Research report

GABAergic neurons of the cat dorsal raphe nucleus express c-fos during carbachol-induced active sleep

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Abstract

Serotonergic neurons of the dorsal raphe nucleus (DRN) cease firing during active sleep (AS, also called rapid-eye-movement sleep). This cessation of electrical activity is believed to play a 'permissive' role in the generation of AS. In the present study we explored the possibility that GABAergic cells in the DRN are involved in the suppression of serotonergic activity during AS. Accordingly, we examined whether immunocytochemically identified GABAergic neurons in the DRN were activated, as indicated by their expression of c-fos, during carbachol-induced AS (AS-carbachol). Three chronically-prepared cats were euthanized after prolonged episodes of AS that was induced by microinjections of carbachol into the nucleus pontis oralis. Another four cats (controls) were maintained 2 h in quiet wakefulness before being euthanized. Thereafter, immunocytochemical studies were performed on brainstem sections utilizing antibodies against Fos, GABA and serotonin. When compared with identically prepared tissue from awake cats, the number of Fos neurons was larger in the DRN during AS-carbachol (35.9 ± 5.6 vs. 13.9 ± 4.4, P < 0.05). Furthermore, a larger number of GABA* Fos* neurons were observed during AS-carbachol than during wakefulness (24.8 ± 3.3 vs. 4.0 ± 1.0, P < 0.001). These GABA* Fos* neurons were distributed asymmetrically with a larger number located ipsilaterally to the site of injection. There was no significant difference between control and experimental animals in the number of non-GABAergic neurons that expressed c-fos in the DRN. We therefore suggest that activated GABAergic neurons of the DRN are responsible for the inhibition of serotonergic neurons that occurs during natural AS.

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Topic: Biological rhythms and sleep

Keywords: Active sleep; REM sleep; Dorsal raphe; Fos; GABA; Serotonin

1. Introduction

Serotonergic (5-HT) neurons comprise one of the most widely distributed neurochemical systems in the vertebrate central nervous system [19]. The dorsal raphe nucleus (DRN) contains the largest number of serotonin containing neurons in the entire brain [49,60]. This nucleus has been implicated in a number of physiological processes, e.g. nociception, analgesia, thermoregulation, as well as being involved in motor and autonomic functions [19,57].

Numerous studies have demonstrated that serotonergic neurons of the DRN are also involved in the regulation of sleep and wakefulness [19,21,40]. With respect to AS, DRN neurons project to brainstem nuclei implicated in the genesis and modulation of this state, such as the laterodorsal and pedunculo-pontine tegmental nuclei (LDT-PPT), the nucleus pontis oralis (NPO) and the locus coeruleus [17,45,46,51,56]. Furthermore, recordings from presumed serotonergic neurons in the DRN of chronic unanesthetized animals have revealed that the activity of these cells varies during the states of sleep and wakefulness. They exhibit a slow tonic rate of discharge during wakefulness (W) and their activity decreases and loses its typical regularity during quiet sleep (QS); these neurons cease firing during AS [7,16,31,47,54]. Decreases in serotonin levels in several brain regions during AS are correlated with the decrease in firing rate that also occurs during this state (reviewed by [40]). It has been postulated that this decrease in activity has a 'permissive' effect on neuronal systems that generate the behavioral and physiological indices of AS [30,41,43].
This suppression of cellular discharge was reversed following the iontophoretic application of GABA<sub>A</sub> antagonists into the DRN during AS [25]. In addition, microdialysis experiments have demonstrated that GABA release in the DRN increases during AS, and that microperfusion of the GABA antagonist picrotoxin decreases the time spent in AS while GABA agonist muscimol increases the duration of this state [37]. These facts suggest that GABAergic inhibition may be responsible for the cessation of activity in serotonergic neurons that accompanies this state. However, the origin of the GABAergic control of DRN neurons during AS is not clear.

