**Social and environmental contexts modulate sleep deprivation-induced c-Fos activation in rats**

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**Highlights**

- Rats were sleep-deprived for 2 h using three voluntary/forced deprivation methods.
- The patterns of c-Fos activation in the brain varied with the deprivation method.
- Voluntary exploration with or without social interaction activated select regions.
- Forced, gentle sensory stimulation was least effective in inducing c-Fos.
- The brain responds to voluntary and forced sleep deprivation differently.

**Abstract**

People often sleep deprive themselves voluntarily for social and lifestyle reasons. Animals also appear to stay awake longer as a result of their natural curiosity to explore novel environments and interact socially with conspecifics. Although multiple arousal systems in the brain are known to act jointly to promote and maintain wakefulness, it remains unclear whether these systems are similarly engaged during voluntary *vs.* forced wakefulness. Using c-Fos immunohistochemistry, we compared neuronal responses in rats deprived of sleep for 2 h by gentle sensory stimulation, exploration under social isolation, or exploration with social interaction, and rats under undisturbed control conditions. In many arousal, limbic, and autonomic nuclei examined (*e.g.*, anterior cingulate cortex and locus coeruleus), the two sleep deprivation procedures involving exploration were similarly effective, and both were more effective than sleep deprivation with sensory stimulation, in increasing the number of c-Fos immunoreactive neurons. However, some nuclei (*e.g.*, paraventricular hypothalamic nucleus and select amygdala nuclei) were more responsive to exploration with social interaction, while others (*e.g.*, histaminergic tuberomammillary nucleus) responded more strongly to exploration in social isolation. In the rostral basal forebrain, cholinergic and GABAergic neurons responded preferentially to exploration with social interaction, whereas resident neurons in general responded most strongly to exploration without social interaction. These results indicate that voluntary exploration with/without social interaction is more effective than forced sleep deprivation with gentle sensory stimulation for inducing c-Fos in arousal and limbic/autonomic brain regions, and suggest that these nuclei participate in different aspects of arousal during sustained voluntary wakefulness.

**Keywords**
Sleep deprivation method; novelty exploration; social interaction; arousal; motivation; immunohistochemistry

## 1. Introduction

Despite the detrimental effects of sleep deprivation (SD) on vigilance and cognitive performance [1], many people voluntarily deprive themselves of sleep for various reasons including shift work and lifestyle choices such as watching television, reading, and socializing [2, 3]. Most studies that investigated the consequences of sleep loss in animals have used forced wakefulness, which is typically induced by gentle, intermittent sensory stimulation (*e.g.*, auditory, visual, and tactile stimulation) for several hours (*e.g.*, [4-6]). Other animal studies have used protocols that take advantage of animals' natural motivation to explore novel environments and interact socially with conspecifics as a way of keeping animals awake (*e.g.*, [7-9]). Such protocols do not require interventions by the experimenter, and are highly effective in eliciting acute periods of 'voluntary' wakefulness particularly during the animal's inactive period.

Wakefulness is promoted and maintained by multiple arousal systems in the brain [10-13]. These systems originate in the brainstem reticular formation, posterior hypothalamus, and basal forebrain, and promote behavioural and cortical activation using different neurotransmitters. Although they are thought to be somewhat redundant in their function, growing evidence suggests that different arousal systems play specific roles in different aspects of wakefulness, such as attention, locomotion, and motivation. For example, basal forebrain neurons increased their firing rates when novel stimuli were presented during SD, suggesting that they may contribute to attentive wakefulness under conditions of increased sleep pressure [14]. The locus coeruleus and anterior cingulate cortex were found to be essential for sustaining wakefulness in rats placed in an environment with novel stimuli, including social interaction [9]. Likewise, tuberomammillary histaminergic neurons in the posterior hypothalamus were shown to be critical for maintaining wakefulness in mice placed in a novel environment [15, 16]. Furthermore, orexin/hypocretin-containing neurons in the lateral hypothalamus were activated much more strongly when cats freely explored a novel environment than when they were forced to stay awake with sensory stimulation or forced locomotion [17, 18]. The latter findings suggest that the orexin/hypocretin system may be mobilized preferentially during voluntary *vs*. forced wakefulness. Whether this is also the case for other arousal systems was unclear.

