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BRAINSTEM GLYCINERGIC NEURONS AND THEIR ACTIVATION DURING ACTIVE (RAPID EYE MOVEMENT) SLEEP IN THE CAT

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Abstract—It is well established that, during rapid eye movement (REM) sleep, somatic motoneurons are subjected to a barrage of inhibitory synaptic potentials that are mediated by glycine. However, the source of this inhibition, which is crucial for the maintenance and preservation of REM sleep, has not been identified. Consequently, the present study was undertaken to determine in cats the location of the glycinergic neurons, that are activated during active sleep, and are responsible for the postsynaptic inhibition of motoneurons that occurs during this state. For this purpose, a pharmacologically-induced state of active sleep (AS-carbachol) was employed. Antibodies against glycine-conjugated proteins were used to identify glycinergic neurons and immunocytochemical techniques to label the Fos protein were employed to identify *activated* neurons.

Two distinct populations of glycinergic neurons that expressed *c-fos* were distinguished. One population was situated within the nucleus reticularis gigantocellularis (NRGc) and nucleus magnocellularis (Mc) in the rostroventral medulla; this group of neurons extended caudally to the ventral portion of the nucleus paramedianus reticularis (nPR). Forty percent of the glycinergic neurons in the NRGc and Mc and 25% in the nPR expressed *c-fos* during AS-carbachol. A second population was located in the caudal medulla adjacent to the nucleus ambiguus (nAmb), wherein 40% of the glycinergic cells expressed *c-fos* during AS-carbachol. Neither population of glycinergic cells expressed *c-fos* during quiet wakefulness or quiet (non-rapid eye movement) sleep.

We suggest that the population of glycinergic neurons in the NRGc, Mc, and nPR participates in the inhibition of somatic brainstem motoneurons during active sleep. These neurons may also be responsible for the inhibition of sensory and other processes during this state. It is likely that the group of glycinergic neurons adjacent to the nucleus ambiguus (nAmb) is responsible for the active sleep-selective inhibition of motoneurons that innervate the muscles of the larynx and pharynx.

Key words: atonia, motoneurons, REM sleep, postsynaptic inhibition, medulla, trigeminal.

The suppression of motoneuron activity by postsynaptic inhibition during active sleep (AS or rapid eye movement (REM) sleep) (Chase et al., 1980; Morales and Chase, 1981; Pedroarena et al, 1994; Pompeiano, 1967) is produced by glycinergic postsynaptic processes (Chase et al., 1989; Soja et al., 1990; Morales et al., 1999; see also Chase and Morales, 2005). The main objective of the present work was to explore glycinergic cell populations in the brainstem vis-à-vis their activation and the subsequent inhibition of motoneurons during AS. Accordingly, we examined the activation of glycinergic neurons in the brainstem of the cat during a state elicited by the microinjection of carbachol in the pons (nucleus pontis oralis) that closely resembles normal AS (Baghdoyan et al., 1987; Lydic and Baghdoyan, 2005); we refer to this state as AS-carbachol. The activation of neurons during this state was assessed by the immunocytochemical detection of the Fos protein.

Not only are somatic motoneurons inhibited during AS, but somatosensory transmission in the brainstem is also suppressed by both pre- and/or postsynaptic inhibitory processes (Cairns et al., 2003; Cairns and Soja, 1998; Pompeiano et al., 1967; Soja et al., 1990, 2001; Taepavarapruk, 2002). In addition, waking-related neurons in brainstem nuclei, such as the raphe and locus coeruleus, appear to be inhibited as well as disfacilitated during this state (Jacobs et al., 1981; Hobson et al., 1983; Siegel, 2005). Consequently, we also examined populations of glycinergic neurons that potentially participate in the inhibition of somatosensory processes in other brainstem nuclei during AS.

Brainstem glycinergic neurons that express *c-fos* during AS were found to be restricted to two cell populations, one within the nucleus reticularis gigantocellularis (NRGc) and nucleus magnocellularis (Mc), including an extension into the nucleus paramedianus reticularis (nPR) in the rostral ventro-medial medulla. A second population surrounded the nucleus ambiguus (nAmb) in the caudal medulla. These groups of neurons, we suggest, are responsible for the diverse inhibitory processes of brainstem origin that occur during AS.

