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Nitrergic ventro-medial medullary neurons activated during cholinergically induced active (REM) sleep in the cat

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Abstract

The rostral ventro-medial medullary reticular formation is a complex structure that is involved with a variety of motor functions. It contains glycinergic neurons that are activated during active (REM) sleep (AS); these neurons appear to be responsible for the postsynaptic inhibition of motoneurons that occurs during this state. We have reported that neurons in this same region contain nitric oxide (NO) synthase and that they innervate brainstem motor pools. In the present study we examined the *c-fos* expression of these neurons after carbachol-induced active sleep (C-AS). Three control and four experimental cats were employed to identify *c-fos* expressing nitrergic neurons using immunocytochemical techniques to detect the Fos protein together with neuronal nitric oxide synthase (nNOS) or NADPH-diaphorase activity. The classical neurotransmitter content of the nitrergic cells in this region was examined through the combination of immunocytochemical techniques for the detection of glutamate, glycine, choline acetyltransferase (ChAT), tyrosine hydroxylase (TH) or GABA together with nNOS. During C-AS, there was a 1074% increase in the number of nitrergic neurons that expressed *c-fos*. These neurons did not contain glycine, ChAT, TH or GABA, but a subpopulation (15%) of them displayed glutamate-like immunoreactivity. Therefore, some of these neurons contain both an excitatory neurotransmitter (glutamate) and an excitatory neuromodulator (NO); the neurotransmitter content of the rest of them remains to be determined. Because some of the nitrergic neurons innervate brainstem motoneurons it is possible that they participate in the generation of tonic and excitatory phasic motor events that occur during AS. We also suggest that these nitrergic neurons may be involved in autonomic regulation during this state. In addition, because NO has trophic effects on target neurons, the present findings represent the first, albeit indirect, evidence for a possible trophic function of this nature during AS.

Keywords

Nitric Oxide; premotor interneurons; reticular formation

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The rostral portion of the ventro-medial medullary reticular formation (VMMR) is a complex region involved in motor, pain and autonomic control (Holstege, 1996; Batten et al., 2000; Mason, 2001). This structure contains a chemically diverse population of neurons that contain glutamate, glycine, GABA, Acetylcholine and several neuropeptides (Fort et al., 1989 and 1990; Holstege, 1996; Morales et al., 1999). In addition, numerous nitrergic neurons, named that way because they express the neuronal isoform of the enzyme nitric oxide synthase (nNOS), have been described in this region (Vincent and Kimura, 1992; Rodrigo et al., 1994; Batten et al., 2000; Pose et al., 2005; Travers et al., 2005).

The present work concentrates on the nitrergic neurons contained in a restricted portion of the rostral VMMR which corresponds to the ventral aspect of the nucleus reticularis gigantocellularis (NRGc) and the nucleus magnocellularis (Mc) and was designed to examine active-sleep related functions of these neurons. Many studies indicate that this region of the reticular formation is involved in the generation of the motor atonia that occurs during the state of active sleep (AS; see for example Morales et al., 1999 and references therein). We have shown that it contains trigeminal premotor neurons that express *c-fos* during carbachol-induced active sleep (C-AS). A subpopulation of these premotor neurons is glycinergic and appears to be responsible for the postsynaptic inhibition of motoneurons during AS (ibid, Morales et al., 2006). These studies were carried out in animals in which carbachol was injected in the Nucleus Pontis Oral (NPO) to evoke long-lasting (1-2 hours) episodes of C-AS (Baghdoyan et al., 1987 and 1989; Morales et al., 1999).

We have previously shown that nitrergic neurons in the rostral VMMR of the cat innervate brainstem motor pools (Pose et al., 2005). Nitrergic neurons which were retrogradely labeled with Cholera toxin subunit b (Ctb) injected into the trigeminal and hypoglossal motor nuclei (mV and mXII respectively) were located in a region which overlaps the location of glycinergic neurons described above.

The afore mentioned observations led us to question if the nitrergic premotor neurons of this region are activated during AS sleep and which neurotransmitter, in addition to NO, is released by them during this state. For this purpose we examined the pattern of *c-fos* expression in the population of nNOS containing neurons of this region after C-AS. In addition, we investigated which “classical” neurotransmitters are contained in these cells. We determined that after C-AS there is a significant increase in *c-fos* expression in this population of nNOS containing neurons and thus that they are activated during this state. On the other hand, these nitrergic cells do not contain glycine, GABA or adrenergic neurotransmitters, whereas a percentage of them are glutamatergic.

Experimental procedures

All experimental procedures were conducted in accord with the *Guide for the Care and Use of Laboratory Animals* (7th edition) and approved by the Chancellor’s Animal Research Committee of the University of California at Los Angeles (UCLA) for the protection of research subjects. All efforts were made to minimize the number of animals and their suffering. Seven adult cats of either sex, weighing between 3.0-3.5 kg, were utilized in the present study. These animals were provided by the UCLA School of Medicine Vivarium. Four animals were studied following prolonged periods of C-AS (experimental animals); three cats were used as controls (control animals). Tissue from an additional animal which had received an injection of Ctb in the trigeminal motor nucleus and studied after a prolonged period of C-AS was also included in this work.