To address this question, we used c-Fos immunoreactivity to identify which nuclei of the arousal, limbic, and autonomic systems in the rat brain are activated after 2 h of SD induced by 3 different procedures: gentle sensory stimulation; exploration of a novel environment under social isolation; and exploration combined with social interaction. Control rats were left undisturbed during this period. This experimental design allowed us to compare the pattern of neuronal activation following an equal duration of forced wakefulness (through gentle sensory stimulation) and voluntary wakefulness (through exploratory behaviour with or without social interaction). Although c-Fos immunohistochemistry was examined as a single label in most brain regions, double immunostaining was also used to examine identified cholinergic and GABAergic neurons in the basal forebrain, and histaminergic neurons in the tuberomammillary nucleus.

## 2. Materials and methods

### 2.1 Animals

Adult male Wistar rats (Charles River Laboratories, Saint-Constant, Quebec, Canada) were housed under a 12:12 light:dark cycle (with lights on at 7:00 AM) in standard housing cages made of clear plastic (40 x 30 x 40 cm) in a colony room. Each cage contained wood shavings bedding, replaced every other day, and food and water were available *ad libitum*. All animals were initially housed in pairs for 1 week. This pair housing helped to reduce aggressive behaviours later for the SD condition that involved social interaction (see below). Thereafter, all animals were singly housed for 2 weeks prior to the experiment. As part of normal maintenance, animals were transferred in clean cages twice a week. The rats weighed 384-500 g on the day of the SD experiment.

Rats were divided into 4 treatment groups (n = 8/group): 1) Home cage controls (HC); 2) SD with gentle sensory stimulation (ST); 3) SD with exploration under social isolation (EI); and 4) SD with exploration and social interaction (ES; modified from [9]). For the ST and ES groups, rats that were initially pair-housed were assigned together to the same group, so that they could be tested together in the SD experiment. Rats in the HC group were left undisturbed in their home cages in a familiar environment (animal colony room), while rats in the ST, EI, and ES groups were sleep-deprived in a novel environment (animal testing room) for 2 h in early light phase (10:00 AM-12:00 PM, a period when nocturnal rats normally spend most of their time asleep) as described below. Either 4 or 6 rats were tested at the same time, with every two animals assigned to one of the 4 treatment conditions. Animal handling procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the Dalhousie University Committee on Laboratory Animals.

### 2.2 Sleep Deprivation

On the day of SD experiment, within the animal colony room, each rat in the ST, EI, and ES groups was transferred from its home cage to another standard housing cage containing clean wood shavings bedding. The animals in these cages were then transferred to the animal testing room containing 4 experimental chambers (53 x 41 x 53 cm), each fitted with a fan and a light. The total time that elapsed between removal from the animal colony and the start of the experiment was 5-10 min.

Under the ST and ES conditions, 2 rats that had been initially pair-housed (see above) were placed, still in individual cages, in a single chamber for 2 h (10:00 AM-12:00 PM), whereas under the EI condition, one rat in its cage was placed next to an empty cage in a single chamber for the same period of time. In the EI and ES conditions, cage tops were removed immediately to allow rats to move to and explore the other cage. In addition, ES rats were allowed to interact with each other (modified from [9]); interaction was mostly non-aggressive, and aggressive behaviours were infrequent and brief in duration. In the ST condition, cage tops remained fastened, and rats were kept awake by using gentle sensory stimuli, such as light tapping on the side of the cage, and by opening and closing the cage top, as required. The stimuli were applied only when the rats showed behavioural signs of sleepiness, for example, when they were immobile with eyes closed or started adopting a sleeping posture. Food and

water were available *ad libitum* for all the conditions. Throughout the entire SD period, rats' behaviour were constantly monitored visually by a single experimenter (N.R.). The HC rats were left undisturbed in their home cages in the colony room.

