Neuroscience 210 (2012) 191–199

THE ANATOMICAL IDENTIFICATION OF SACCADIC OMNIPAUSE NEURONS IN THE RAT BRAINSTEM

M. HITTINGER[a1] AND A. K. E. HORN[b*]

[a]*Department of Clinical Radiology, Ludwig-Maximilians-University, D-81377 Munich, Germany*

[b]*Institute of Anatomy, Department I, Ludwig-Maximilians-University, D-80336 Munich, Germany*

Abstract—Omnipause neurons (OPNs) represent a crucial component for the generation of saccadic eye movements. They inhibit saccadic premotor neurons in the paramedian pontine reticular formation (PPRF) as well as in the rostral interstitial nucleus of the medial longitudinal fascicle (RIMLF) during the intersaccadic interval. In turn, inhibition of OPNs is a prerequisite in order to generate saccadic eye movements. Although the anatomy of the saccadic system including the OPNs has been extensively studied in primates and cats, no detailed anatomical description of these neurons in rats has been performed so far. The aim of the present study was the identification of putative OPNs in the rat brainstem based on their projection target, localization, and histochemical characteristics. Stereotactic tract-tracer injections into the rostral mesencephalon including the RIMLF in rat resulted in back-labeling of a neuron group adjacent to the midline at the level of traversing fibers of the abducens nerve, which are considered as OPNs lying in the nucleus raphe interpositus. Combined immunohistochemical staining for various markers revealed in these neurons the expression of parvalbumin, chondroitin sulfate proteoglycan, and glycine, but a lack of serotonin. The results of our study demonstrate the striking similarity between individual elements of the premotor saccadic network in rats and primates. The exact knowledge of their location in rats provides a basis for *in vitro* studies of the OPNs in rat brainstem slices.

Key words: oculomotor, eye movements, raphe nuclei, reticular formation, rodents.

Saccades are fast conjugate eye movements that direct the eyes to an object of interest. Voluntary saccades are primarily present in foveate vertebrates, such as monkeys (Stahl, 2004; Johnston and Everling, 2008), but spontaneous saccades used for gaze shifting and not for quick phases of compensatory eye movements have also been observed in mice and rats (McHaffie and Stein, 1982; Chelazzi et al., 1989; Bähring et al., 1994; Sakatani and Isa, 2007). With recording and tract-tracing studies the premotor circuitry for the generation of saccades has extensively been studied in cats and non-human primates (Moschovakis et al., 1996; Scudder et al., 2002; Shinoda et al., 2008). The quick phases of optokinetic and vestibulo-ocular reflexes have similar characteristics to saccades, and use similar brainstem pathways (Leigh and Zee, 2006). These reflexes are well developed in rats (Cazin et al., 1980, 1984; Hess et al., 1985), but little information on the saccadic neural circuitry for their generation is available for rats (Cooper and Phillipson, 1993).

Saccades, including quick phases of optokinetic and vestibulo-ocular reflexes, are generated by the coordinated action of two different types of neurons in the reticular formation: burst neurons (BNs) and omnipause neurons (OPNs) (Scudder et al., 2002; Leigh and Zee, 2006; Horn and Leigh, 2011). Premotor BNs exhibit a high-frequency burst only during a saccade, thereby activating the motoneurons of extraocular muscles monosynaptically (Fuchs et al., 1985; Moschovakis et al., 1996). During fixation and slow eye movements OPNs exert a tonic inhibition on premotor BNs, which is released just before a saccade by inhibition of the OPNs. In cats and monkeys excitatory BNs for horizontal saccades are located in the paramedian pontine reticular formation (PPRF) in the caudal pons, those for vertical saccades in the rostral interstitial nucleus of the medial longitudinal fascicle (RIMLF) in the rostral mesencephalon (for review Horn, 2006; Horn and Leigh, 2011). With tracer injections including intracellular tract-tracing methods the OPNs were shown to project to saccadic premotor BNs in the PPRF as well as to the BNs in the RIMLF (Strassman et al., 1987; Ohgaki et al., 1989; Horn et al., 1994; Horn and Büttner-Ennever, 2008).

The OPNs are located at the midline of the PPRF between the traversing fibers of the abducens nerve (NVI). In monkeys they form a circumscribed nucleus of two compact paramedian cell columns, which was termed nucleus raphe interpositus (RIP) (Büttner-Ennever et al., 1988). The neurons of the RIP are medium-sized with horizontally oriented

[1] Present address: Institute of Neuroradiology, University of Lübeck, Ratzeburger Allee 160, D-23538 Lübeck, Germany.