Surgical procedures

These procedures were similar to those reported in previous work (see for example Morales et al., 1999). Animals were prepared for recording states of sleep and wakefulness and for the delivery of substances within the brainstem. Before anesthesia, the animals were premedicated with atropine (0.4 mg/kg, im) and Xylazine (2mg/kg, im). Anesthesia was first induced with Ketamine (15mg/kg, im) and maintained with a gas mixture of halothane in oxygen (2-3%).

The head of the cat was positioned in a heavy duty stereotaxic frame, and the calvarium was exposed. Stainless steel screw electrodes were threaded into the frontal and parietal bones and into the orbital portion of the frontal bone to record EEG and electro-oculogram (EOG) waves, respectively. A Winchester plug that was connected to these electrodes and a chronic head-restraining device were bonded to the calvarium with acrylic cement. An opening 3.0 - 4.0 mm in diameter was drilled in the calvarium overlying the cerebellar cortex. This craniotomy was covered with bone wax and provided later access to the NPO for the injection of carbachol. During the recovery period from surgery, an analgesic (Buprenex, 0.01 mg/kg, im) was administered. An antibiotic (Cephazolin) was administered parenterally for 4 days. The wound margins were regularly cleaned and covered with antibiotic ointment. The animals were allowed to recover from these surgical procedures for 2 weeks. After this period, they were adapted to the head-restraining device until they exhibited episodes of naturally occurring sleep.

One additional animal was injected with Ctb within the trigeminal motor nucleus to retrogradely label premotor neurons in the brainstem. For this purpose, a three barreled micropipette assembly, consisting of a carbon fiber-recording microelectrode and two side barrels containing Ctb (List Biological Laboratories, Campbell, CA), was lowered into the trigeminal motor pool. As described in previous communications, Ctb was injected by iontophoresis (2 μ A positive current pulses, 7 sec on and 7 sec off, for 20 min in each barrel) at the site where the antidromic field potential was largest (4-5 mV), (Morales et al., 1999; Pose et al., 2005). The cat was allowed to survive for 10-14 days, adapted to the head-restraining device and afterwards treated as the other experimental animals as described below.

Experimental and control animals

During the sleep studies, the EEG, electromyogram (EMG), and electro-oculogram (EOG) activity were recorded to score sleep states. On the day of the final experiment the cannula of a 1 μ l Hamilton syringe filled with a solution of carbachol (88 mM in saline), was lowered through the access hole in the occipital bone to inject carbachol at the stereotaxic coordinates posterior 3.0, lateral 1.3, vertical -3.5 (Berman, 1968). Many previous studies have shown that a prolonged AS-like state occurs only if carbachol is injected into a restricted area of the rostral pontine tegmentum (Baghdoyan et al., 1987, 1989; Vanni Mercier, 1989). In our studies the stereotaxic boundaries of this region according to Berman (1968) have been posterior 2-3.9, lateral 1.2-2, vertical -3.5-7 (see for example Morales et al., 1987 for histological confirmation of injection sites). In the present work four cats were injected with 0.1 μ l of carbachol solution (equivalent to 1.6 μ g of carbachol). One to 3 minutes were usually required to induce C-AS. These experimental animals remained in C-AS 90% of the recording time. Three cats were employed as control animals; in these cats the same procedures as in the experimental animals were employed except that saline was substituted for carbachol. During the recording session, these control animals remained in a state of quiet wakefulness with intermittent episodes of quiet (non-REM) sleep.

Following a two hour recording session, the animals were given an overdose of sodium pentobarbital (60 mg/kg, i.p.) and perfused transcardially with 1 liter of heparinized saline followed by 2.5 liters of a solution of 4% paraformaldehyde, 15% saturated picric acid and 0.25 % glutaraldehyde in 0.1M phosphate buffer (PB) at pH 7.4. The brainstem was removed and immersed for a 24 hour post-fixation period in a solution consisting of 2% paraformaldehyde and 15% saturated picric acid in 0.1M PB at pH 7.4. Following post-fixation, the tissue was kept in a solution of sucrose (30%) in 0.1M PB at pH 7.4. Forty-eight to seventy-two hours later, the brainstem was frozen and cut into 15 μ m-thick coronal sections using a cryostat. Each section was placed in one well of a 36 well tray containing a buffered solution of 0.1M PBS, 0.3% Triton X-100 (PBST) and 0.1% sodium azide. 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. Therefore, each well contained a sample of the entire brainstem consisting in a set of sections separated by 540 μ m from one another (*i.e.*, 15 μ m $\times$ 36). Using this procedure, neighboring wells contained adjacent sections. The sections contained in different wells were then processed using different antibodies.