## 2.3 Perfusion, Fixation, and Brain Sectioning

At the end of SD/control period, rats were injected with an overdose of anesthetics (208 mg/kg ketamine, 9.6 mg/kg xylazyne, and 1.8 mg/kg acepromazine, i.p.), and perfused intracardially with 100 ml of 0.1 M phosphate-buffered saline (PBS; pH = 7.4) followed by 400 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed, postfixed for 4-6 h in the same fixative solution, and then transferred to 30% sucrose in 0.01 M phosphate buffer for 2 days at 4ºC. Coronal brain sections (40 µm) were cut on a freezing microtome and collected in 5 serial sets in 0.05 M Tris-buffered saline (TBS, pH 7.4). Four sets were used in the present experiment.

## 2.4 Immunohistochemistry

One set of sections through the entire brain was single-immunostained for c-Fos protein, as described previously [19]. Briefly, sections were incubated with a rabbit polyclonal anti-c-Fos antibody (1:15,000; Catalog No. PC38; Oncogene Research Products, Cambridge, MA) for 2 days at 4°C or overnight at room temperature. The anti-c-Fos antibody was raised against the N-terminal sequence (residues 4-17) of c-Fos protein that is conserved among human, mouse, and rat. On Western blot, this antibody recognized a ~55 kDa band corresponding to the expected molecular weight of c-Fos (manufacturer's technical information). Sections were subsequently incubated for 1 h with a biotinylated donkey anti-rabbit IgG antibody (1:1000, Jackson ImmunoResearch Laboratories, West Grove, PA) followed by a standard avidin-biotin-horseradish peroxidase complex (ABC; Vector Laboratories, Burlingame, CA) for 1 h. Sections were then placed in 0.02% solution of diaminobenzidine (DAB; Sigma) and 0.65% nickel, and then 0.006% hydrogen peroxide was added to obtain a black-purple nuclear precipitate for c-Fos.

Sections through the basal forebrain from a second series of sections were double-immunostained for c-Fos and vesicular acetylcholine transporter (VAChT) to distinguish between c-Fos activation of cholinergic and non-cholinergic neurons. These sections were processed to visualize c-Fos first, as described above, and then VAChT, as reported previously [19], using a goat polyclonal antibody to VAChT (1:10,000; Catalog No. AB1578; Chemicon International, Temecula, CA), a biotinylated donkey anti-goat IgG antibody (1:1000, Jackson), ABC, and DAB (without nickel) as a chromogen to produce a brown cytoplasmic reaction product. The anti-VAChT antibody was raised against a synthetic peptide representing amino acids 511-530 from the cloned rat VAChT, and recognized a 65-70 kDa band corresponding to VAChT protein on Western blot (see [20]). The immunohistochemical specificity of VAChT was confirmed previously [19].

A third set of sections was used to provide sections through the basal forebrain that were double-immunostained for c-Fos and glutamic acid decarboxylase (GAD67) to identify c-Fos activation of GABAergic and non-GABAergic neurons. These sections were processed to visualize GAD67 first, and then c-Fos, as described above. This

order of staining was necessary because Triton X-100 (a permeabilizing agent) was omitted from incubation with the anti-GAD67 antibody to enhance levels of GAD staining in cell bodies, as per manufacturer's recommendation (Triton X-100 was added for the rest of the procedures). A mouse monoclonal anti-GAD67 antibody (1:1000; Catalog No. MAB5406; Millipore, Temecula, CA) was used, with a biotinylated donkey anti-mouse IgG antibody (1:1000, Jackson), ABC, and DAB, as described above. The anti-GAD67 antibody was directed against the recombinant fusion protein from amino acids 1-101 of human protein containing the N-terminal region of GAD67 (see [20]). This antibody recognized a ~67 kDa band in the rat cerebellar cortex [21], and has been frequently used to label GABAegic neurons in the basal forebrain (*e.g.*, [22, 23]).

