Neuroscience 138 (2006) 55–68

NEUROFILAMENT PROTEINS ARE PREFERENTIALLY EXPRESSED IN DESCENDING OUTPUT NEURONS OF THE CAT THE SUPERIOR COLLICULUS: A STUDY USING SMI-32

V. FUENTES-SANTAMARIA,* B. E. STEIN AND J. G. McHAFFIE

Department of Neurobiology and Anatomy, Wake Forest University School of Medicine, Winston-Salem, NC 27157-1010, USA

Abstract—Physiological studies indicate that the output neurons in the multisensory (i.e. intermediate and deep) laminae of the cat superior colliculus receive converging information from widespread regions of the neuraxis, integrate this information, and then relay the product to regions of the brainstem involved in the control of head and eye movements. Yet, an understanding of the neuroanatomy of these converging afferents has been hampered because many terminals contact distal dendrites that are difficult to label with the neurochemical markers generally used to visualize superior colliculus output neurons. Here we show that the SMI-32 antibody, directed at the non-phosphorylated epitopes of high molecular weight neurofilament proteins, is an effective marker for these superior colliculus output neurons. It is also one that can label their distal dendrites. Superior colliculus sections processed for SMI-32 revealed numerous labeled neurons with varying morphologies within the deep laminae. In contrast, few labeled neurons were observed in the superficial laminae. Neurons with large somata in the lateral aspects of the deep superior colliculus were particularly well labeled, and many of their secondary and tertiary dendrites were clearly visible. Injections of the fluorescent biotinylated dextran amine into the pontine reticular formation revealed that approximately 80% of the SMI-32 immunostained neurons also contained retrogradely transported biotinylated dextran amine, indicating that SMI-32 is a common cytoskeletal component expressed in descending output neurons. Superior colliculus output neurons also are known to express the calcium-binding protein parvalbumin, and many SMI-32 immunostained neurons also proved to be parvalbumin immunostained. These studies suggest that SMI-32 can serve as a useful immunohistochemical marker for detailing the somatic and dendritic morphology of superior colliculus output neurons and for facilitating evaluations of their input/output relationships.

Key words: output neurons, reticular formation, immunocytochemistry, cytoskeletal protein.

In recent years, a group of cytoskeletal proteins (i.e. neurofilament proteins, see Sternberger and Sternberger, 1983; Lee et al., 1988) that participate in the formation and stabilization of neuronal cytoarchitecture has been used to examine morphological characteristics of different subsets of neurons in the CNS (Campbell and Morrison, 1989; Del Rio and DeFelipe, 1994; Hof et al., 1995; Bickford et al., 1998; Carden et al., 2000). An antibody against these proteins, referred to as SMI-32, is of particular interest here. This antibody recognizes the non-phosphorylated epitope on the high molecular weight subunit of the neurofilament protein that is located mainly in dendrites but also is present in the somata, and occasionally, in thick axons (Sternberger and Sternberger, 1983). Interestingly, these neurofilament proteins are often present in the dendrites of neurons with rapidly-conducting long-projecting axons (Hoffman et al., 1987; Campbell et al., 1991; Bourne and Rosa, 2003). This morphological profile is typical of deep layer superior colliculus (SC) output neurons in a variety of species that co-express parvalbumin and glutamate (Mize et al., 1992; Jeon et al., 1997; McHaffie et al., 2001).

In the cat SC, responses to multisensory stimuli are found preferentially on output neurons whose axons exit via the tectoreticular pathway (Meredith and Stein 1986; Wallace et al., 1993) en route to the contralateral brainstem through the dorsal tegmental decussation and predorsal bundle (Grantyn and Grantyn, 1982; Huerta and Harting, 1982; Moschovakis and Karabelas, 1985; Munoz and Guitton, 1991; Guitton and Munoz, 1991; Meredith et al., 2001). Because this SC output pathway is integral in the control of head and eye movements, amplifying or depressing the activity of its constituent neurons has substantial effects on overt behavior (see Stein et al., 1988, 1989; Wilkinson et al., 1996; Jiang et al., 2002; Jiang and Stein 2003; Burnett et al., 2004). Given that the activity of these SC multisensory neurons depends on the coordination of inputs from a number of morphologically and functionally diverse structures (see Stein 1998), the microanatomy of this convergence is of considerable interest. However, detailing the dendritic morphology of these multisensory neurons as well as the synaptology of their tectopetal afferents has been hampered by the fact that many afferents (particularly from the cortex) terminate on higher order dendrites that typically are not labeled with neurochemical markers expressed in the soma of SC output neurons (Cork et al., 1998; McHaffie et al., 2001; Soares et al., 2001; Behan et al., 2002).