Immunohistochemical procedures

Free floating sections were processed for the combined detection of Fos immunoreactivity together with either neuronal nitric oxide synthase (nNOS) immunoreactivity or NADPH-diaphorase (NADPH-d) activity. Immunocytochemistry for Fos, nNOS and/or NADPH-d histochemistry were also combined with immunocytochemical procedures to detect several neurotransmitters. Unless otherwise stated the tissue was processed first for Fos detection and afterwards for neurotransmitter detection.

For Fos immunostaining the sections were incubated overnight in a polyclonal rabbit antibody (Fos Ab5, Oncogene Research Products, Calbiochem, MA) at a dilution of 1:20,000 by PBST-azide. The sections were then rinsed in PBST for 30 minutes and incubated for 90 minutes in biotinylated donkey anti-rabbit IgG (Jackson Immuno Research Laboratories, West Grove, PA) diluted 1:300 in PBST containing 1.5% normal donkey serum (NDS). After rinsing for 30 minutes, the sections were incubated for 90 minutes in a standard ABC complex (Vector Standard Elite kit, Vector Laboratories, Burlingame, CA) at a dilution of 1:200. Peroxidase activity was visualized by processing the sections with the Nickel Ammonium Sulfate enhanced diaminobenzidine (DAB) method consisting of immersion in 0.6% Nickel Ammonium Sulfate, 0.02% DAB and 0.015% hydrogen peroxide in 50ml of 50mM Tris-buffered saline (pH = 7.6) for 15-30 minutes.

For nNOS immunocytochemistry, free floating sections were incubated overnight in a polyclonal antibody directed against nNOS (Accurate Chemical and Scientific Corp. Westbury, NY) dilution 1:500. After rinsing in PBST, the tissue was incubated for 90 minutes in biotinylated donkey anti-rabbit serum (Jackson Immuno Research Laboratories) at a dilution of 1:500. The sections were then rinsed in PBST and treated with the ABC complex (Vector Standard Elite kit). Peroxidase activity was visualized with the DAB/hydrogen peroxide method as described above except that a non-intensified (absence of nickel ions) reaction was employed to produce brown immunoreactivity. A number of sections were processed for nNOS by immunofluorescent methods. In these cases after treatment with the primary antibody the sections were exposed to Alexa Fluor 594 donkey anti-rabbit IgG 1:200 (Molecular Probes, Eugene, OR, USA).

NADPH-diaphorase (NADPH-d) chemical activity was examined using nitro blue tetrazolium (NBT). To detect NADPH-d chemical activity, sections were incubated in a solution of 0.1M PBS, pH 7.4, 0.3% Triton, 0.1 mg/ml NBT and 1.0 mg/ml of beta-NADPH

for 30-60 minutes at 37°C. It has been demonstrated that after tissue fixation with aldehyde fixative solutions, the only remaining NADPH-d histochemical activity in the tissue corresponds to that of the nNOS enzyme (Dawson et al., 1991; Hope et al., 1991).

For glutamate labeling, the sections were preincubated in 6% NDS in PBST; they were then exposed to mouse anti-glutamate antibody (Swant, Bellinzona, Switzerland) 1:1500, 3% NDS in PBST and incubated in donkey anti-mouse serum (Jackson Immuno Research Laboratories) 1:200, 3% NDS. Subsequently, the sections were incubated in standard ABC (Vector Laboratories) 1:200. Finally, they were treated by the DAB method without nickel-enhancement. For the immunofluorescent detection of glutamate after exposure to the primary antibody the sections were treated with Alexa Fluor 488 donkey anti-mouse IgG (Molecular Probes). In a subset of experiments glutamatergic neurons were identified employing an antibody for the glutamate transporter VGLUT2. The sections were treated with 5% NDS with 2% bovine serum albumin (BSA) for 1 hr in order to pre-block non-specific binding sites in the tissue. Subsequently, the sections were incubated with a mouse monoclonal antibody directed against VGLUT2 (MAB5504, Millipore, Temecula, CA) 1:350 in 3% NDS, PBST, and 0.1% sodium azide, first at room temperature overnight and then at 4° C for 48 hours. The tissue sections were then incubated with a donkey anti-mouse IgG secondary antibody (Jackson Immuno Research Laboratories) 1:400 in PBST for 90 min at room temperature. To visualize VGLUT2 immunoreactivity, a similar ABC technique (1:400 in PBST; 1 hr) as described above was used. Both of the immunohistochemical techniques employed to identify glutamatergic neurons yielded similar results.

For glycine labeling, sections were pretreated with 0.1 M sodium-borohydride for 20 min, followed by NDS 3% and then transferred to a solution containing a rabbit anti-glycine polyclonal antibody (Chemicon International, Temecula, CA, USA) 1:5000 in 6% NDS, 2% BSA, 0.1% sodium azide in PBS for 72 hours. Afterwards the sections were rinsed in PBS and immersed in a solution of 1:500 biotinylated donkey antirabbit IgG (Jackson Immuno Research Laboratories) in 3% NDS, 2% BSA, PBS for 90 min. Subsequently the sections were rinsed in PBS, treated with the ABC complex (Vector Standard Elite kit) 1:500 for 60 min and processed with the DAB/hydrogen peroxide method.