Sections through the tuberomammillary nucleus from a fourth set of sections were double-immunostained for c-Fos and adenosine deaminase (ADA; [24]) to determine whether histaminergic neurons, which selectively express ADA in this particular brain region, expressed c-Fos following the behavioural treatments. These sections were processed to visualize c-Fos first, as described above, and then ADA. A rabbit polyclonal anti-ADA antibody was used, with a biotinylated donkey anti-rabbit IgG antibody (1:1000, Jackson), ABC, and DAB, as described above. The anti-ADA antibody was raised against calf spleen ADA (1:200; Catalog No. AB1815; Millipore) was used. The pattern of staining in the tuberomammillary nucleus obtained with this antibody was similar to that reported in previous studies with antibodies against ADA or histidine decarboxylase, the synthetic enzyme for histamine [19, 24, 25].

## 2.5 Cell Counts

An experimenter, blind to the treatment conditions with respect to individual brain sections, counted single- and double-immunoreactive cells directly under a bright-field microscope (Olympus) at 156.25x (single-immunostained sections) or 312.50x (double-immunostained sections). These counts represented relative numbers of profiles, not absolute numbers of cells or their estimates. The nomenclature and boundaries of nuclei were according to the rat brain atlas by Paxinos and Watson [26], and the rostrocaudal coordinates (in mm from Bregma) indicated below were taken from that atlas.

*2.5.1 Single-immunostained sections.* Single c-Fos-positive cells were counted in 16 brain regions, including those with arousal, limbic, and autonomic functions, using sections singly immunostained for c-Fos. Counting boxes were used for all regions, except the dentate gyrus. The size and placement of counting box are described below for each region of interest.

In the prelimbic cortex (posterior part; A2.70-A2.20), a counting box (770 μm x 770 μm) was centered between the midline of the brain and the medial border of the corpus callosum, and placed ventral to the level of the dorsal surface of the corpus callosum.

In the anterior cingulate cortex (A1.0-A1.2), a box (770 μm x 770 μm) was placed with its medial segment touching the midline of the brain, and its ventrolateral corner touching the corpus callosum.

For the accumbens nucleus (A1.6-A1.2), cell counts were obtained separately for the shell and core divisions. For the shell division, a box (500 μm x 1000 μm) was

positioned dorsal to the level of the ventral surface of the anterior commissure and lateral to the clearly visible, major division of the Islands of Calleja. For the core division, a box (500 μm x 1000 μm) was placed dorsal to the level of the ventral surface of, and medial to, the anterior commissure.

In the ventrolateral preoptic nucleus (P0.3-P0.4), a box (310 μm x 310 μm) was placed dorsal to the ventral surface of the brain and 150 μm lateral to the optic chiasm.

In the paraventricular hypothalamic nucleus (P1.8-P1.9), a box (420 μm x 230 μm) was centered at the parvicellular division, which was clearly visible along the wall of the third ventricle.

For the amygdala (P2.1-P2.3), separate cell counts were obtained for the central, basolateral, and medial nuclei. In the central nucleus, a box (690 μm x 620 μm) was positioned 80 μm dorsal to the clearly visible intercalated nucleus and medial to the commissural stria terminalis. In the basolateral nucleus, a box (620 μm x 690 μm) was positioned 150 μm lateral to the intercalated nucleus, and dorsal to the level of the ventral border of the latter nucleus. In the medial nucleus, a box (770 μm x 460 μm) was placed dorsal to the clearly visible bed nucleus of the accessory olfactory tract and parallel to the optic tract.

In the perifornical hypothalamic area (P2.8-P3.3), a box (770 μm x 540 μm) was placed so that the midpoint of its ventral segment was centered at the fornix.

In the dentate gyrus of the dorsal hippocampus (P3.3-P3.6), separate cell counts were obtained from the granule cell layer in the superior and inferior blades [27, 28]. Cells were counted over the entire length of each blade.

In the paraventricular thalamic nucleus (posterior division; P3.3-P3.6), a box (1000 μm x 500 μm) was centered in the nucleus, with the dorsal border of the box touching the ventral surface of the third ventricle.

