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Diurnal Variation of c-Fos Expression in Subdivisions of the Dorsal Raphe Nucleus of the Mongolian Gerbil *(Meriones unguiculatus)*

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ABSTRACT

Recent studies suggest that the dorsal raphe nucleus (DRN) of the brainstem contains several subdivisions that differ both anatomically and neurochemically. The present study examined whether variation of c-Fos expression across the 24-hour light-dark cycle may also be different in these subdivisions. Animals were kept on a 12:12 light-dark cycle, were perfused at seven different time points, and brain sections were processed by using c-Fos immunocytochemistry. At all coronal levels of the DRN, c-Fos expression reached a peak 1 hour after the light-dark transition (lights-off) and reached its lowest levels in the middle of the light period. In contrast to the light-dark transition, c-Fos levels did not change significantly after the dark-light transition (lights-on). One-way analysis of variance (ANOVA) revealed that the diurnal variation of c-Fos expression was highly significant in the caudal ventral DRN. Similar variation in c-Fos expression also was observed in the other DRN subdivisions, but this variation appeared to gradually diminish in the caudal-to-rostral and ventromedial-to-dorsomedial directions. Double-label immunocytochemistry revealed that, 1 hour after lights-off, only 11% of c-Fos-positive neurons in the caudal ventral DRN were serotonin (5-HT)-immunoreactive. These results suggest that DRN subdivisions may differ functionally with regard to the diurnal cycle, and that these differences may be reflected in the activity of nonserotonergic cells in the DRN. J. Comp. Neurol. 440: 31–42, 2001.

Indexing terms: brainstem; topography; serotonin (5-HT); GABA; circadian; immediate early gene

The gerbil dorsal raphe nucleus (DRN) is composed of several subdivisions that have been identified based on their efferent projections to the superior colliculus (SC) and 5-hydroxytryptamine (5-HT, serotonin) neuronal immunoreactivity (Janušonis et al., 1999). Neurons projecting to the SC, lateral geniculate nucleus, and visual cortex also are found in some subdivisions of the rat DRN but not in others (Waterhouse et al., 1986, 1993). DRN subdivisions differ in their neurotransmitter and/or neuromodulator content, as has been demonstrated in the rat (Petit et al., 1995; Xu and Hökfelt, 1997) and squirrel monkey (Charara and Parent, 1998). Therefore, it is of interest to know whether these subdivisions also may be different functionally. Electrophysiological and microdialysis studies usually lack anatomical precision with regard to specific DRN subdivisions. Immediate early gene (*c-fos, c-jun, zif268*) expression offers a promising alternative, because it can be analyzed cell by cell, in many small anatomically well-defined areas in the brain of the same animal. However, c-Fos expression in the DRN is extremely sensitive to various stressful stimuli (Silveira et al., 1993; Matsuda et al., 1996; Campeau and Watson, 1997; Grahn et al., 1999); therefore, control animals exposed to comparable levels of stress are essential to demonstrate the specificity of the stimulation. This problem does not arise if baseline levels of c-Fos expression are studied, and variation of c-Fos expression across the 24-hour light-dark cycle is such a case.

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DRN neurons synthesize and colocalize many neurotransmitters and neuromodulators (Verge and Callas, 2000). It is estimated that some 50–70% of neurons in the DRN are nonserotonergic (Wiklund et al., 1981; Descarries et al., 1982). DRN neurons have been shown to contain gamma aminobutyric acid (GABA; Stamp and Semba, 1995; Charara and Parent, 1998), glutamate (Clements and Grant, 1990), enkephalins (Leger et al., 1986), substance P (Chan-Palay et al., 1978; Charara and Parent, 1998; Sergeyev et al., 1999), galanin, and nitric oxide synthase (Xu and Hökfelt, 1997). Some of these neurotransmitters and neuromodulators may be colocalized with serotonin. However, little is known about which of these cell types exhibit diurnal variation in their activity and whether they occur in anatomically localized subdivisions of the DRN.

In rats, DRN serotonin levels reach a trough during the dark phase and a peak during the early light phase; this peak shifts considerably when animals are kept in constant darkness (Cagampang et al., 1993). In hamsters, DRN serotonin levels peak during the second half of the light phase (Wesemann et al., 1989; Ozaki et al., 1993). Serotonin levels in the DRN also fluctuate as a function of the sleep-wake cycle, with the highest levels occurring when cats and rats are awake and the lowest when they are in the rapid eye movement (REM) stage of sleep (Portas and McCarley, 1994; Portas et al., 1998, 2000). The firing rate of serotonergic neurons in the cat DRN is also highest when animals are awake and lowest when animals are in REM sleep (Fornal and Jacobs, 1988). Recent studies have shown that the activity of GABAergic neurons in the DRN also changes in rats and cats during REM sleep (Yamuy et al., 1995; Nitz and Siegel, 1997; Maloney et al., 1999; Torterolo et al., 2000), suggesting that the sleep-wake cycle may depend on interaction between serotonergic and GABAergic neurons.

In the present study, diurnal variation of c-Fos expression was studied in the subdivisions of the DRN of the Mongolian gerbil (*Meriones unguiculatus*). The Mongolian gerbil provides a good model to study DRN subdivisions in relation to the light-dark cycle because its DRN subdivisions have been mapped (Janušonis et al., 1999) and its DRN receives direct retinal afferents whose density varies in different DRN subdivisions (Fite et al., 1999). Also, the diurnal activity and sleep patterns of the Mongolian gerbil have been previously studied (Pietrewicz et al., 1982; Susic and Masirevic, 1986).

MATERIALS AND METHODS

Animals and histology

All experiments were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Amherst in accordance with the NIH and USDA guidelines. Adult male gerbils were kept on a 12-hour light; 12-hour dark (12L:12D) cycle, 3–5 animals per cage. Gerbils were perfused at the following seven zeitgeber times (ZTs: lights-on at ZT 0 [7:00], lights-off at ZT 12 [19:00]): ZT 1 (08:00), ZT 6 (13:00), ZT 11 (18:00), ZT 13 (20:00), ZT 15 (22:00), ZT 18 (01:00), and ZT 23 (06:00). At each time, 4–7 animals were used (total n = 35). Gerbils were not handled or disturbed prior to anesthetization, and animals killed on the same day were taken from different cages. Gerbils were quickly anesthetized (i.p.) with a mixture of ketamine (200 mg/kg) and xylazine (20 mg/kg). The exposed heart was injected with 0.3 ml of a heparin solution (5,000 USP units/ml), and animals were perfused with saline followed by 400 ml of chilled 4% paraformaldehyde in phosphate buffer (PB, pH 7.2). The brain was removed and immersed in 30% sucrose in PB at 4°C overnight. The next day, serial coronal sections were cut throughout the mesencephalon on a freezing microtome at 40-μm thickness and stored in cryoprotectant (30% sucrose containing 1% polyvinylpyrrolidone and 30% ethylene glycol).