To label cholinergic neurons, immunohistochemistry for choline acetyltransferase (ChAT) was performed. Sections were sequentially incubated in a goat anti-ChAT polyclonal antibody (Chemicon International) 1:500; donkey anti-goat IgG (Jackson Immuno Research Laboratories) 1:300 in PBST and ABC, 1:200. Subsequently, the tissue was treated with the DAB method. Alternatively, for the detection of ChAT by immunofluorescent methods, after exposure to the primary antibody the sections were incubated in Alexa Fluor 488 donkey anti-goat IgG (Molecular Probes) 1:200.

For the detection of tyrosine hydroxylase (TH), the sections were first incubated in PBS, 50mM glycine, 0.1% BSA followed by immersion in sheep anti-TH antibody (Pell-Freez, Rogers, AR, USA) 1:5000 and exposed to Alexa Fluor 488 donkey anti-sheep IgG (Molecular Probes) 1:1000.

To detect gabaergic cells, we used glutamate decarboxylase (GAD) labeling. Sections were incubated in 0.5% sodium borohydride and thereafter, they were incubated in PBS, 50mM glycine, 0.1% BSA, followed by 6% BSA in PBST; they were then exposed to mouse anti-GAD67 serum (Chemicon International) 1:1000, PBST, 4% BSA. Thereafter, the sections were incubated in Alexa Fluor 555 donkey anti-mouse IgG (Molecular Probes) 1:500.

For CTb immunocytochemistry, sections which had been processed for Fos detection were incubated in goat serum raised against CTb (List Biological Laboratories, CA.) at a dilution

of 1:20,000 in PBS-.25% Triton-.1% Na Azide (PBST-AZ). They were then rinsed over a 30 minute period and placed for 90 minutes in biotinylated donkey anti-goat serum (Jackson Immuno Research Laboratories) diluted 1:2,000 with PBST. After rinsing for 30 minutes, the sections were incubated in standard ABC complex (Vector Laboratories) for 90 minutes at room temperature at a dilution of 1:400, rinsed for 30 minutes and then processed with the DAB method.

To confirm antibody specificity, preadsorption and omission tests were performed for nNOS, whereas omission tests were carried out for GAD, TH, ChAT, Glutamate, and glycine. No positive immunoreactivity was detected in these experiments.

Analysis

Histological sections were examined using an Olympus BX60 microscope (Olympus Optical, Tokio, Japan). Photomicrographs for neuronal counting and illustrations were obtained by means of a digital camera attached to the microscope and connected to a microcomputer with Photoshop software (Adobe Systems, San Jose, CA, USA). A Nikon Eclipse E600 FN epifluorescence microscope (Nikon Instrument, Inc. Melville, NY, USA) equipped with the appropriate filter set for each probe was used to analyze sections stained with fluorescent antibodies. Images were obtained with a digital camera and the localization of signals was determined with Photoshop software.

Sections containing the nitroergic population in the ventro-medial reticular formation were selected for analysis according to our previous work (Pose et al., 2005). Ten nonadjacent sections of the brainstem of each cat were used to count the number of nitroergic cells and the number of nitroergic cells with Fos⁺ nuclei. The mean percentage of Fos⁺ nitroergic neurons per section per cat was calculated. The means for C-AS and control cats were compared using a Student's *t* test. The following formula was used to express the percent increase in the number of nitroergic *c-fos* expressing neurons during C-AS: $(AS-C/C) \times 100$ where AS and C are the mean percentage of Fos⁺ nitroergic neurons per section in C-AS and control experiments respectively. The sections of the Ctb injected cat were not included in these calculations.

Results

After carbachol microinjection, the experimental animals remained for 1 to 2 hours (1 hr 38 min $\pm$ 7 min) in an active sleep-like state. Control animals remained in a state of quiet wakefulness with intermittent episodes of quiet (non-REM sleep).

We examined the pattern of *c-fos* expression of the population of nitroergic neurons located in the rostral portion of the VMMR within the Mc and the ventral aspects of the NRGc. As previously described (Pose et al., 2005), the stereotaxic boundaries according to the Berman atlas (Berman, 1968) of the region occupied by this population are: P7 to P13.5, L1.0 to L2.6 and H6.4 to H8.5. Nitroergic neurons were identified by the presence of nNOS immunoreactivity or NADPH-d reactivity.