In the supramammillary nucleus (P4.3-P4.5), 2 boxes were used (770 μm x 380 μm each), and cell counts were combined. One box was centered in the anterior division between the bilateral principal mammillary tracts, so that the midpoints of the two lateral segments of the box overlay the dorsal border of the principal mammillary tracts. The other box was centered in the posterior division, which is located dorsal to the principal mammillary tracts.

In the ventral tegmental area (P5.2-P5.3), a box (770 μm x 380 μm) was placed dorsal to the mammillary peduncle and lateral to the medial lemniscus.

In the dorsal raphe nucleus (P7.8-P8.0), a box (1540 μm x 460 μm) was centered in the nucleus so that the dorsal border of the box touched the ventral surface of the cerebral aqueduct.

In the locus coeruleus (P9.7-P10.0), 2 boxes were used, one (310 μm x 150 μm) centered in the anterior division, and the other (540 μm x 150 μm) centered in the posterior division; the two cell counts were combined. The locus coeruleus was easily discernible as a dense neuronal cluster ventrolateral to the fourth ventricle.

*2.5.2 Double-immunostained sections.* The basal forebrain and tuberomammillary nucleus were analyzed using sections dually immunostained for c-Fos and transmitter markers.

In the basal forebrain, cell counts were obtained in 6 nuclei: the medial septum (A0.5-A0.2); nuclei of the vertical (A0.5-A0.2) and horizontal (A0.5-P0.8) limbs of the

diagonal band of Broca; magnocellular preoptic nucleus (P0.4-P0.8); substantia innominata (P1.6-P1.8); and magnocellular basal nucleus (P1.6-P1.8). The same counting box (400 μm x 400 μm) was used for all the nuclei. As the boundaries of these basal forebrain nuclei are traditionally defined by the presence of cholinergic neurons [29], the box was first placed inside each nucleus in sections double-immunostained for c-Fos and VAChT, so that the box contained the largest number of VAChT-immunopositive (VAChT+) neurons. The box was then placed approximately at the same location in adjacent sections that were double-immunostained for c-Fos and GAD.

In the tuberomammillary nucleus (P3.8-P4.3), double-labeled cells were counted in sections immunostained for c-Fos and ADA using 2 counting boxes, one (200 μm x 200 μm) in the dorsal division, and the second in the ventral division (200 μm x 400 μm); each box was placed so that it contained the largest number of ADA-immunoreactive neurons. A separate count was obtained from each division.

For each structure, cell counts were obtained from 2 sections (except for 4 sections for the horizontal diagonal band, and 1 section each for the prelimbic and anterior cingulate cortices) within the rostrocaudal levels as indicated above. The 2 sections were ~160 μm apart rostrocaudally. Counts were done separately on either side of the brain for each structure. Counts were then averaged for a mean count per side of the brain per section for each structure. Cells counts were obtained from all the rats (n = 8 per group) for all the brain areas under study, except for two brain regions for which either one or two rats did not provide data: the prelimbic cortex (n = 5 or 6/group) because of incomplete sets of sections, and the ventrolateral preoptic nucleus (n = 7 or 8/group) because of damaged sections.

Sections were digitally photomicrographed using Zeiss microscope. The contrast and brightness of images were adjusted using Adobe Photoshop CS4 (Adobe Systems, San Jose, CA).

## 2.6 Statistical Analysis

Data analyses were conducted using Statview 5.0 (SAS Institute Inc., Cary, NC). Cell counts were analyzed using separate one-way analyses of variance with Group as a factor. When group variances were not statistically equal, logarithmic transformation [log (X+1)] was used. If a significant main effect was found, multiple pairwise comparisons were performed to compare each treatment group with each other using Tukey-Kramer post hoc tests. *P* values < 0.05 were considered statistically significant. Values are expressed as means ± SEM.