We and others have used the expression of c-fos during AS induced by microinjection of carbachol into the NPO of the cat to examine neural activity during this state [9,33,34,48,62–64]. The AS-carbachol state resembles naturally occurring AS polygraphically, electrophysiologically and behaviorally [3,12,32,55]. Furthermore, in a similar model, carbachol microinjection into the NPO was found to produce a cessation of firing of serotonergic neurons in the DRN, which is similar to that which occurs during natural AS [50]. Using the AS-carbachol model, we previously demonstrated that the DRN has a larger number of c-fos-expressing neurons during AS-carbachol than during wakefulness and that the majority of these neurons are not serotonergic [63].

In the present study, using a double-labeling procedure for GABA and Fos, we found that the number of activated GABAergic neurons in the DRN increases during AS-carbachol. We therefore hypothesize that these cells may be GABAergic neurons that inhibit serotonergic neurons of the DRN during this state. Preliminary results of this study have been presented in abstract form [53].

2. Material and methods

2.1. Animals

Seven male adult cats (three experimental and four controls) were used in this study. The animals were obtained from and determined to be in good health by the UCLA Division of Laboratory Animal Medicine. All of the experimental procedures were conducted in accord with the Guide for the Care and Use of Laboratory Animals (7th edition, National Academy Press, Washington DC, 1996) and approved by the Chancellor’s Animal Research Committee of the UCLA Office for Protection of Research Subjects.

2.2. Surgical procedures

Before anesthesia, the animals were premedicated with Xylazine<sup>©</sup> (2.2 mg/kg, i.m.), atropine (0.04 mg/kg, i.m.) and antibiotics (Cefazolin<sup>®</sup> and Bicillin<sup>®</sup>, i.m.). Anesthesia was first induced with ketamine (15 mg/kg, i.m.) and maintained with a gas mixture of halothane (1–3%) in oxygen. Thereafter, the head was positioned in a stereotaxic frame and the calvarium was exposed. Stainless steel screw electrodes were placed in the frontal and parietal bones for recording the electroencephalogram (EEG) and into the orbital portion of the frontal bone to record eye movements (EOG). Deep bipolar electrodes were implanted in both lateral geniculate nuclei in order to monitor ponto-geniculo-occipital waves (PGO). A Winchester plug (connected to these electrodes) and a chronic head-restraining device were bonded to the calvarium with acrylic cement. A small hole, 5 mm in diameter, which was made in the calvarium overlying the cerebellar cortex, was then filled with bone-wax. This hole was subsequently used to provide access for drug administration. At the end of these surgical procedures, an analgesic (Buprenex<sup>®</sup>, 0.01 mg/kg, i.m.) was administered. Incision margins were kept clean and a topical antibiotic was administered on a daily basis. After the animals had recovered from these surgical procedures, they were adapted to the head-restraining device for 2 weeks.

2.3. Experimental procedures

In the experimental animals, carbachol (0.8 μg in 0.2 μl of saline) was microinjected into the NPO (AP −2 to −3, L 1 to 2, H −3.5 to −5, according to Berman’s atlas [6]) using a 2-μl Hamilton syringe. After the animals had spent approximately 2 h in the AS-carbachol state, they were deeply anesthetized with sodium pentobarbital i.p. (60 mg/kg) and perfused for immunocytochemistry (see below). Within this time period, the c-fos protein product (Fos) is known to reach optimal concentration [9,34,62].

The control animals underwent the same surgical and habituation procedures as the experimental animals. All of them remained for 2 h in a state of quiet wakefulness before they were euthanized. In one control cat (C4), saline (0.2 μl) was microinjected into the NPO 2 h before the animal was euthanized. The EEG, EOG, PGO and neck electromyogram (EMG, obtained using acutely placed bipolar electrodes) were recorded and stored in a Power Macintosh G3 microcomputer using SUPERSCOPE<sup>®</sup> software.