*Corresponding author. Tel: +1-336-713-0150; fax: +1-336-716-4534.
E-mail address: vfuentes@wfubmc.edu (V. Fuentes-Santamaria).
Abbreviations: BDA, biotinylated dextran amine; NHS, normal horse serum; PB, phosphate buffer; PBS, phosphate-buffered saline; SAI, stratum album intermedium; SAP, griseum album profundum; SC, superior colliculus; SGI, stratum griseum intermediale; SGP, stratum griseum profundum; SGS, stratum griseum superficiale; SO, stratum opticum; SZ, stratum zonale; Tx, Triton X-100.

0306-4522/06$30.00+0.00 © 2005 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2005.11.045

In the present study, a combination of retrograde labeling and immunohistochemistry techniques was used to determine if SC neurons projecting to the reticular formation express the neurofilament protein SMI-32 and, if so, to document the morphology, distribution and proportion of such neurons. Furthermore, in order to determine to what extent SMI-32 immunostained neurons are colocalized with other chemical markers of SC neurons, we sought to determine if SMI-32 is co-expressed with parvalbumin, a calcium binding protein present in many cat SC output neurons (Mize et al., 1992). Preliminary results of aspects of these data have been presented briefly in abstract form (Fuentes-Santamaria et al., 2003).

EXPERIMENTAL PROCEDURES

Experimental animals

Nine cats were used in the present study. All the experimental procedures were conducted following the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" (NIH Publications No. 80–23, revised 1996) and approved by the Institutional Animal Care and Use Committee at Wake Forest University School of Medicine. Efforts were made to minimize the animals' discomfort and the number of animals used.

Nonfluorescent immunocytochemistry

Animals ($n=4$) were sedated with ketamine hydrochloride (30 mg/kg, i.m.) and acepromazine maleate (0.05–0.1 mg/kg, i.m.). Thirty minutes later, they were anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and, following the loss of pinna withdrawal reflexes, were perfused transcardially with 0.9% saline wash followed by a fixative solution of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.3. The brains were blocked *in situ*, removed, and stored overnight at 4 °C in the same fixative solution. On the following day, the brainstem was sectioned at 50 μm on a vibratome in a coronal plane. Sections were rinsed several times in phosphate-buffered saline (PBS) containing 0.2% Triton X-100 (Tx) and, in order to reduce nonspecific binding, were blocked for 1 h in PBS-Tx (0.2%) containing 10% normal horse serum (NHS). Sections then were incubated overnight at 4 °C with SMI-32 monoclonal antibody (1:10,000; Sternberger Monoclonals Inc., Jarrettsville, MD, USA) in a solution containing PBS-Tx (0.2%), pH 7.4. After four 15 min rinses in PBS-Tx (0.2%), sections were incubated for 1 h in biotinylated anti-mouse secondary antibody (1:200; Vector Laboratories, Burlingame, CA, USA). The Vector biotin–avidin procedure (Hsu et al., 1981) was used to link the antigen–antibody complex to HRP, which was then visualized with diaminobenzidine (DAB; 3,3 diaminobenzidine tetrahydrochloride) histochemistry. Finally, sections were mounted onto gelatin-coated slides, coverslipped using Cytoseal (Stephens Scientific, Riverdale, NJ, USA) and dried overnight at 4 °C. The specificity of this particular antibody has been verified previously in the cat (van der Gucht et al., 2001).