## 3. RESULTS

### 3.1 Behaviours during Sleep Deprivation

Rats undergoing SD by gentle sensory stimulation (ST group) were behaviourally active during the first 20-30 min after placement in the chamber, and then became less active gradually, spending more time in 'quiet' wake. To keep these animals awake, repeated interventions (light tapping on the side of the cage, and opening and closing of the cage top) were required, especially in the second hour (5.0 ± 2.0 [mean ± SEM] interventions in the first hour *vs.* 36.0 ± 5.6 interventions in the second hour; the

numbers indicate those required to keep two rats awake that were concurrently tested next to each other but in separate cages, as explained in the Materials and Methods). In contrast to the ST group, rats that were sleep-deprived using exploration under social isolation (EI group) or exploration with social interaction (ES group) remained spontaneously awake and largely active during most of the 2 h period. These animals typically explored the other cage and, in the case of the ES rats, interacted with the other rat tested concurrently, with sniffing, following and playing with each other. The EI and ES rats required only 1-2 or no interventions by the experimenter during the entire 2 h period.

**3.2 Patterns of c-Fos Immunoreactivity in Selected Brain Regions**

Neurons immunoreactive for c-Fos (c-Fos+) were counted in 16 brain regions, including arousal, limbic, and autonomic nuclei, in sections singly immunostained for c-Fos. Figure 1 shows examples of c-Fos immunoreactivity in the brains of rats sleep-deprived under different conditions.

In most brain regions examined, there was a large increase in c-Fos labeling under all the SD conditions relative to the home cage control condition, whereas in a few areas there were no significant differences between the 4 conditions (Supplementary Table 1).

In the brain regions that showed statistically significant differences between the 4 treatment groups, two main patterns of c-Fos labeling that varied with the SD methods were observed. In the first pattern (Fig. 2), which was more common, the number of c-Fos+ neurons was increased significantly in response to exploration regardless of the social context, while only moderate c-Fos labeling occurred after SD with sensory stimulation (EI and ES > HC; intermediate values in the ST group were typically not significantly different from values in any other groups). This pattern was seen in 7 regions: the prelimbic cortex ($F_{3,17} = 4.96$, $P = 0.012$; Fig. 2A), anterior cingulate cortex ($F_{3,28} = 3.33$, $P = 0.021$; Figs. 1A and 2B), paraventricular thalamic nucleus ($F_{3,28} = 3.55$, $P = 0.027$; Figs. 1B and 2C), supramammillary nucleus ($F_{3,28} = 6.50$, $P = 0.0018$; Fig. 2D), ventral tegmental area ($F_{3,28} = 8.14$, $P = 0.0005$; Fig. 2E), dorsal raphe nucleus ($F_{3,28} = 6.50$, $P = 0.0017$; Fig. 2F), and locus coeruleus ($F_{3,28} = 28.73$, $P < 0.0001$; Figs. 1C and 2G). In the locus coeruleus, the increase in the ST group *vs*. the HC group was also significant ($P < 0.05$). In addition, there were statistical trends of an increase in both the EI and ES groups *vs*. the HC group in the shell division of the accumbens nucleus and in the perifornical hypothalamic area ($P = 0.065$ and $0.067$, respectively; Supplementary Table 1).

In the second pattern of response to SD (Fig. 3), a significant increase occurred only after SD with exploration and social interaction, while SD with exploration without social interaction, and SD with sensory stimulation resulted in moderate c-Fos labeling (ES > HC; intermediate values in the ST and EI groups were not significantly different from each other or from values in the ES and HC groups). This pattern was observed in 4 brain regions: the core division of the accumbens nucleus ($F_{3,28} = 2.95$, $P = 0.049$; Fig. 3A), paraventricular hypothalamic nucleus ($F_{3,28} = 4.87$, $P = 0.0075$; Figs. 1D and 3B), and medial ($F_{3,28} = 6.25$, $P = 0.0022$; Figs. 1E and 3C) and basolateral ($F_{3,28} = 3.01$, $P = 0.047$; Fig. 3D) nuclei of the amygdala. The central nucleus of the amygdala showed a similar trend ($P = 0.067$; Supplementary Table 1).