2.4. Histological procedures

The animals were perfused under deep anesthesia with 1 l of heparinized saline followed by 1.5 l of a solution of 4% paraformaldehyde, 15% saturated picric acid and 0.5% glutaraldehyde in phosphate buffer (PB, 0.1 M, pH 7.4). Subsequently, perfusion with 500 ml of the same solution with 10% sucrose was carried out. The brainstem was removed and immersed in a postfixative solution for 24 h; the solution consisted of 2% paraformaldehyde, 15% saturated picric acid and 10% of sucrose in PB. Following postfixation, the tissue was kept for 3 days in a solution of sucrose (25%) and sodium azide (0.1%) in PB. Thereafter
the brainstem was frozen and cut into 14 μm-thick sections using a Reichert–Jung cryostat. The sections were stored in a solution of 0.1% sodium azide, 0.25% in phosphate buffered saline (PBS, 0.1 M). Brainstem sections were first immunostained for Fos. For this purpose, free-floating sections from selected brain stem sections were incubated overnight in a rabbit polyclonal Fos antiserum (Fos Ab5; Oncogene Research Products/Calbiochem, La Jolla, CA, USA) at a dilution of 1:40 000 in PBS. The sections were rinsed four times in PBS for a total duration of 30 min and then incubated for 90 min in a biotinylated donkey anti-rabbit immunoglobulin G (1:700). Subsequently, the sections were incubated with the ABC complex (Vector ABC Elite kit, 1:500) for 60 min. After rinsing again, the tissue was reacted for 10−20 min with 0.6% nickel ammonium sulfate, 0.02% diaminobenzidine tetrahydrochloride (Sigma) and 0.015% hydrogen peroxide in 50 ml of 50 mM tris buffer, pH 7.5.

In order to identify GABAergic neurons, polyclonal guinea pig antibodies raised against GABA-keyhole limpet hemocyanin conjugated with glutaraldehyde (NT-108, Protos) were employed. After the sections were treated for Fos immunostaining, they were incubated overnight with GABA antibody (1:3500) and normal donkey serum (NDS; 3%). Then, after the sections were rinsed, they were incubated for 60 min with biotinylated donkey anti-guinea pig antibody (1:300) plus NDS. After another rinse, the tissue was incubated in the ABC complex (1:200) for 60 min. Finally, the tissue was exposed to diaminobenzidine (without nickel enhancement).

Serotonin immunocytochemistry was performed on brainstem sections of both an AS-carbachol cat and an awake cat (which were also both employed for GABA and Fos analysis), in order to determine the distribution of serotoninergic neurons. Following Fos immunocytochemistry, the sections were incubated overnight with polyclonal goat serotonin antiserum (Incstar, 1:10 000) and NDS. Subsequently, the tissue was incubated with biotinylated donkey anti-goat immunoglobulin (1:1000) followed by incubation with ABC complex (1:400). Finally, the tissue was exposed to diaminobenzidine (without nickel enhancement).

In all procedures, four final rinses with 0.01 M PBS preceded the mounting of sections on slides. Control omission experiments were performed without the exposure of the tissue to the primary antibody. In representative sections, Fos immunocytochemistry was followed by a Pyronin-Y counterstain.

2.5. Data analysis

Brainstem coronal sections from approximately A 0.5 (which corresponds to the rostral DRN) to approximately P −2.5 (which corresponds to the caudal DRN) were utilized. Counts of the different types of cells were carried out in seven sections for each animal (one section every 500 μm). The mean number of immunostained neurons (Fos+, GABA+ Fos+ and GABA− Fos+) on each side of the brain and for each animal were then calculated. These means were used to compare stained neurons during AS-carbachol and wakefulness (n=3 and n=4, respectively).

The serotoninergic area extends beyond the cytoarchitectonic limits of the DRN [42,60]. In order to standardize the area of analysis, the lateral borders were defined by a parasagittal plane traced from the lateral border of the main fascicle of the medial longitudinal fascicle (mlf, see Fig. 3B). The dorsal and ventral borders of the DRN were determined according to standard criteria [52]. Serotonergic neurons were observed throughout the defined area of analysis. In caudal sections, special care was taken to avoid including neurons of the dorsal segmental nucleus of Gudden in this analysis.

Photomicrographs were obtained using a Spot digital camera attached to an Olympus B×60 microscope. Images were analyzed using Adobe PHOTOSHOP® software with a Power Macintosh G3 computer. Conventional and Nomarsky optics were employed. A 100× oil immersion objective lens was used to measure the diameter of soma profiles. The distribution of labeled neurons was determined from drawings using a camera lucida attachment.