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Abbreviation: AS, active sleep; BSA, bovine serum albumin; CTb, cholera toxin subunit B; DAB, diaminobenzidine tetrahydrochloride; DMn, deep mesencephalic nucleus; IgG, immunoglobulin G; Mc, nucleus magnocellularis; nAmb, nucleus ambiguous; N_d, neuron "density"; NPC, nucleus reticularis pontis caudalis; NPO, nucleus reticularis pontis oralis; nPR, nucleus paramedianus reticularis; NRGc, nucleus reticularis gigantocellularis; OSA, obstructive sleep apnea; PAG, periaqueductal gray; PBS, phosphate buffer saline; PBST, phosphate buffer saline, 0.3% Triton X-100; PcRF, parvocellular reticular formation; REM, rapid eye movement.

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EXPERIMENTAL PROCEDURES

Thirteen adult cats of both sexes, weighing between 3.0–3.5 kg, were utilized in the present study. Six animals were studied in conjunction with AS-carbachol (experimental animals); seven cats, in which saline was injected into the nucleus reticularis pontis oralis (NPO), were used as controls; four were killed during quiet sleep; three were killed during quiet wakefulness.

Initial surgical procedures

All experimental procedures were approved by the Chancellor's Animal Research Committee (ARC) of the UCLA Office for the Protection of Research Subjects. The surgical procedures to prepare "chronic" cats for behavioral state recordings and the delivery of substances into the brainstem have been previously described (Morales et al., 1999). All efforts were made to use only the number of animals necessary to produce reliable scientific data and to minimize animal suffering.

Two weeks after surgery, the cats were sleep-adapted in a head-restraining device. One day before the final experiment, each animal was anesthetized with halothane, a bone wax plug covering an access hole in the calvarium was removed, and the underlying dura mater was cut. On the day of the experiment, EEG, EMG, and EOG activity were monitored. Following a baseline recording period of one hour, the cannula of a 1 μl Hamilton syringe (Hamilton, Reno, NV, USA), which was filled with a solution of carbachol (16 μg in 1 μl of saline), was lowered through the access hole in the occipital bone in order to inject carbachol at the following stereotaxic coordinates: posterior 3.0, lateral 1.3, vertical −3.5 (Berman, 1968). The reader is referred to diagrams of previous publications where the site of injections are shown (Morales et al., 1987; and Xi et al., 2004). Six cats were injected with 0.1 μl of this solution. After a short latency (1–4 min), these animals entered into AS-carbachol; this state lasted for an average of 1 h and 40 min.

In seven control cats, the preceding procedures were followed, except that saline was injected instead of carbachol. In four animals, during the recording session, the (control) animals remained in a state of quiet sleep. Three animals were kept awake before killing them. Two experimental and two control cats were also prepared for retrograde tracing experiments using cholera toxin subunit B (CTb), as previously described (Morales et al., 1999). To identify premotor trigeminal interneurons, CTb was injected by iontophoresis (2 μA positive current pulses, 7 s on, 7 s off, for 20 minutes in each barrel) into the masseter nucleus at a point where the antidromic field potential evoked by i.m. electrical stimulation of masseter nerve fibers was largest (4–5 mV; Castillo et al., 1991). These cats were allowed to survive for 10–14 days before the final experimental procedure. The experiments dealing with premotor neurons were included in order to confirm and expand the data presented in our previous publication (Morales et al., 1999).

Following the experimental recording sessions, the animals were injected with an overdose of sodium pentobarbital (60 mg/kg, i.p.) and perfused transcardially with 1 l of saline followed by 2.5 liters of a solution of 4% paraformaldehyde, 15% saturated picric acid and 0.25% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4. The brainstem was removed and immersed for a 24 h post-fixation period in a solution consisting of 2% paraformaldehyde and 15% saturated picric acid in a 0.1 M phosphate buffer at pH 7.4. Following post-fixation, the tissue was kept for 48 h in a solution of sucrose (30%) in a 0.1 M phosphate buffer at pH 7.4 for cryoprotection.