Figure 1 (A to F) depicts microphotographs of the region under study from an experimental animal. The tissue was treated for the combined detection of the Fos protein (dark brown nuclei) and nNOS immunostaining (brown cytoplasm). Numerous nNOS reactive cells expressed *c-fos* (signaled by arrows in Figure 1B, C, D, E and F) during C-AS. These neurons were intermingled with other nitroergic cells that did not express *c-fos* (arrowheads in Figure 1A, B, C, E and F) as well as with *c-fos* expressing cells that were not nitroergic (asterisks in Figure 1B). The diagram in Figure 1G corresponds to a representative brainstem section including the region of interest to show the location of the nitroergic cells.

Empty circles represent Fos⁻/nitrergic neurons, filled circles represent Fos⁺/nitrergic neurons. Fos immunostaining was also combined with NADPH-d histochemistry to compare the pattern of *c-fos* expression of nitrergic neurons between control and experimental animals. Microphotographs of sections that were processed in this way are depicted in Figure 2, where A and B correspond to sections of a control cat and C and D to sections of a C-AS animal. Figure 2E demonstrates that nitrergic premotor neurons express *c-fos* during carbachol evoked active sleep. This microphotograph belongs to tissue of the Ctb injected cat; the section was processed for the combined detection of Fos, Ctb (cytoplasmic granules) and NADPH-d.

In C-AS cats $31.7 \pm 1.5\%$ (mean $\pm$ SEM) of the nitrergic cells expressed *c-fos*, whereas in control animals the percentage was $2.7 \pm 0.8\%$ ($p < 0.0001$). There was therefore a 1074% increase in the number of nitrergic cells that expressed *c-fos* in the region studied in C-AS cats when compared to control animals. The bar diagram in Figure 2F represents these results.

Neurotransmitter phenotype

nNOS colocalizes with classical neurotransmitters, such as glutamate or acetylcholine (Vincent et al., 1986, Maqbool et al., 1995). In order to determine which neurotransmitter is colocalized in the nitrergic *c-fos* expressing neurons described in this report several immunocytochemical techniques were combined.

Figure 3 (A, B) shows microphotographs of the same section that was processed for the immunofluorescent detection of ChAT (green, figure 3A) and nNOS (red, figure 3B). It is apparent that the region occupied by nitrergic neurons contains cholinergic cells. Arrows in Figure 3A point to two cells which were positive to ChAT; these same cells also showed nNOS immunoreactivity as can be seen in Figure 3B (arrows). Arrowheads in Figure 3B point to nNOS reactive cells in the same field which did not contain ChAT. Cholinergic cells were much fewer in number than the nitrergic ones; many of them contained nNOS. When considering the whole population of nitrergic neurons, we found that approximately 12% of them also contained ChAT. However, the combination of Fos processing together with ChAT processing revealed that the cholinergic neurons in this region do not express *c-fos* during C-AS. Examples are shown in Figure 3C and 3D. Cholinergic neurons that also contain nNOS may correspond to those nitrergic neurons that do not express *c-fos* during C-AS, that we also observed (see examples in Figures 1 and 2).

We have previously shown (Morales et al., 1999) that glycinergic neurons that express *c-fos* during AS are located in the same region that is the focus of our present report. However, when NADPH-d histochemistry was combined with glycine immunocytochemistry it was observed that glycine does not colocalize with NADPH-d reactivity. This is shown in the examples in Figure 4A and 4B. Further evidence is shown in Figure 4C which is an example of tissue from an experimental animal processed for the detection of glycine, Fos and NADPH-d. Glycine immunoreactive *c-fos* expressing cells are present, they do not exhibit NADPH-d reactivity. One NADPH-d reactive neuron is visible in the same field. We conclude that the nitrergic neurons that express *c-fos* are not glycinergic. It is noteworthy that there is close apposition between glycinergic and nitrergic cell bodies and processes in this region.

Numerous neurons in the region studied displayed glutamate-like immunoreactivity. Double-staining procedures for glutamate and nNOS revealed that approximately 15% of the nNOS positive neurons were also positive for glutamate. An example of double immunofluorescent staining for glutamate (green) and nNOS (red) is shown in Figure 5 (A, B, C). A green fluorescent glutamate immunoreactive neuron can be observed in Figure 5A.

The same field, with immunofluorescence for nNOS in red shows three labeled neurons (Figure 5B). A double-labeled neuron becomes evident in the superimposition shown in Figure 5C. The single-labeled nNOS positive cells are still evident in Figure 3C (arrowheads). Glutamate immunoreactivity was also observed in NADPH-d reactive neurons that expressed *c-fos*. Figure 5 (D, E, F, G) depicts several microphotographs from tissue of an experimental cat that was processed to detect glutamate (brown cytoplasm), Fos protein (dark brown nuclei) and NADPH-d activity (blue cytoplasm). In D and E several *c-fos* expressing neurons that display glutamate immunoreactivity together with NADPH-d activity are visible (long arrows). The same section contained other glutamate reactive neurons that were not nitrenergic; some expressed *c-fos* (example in A, short arrow) and others did not (examples in D). Fos positive nuclei of non nitrenergic, non glutamatergic neurons are also visible (C).