Tracer injection in the reticular formation

Animals ($n=3$) were rendered tractable by pretreatment with ketamine hydrochloride (30 mg/kg, i.m.) and acepromazine maleate (0.05–0.1 mg/kg, i.m.), intubated through the mouth, and then anesthetized for surgery with isoflurane (0.5–3%). Each animal was positioned in a stereotaxic head-holder, and a craniotomy was performed to provide access to the area of interest. The tracer biotinylated dextran amine (BDA) conjugated to Alexa-594 (Molecular Probes, Eugene, OR, USA) was dissolved in PB to yield a concentration of 10%. Three injections were made into the pontine reticular formation at different anteroposterior positions and depths using a 30° posterior approach (AP=−7.7, ML=−1, D=6; AP=−6, ML=−1, D=5; AP=−4, ML=−1, D=5.5). The coordinates of the injection sites were determined according to locations of terminal labeling seen in previous studies (Stein et al., 1982, 1984). The tracer was pressure injected using a 10 μl Hamilton syringe, yielding a deposit of approximately 3 μl of BDA at each site. Following the injection, the syringe was left in place for 15 min to minimize the migration of the tracer up the needle tract during its removal. The choice of BDA as tracer to retrogradely label neurons in the SC was based mainly on its ability to fill completely cell bodies, dendritic arborization, and also axon arbors allowing us to perform suitable morphometric studies. In addition, this tracer has been shown to be more sensitive than HRP for retrograde labeling and even more reliable that biocytin or neurobiotin (Lapper and Bolam, 1991). In the present study, a 10-day survival time was found optimal to label the tectoreticular pathway of the cat, although it may vary depending on the length of the neuronal pathway being studied as well as on the size of the animal (Vercelli et al., 2000). While this tracer has not been found to result in transynaptic labeling, it has been reported to label broken fibers of passage and surrounding intact fibers as well (Vercelli et al., 2000).

Fluorescent immunocytochemistry

Following a 10-day survival period, animals were anesthetized as indicated above and perfused transcardially with 0.9% saline wash followed by a fixative solution of 4% paraformaldehyde in 0.1 M PB, pH 7.3. The brains were sectioned at 50 μm on a vibratome in a coronal plane, and sections were examined under fluorescent microscopy to confirm the presence of retrogradely labeled neurons in the SC BDA-fluorescent sections were rinsed several times in PBS containing 0.2% Tx and, in order to reduce nonspecific binding, were blocked for 1 h in PBS-Tx (0.2%) containing 10% NHS. Sections were incubated overnight at 4 °C with SMI-32 monoclonal antibody (1:2500; Sternberger Monoclonals Inc.) in a solution containing PBS-Tx (0.2%), pH 7.4. After four 15 min rinses in PBS-Tx (0.2%), sections were incubated for 1 h in biotinylated anti-mouse secondary antibody (Vector Laboratories); the staining was visualized by incubation in a solution of PBS-Tx (0.2%) and streptavidin conjugated to Alexa 350 (Molecular Probes). Finally, sections were mounted onto gelatin-coated slides, coverslipped using Gel Mount mounting medium (Biomeda, Foster City, CA, USA), and dried overnight at 4 °C.

Parvalbumin and SMI-32 double labeling

Animals ($n=2$) were perfused as described above. Sections were rinsed four times in PBS-Tx (0.2%) and blocked for 1 h in PBS-Tx (0.2%) containing 10% NHS. They then were incubated overnight in a cocktail of SMI-32 (1:2500 Sternberger Monoclonals Inc.) and parvalbumin (1:1000, Affinity BioReagents, Golden, CO, USA) primary antibodies. Following four 15 min rinses in PBS-Tx (0.2%), sections were incubated in fluorescently labeled secondary antibodies for 2 h at room temperature (1:200, anti-mouse conjugated to Alexa 488 for SMI-32; 1:200, anti-rabbit conjugated to Alexa 594 for parvalbumin; Molecular Probes). Finally, the sections were rinsed in PBS, mounted, coverslipped, and maintained in the refrigerator.

Anatomical boundaries

A series of adjacent sections stained with Cresyl Violet were used to determine laminar boundaries within the SC in accordance with previous terminology (Kanaseki and Sprague, 1974). Briefly, the SC is generally divided into superficial and deep laminae. The superficial laminae consist of the stratum zonale (SZ), stratum griseum superficiale (SGS) and stratum opticum (SO), while the deeper layers (an amalgam of the intermediate and deep layers)