Immunocytochemistry

Sections were rinsed 3 times (5 minutes each) in 0.1 M phosphate-buffered saline (PBS, pH 7.0); incubated in 0.3% H_2O_2 in PBS for 30 minutes; rinsed 2 times (5 minutes each) in PBS; incubated in 3% normal goat serum (NGS, Vector Laboratories, Burlingame, CA) and 0.25% Triton X-100 (TX) in PBS for 2 hours; and incubated in a rabbit anti-c-Fos IgG (Oncogene Research Products, San Diego, CA, cat. no. PC38, diluted 1:10,000–1:6,700) solution containing 3% NGS and 0.25% TX in PBS for 3 days at 4°C. Sections were rinsed 6 times (10 minutes each) in PBS; incubated in goat anti-rabbit IgG (Vector Laboratories, 5 μg/ml) with 3% NGS and 0.25% TX in PBS for 2 hours; rinsed 3 times (10 minutes each) in PBS; incubated in a 1:100 avidin-biotin-peroxidase (ABC Elite, Vector Laboratories) solution containing 0.5% TX in PBS (TX was added to the prepared ABC solution several minutes before the incubation); rinsed 2 times (10 minutes each) in PBS; preincubated in a 0.05% 3,3'-diaminobenzidine (DAB) solution in PBS for 1 minute; and reacted by adding 0.01% H_2O_2 to the solution. Following incubation for 8 minutes, sections were rinsed 3 times (5 minutes each) in PBS, mounted on chromium-subbed slides, allowed to air-dry at room temperature, cleared with Hemo-De, and coverslipped with Permount. In addition, two gerbils were used for 5-HT immunocytochemistry. Animals were perfused and serial sections throughout the mesencephalon were reacted for 5-HT as described previously (Fite et al., 1999).

Definitions of DRN subdivisions

The borders of DRN subdivisions were defined in relation to topographic landmarks that were readily distinguishable. These subdivisions were described in a previous study, where 5 rostrocaudal levels of the DRN were delineated (Janušonis et al., 1999). In sections stained for c-Fos, the two most rostral levels (Level 1 and Level 2) could not be readily distinguished and, therefore, were analyzed as one level (Levels 1–2). Each level was delineated as follows:

Levels 1–2. The rostral extent of Levels 1–2 was delimited by the Edinger-Westphal nucleus and the caudal extent by the caudal pole of the oculomotor (III) nucleus. The medial DRN was delimited by the medial edge of the oculomotor nuclei laterally, by the ventral edge of the aqueduct dorsally, and by the dorsal pole of the oculomotor nucleus ventrally. The medial DRN was divided into equal dorsomedial and ventromedial subdivisions. The lateral DRN at Levels 1–2 was delimited by the lateral edge of the oculomotor nuclei laterally, by the ventral edge of the aqueduct dorsally, and by the parvicellular part of the oculomotor nucleus ventrally.

Level 3. The rostral extent of Level 3 was defined by the caudal pole of the oculomotor nuclei and its caudal extent by the rostral pole of the dorsal tegmental nuclei (DTgs). The medial DRN was divided into equal dorsomedial and ventromedial subdivisions.

Level 4. The rostral extent of Level 4 was defined by the rostral pole of the DTg (they are just dorsal to the medial longitudinal fasciculi (MLF) at this level), and its caudal extent by the rostral-caudal level at which the centers of the DTgs are located halfway between the ventral edge of the aqueduct and the MLF. The dorsomedial subpopulation was delimited by the ventral edge of the aqueduct dorsally and by the midpoint between the ventral edge of the aqueduct and the dorsal pole of the DTg ventrally. The ventromedial subpopulation was delimited by the dorsomedial DRN dorsally and by the dorsal edge of the DTg ventrally. The interfascicular DRN was delimited by the ventral edge of the DTg dorsally and by the MLF ventrally. The lateral extent of these populations was easily defined due to low c-Fos immunoreactivity in areas located lateral to the DRN. The lateral DRN subpopulations at Level 4 are small, and their serotonergic neurons intermingle with adjacent nuclei (Janušonis et al., 1999); therefore, they were not analyzed in the present study.

Level 5. The rostral extent of Level 5 was defined by the level at which the centers of the DTgs lie midway between the ventral edge of the aqueduct and the MLF, and the caudal extent by the caudal pole of the inferior colliculus. The dorsomedial DRN and ventromedial DRN were located dorsal and ventral to the DTg, respectively, and were well-defined due to low c-Fos immunoreactivity in the areas adjacent to the DRN at this level.

Analysis

Grayscale digital images of the DRN from alternate sections at each of the four rostral-caudal DRN levels were captured by using a CCD72 camera mounted on an Olympus microscope (10× objective) and Macintosh Scion Image 1.57. Care was taken to obtain images of comparable brightness and contrast. The boundaries of DRN subpopulations were marked in Photoshop 5.0, as follows: for Levels 1–2, the oculomotor nuclei were used as guides to draw lines marking the medial and lateral DRN; for Level 3, a representative section stained for 5-HT was scanned, the right lateral DRN and the medial DRN were marked by drawing lines in Photoshop, and these lines were overlaid on digital images of sections stained for c-Fos; at Levels 4 and 5, DRN subdivisions could be readily distinguished in sections stained for c-Fos due to substantially lower c-Fos levels in adjacent areas. For all sections, a brightness threshold of 50% of background level was chosen; cell nuclei darker than the threshold value were considered to be c-Fos-positive and were counted in each DRN subdivision. This threshold was selected because in representative sections it yielded numbers of c-Fos-positive nuclei closely matching those obtained by visual counting. For Levels 4 and 5, the numbers of c-Fos-positive cells obtained by this method were checked by visual counting of c-Fos-immunoreactive cells in the actual sections. Similar thresholding techniques have been used by other researchers (Kaufman et al., 1993; Moratalla et al., 1996; Canales and Graybiel, 2000). c-Fos-positive nuclei were counted separately in each section, and the mean number obtained for each DRN subdivision, in each animal, was used for statistical analysis (SYSTAT 8.0). One-way analysis of varaiance (ANOVAs) were used to test the effect of ZT on c-Fos expression in each DRN subdivision; and Tukey tests were used for post-hoc pairwise comparisons. In all tests, $p < 0.05$ was selected as the significance level.

Double-label immunocytochemistry

Following the analysis of c-Fos-positive nuclei at different ZTs, two gerbils were perfused at 20:00 and were used for double-label (c-Fos and 5-HT) immunocytochemistry. They were perfused and brain sections were processed for c-Fos as describe above (the rabbit anti-c-Fos IgG was diluted 1:10,000). Following the DAB reaction and 3 rinses in PBS (pH 7.0), sections were transferred to 0.1 M PBS with a pH of 7.4 which was used for all further rinses and incubations. Sections were rinsed 4 times (5 minutes each) in PBS; incubated in 0.3% H_2O_2 in PBS for 20 minutes (this step was omitted in half the sections, with no detectable change in staining); rinsed 4 times (5 minutes each) in PBS; incubated in 3% NGS, 1% bovine serum albumin (BSA, Sigma) and 3% TX in PBS for 30 minutes; and incubated in 1:1,500 rabbit anti-5-HT IgG (Protos Biotech Corporation, New York, NY, cat. no. NT102) with 3% NGS, 1% BSA, and 3% TX in PBS for 4 days at 4°C. Sections were rinsed 4 times (5 minutes each) in PBS, incubated in biotinylated goat anti-rabbit IgG (Vector Laboratories) at 5 μg/ml with 1.5% NGS and 1% TX for 90 minutes, rinsed 3 times (10 minutes each) in PBS, incubated in ABC (1:100, Vector Laboratories) in PBS for 90 minutes, rinsed 3 times (5 minutes each) in PBS, and developed in Vector SG (Vector Laboratories, cat. no. SK-4700) in PBS for 5 minutes. Sections were rinsed once in PBS (5 minutes), in distilled water (30 seconds), and again in PBS (2 times, 5 minutes each). They were mounted on chromium-subbed slides, allowed to air-dry, and coverslipped with Permount.

The product of the DAB reaction was brown or dark brown and the product of the SG reaction was blue-gray. This provided good contrast (also recommended by Vector Laboratories), and SG-stained 5-HT somata did not obscure DAB-stained c-Fos nuclei. Similar immunocytochemistry protocols have been used and discussed by other researchers (Sternberger and Joseph, 1979; Yamuy et al., 1995; Maloney et al., 1999; Torterolo et al., 2000).