Nitrenergic neurons in the VMMR displayed immunoreactivity neither for glycine, GAD nor for TH.

Discussion

In the present study we demonstrate that there is a significant increase in the number of nNOS containing neurons of the VMMR that express *c-fos* during C-AS. A subpopulation of these neurons is glutamatergic. In addition, we provide evidence indicating that these *c-fos* expressing nitrenergic neurons do not contain glycine, ChAT, TH or GABA. These findings together with previous data (see below) indicate that motoneurons and possibly other cells will be exposed to the neuromodulatory actions of NO during the state of AS. NO is therefore probably involved in the motor as well as in other functions controlled by the VMMR during this sleep stage.

Methodological considerations

The state induced by carbachol is indistinguishable from normal AS (Baghdoyan et al., 1987 and 1989; Morales et al., 1987; Kolhmeier et al., 1996; Maloney et al., 1999). Its longer duration, however, makes it possible for the Fos protein to reach detectable levels in the nucleus of activated cells (Yamuy et al., 1993; Shiromani et al., 1995). On this issue, as well as on the potential caveats related to the use of *c-fos* expression, we refer the reader to previous papers (Herrera and Robertson, 1996; Chaudhury, 1997; Maloney et al., 1999; Morales et al., 1999). In the present work the control animals were kept in a state of wakefulness. The surgical and injection procedures were the same as for experimental animals except for the fact that saline was injected instead of carbachol. Therefore, the increase in the number of *c-fos* expressing cells seen in the VMMR most likely resulted from synaptic activation of these neurons related to the state of active sleep.

As in our previous reports (Pose et al., 2005), nitrenergic neurons were identified by the use of two independent techniques: the detection of the enzyme nNOS by immunocytochemistry and the histochemical detection of NADPH-d activity. The latter has been shown to correspond to the nNOS enzyme in brain tissue (Dawson et al., 1991; Hope et al., 1991). We have reported (Pose et al., 2005) as have others (Batten et al., 2000) that both techniques label the same set of neurons in the medullary reticular formation.

Classical neurotransmitters in nitrenergic neurons

Several different neurotransmitters are contained in the neurons of the VMMR (Fort et al., 1989, 1990; Holstege et al., 1996; Morales et al., 1999; Batten et al., 2000; Travers et al., 2005), and nNOS has been shown to colocalize with diverse neurotransmitters and neuropeptides in the central nervous system (Vincent et al., 1986; Maqbool et al.,

1995;Batten et al., 2000). For these reasons we sought to determine which neurotransmitter/s are contained in the nitrergic neurons that become activated during AS.

Some nitrergic neurons in the brainstem, typically those in the pedunculo pontine tegmental nucleus (PPT) and laterolateral tegmental nucleus (LDT) are cholinergic (Vincent et al., 1986). Some of these cells are active during AS (Yamuy et al., 1993; Shiromani et al., 1995). In the medullary reticular formation, a small proportion of colocalization for ChAT and nNOS has been described (Travers et al., 2005) although in a more dorsolateral location, namely in the intermediate reticular formation. In this study we describe a relatively small population of cholinergic neurons located in the same region occupied by the nitrergic cells. Many of these cholinergic neurons contained nNOS. However, because *c-fos* expression was not observed in them we conclude that the nitrergic neurons which are activated during AS are not cholinergic.

In accordance with our previous studies (Morales et al., 1999,2006), glycinergic neurons which expressed *c-fos* during AS were observed in the VMMR. The AS activated nitrergic neurons described in the present work are located in an overlapping region with these glycinergic cells. Our doublestaining experiments indicate that these nitrergic neurons were not glycinergic.

Our studies lead us to conclude that glutamate is one neurotransmitter utilized by the *c-fos* expressing nitrergic neurons in the VMMR and to rule out such a role for ACh, Glycine, GABA and bioamines. It follows that the neurotransmitter content of many of these nitrergic neurons still remains to be identified. This last conclusion is in agreement with the studies of other authors regarding nitrergic neurons in this portion of the medulla oblongata (Dun et al., 1994; Batten et al., 2000).

In our study we did not investigate the presence of neuropeptides in the nitrergic neurons. It is possible that some may contain somatostatin because Maqbool et al., (1995) reported the co-localization of this peptide in medullary nitrergic neurons, some of which may correspond to the nitrergic neurons of our study. It is noteworthy however that the study by Maqbool et al. and others have shown that nNOS or NADPHd expressing neurons in the VMMR *do not* contain several other neuropeptides which were present in other neurons with overlapping distributions. This is the case for neuropeptide Y, colecystokinin, calbindin, calretinin, and parvalbumin (Maqbool et al., 1995, Batten et al., 2000).

Functional considerations

Whereas several authors have described the existence of nitrergic neurons in the portion of the VMMR which is the focus of the present study (Vincent and Kimura, 1992; Dun et al., 1994; Rodrigo et al., 1994), there is less information regarding the functions in which these particular neurons participate. As far as we know there are no previous studies about this nitrergic population in relation to the state of AS.