The level of statistical significance of the difference between the mean number of immunoreactive neurons in control and AS-carbachol cats was evaluated using the Student’s t-test. The criterion chosen to discard the null hypothesis was $P<0.05$.

3. Results

Following the administration of carbachol, all the experimental animals exhibited the AS-carbachol state. The latency to the onset of AS-carbachol was 2.0±1.3 min (mean±S.E.M) and the duration of this induced state was 107.8±4.8 min. Table 1 presents these data for each animal.

3.1. Characteristic of immunostained neurons

As illustrated in Fig. 1, Fos immunoreactivity was restricted to the neuronal nuclei. Fos+ neurons were identified by their dark-stained nuclei.

Serotonergic immunoreactive neurons exhibited brown-

<table>
<thead>
<tr>
<th>Cat</th>
<th>Duration (min)</th>
<th>Latency (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS-C 1</td>
<td>108.0</td>
<td>4.5</td>
</tr>
<tr>
<td>AS-C 2</td>
<td>99.3</td>
<td>1.0</td>
</tr>
<tr>
<td>AS-C 3</td>
<td>116.0</td>
<td>0.5</td>
</tr>
</tbody>
</table>
stained soma and dendrites (Fig. 1A). The distribution of serotonergic neurons observed in the present study was similar to that previously reported in the cat [20]. As illustrated in the photomicrograph of Fig. 1A, during AS-carbachol, \( c-fos \) expression was observed mainly in non-serotonergic neurons. We have previously analyzed \( c-fos \) expression in serotonergic neurons [63], therefore this analysis was not carried out in the present study.

GABA immunoreactive neurons, which were observed in the DRN, exhibited a diffuse brown-stained soma (Fig.
1). Other structures that are known to contain GABAergic neurons, e.g. cerebellum, globus pallidus, dorsal nucleus of the lateral lemniscus, etc, also exhibited strong immunostaining [35]. No GABA immunoreactivity was observed in control omission experiments.

In brainstem sections immunostained for GABA and Fos, double-labeled neurons (GABA+ Fos+ neurons) displayed a brown cytoplasm and a black nucleus. Non-GABAergic neurons that expressed c-fos (GABA− Fos+ neurons) were also observed. In about 4% of the Fos+ neurons it was not possible to determine whether or not they were GABAergic because of their small size and the intensity of background staining. These neurons were counted as Fos+, but were not classified as either GABA+ Fos+ or GABA− Fos+. Representative examples of GABA+ Fos+, GABA− Fos+ and GABA+ Fos− neurons are presented in Fig. 1.

### Table 2
Mean cell counts from individual cats

<table>
<thead>
<tr>
<th>Cat</th>
<th>GABA+ Fos+</th>
<th>GABA− Fos+</th>
<th>Total Fos</th>
</tr>
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<tbody>
<tr>
<td>C1</td>
<td>6.2</td>
<td>9.5</td>
<td>16.7</td>
</tr>
<tr>
<td>C2</td>
<td>3.4</td>
<td>1.6</td>
<td>5.0</td>
</tr>
<tr>
<td>C3</td>
<td>3.3</td>
<td>15.3</td>
<td>18.9</td>
</tr>
<tr>
<td>C4</td>
<td>3.1</td>
<td>11.0</td>
<td>15.1</td>
</tr>
</tbody>
</table>

AS-carbachol

| AS-C1 | 30.7 | 14.0 | 46.6 |
| AS-C2 | 24.3 | 7.1  | 33.1 |
| AS-C3 | 19.4 | 7.3  | 27.9 |

*Each value represents an average of neurons in seven sections (see Material and methods). The ‘Total Fos’ group included Fos+ neurons that were impossible to identify either as GABA+ or as GABA− (see text).

### 3.2. Fos immunoreactivity during AS-carbachol

The mean number of Fos+ neurons (GABA+ Fos+ and GABA− Fos+) was larger during AS-carbachol compared to wakefulness (35.9±5.6 vs. 13.9±4.4, *P*<0.05, see bar chart in Fig. 2A). Table 2 presents the mean cell counts from individual cats. The photomicrograph in Fig. 3A, illustrates the distribution of Fos+ neurons in the dorsal raphe in a section counterstained with Pyronin-Y. Fos+ neurons were located adjacent to the mlf and in the lateral portions of the raphe nucleus. They were scarce in the central raphe, where the largest concentration of serotonergic neurons is located.