Subsequently, the brainstem was frozen and cut in 15 μm thick sections using a cryostat. Each section was placed in one well of a 36 well tray containing a phosphate buffered solution (phosphate buffer saline, PBS). The first section obtained was placed in the first well of the tray and consecutive sections were placed in the remaining wells in serial order. Section number 37 was placed in well 1 and the procedure was repeated until the entire brainstem was sectioned. Each well contained a sample of the entire brainstem with each section in the well separated by 540 μm (i.e. 15 μm$\times$36). By means of this procedure, neighboring wells that contained pairs of adjacent sections could be processed using different immunohistochemical procedures. After sectioning, the tissue was stored in 0.1 M phosphate buffer saline containing 0.3% Triton X-100 (PBST) and 0.1% sodium azide.

Immunocytochemistry

A polyclonal rabbit antibody was used for Fos immunostaining (Fos Ab5, Oncogene Research Products/Calbiochem, Cambridge, MA, USA). Free-floating sections were incubated overnight in this antiserum at a dilution of 1:20,000 by PBST–azide. The sections were then rinsed in PBST for 30 min and incubated for 90 min in biotinylated donkey anti-rabbit immunoglobulin G (IgG) diluted 1:300, that contained 1.5% normal donkey serum. After rinsing for 30 min, the sections were incubated for 90 min in the ABC complex at a dilution of 1:200. The tissue was then rinsed and the peroxidase activity was visualized by the diaminobenzidine tetrahydrochloride (DAB) method described above. After several rinses of PBST, control sections were mounted onto Superfrost Plus slides (Erie Scientific, Portsmouth, NH, USA) from a PBST diluted to 0.01%. In those neurons that expressed *c-fos*, Fos immunostaining was restricted to the cell nuclei (Fos^+ neurons). Immunostaining was never observed in the cytoplasm of these cells or in Fos^- neurons.

The tissue was processed to detect glycine-like immunoreactivity after processing for Fos immunolabeling. The following different rabbit antibodies, raised against glycine-conjugated proteins, were used initially: rabbit anti-glycine polyclonal from Chemicon (Temecula, CA, USA); rabbit anti-glycine polyclonal from SFRI Berganton, France or the antibody previously employed by Rampon et al. (1966a,b). We use the term glycine-like immunoreactivity, see below, to reflect the fact that the antibody is fabricated against a protein conjugated with glycine, not against glycine itself. Although the immunostaining achieved with the latter two antibodies was qualitatively similar to that obtained using the antibody from Chemicon, the results employing the Chemicon antibody were superior in terms of the intensity and specificity of the staining. Therefore, in the present report we only refer to data obtained with the Chemicon antibody. The procedures employing this antibody were as follows: sections were first treated with 0.1 M sodium-borohydride for 20 min, rinsed with four changes of doubled-distilled water and three changes of PBS pH 7.4. They were then placed into a solution of 3% normal donkey serum for 30 min and transferred to a solution containing the glycine antibody (1:5000) in 6% normal donkey serum and 2% bovine serum albumin (BSA), 0.1 M PBS pH 7.4 containing 0.1% sodium azide, for 72 h. After the sections were rinsed in PBS, they were immersed in a solution of 1:500 biotinylated donkey anti-rabbit IgG (Jackson Laboratories, West Grove, PA, USA) in 3% normal donkey serum (2% BSA, PBS) for 90 min. The sections were then incubated in a solution of 1:500 ABC (Vector Laboratories, Burlingame, CA, USA) for 60 min. Thereafter, the sections were reacted in a solution of DAB/hydrogen peroxide for 15–20 min. After a final washing, they were mounted on Superfrost Plus slides, dehydrated, cleared and coverslipped.

Neurons displaying glycine-like immunoreactivity were consistently observed in regions in which other researchers, in studies that did not involve behavioral analyses, also described the existence of glycinergic cells (Fort et al., 1993; Li et al., 1996). In addition, neurons which are known *not* to employ glycine as a neurotransmitter remained unstained (for example see cells in Fig. 1).

A preabsorption test was performed to further evaluate the specificity of the glycine antibody; these procedures were sim-