A possible function of these nitrergic neurons may be related to the motor phenomena that occur in somatic motoneurons during AS. Multiple evidence indicates that this part of the ventro-medial medulla is involved in mediating the atonia of AS through the activity of glycinergic neurons that it contains (see for example Morales et al., 1999,2006). Our present study indicates that the nitrergic neurons activated during C-AS are not the glycinergic neurons of this area, but we also observed that both types of neurons are in close proximity to each other. NO acts as a neuronal and synaptic neuromodulator and exerts excitatory actions on many targets (see for example, Southman and Garthwaite 1993;Garthwaite and Boulton 1995;Abudara et al 2002). The close apposition of the *c-fos* expressing nitrergic and glycinergic neurons raises the possibility that NO may contribute, through local actions, to

the activation of glycinergic neurons and, consequently, to the postsynaptic inhibition observed in motoneurons during AS.

In addition we have shown, in a previous paper (Pose et al., 2005) and in the present study, that nitroergic neurons in the VMMR innervate brainstem motor pools. Therefore at least part of the *c-fos* expressing nitroergic neurons described here may have direct motor actions during active (REM) sleep. During this state there is, in addition to tonic inhibition, a phasic increase of excitability of motoneurons during rapid eye movements that could be mediated by NO. Taking our data together our conclusion is that nitroergic premotor neurons act during the “tonic” and “phasic” episodes of AS to modulate motoneuron properties and/or their synaptic inputs. Finally, it is possible that some of the nitroergic neurons project to motoneurons of the spinal cord, although we did not conduct the experiments to prove this. If this is the case it is reasonable to assume that NO participates in the control of their activity during AS.

It is known that NO has trophic effects on motoneurons (Inglis et al., 1998); these effects may thus also be exerted on motor pools innervated by these nitroergic fibers. The present study was not designed to obtain data related to this issue.

Which other possible functions could these neurons be mediating during C-AS?

Autonomic functions, including cardiovascular and respiratory regulation, are characteristically variable during AS (Parmeggiani, 2005). It has been proposed that this depends on a central variability of visceral control associated with variations in feedback regulatory loops (ibid). On the other hand, many anatomical and physiological studies indicate that NO is involved in modulating central cardiovascular, respiratory, gastrointestinal and other autonomic pathways in the medulla (for a comprehensive review see Batten et al., 2000). The source of NO may come from peripheral afferents or other brain regions and also from the medulla itself. In this regard, nitroergic projections from within the medulla to autonomic structures such as the nucleus ambiguus (Amb), the nucleus tractus solitarius (NTS), and dorsal complex of the vagus have been described. With respect to the reticular formation, several of its divisions project to the NTS (Esteves et al., 2000; Batten et al., 2000). Interestingly, a recent study by Babic et al. (2008) in the rat describes a population of nitroergic neurons which project both to the NTS and the Amb and are located ventro-medially along the rostrocaudal extent of the facial nucleus starting at the level of the rostral pole of the inferior olive. The nitroergic neurons of our present study in the cat occupy a very similar region although with a more caudal extension. It is thus possible that part of the *c-fos* expressing nitroergic neurons described here project to these nuclei and that their activation during AS is related to the several autonomic phenomena which characterize this state.

Another function to consider relates to pain modulation, because NO has been implicated in central nociception (Luo and Cizkova, 2000). In addition, behavioral responses to painful stimulation vary across the sleep-wake cycle and are thus believed to be modulated by central influences (Foo and Mason, 2003). Structures in the rostral part of the ventro-medial medulla, notably the raphe magnus and adjacent reticular formation, are amply recognized to be involved in central pain modulation exerting both inhibitory, as well as facilitatory effects (Mason, 2001). In relation to our study, Coutinho et al. (1998) have provided evidence indicating that nitroergic neurons in the medulla participate in hyperalgesia. The cells described by these authors are located in the midline and in the lateral paragigantocellular nucleus. Therefore, they do not seem to correspond to the nitroergic neurons of our study, although the possibility cannot be fully discarded. More specific

experiments are needed to ascertain if the activation during AS described here is related to central pain modulation during this state.

We conclude that nitrergic ventro-medial medullary neurons have a role in the motor control exerted during active sleep and may also participate in autonomic functions during this state.

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Abbreviations

Amb	nucleus ambiguous
AS	active sleep
BSA	bovine serum albumin
ChAT	Choline acetyltransferase
CTb	cholera toxin subunit B
C-AS	carbachol induced active sleep
DAB	diaminobenzidine tetrahydrochloride
GAD	glutamate decarboxylase
IgG	immunoglobulin G
Mc	nucleus magnocellularis
NADPH	nicotinamide adenine dinucleotide phosphate
NADPH-d	NADPH diaphorase
NDS	normal donkey serum
NO	nitric oxide
nNOS	neuronal nitric oxide synthase
NRGc	nucleus reticularis gigantocellularis
NTS	nucleus tractus solitarii
PB	phosphate buffer
PBS	phosphate buffered saline
PBST	phosphate buffered saline, 0.3% Triton X-100
REM	rapid eye movement
TH	tyrosine hydroxylase
VMMR	ventro-medial medullary reticular formation

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Figure 1.

