antibody (diluted 1:250; Cell Signaling Tech, Beverly, MA, USA) as the primary antibody. The MOBs were cut coronally into 28-μm sections and thaw-mounted onto slides. The sections were washed three times in PBS, permeabilized by a 15 min incubation with 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) in PBS, blocked for 30 min with 10% normal goat serum/0.2% Triton X-100 in PBS, and subsequently incubated 20 h at 4 °C with the primary antibody/10% normal goat serum/0.2% Triton X-100 in PBS. After three washes in 0.2% Triton X-100 in PBS, the immunoreactivity was visualized by incubation for 3 h with Alexa 546-conjugated goat anti-rabbit IgG (diluted 1:300; Molecular Probes Inc.). After washing three times in PBS, the sections were counterstained with 0.3 μM bisbenzimide for 5 min.

Double-labeling for activated caspase-9 and neuronal nuclei (NeuN)

To characterize the activation of caspase-9 in the GCL, double-labeling for cleaved caspase-9 and the neuronal marker NeuN was performed on 12-μm sections. The sections were treated as described for immunohistochemistry for caspase-9 except that they were incubated simultaneously with mouse anti-NeuN monoclonal antibody (diluted 1:250; Chemicon, Temecula, CA, USA) and rabbit anti-caspase-9 polyclonal antibody for 15 h at 4 °C.