*Correspondence to: A. K. E. Horn, Institute of Anatomy, Dept. I, Ludwig-Maximilians-University, Pettenkoferstrasse 11, D-80336 Munich, Germany. Tel: +49-89-5160-4880; fax: +49-89-5160-4857.
E-mail address: Anja.Bochtler@med.uni-muenchen.de (A. K. E. Horn).

Abbreviations: BNs, burst neurons; CSPG, chondroitin sulfate proteoglycan; CTB, choleragenoid toxin subunit B; CTB-AU, choleragenoid toxin subunit B conjugated to gold; Cy, carbocyanine; GLY, glycine; GLYT2, glycine transporter 2; INC, interstitial nucleus of Cajal; LGNd, dorsal lateral geniculate nucleus; LGNv, ventral lateral geniculate nucleus; LSO, lateral superior olive; MGN, medial geniculate nucleus; ML, medial lemniscus; MLF, medial longitudinal fascicle; MNTB, medial nucleus of the trapezoid body; MSO, medial superior olive; NRPC, nucleus reticularis pontis caudalis; nVI, abducens nucleus; NVI, abducens nerve; NVII, facial nerve; nVm, motor trigeminal nucleus; OPN, omnipause neuron; PB, phosphate buffer; PC, posterior commissure; PDTg, posterodorsal tegmental nucleus; PPRF, paramedian pontine reticular formation; PPT, posterior pretectal nucleus; PR, prerubral field; PV, parvalbumin; PY, pyramidal tract; RIMLF, rostral interstitial nucleus of the medial longitudinal fascicle; RIP, nucleus raphe interpositus; RM, nucleus raphe magnus; SN, substantia nigra; TR, tractus retroflexus; WGA-HRP, wheat germ agglutinin conjugated to horseradish peroxidase; ZI, zona incerta; 5-HT, serotonin.

0306-4522/12 $36.00
10.1016/j.neuroscience.2012.02.048

dendrites, which reach across the midline. Unlike the ventrally and dorsally bordering nucleus raphe magnus (RM) and nucleus raphe pontis (RP) the OPNs are not serotoninergic, but use glycine as a transmitter (Horn et al., 1994). Presumably on account of their high rate of metabolic activity, OPNs contain high concentrations of cytochrome oxidase (COX) and the calcium-binding protein parvalbumin (PV), and they are ensheathed by perineuronal nets; these properties can be used to identify OPNs in cats and humans as well (Büttner-Ennever et al., 1988; Horn et al., 1994, 2003; Horn and Adamczyk, 2011).

In the present paper we provide anatomical data on the location of OPNs based on their projections to the RIMLF and their histochemical properties, for example, immunoreactivity for glycine markers, parvalbumin, perineuronal nets and their lack of serotonin.

EXPERIMENTAL PROCEDURES

Eleven pigmented rats were used for this study, and all experiments were performed in accordance with state regulations and with approval of the state and university animal care committees. For immunocytochemical staining with antibodies against PV, glycine-transporter 2 (GLYT2)- and chondroitin sulfate proteoglycan (CSPG)-based perineuronal nets, four rats were killed with an overdose of pentobarbital, and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After removal from the skull the brains were immersed in 10% sucrose in 0.1 M phosphate buffer and transferred to 30% sucrose for frozen sectioning. One case was cut using a vibratome.