Nitroergic neurons in the ventro-medial medullary reticular formation express *cfos* during AS-carbachol. A to F: Microphotographs of sections obtained from a C-AS animal that were processed for the detection of both nNOS (brown cytoplasm) and Fos immunoreactivity (dark brown nuclei). Arrows (in B, C, D, E and F) point to nNOS reactive cells which express *cfos*. Arrowheads (in A, B, C, E and F) indicate nNOS reactive cells that do not express *cfos* and asterisks (in B) indicate *cfos* expressing cells that are not nitroergic. G: Diagram of a representative brainstem section of an experimental animal containing the region of interest. Empty circles represent Fos⁻/nitroergic neurons, filled circles represent Fos⁺/ nitroergic neurons.

Figure 2. *c-fos* expression in nitrenergic neurons in control and experimental animals. A to D: Microphotographs of sections that were processed for the detection of NADPH-d activity (blue cytoplasm), and Fos immunostaining (brown nuclei). A and B correspond to sections of control animals, C and D to sections of experimental animals; arrows point to nitrenergic/*c-fos* expressing cells; in C, an arrowhead points to a neuron expressing *c-fos* which is not nitrenergic. E: Nitrenergic *c-fos* expressing neurons innervating trigeminal motoneurons. The tissue was processed for the detection of NADPHd, CTb (black granules), and Fos. Triple arrowheads point to a Fos⁺/CTb⁺/NADPHd reactive cell. The single arrowhead points to a premotor neuron which is not nitrenergic. Double arrows point to a premotor neuron which is neither nitrenergic nor expresses *c-fos*. F: Results for all animals. Each bar represents the mean percentage of nitrenergic cells that expressed *c-fos*, per section, per cat, within the studied region. Data obtained from 4 experimental (C-AS) and 3 control cats. Mean ± SEM values: C-AS 31.7 ± 1.5 %; control 2.7 ± 0.8%.

Figure 3.

A, B: Some nitrergic neurons in the ventro-medial medulla are cholinergic.

Microphotographs of the same section processed for the detection of both ChAT (A, green) and nNOS (B, red) immunoreactivities by fluorescent methods. Two ChAT and nNOS immunoreactive neurons are indicated by arrows in A and B respectively. Two single-labeled nNOS reactive neurons are also visible in 3B (arrowheads).

C, D. Cholinergic neurons in the ventro-medial medulla do not express *cfos* during C-AS.

Microphotographs of the same section processed for the detection of ChAT (brown cytoplasm) and Fos (black nuclei) immunoreactivity. 3C shows a neuron displaying ChAT immunoreactivity in its cytoplasm; its nucleus is not stained for Fos. 3D shows a Fos positive nucleus of a non-cholinergic neuron from the same section.

Figure 4.

A, B. Glycinergic neurons in the ventro-medial medulla do not contain nNOS.

Microphotographs of the same section which was processed for the detection of NADPH-d (blue) and glycine immunoreactivity (brown). A. Two neurons showing NADPH-d reactivity (blue cytoplasm). B. One neuron displaying glycine immunoreactivity (brown cytoplasm) in close apposition to a NADPH-d reactive cell. No evidence for double-labeling was observed.

C. The ventro-medial medulla contains non-nitroergic glycinergic cells that express *c-fos* during AS-carbachol. This section from an experimental animal was processed for the detection of NADPH-d activity (blue) and of glycine (brown cytoplasm) and Fos (dark brown nuclei) immunoreactivities. Several glycine immunoreactive *c-fos* expressing cells can be observed, they do not exhibit NADPH-d reactivity. One NADPH-d reactive neuron is visible in the same field.

Figure 5.

A, B. Microphotographs of the same section processed for double immunofluorescent detection of glutamate (A, green, arrow) and nNOS (B, red, arrow and arrowheads). C. Superimposition of A and B showing co-localization in one neuron (arrow). D to G. Microphotographs of a section from an experimental animal processed for the combined detection of NADPH-d activity (blue) and of glutamate (brown cytoplasm) and Fos (dark brown nuclei) immunoreactivities. D. Long arrow: Fos⁺ cell displaying both NADPH-d and glutamate reactivity. Short arrow: Fos⁺ cell displaying glutamate immunoreactivity but not NADPH-d activity. E. Fos⁺ cells displaying both NADPH-d and glutamate reactivity (long arrows). F. Fos⁺ nuclei of cells that do not display either NADPH-d activity or glutamate immunoreactivity (arrowheads). G. Glutamate immunoreactive cells that do not express *c-fos* and also do not exhibit NADPH-d activity (short arrows).