**3.3 Patterns of c-Fos Immunoreactivity in the Basal Forebrain**

To study c-Fos labeling in the basal forebrain, we used additional VAChT and GAD immunostaining to identify cholinergic and GABAergic neurons. The regional pattern of c-Fos labeling across the basal forebrain nuclei was similar regardless of whether the transmitter phenotype label was VAChT or GAD. However, the total number of c-Fos+ neurons (*i.e.*, combined numbers of single- and double-labeled neurons) in sections immunostained for c-Fos first and then VAChT was, on average, 10-45% higher than in those immunostained for GAD then c-Fos, suggesting that the timing of c-Fos processing affected c-Fos detection (see Materials and Methods for sequential double labeling procedures). For this reason, c-Fos+ cell counts obtained from sections immunostained for c-Fos first and then VAChT were used to analyze the total c-Fos+ cell counts described below.

*3.3.1 Total c-Fos+ neurons.* The rostral and middle nuclei of the basal forebrain were responsive to SD, particularly when it was imposed with exploration without social interaction, but the caudal nuclei of the basal forebrain did not respond to SD with any method. Specifically, in the rostral-middle basal forebrain, the increase was significant in both the EI and ES groups *vs.* the HC and/or ST groups for the medial septum ($F_{3,28} = 10.95$, $P < 0.0001$; EI and ES > HC, and EI > ST, $P < 0.05$; Fig. 4A), while the increase was significant only in the EI group *vs.* the HC group for the vertical and horizontal diagonal band nuclei ($F_{3,28} = 5.06$ and $4.74$, $P = 0.0063$ and $0.0085$, respectively; EI > HC, $P < 0.05$ for both nuclei; Figs. 4B and C) and the magnocellular preoptic nucleus ($F_{3,28} = 2.96$, $P = 0.049$; EI > HC, $P < 0.05$; Fig. 4D); there was a trend of an increase in the ST group *vs.* the HC group in each of these rostral-middle subnuclei (P > 0.05 for each comparison). In contrast, in the caudal basal forebrain, there was no significant effect of SD in either the substantia innominata ($F_{3,28} = 2.37$, $P = 0.092$; Fig. 4E) or the magnocellular basal nucleus ($F_{3,28} < 1$; Fig. 4F).

*3.3.2 Cholinergic neurons.* The total number of VAChT+ neurons (with and without c-Fos) in any basal forebrain nucleus was not affected by SD (Supplementary Table 2), indicating that the areas of quantification (using counting boxes) and staining intensities were fairly uniform across animals and conditions.

Similar to the results of total c-Fos+ neurons described above, the percentage of VAChT+/Fos+ neurons out of all VAChT+ neurons in the medial septum, although fairly low, was increased significantly in the ES group, compared with the HC and ST groups ($F_{3,28} = 5.45$, $P = 0.0044$; ES [4.3%] > HC [0.7%] and ST [1.6%], $P < 0.05$; values in the EI group were intermediate [2.3%]; Fig. 5A). There were no significant effects on the percentages of VAChT+/Fos+ neurons in the other basal forebrain nuclei studied (*Ps* = 0.12-0.88). Similar results were obtained from the analysis using raw numbers of VAChT+/Fos+ neurons (Supplementary Table 2). Examples of c-Fos immunoreactivity in VAChT+ and VAChT-immunonegative (VAChT−) neurons in the medial septum of an ES rat are shown in Figure 1F.

In parallel with the results with the total number of c-Fos+ neurons (see above), VAChT− neurons in the rostral and middle, but not caudal, nuclei of the basal forebrain

showed an increase in c-Fos labeling after SD, in particular after SD with exploration without social interaction (Supplementary Table 2).

*3.3.3 GABAergic neurons.* Throughout the basal forebrain, GAD+ neurons were present in the areas where VAChT+ neurons were located. The total number of GAD+ neurons was 20-70% higher than VAChT+ neurons in all the basal forebrain nuclei studied, and did not differ among the 4 treatment groups (Supplementary Table 3). The greater abundance of GABAergic *vs.* cholinergic neurons is consistent with previous reports [30, 31].