RESULTS

Diurnal variation of c-Fos expression

The observed levels of c-Fos immunoreactivity in DRN subdivisions at Levels 1–2, Level 3, Level 4, and Level 5 are summarized in Figures 1, 2, 3, and 4, respectively. The results of ANOVA analysis (F-values and levels of significance) are shown in Table 1. In almost all DRN subdivisions at all coronal levels, c-Fos expression reached a peak 1 hour after the light-dark transition (20:00) and reached its lowest levels in the middle of the light period (13:00). However, this diurnal variation in the number of c-Fos-positive nuclei was especially pronounced in the caudal DRN (Figs. 3–5). One hour after lights-off (20:00), the mean number c-Fos-positive nuclei in the interfascicular population at Level 4 showed a sevenfold increase compared with the mean number at the midpoint of the light period (13:00). The c-Fos levels at 20:00 were significantly higher than the c-Fos levels at 01:00, 06:00, 08:00, and 13:00 (Fig. 3D). A similar statistically significant variation

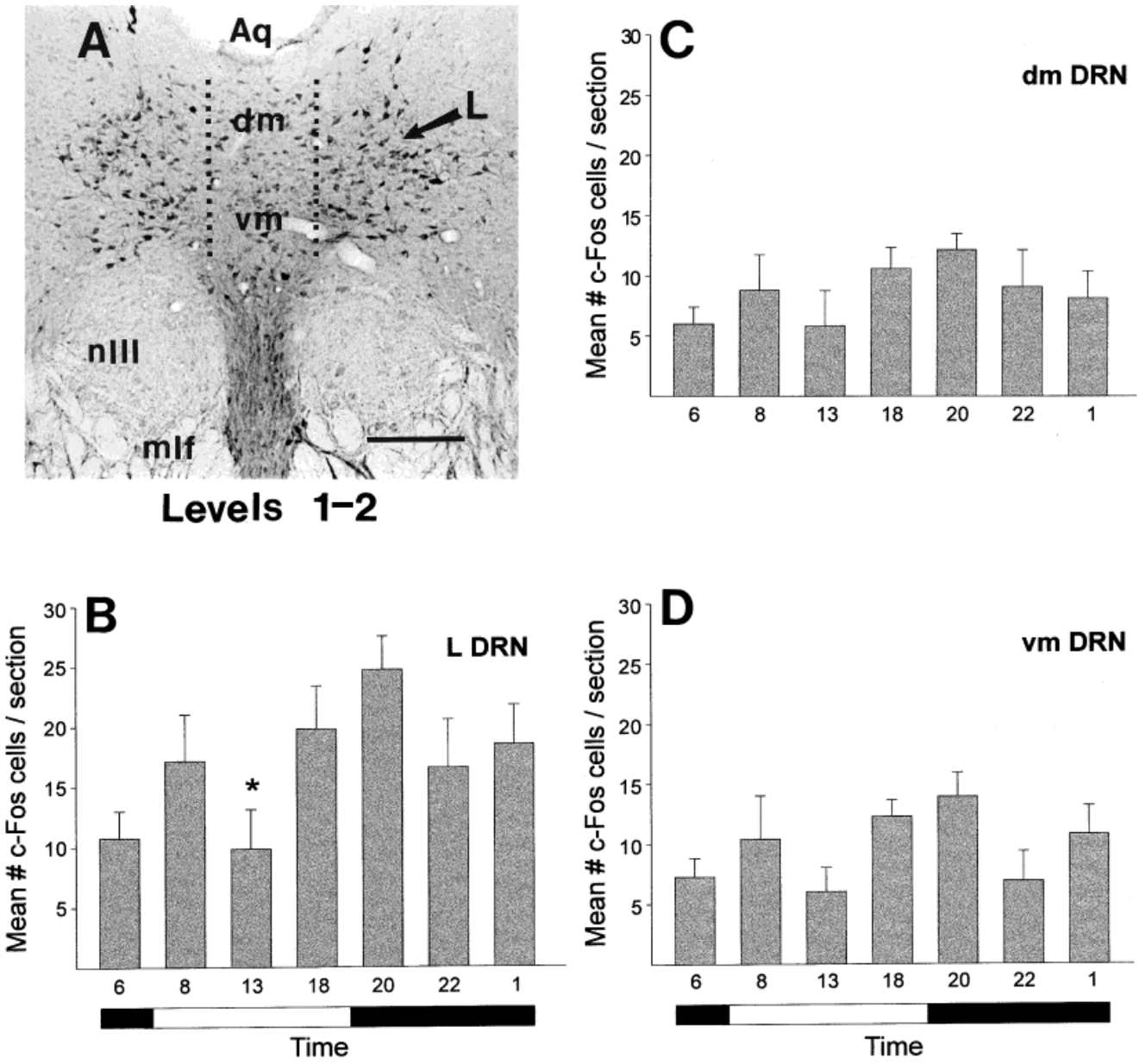


Fig. 1. Levels 1–2. **A:** Serotonin (5-HT)-immunoreactive cells in the dorsal raphe nucleus (DRN). Dashed lines delineate the medial DRN. B–D: Numbers (mean ± S.E.M.) of c-Fos-immunoreactive cells at selected times in the lateral (L) DRN (**B**), dorsomedial (dm) DRN (**C**), and ventromedial (vm) DRN (**D**). The light and dark bars represent the light and dark periods of the diurnal cycle (lights on at 7:00 and off at 19:00). Asterisk indicates a significant difference ($P < 0.05$) compared with the 20:00 point. Aq, aqueduct; nIII, oculomotor nucleus; mlf, medial longitudinal fasciculus. Scale bar = 200 μm.

also was observed in the ventromedial subdivision at Level 5, with the c-Fos levels at 20:00 showing a twofold increase over the c-Fos levels at 13:00 (Figs. 4C, 5). The ventromedial subdivision at Level 4 and the dorsomedial subdivision at Level 5 also showed statistically significant diurnal variation in c-Fos expression, with a peak occurring at 20:00 (Figs. 3C, 4B); however, post-hoc analysis revealed that their c-Fos levels at 20:00 were significantly different only from the time with the lowest c-Fos levels (13:00). The numbers of c-Fos-positive nuclei in the caudal ventral subdivisions of the DRN appeared to begin to rise at least 1 hour before the onset of darkness (Figs. 3D, 4B,C). More rostrally, c-Fos levels in the medial DRN subdivisions also appeared to reach a peak at 20:00, but this variation fell short of statistical significance ($P > 0.1$) for both the dorsomedial and ventromedial DRN at Level 3, and for the ventromedial DRN at Levels 1–2. It was nonsignificant ($P > 0.4$) for the dorsomedial DRN at Levels 1–2.

The change in c-Fos expression was clearly different at the onset of darkness compared with the onset of light. Whereas a dramatic increase in c-Fos expression in the