For combined tract-tracing and immunocytochemistry six rats received a tracer injection into the RIMLF. The animals were deeply anaesthetized with equithesin (2.5 ml/kg body weight) and placed into a stereotaxic frame (Stoelting). After a skin cut the skull was exposed and the bone landmarks (lambda and bregma) were used to determine the coordinates of the location of RIMLF (bregma −4.5 mm, lateral 0.5 mm, depth 7.2 mm) for tracer injection according to the atlas (Paxinos and Watson, 1997). The skull was trepanned regionally and the tracer was injected using a fine glass pipette mounted on a Hamilton syringe that was attached to the stereotactic frame. Four animals received an injection of Wheat germ agglutinin-horseradish peroxidase (WGA-HRP; Sigma; L3892; 2.5% in 0.9% saline), two animals received an injection of non-toxic 0.5 μl choleragenoid toxin subunit B conjugated with colloidal gold (CTB-AU Biological List Laboratories # 1085; 10% in aqua bidest) and two animals were injected with unconjugated CTB-AU (1% in aqua bidest) (see Table 1). The pipette was left in place for 10 min before being carefully retracted. The animals were allowed to wake up and they were housed individually until recovery, and all efforts were made to minimize their discomfort. After a survival time of 3 days (WGA-HRP or CTB) or 7 days (CTB-AU) the animals were killed with an overdose of sodium-pentobarbital and were transcardially perfused with 0.9% saline followed by either a mixture of paraformaldehyde and glutaraldehyde in 0.1 M phosphate buffer (PB) (pH 7.4) or 4% paraformaldehyde in 0.1 M PB (pH 7.4) (see Table 1). The brain was removed and equilibrated in increasing concentrations of sucrose in 0.1 M PB (10%–30%) for freeze cutting. The brainstems were cut at 40 μm in the transverse stereotaxic plane. WGA-HRP was visualized with the tetramethylbenzidine method with stabilization in ammonium heptamolybdate and a subsequent reaction with diaminobenzidine-cobalt (DAB-Co) in the presence of 0.01% H_2O_2 (Horn and Hoffmann, 1987). CTB was detected with immunoperoxidase methods as described previously (Büttner-Ennever et al., 2001). CTB-AU was visualized according to the protocol of Llewellyn-Smith et al. (1990). In brief, slices were collected and washed in 0.05 M citric acid buffer (pH 5.5). In order to enhance the permeability, slices were incubated in 50% ethanol for 30 min before being washed in citric acid buffer (pH 5.5) followed by treatment with two changes of freshly prepared IntenSE kit (Amersham Biosciences, Little Chalfont, UK) in the dark for 70 and 40 min, respectively. After three 5-min washes in citric acid buffer followed by three 5-min washes in 2% sodium acetate sections were gold-toned by an incubation in 0.05% chloroauric acid for 7 min. After washing in sodium acetate sections were fixed in three 5-min changes of 2.5% Na thiosulfate and several rinses in sodium acetate.

After tracer visualization free-floating sections were processed for the immunocytochemical detection of either PV, serotonin (5-HT)), GLYT2 or glycine (GLY). The sources and dilutions of primary antibodies are listed in Table 2. The locations of antibody binding sites were detected with incubations in biotinylated anti-mouse (1:200; Vector Lab) for PV or anti-goat (1:200; Vector Lab) for GLYT2, or anti-rabbit (1:200; Vector Lab) for 5-HT and GLY followed by a 1-h incubation in extravidin-peroxidase (1:1000; Sigma) and visualized with 0.05% diaminobenzidine (DAB) and 0.01% hydrogen peroxide in Tris buffer (pH 8) resulting in a homogeneous brown staining of immunopositive cell bodies. Selected sections of two cases were treated with 2% normal donkey serum in 0.1 M TBS (pH 7.4) containing 0.3% Triton-X 100 for 1 h and then incubated in a cocktail containing rabbit anti-CSPG (1:1000) and mouse anti-PV (1:1000) over night. After three washes the sections were treated with a mixture of donkey carbocyanine 3 (Cy3)-tagged anti-rabbit IgG (1:200; Dianova, Hamburg Germany) and donkey Cy2-tagged anti-mouse IgG (1:200; Dianova, Hamburg Germany) for 2 h.

Analysis of stained sections

The slides were examined with a microscope equipped for bright-field and fluorescence illumination (Leica DMRB, Bensheim, Germany). Photographs were taken with a digital camera (Pixera Pro 600 ES; Klughammer, Markt Indersdorf, Germany) mounted on the microscope. The images were captured on a computer with Pixera Viewfinder software (Klughammer) and processed with Photoshop 7.0. After conversion into black and white, the sharp-

Table 1. List of experimental cases and injection parameters

Case	Injection site	Tracer	Volume	Survival time	Fixation
RMH1	RIMLF	(10%) CTB-AU	0.5 μl	7 d	0.5% GA/2% PFA
RMH2	RIMLF	2.5% WGA-HRP	0.3 μl	3 d	4% PFA
RMH3	RIMLF	(10%) CTB-AU	0.5 μl	7 d	4% PFA
RMH3*	RIMLF	2.5% WGA-HRP	0.3 μl	3 d	4% PFA
RMH4	RIMLF	2.5% WGA-HRP	0.3 μl	3 d	1% PFA/2.5% GA
RMH5	RIMLF	2.5% WGA-HRP	0.3 μl	3 d	1% PFA/2.5% GA
RMH6	Dorsocaudal to RIMLF	1% CT-B	0.5 μl	3 d	4% PFA
RMH7	Caudal to RIMLF	1% CT-B	0.5 μl	3 d	4% PFA