### 3.3. GABAergic neurons (GABA+)

The camera lucida drawing in Fig. 3B depicts the distribution of GABAergic neurons in the DRN. These neurons were mainly located in the lateral regions of the DRN; very few were found in the central region. As shown in Fig. 3A and B, the distribution of Fos+ neurons was similar to the distribution of GABAergic neurons.

There was a 620% increase in the number of GABA+ Fos+ neurons during AS-carbachol compared to control (24.8±3.3 vs. 4.0±1.0, *P*<0.001), as illustrated in Fig. 2B (see also Table 2). Of the total number of Fos+ neurons, 69% were GABAergic in the AS-carbachol animals and only 29% in awake animals (24.8 GABA+ Fos+ out of

![Fig. 2](image-url)
of 8.7±0.3 μm. GABA+ Fos+ neurons were also heterogeneous in shape. The majority (51%) of these neurons were oval-shaped (Fig. 1B,C and F), 39% were fusiform (Fig. 3E) and 10% were multipolar (Fig. 3D).

Fig. 4A consists of camera lucida drawings from waking and AS-carbachol cats. Note the larger number of GABA+ Fos+ neurons that are present in the section obtained from the AS-carbachol cat.

The number of GABA+ Fos+ neurons in the AS-carbachol cats was larger ipsilateral to the injection site than on the contralateral side (17.3±2.2 vs. 7.5±1.2, P<0.05; Fig. 4B). No significant differences were observed between the right and left side of the sections obtained from cats that were awake. In the saline-injected animal, the number of GABA+ Fos+ neurons was similar between the ipsilateral (1.4±0.7, average of seven sec-

Fig. 3. Distribution of Fos+ and GABA+ neurons in the DRN. (A) This photomicrograph illustrates Fos immunostaining with Pyronin-Y counterstaining in the ventromedial region of the DRN during AS-carbachol. c-fos-expressing neurons were mainly distributed in the periphery close to the medial longitudinal fasciculus (examples are indicated by arrowheads). Fos immunoreactivity was not observed in the large, centrally located neurons. The photomicrograph was obtained from a 14-μm thick section and processed with the diaminobenzidine method enhanced with nickel. Calibration bar=100 μm. (B) Camera lucida drawing that depicts the distribution of neurons immunostained for GABA in a representative section (P =1.5) of the DRN. Each dot represents one immunolabeled neuron. Note that GABAergic neurons were concentrated mainly in the lateral regions of the DRN. Dashed lines indicate the borders of the area that was included in the present analysis. (A) and (B) were obtained from adjacent sections of the same cat. 4V, fourth ventricle; mlf, medial longitudinal fasciculus.

35.9 Fos+ neurons, and 4.0 out of 13.9, respectively). GABA+ Fos+ neurons were small, with an average major diameter of 15.7±0.6 μm and an average minor diameter
tions) and the contralateral sides (1.7±0.8, average of seven sections).

During AS-carbachol, the number of GABA+ Fos+ neurons was significantly larger on both in the ipsilateral (17.3±2.2, $P<0.001$) and contralateral sides (7.5±1.2, $P<0.005$) compared to sections from the animals that were awake (1.9±0.6, right side; 2.1±0.5, left side).

3.4. Non-GABAergic neurons (GABA−)

Compared to control conditions, no statistically significant changes in the number of GABA−Fos+ neurons were observed in AS-carbachol cats (9.5±2.3 vs. 9.3±4.1; Fig. 2C, see also Table 2). The photomicrographs of Fig. 1 are examples of GABA− Fos+ neurons in brainstem sections from AS-carbachol cats (Fig. 1C, G and H) and from a cat during wakefulness (Fig. II). These neurons were intermingled with GABA+ Fos+ (Fig. 1C) as well as GABA+ Fos− (Fig. II) neurons.