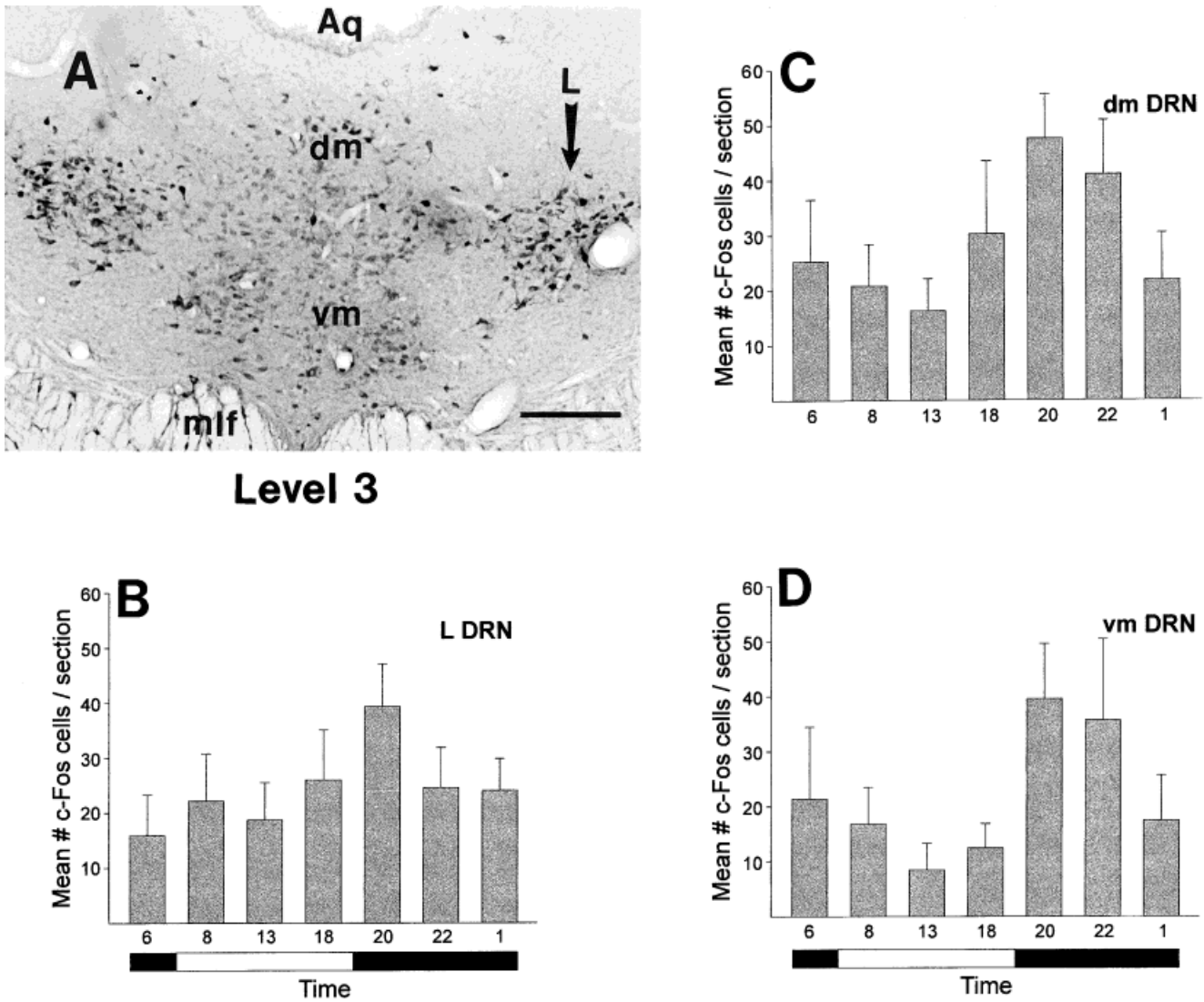


Fig. 2. Level 3. **A:** Serotonin (5-HT)-immunoreactive cells in the dorsal raphe nucleus (DRN). B– D: Numbers (mean ± S.E.M.) of c-Fos-immunoreactive cells at selected times in the lateral (L) DRN (**B**), dorsomedial (dm) DRN (**C**), and ventromedial (vm) DRN (**D**). The light and dark bars represent the light and dark periods of the diurnal cycle (lights on at 7:00 and off at 19:00). Aq, aqueduct; mlf, medial longitudinal fasciculus. Scale bar = 200 μm.

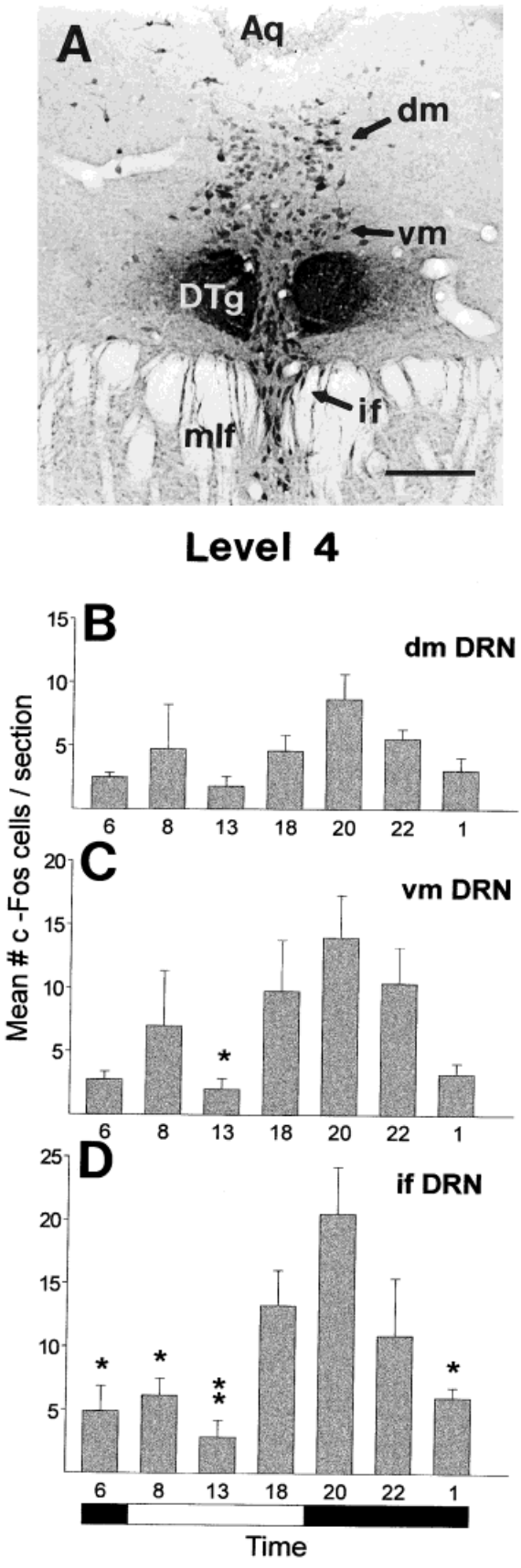
caudal DRN was observed at the onset of darkness, only a relatively small change of c-Fos expression was observed at the onset of light.

The lowest levels of c-Fos expression were observed in the middle of the light period (13:00) in virtually all DRN subdivisions. The ventromedial DRN at the most caudal level (Level 5) was an exception, where the c-Fos levels at 13:00 appeared to be the same as those at 01:00, 06:00, 08:00, and 22:00 ($P > 0.99$). Pairwise post-hoc comparisons showed no significant differences between the number of c-Fos nuclei at 13:00 and those at any other time in all DRN subdivisions ($P > 0.09$), with the exception of the c-Fos levels at 18:00 and 20:00 already discussed.

The lateral DRN at both Levels 1–2 and Level 3 showed an increase in c-Fos levels at 20:00; however, this variation was significant only in the lateral DRN at the most rostral level, Levels 1–2. The lateral DRN at Levels 1–2 also was the only DRN subdivision located rostral of Level 4 for which the diurnal variation of c-Fos expression was significant. These results of the statistical analysis are graphically summarized in Figure 6.

Double-label (c-Fos and 5-HT) immunocytochemistry

Double-label (c-Fos and 5-HT) immunocytochemistry showed that the vast majority of DRN neurons expressing c-Fos at 20:00 were not 5-HT-immunoreactive. In all DRN subdivisions at all coronal levels, c-Fos-positive cells were intermingled with 5-HT-immunoreactive neurons (Fig. 7A–C). However, in every DRN subdivision, few cells (0–8 per section) were clearly double-labeled under high (400–1,000×) magnification, with a dark brown nucleus contained entirely within the blue-stained cell body (Fig. 7B,D,E). Because the numbers of double-labeled cells were



small, the percentage of double-labeled cells in the caudal ventral region of the DRN was calculated by combining the ventromedial and interfascicular DRN at Level 4, and the ventromedial DRN at Level 5. These calculations showed that only 11% of c-Fos-positive cells in this region were 5-HT-immunoreactive. Proportionally, approximately 41% of c-Fos-positive cells were double-labeled for 5-HT in the dorsomedial DRN at Levels 4 and 5; however, it should be noted that this percentage is based on a very small total number of c-Fos-positive cells (approximately 8 per section) in these subdivisions (Figs. 3B, 4B). Also, at Levels 4 and 5, the populations of c-Fos-positive cells in the DRN appeared to extend more laterally than the populations of 5-HT-immunoreactive cells (Fig. 7A). In the more rostral (Levels 1–2 and Level 3) DRN subdivisions, the percentages of c-Fos-labeled cells that were 5-HT-immunoreactive varied from 5% to 16%. By combining all double-labeled and c-Fos-labeled cells in all DRN subdivisions at all levels, it was estimated that, overall, only 12% of c-Fos-labeled cells in the DRN were 5-HT-immunoreactive at 20:00.

DISCUSSION

Diurnal variation of c-Fos expression in the DRN

In general, the greatest mean number of c-Fos-positive (c-Fos+) nuclei occurred 1 hour after the onset of the dark period (20:00), whereas the lowest mean number of c-Fos+ nuclei occurred in the middle of the light period (13:00) in virtually all DRN subdivisions. However, statistical analysis showed that this diurnal variation was significant only in the caudal DRN. The amount of variation (measured as significance levels in one-way ANOVA) appeared to form a gradient, with the greatest diurnal variation of c-Fos expression occurring in the caudal ventromedial and interfascicular subdivisions of the DRN, and the smallest diurnal variation occurring in the rostral dorsomedial DRN (Fig. 6).

In diurnal and nocturnal rodents, several brain areas have been previously demonstrated to exhibit diurnal variation of c-Fos expression (Kononen et al., 1990; Nunez et al., 1999). The suprachiasmatic nucleus (SCN) in both diurnal and nocturnal species shows the same temporal pattern of c-Fos expression, with a peak occurring at the onset of the light period (Kononen et al., 1990; Katona et al., 1998; Nunez et al., 1999). Therefore, it has been suggested that the diurnal/nocturnal behavioral pattern of the animal species arises outside the SCN (Nunez et al., 1999). In brain areas outside the SCN, diurnal c-Fos variation may be species-specific (Nunez et al., 1999), and even one brain area may show diurnal c-Fos variation that

Fig. 3. Level 4. **A:** Serotonin (5-HT)-immunoreactive cells in the dorsal raphe nucleus (DRN). B– D: Numbers (mean ± S.E.M.) of c-Fos-immunoreactive cells at selected times in the dorsomedial (dm) DRN (**B**), ventromedial (vm) DRN (**C**), and interfascicular (if) DRN (**D**). The light and dark bars represent the light and dark periods of the diurnal cycle (lights on at 7:00 and off at 19:00). Asterisks indicate a significant difference ($*P < 0.05$ and $**P < 0.01$) compared with the 20:00 point. Aq, aqueduct; DTg, dorsal tegmental nucleus; mlf, medial longitudinal fasciculus. Scale bar = 200 μm.

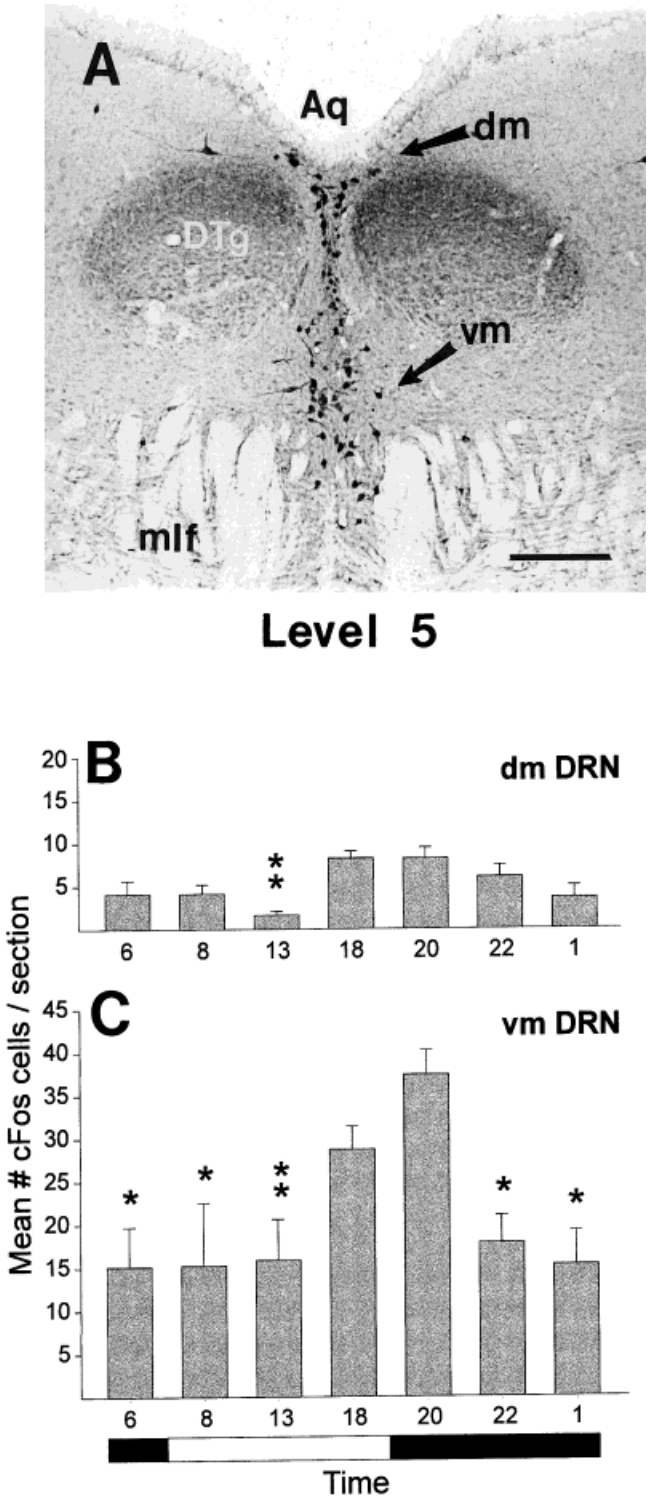


Fig. 4. Level 5 . **A:** Serotonin (5-HT)-immunoreactive cells in the dorsal raphe nucleus (DRN). B and C: Numbers (mean ± S.E.M.) of c-Fos-immunoreactive cells at selected times in the dorsomedial (dm) DRN (**B**) and ventromedial (vm) DRN (**C**). The light and dark bars represent the light and dark periods of the diurnal cycle (lights on at 7:00 and off at 19:00). Asterisks indicate significant differences (*$P <$ 0.05 and **$P < 0.01$) compared with the 20:00 point. Aq, aqueduct; DTg, dorsal tegmental nucleus; mlf, medial longitudinal fasciculus. Scale bar = 200 μm.

TABLE 1. Analysis of Variance (ANOVA) of Diurnal c-Fos Variation in Dorsal Raphe Nucleus (DRN) Subdivisions

Level	DRN subdivision	F[1]	P[2]
1–2	Dorsomedial	1.028	0.428
1–2	Ventromedial	1.972	0.104
1–2	Lateral	2.801	**0.029**
3	Dorsomedial	1.978	0.103
3	Ventromedial	1.736	0.149
3	Lateral	1.151	0.360
4	Dorsomedial	2.361	0.058
4	Ventromedial	2.909	**0.026**
4	Interfascicular	5.007	**0.001**
5	Dorsomedial	5.163	**0.001**
5	Ventromedial	4.815	**0.002**

[1]One-way ANOVAs F-value ($F_{6,27}$ for Level 4 and $F_{6,28}$ for all other levels).
[2]Level of significance. Significant variation is shown in bold.

may be 180° out of phase with the variation in another brain area of the same species (Novak et al., 2000).