4. Discussion

The present results indicate that compared to wakefulness, GABAergic neurons of the DRN are activated during AS-carbachol, whereas the activity of non-GABAergic neurons was unchanged.

GABAergic terminals establish synaptic contacts on serotonergic DRN neurons [15,58]; GABA_A receptors are also located in these cells [11]. An inhibitory effect of GABA on serotonergic neurons has been demonstrated both in vivo and in vitro [24,38]. During AS, microdialysis studies have shown that GABA levels in the DRN increase compared to the levels present during other behavioral states [37]. Recently, Luppi et al. reported that the iontophoretic application of the GABA_A antagonist bicuculline abolishes the suppression of activity of serotonergic neurons during AS, confirming the importance of GABAergic input in the cessation of serotonergic neuronal discharge during this state [25].

GABAergic neurons have been previously described in the DRN [4,5,15,35,36]. A small proportion of these GABAergic neurons project to the hypothalamus [10]; however, it remains to be determined if these neurons also innervate serotonergic DRN neurons. The GABAergic neurons that were activated during AS-carbachol were small in size and located mainly in the lateral areas of the DRN. Electrophysiological studies in the DRN have indicated the presence of neurons with characteristics different from the typical wide action potentials and clock-like discharge patterns of serotonergic neurons [2]. Some of these (presumably) non-serotonergic neurons increase their discharge during AS [1,7,23,47]. These neurons may be the GABAergic neurons (GABA+ Fos+) that are described in the present report.

The presence of activated GABAergic neurons during AS also support the previously reported increase in metabolic activity that has been described in the DRN during this state [27]. In addition, Maloney et al. [28] have shown that the number of GABAergic neurons that express c-fos in the DRN of the rat was positively correlated with the percent time spent in AS during recovery from AS deprivation.

In contrast to the GABAergic neurons, there was no significant difference in the number of non-GABAergic Fos+ (GABA− Fos+) neurons in AS-carbachol and awake cats.

An important question involves the source of the activation of GABAergic neurons of the DRN during AS. We suggest that neurons in the NPO may be involved in this process for the following reasons. Electrical stimulation of the NPO produces an inhibitory response in DRN neurons that is blocked by GABA_A antagonists, but not by glycine antagonists [59]. Neurons in the NPO are also activated during natural AS and directly by carbachol [13,14,18,29]. There are also fiber projections to the DRN from the NPO and surrounding areas [22,42,44]. Therefore, it is also possible that GABAergic DRN neurons are activated by cholinceptive AS-on neurons in the NPO. In addition, a stronger ipsilateral NPO-DRN projection may explain why, in the present study, a larger number of GABA+ Fos+ neurons were located ipsilateral to the injection site. It is possible that GABAergic DRN neurons are also driven by direct cholinergic input from the LDT-PPT during naturally occurring AS; the LDT-PPT nuclei are implicated in the generation of AS and projections from these nuclei to the DRN have been described [8,21,61].

A population of GABAergic neurons that did not express c-fos during AS-carbachol was also observed. These neurons may be activated in natural AS, however, it is also possible that there is a population of GABAergic neurons within the DRN with a function unrelated to the inhibition of serotonergic neurons during AS.

The data in this report suggest that the suppression of activity in serotonergic neurons during AS may be due to the activity of GABAergic neurons within the DRN. In addition, other GABAergic inputs (from outside the DRN) may also play a role in the suppression of serotonergic activity during AS [26]. GABAergic projection to the DRN from the lateral preoptic area, lateral hypothalamic area, periaqueductal gray, substantia nigra, dorsal paragigantocellular nucleus and the ventral tegmental area have been described [26]. However, it is unlikely that the source of the state-dependent, GABAergic inhibition of serotonergic DRN neurons is rostral to the midbrain, because DRN units have been demonstrated to retain their AS-off pattern of firing in cats that had been descerebrated at the precollicular level [39].

In conclusion, the present study indicates that GABAergic neurons of the DRN are active during AS. It is also suggested that these GABAergic neurons inhibit the dis-
charge of serotonergic neurons within the DRN during this state.

Acknowledgements

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