The Mongolian gerbil is a crepuscular species, with peaks of activity occurring at dawn and dusk (Pietrewicz et al., 1982). The peak of c-Fos expression occurring 1 hour after lights-off could be related to locomotor activity, because, in rats, artificially induced locomotor activity increases c-Fos expression in the DRN (Brudzynski and Wang, 1996). However, the diurnal variation of c-Fos expression was not the same in all DRN subdivisions in gerbils, whereas artificially induced locomotor activity appeared to elevate c-Fos levels in the whole DRN in rats (Brudzynski and Wang, 1996). Also, in the present study, the peak of c-Fos expression occurred *only* at the light-dark transition, whereas little change in c-Fos expression was observed at the dark-light transition. In contrast, the activity levels (locomotion, digging, scrabbling, eating, grooming) of gerbils are virtually the same at the dark-light and the light-dark transitions (Pietrewicz et al., 1982), suggesting that the observed variation of c-Fos expression in the DRN may depend on other factors.

The diurnal variation of c-Fos expression may correlate with the sleep-wake state of the animals. The sleep-wake cycle has been extensively studied with respect to the activity of serotonergic cells in the DRN. Serotonin levels in the DRN of rats and cats are highest when animals are awake and lowest when animals are in the REM stage of sleep (Portas and McCarley, 1994; Portas et al., 1998, 2000). Also, the activity of serotonergic neurons in the DRN is invariant across the light-dark cycle if recordings of neurons are made during the same behavioral state (REM sleep or quiet waking) in cats, suggesting that the activity of DRN serotonergic neurons may be a function of the sleep-wake cycle rather than a function of the light-dark cycle (Trulson and Jacobs, 1983; Jacobs and Azmitia, 1992). Rats recovering from REM sleep deprivation show higher levels of serotonergic (5-HT+) cells expressing c-Fos than controls (Maloney et al., 1999). In contrast, the number of c-Fos+ serotonergic neurons does not change in cats during carbachol-induced REM sleep (Yamuy et al., 1995).

Many c-Fos-expressing cells observed in this study appeared to be nonserotonergic (5-HT−). These cells exhibited diurnal variation of c-Fos expression, because the large increase in the number of c-Fos+ cells in the caudal ventral DRN could not be accounted for by a probable increase in the number of c-Fos+ serotonergic cells only, which constituted only 11% of c-Fos+ cells when the num-

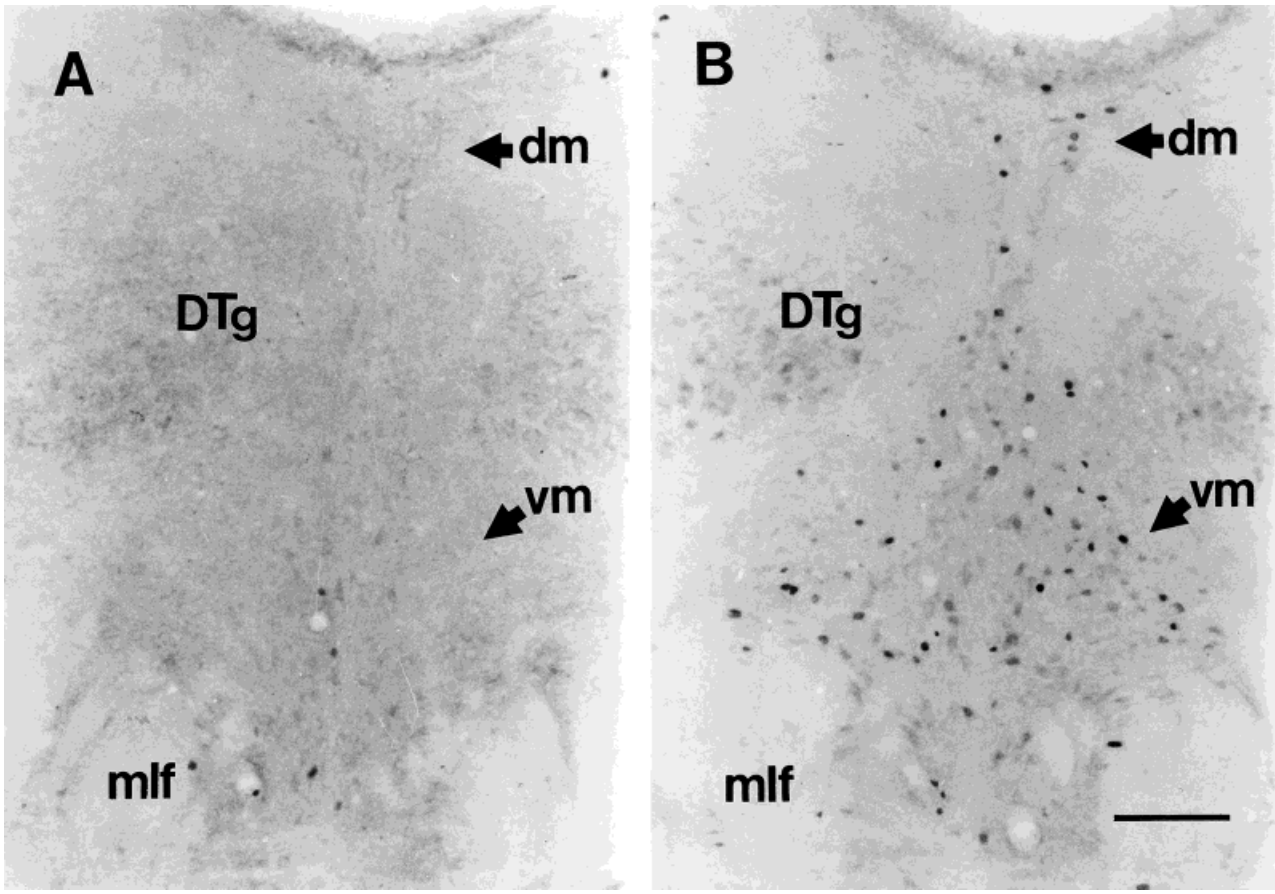


Fig. 5. c-Fos-positive nuclei in the dorsal raphe nucleus (DRN) at Level 5 at 13:00 (**A**) and 20:00 (**B**). Aq, aqueduct; dm, dorsomedial DRN; DTg, dorsal tegmental nucleus; mlf, medial longitudinal fasciculus; vm, ventromedial DRN. Scale bar = 100 μm.

bers of c-Fos+ cells reached their maximum (20:00). The neurochemical identity of these cells is unclear. They may be GABAergic (GABA+) because many neurons in the DRN contain GABA, and very few GABAergic neurons in the rat DRN colocalize 5-HT (Stamp and Semba, 1995). Yamuy et al. (1995) also have shown that, in cats, carbachol-induced REM sleep elevates c-Fos levels in the DRN, but that only some 6% of c-Fos-expressing cells are serotonergic. The number of c-Fos+ /5-HT+ cells was the same in the DRN of carbachol-treated and saline-treated awake animals (Yamuy et al., 1995), whereas the number of c-Fos+/GABA+ cells in carbachol-treated animals showed a 620% increase compared with control animals (Torterolo et al., 2000). Elevated levels of c-Fos+/GABA+ cells also were observed in rats recovering after REM sleep deprivation (Maloney et al., 1999). These data suggest that, in the DRN, the sleep-wake cycle may correlate with c-Fos expression in GABAergic cells. This is further supported by a microdialysis study showing that GABA levels in the DRN are higher when cats are in the REM stage of sleep (Nitz and Siegel, 1997).

The results of the previous research and the present study suggest that the nonserotonergic component of the DRN plays an important role in the sleep-wake and/or the light-dark cycle. However, Mongolian gerbils are active and less likely to sleep at the light-dark transition (Pietrewitcz et al., 1982; Susic and Masirevic, 1986), the time when c-Fos levels in the DRN are highest. This suggests that c-Fos+/5-HT− cells observed in this study may be different from c-Fos+/GABA+ cells observed in cats and rats with induced REM sleep. These nonserotonergic c-Fos-expressing cells may be different GABAergic cells, or may be non-GABAergic cells that contain neurotransmitters other than 5-HT or GABA.

The observed diurnal variation of c-Fos expression in the gerbil DRN may be modulated by light, either through the lateral habenula or directly from the retina. In rats and Mongolian gerbils, the retina projects to the lateral habenula (Qu et al., 1996; unpublished observations) which in turn projects to all subdivisions of the DRN (Peyron et al., 1998). Light also may modulate this variation via the direct projection from the retina to the DRN that has been described in rats, Mongolian gerbils, tree shrews, and Chilean degus (Shen and Semba, 1994; Fite et al., 1999; Reuss and Fuchs, 2000; Fite and Janušonis, 2001). Interestingly, the density of direct retinal afferents is highest in the dorsal and lateral DRN in gerbils and Chilean degus (Fite et al., 1999; Fite and Janušonis, 2001), and they occur almost exclusively in the lateral DRN in rats (Fite et al., 1999). In contrast, the most

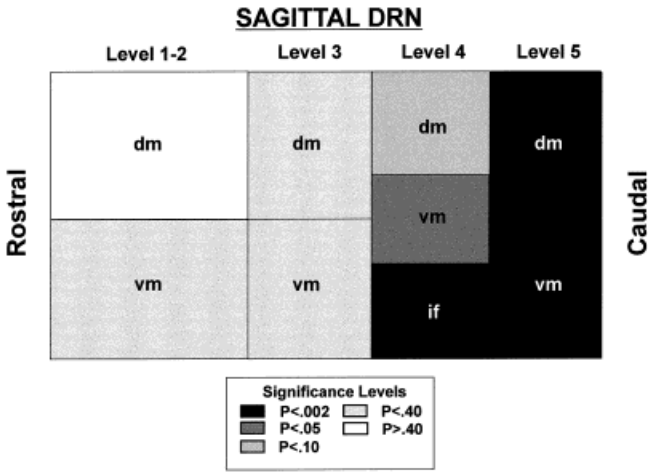


Fig. 6. A highly schematic diagram of the medial dorsal raphe nucleus (DRN) subdivisions in the sagittal plane. The intensity of shading indicates the significance level of diurnal variation of c-Fos expression, as measured by the one-way analysis of variance (ANOVA). dm, dorsomedial DRN; vm, ventromedial DRN; if, interfascicular DRN.

prominent variation in c-Fos expression occurs in the caudal ventromedial DRN. This suggests that either the observed variation of c-Fos expression does not directly depend on the light input from the retina, or that the caudal ventromedial DRN is modulated by afferents from the lateral DRN. The hypothesis that light may modulate c-Fos expression in the DRN is indirectly supported by some preliminary data (Janušonis et al., 1998; Fite et al., 2001) showing that flashing light may reduce the number of c-Fos+ nuclei in the DRN. However, the anatomical connections between DRN subdivisions are virtually unexplored, because it is difficult to obtain tracer injections restricted to only one DRN subdivision.

It is not clear how the activity of gerbil c-Fos-immunoreactive cells observed in the caudal ventral DRN project is synchronized. All of these cells may receive input from the same source, or neurons in the caudal ventral DRN may be electrically coupled, because many DRN neurons show dye-coupling when injected with biocytin (Stezhka and Lovick, 1995), and some DRN neurons show correlated activity (Richards et al., 1997).

Caudal ventral DRN may be anatomically and functionally unique

This study revealed a pronounced variation of diurnal c-Fos expression in the caudal ventral DRN. These results are supported by anatomical studies that suggest that the caudal ventral region of the DRN, in fact, may be a distinct DRN subdivision. In the Mongolian gerbil, serotonergic neurons in the caudal ventromedial DRN and interfascicular DRN (Levels 4 and 5) project to the superior colliculus (SC); whereas at more rostral levels very few, if any, ventromedial neurons project to the SC (Janušonis et al., 1999). In rats, the ventromedial DRN also projects to the visual cortex (Waterhouse et al., 1986) and the piriform cortex (Datiche et al., 1995). In the Mongolian gerbil, neurons projecting to the primary visual cortex (V1) appear to be localized in the ventromedial subdivisions of the DRN as well (Janušonis et al., 2000). In hamsters and rats, the caudal DRN projects to the deep pineal gland (Leander et al., 1998; Moller and Hay-Schmidt, 1998).

Most electrophysiologal studies have focused on neuronal firing or c-Fos expression in the DRN as a whole, without specifying DRN subdivisions. However, DRN cells that increased their firing rate in response to the stimulation of the head, neck, and face in the cat appeared to be intermixed with unresponsive cells, and tended to be localized in the ventromedial DRN (Fornal et al., 1996). Grahn et al. (1999) reported that c-Fos levels the middle and caudal areas of the rat DRN were twice as high following inescapable tail-shock compared with escapable tail-shock, whereas c-Fos levels in the rostral DRN were the same. Lowry et al. (2000) demonstrated that corticotropin-releasing factor (CRF), a stress-related neuropeptide, rapidly increased firing rates of a subpopulation of serotonergic neurons in the ventral DRN in rats. Stress associated with isolation and daily restraint potentiated these responses in the ventral portion of the caudal DRN (Lowry et al., 2000). Therefore, it appears that different DRN subdivisions may play somewhat different functional roles, and the caudal ventral DRN may be sensitive to some stressful and/or somatosensory stimulation. However, it remains unclear whether the population of c-Fos-positive cells that showed diurnal variation in the present study was the same population of cells that were activated in experiments that involved stress or other stimulation.

Each DRN subdivision may contain several cell types

Recent studies suggest that each DRN subdivision contains several types of cells that are intermixed and are not segregated into still smaller clusters. In the present study, the majority of c-Fos+ nuclei were shown to be nonserotonergic and were intermingled with serotonergic cells. Many serotonergic cells occur in the gerbil caudal ventromedial and interfascicular DRN, and, in these subdivisions, up to one-third of neurons projecting to the superior colliculus are nonserotonergic (Janušonis et al., 1999). Other neutransmitters and neuromodulators are present in different DRN subdivisions, and some of them may be colocalized. For example, in the squirrel monkey, SP-immunoreactive neurons occur almost in all subdivisions of the DRN, rostrally; but are confined to the area between the two medial longitudinal fasciculi, caudally (Charara and Parent, 1998). Xu and Hökfelt (1997) have shown that the proportion of serotonergic cells that do not colocalize galanin or nitric oxide synthase (NOS) is higher in the caudal ventromedial DRN than in the rostral ventromedial DRN. In the human DRN, the binding of the 5-HT_{1A} receptor is high in the interfascicular subdivision of the rostral DRN, and decreases in the ventrodorsal and rostrocaudal directions (Stockmeier et al., 1996). In the rat, the expression of serotonin transporter (SERT) is higher in the ventromedial DRN (Rattray et al., 1999); the same difference may exist at the rostral level of the human DRN (Stockmeier et al., 1996).

These studies suggest that each DRN subdivision may be neurochemically unique. However, it may be not a specific cell type, but rather a specific mixture of several cell types that determines the function(s) of a given DRN subdivision. This hypothesis is supported by the evidence that: (1) a substantial topographic overlap exists between

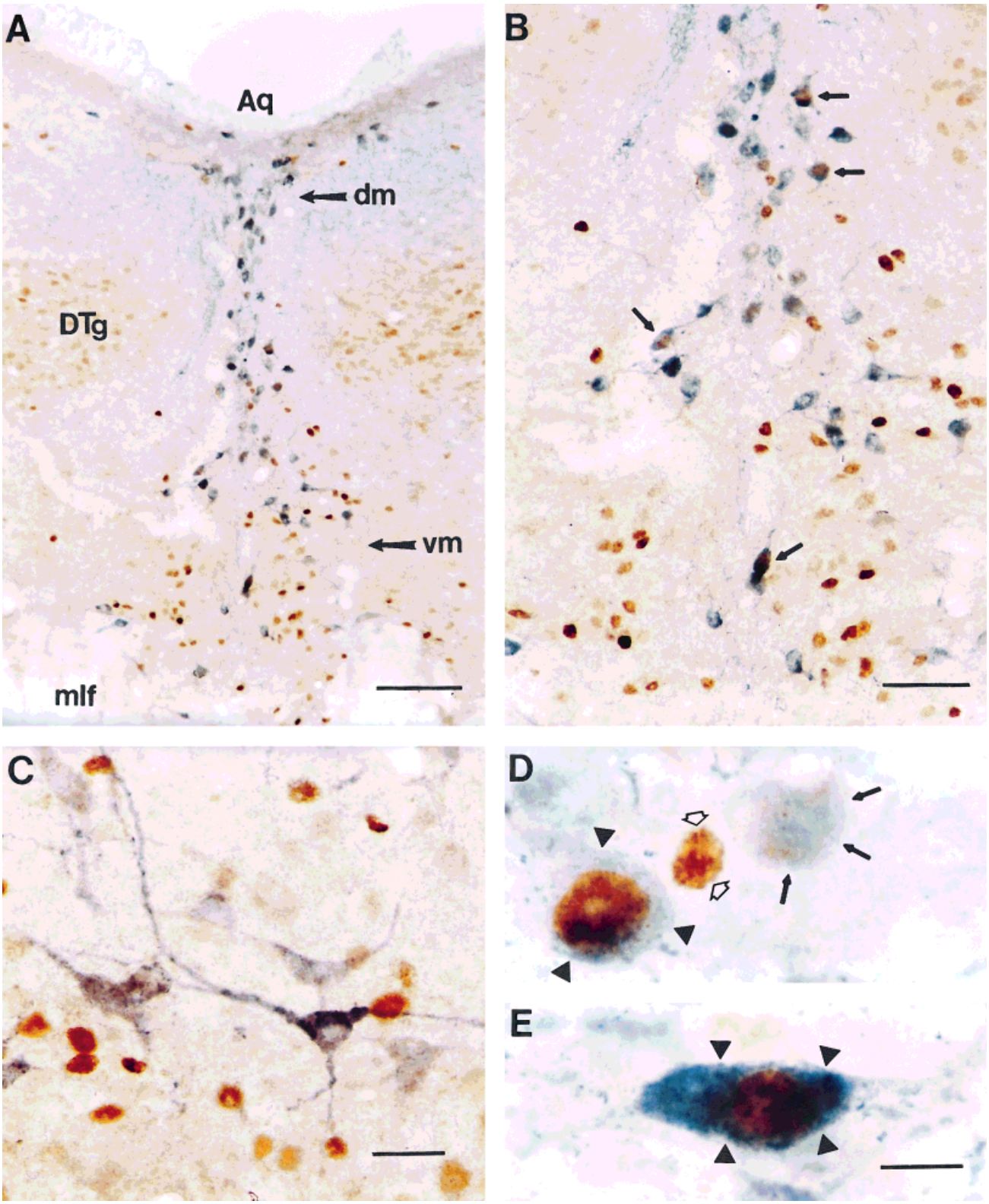


Fig. 7. Double-labeling for c-Fos (brown cell nuclei) and serotonin (5-HT; blue-gray cells) in the dorsal raphe nucleus (DRN) at 20:00. **A:** c-Fos-positive and 5-HT-positive cells are intermingled in the DRN at Level 5. **B:** Higher magnification of the ventromedial DRN at Level 5 (see A). Arrows indicate double-labeled cells. Note that most c-Fos-positive cells are not double-labeled. **C:** Ventrolateral DRN at rostral Level 1 showing 5-HT+/c-Fos− cells surrounded by c-Fos+ nuclei. Lateral is to the left of the figure, medial is to the right. **D:** A putative double-labeled cell (black arrowheads) in the dorsomedial DRN at the transition between Level 3 and Level 4, with a c-Fos+/5HT− cell (open arrows) and a c-Fos−/5-HT+ cell (small arrows). **E:** A putative double-labeled cell (arrowheads) located immediately under the aqueduct in the dorsomedial DRN at Level 4. Aq, aqueduct; dm, dorsomedial DRN; DTg, dorsal tegmental nucleus; mlf, medial longitudinal fasciculus; vm, ventromedial DRN. Scale bars = 100 μm in A, 50 μm in B, 20 μm in C, 10 μm in E (applies to D,E).

various neurotransmitters and neuromodulators in the DRN (Charara and Parent, 1998; Xu and Hökfelt, 1997); (2) at least in some cases, these neurotransmitters and neuromodulators are clearly localized in different individual cells (Stamp and Semba, 1995; Xu and Hökfelt, 1997); and (3) at least two types of DRN cells (serotonergic and nonserotonergic) often project to the same brain area (Koh et al., 1991; Janušonis et al., 1999). Even when the projection appears to be exclusively serotonergic (Mize and Horner, 1989; Gonzalo-Ruiz et al., 1995), several types of serotonergic cells may exist (Mize and Horner, 1989), each having a different complement of colocalized neurotransmitters and neuromodulators.

Understanding DRN subdivisions is potentially important in clinical neuroscience, because recent studies indicate that some mental disorders may be correlated with alterations in certain DRN subdivisions. For example, in suicidal victims with major depression, an increase in the binding of 5-HT_{1A} receptors has been observed, which was localized to the dorsal and ventrolateral DRN subdivisions (Stockmeier et al., 1998). These DRN subdivisions express lower levels of SERT (Rattray et al., 1999); therefore, antidepressants that target SERT (e.g., fluoxetine, sertraline) are likely to have the most significant effect not on these DRN subdivisions, but on the ventromedial DRN which has the highest levels of SERT (Stockmeier et al., 1996; Rattray et al., 1999). This could potentially lead to altered activity in the visual cortex, because, at least in rodents, the ventromedial DRN projects to the primary visual cortex (Waterhouse et al., 1986, 1993; Janušonis et al., 2000). In humans, fluoxetine and sertraline, SERT-targeting antidepressants, have been reported in some cases to induce simple visual hallucinations (lines, circles; Bourgeois et al., 1998), suggesting induced abnormal activity in the primary visual cortex. On the other hand, light therapy may be effective in patients with this pattern of altered levels of the 5-HT_{1A} receptor, because, at least in highly visual rodents, the lateral DRN receives the most prominent direct retinal innervation (Fite et al., 1999; Fite and Janušonis, 2001). Light therapy has been used effectively to treat some forms of depression, such as seasonal affective disorder (SAD; Lam and Levitan, 2000). Interestingly, it has been suggested that SAD is a biologically heterogeneous condition (Lam and Levitan, 2000), which might be due to alterations occurring in different subdivisions of the DRN. Further comparative studies of the DRN, including those in primates and humans, should provide valuable insights into the internal neuronanatomical and functional organization of this major component of the mammalian serotonin system.

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