The occurrence of c-Fos induction in GABAergic neurons was restricted to the rostral and, to some extent, the middle level of the basal forebrain. Similar to the results with cholinergic neurons, the percentage of GAD+/Fos+ neurons out of all GAD+ neurons in the medial septum was increased significantly in the ES group. However, unlike with the cholinergic neurons, the other two methods of SD also significantly increased the percentage of GAD+/Fos+ neurons ($F_{3,28} = 15.05$, $P < 0.0001$; ST [7.2%], EI [8.5%] and ES [12.1%] > HC [2.2%], $P < 0.05$); as in the cholinergic neurons, the percentage was significantly higher in the ES group than in the ST group (ES > ST, $P < 0.05$; Fig. 5B). Slightly more caudally, the percentage of GAD+/Fos+ neurons in the diagonal band nuclei was also increased to similar levels by all the SD methods (vertical diagonal band: $F_{3,28} = 5.29$, $P = 0.0051$; ST [4.6%] and ES [4.8%] > HC [1.1%], $P < 0.05$; and horizontal diagonal band: $F_{3,28} = 6.17$, $P = 0.0023$; ST [6.0%], EI [5.5%] and ES [5.4%] > HC [1.2%], $P < 0.05$; Fig. 5B). There were no significant group differences in the more caudal basal forebrain nuclei ($Ps = 0.19$-$0.92$), as was the case for cholinergic neurons. The same results were obtained with the raw numbers of GAD+/Fos+ neurons (Supplementary Table 3). Examples of c-Fos immunoreactivity in GAD+ and GAD− neurons in the medial septum of an ES rat are shown in Figure 1G.

GAD− neurons within the rostral and middle, but not caudal, basal forebrain nuclei showed an increase in c-Fos labeling after SD (Supplementary Table 3), in a pattern similar to that of the total c-Fos+ neurons described above.

## 3.4 Patterns of c-Fos Immunoreactivity in the Tuberomammillary Nucleus

For c-Fos labeling in the tuberomammillary nucleus, we focused on histaminergic neurons identified by the presence of ADA immunoreactivity [24]. Unexpectedly, the total number of ADA+ neurons in the ventral division was higher (by 54%) in the EI group than in both the HC and ES groups ($F_{3,28} = 4.94$, $P = 0.007$, EI > HC and ES, $P < 0.05$); a similar increase was seen in the dorsal division, but this difference was not significant ($F_{3,28} = 1.58$, $P = 0.22$; Supplementary Table 4).

Examples of c-Fos labeling in ADA+ and ADA− neurons in an EI rat are shown in Fig. 1H. Although relatively low, the percentage of ADA+/Fos+ neurons out of all ADA+ neurons was significantly higher in the EI group *vs.* the HC group for both the dorsal ($F_{3,28} = 3.42$, $P = 0.031$; EI [9.3%] > HC [1.1%], $P < 0.05$; Fig. 6A) and the ventral division ($F_{3,28} = 3.04$, $P = 0.046$; EI [13.1%] > HC [4.1%], $P < 0.05$; Fig. 6B); intermediate values were found in the ST group (dorsal: 5.3%; ventral: 7.8%) and the ES group (dorsal: 6.4%; ventral: 7.3%). Similar results were found with the raw numbers of ADA+/Fos+ neurons (Supplementary Table 4), despite the differences in the total numbers of ADA+ neurons across experimental conditions.

## 4. Discussion

We found that 2 h of SD induced under 3 different environmental and social conditions (gentle sensory stimulation, exploration of a novel environment under social isolation, and exploration with social interaction) resulted in condition-dependent patterns of neuronal activation, as indexed by c-Fos immunoreactivity, in selected arousal, limbic, and autonomic brain areas and neuronal groups (summarized in Supplementary Table 5). In the rostral basal forebrain, cholinergic and GABAergic neurons showed different condition-dependent patterns compared to unidentified neurons in the same nuclei. These results indicate that the extent of c-Fos responses to SD varies depending on whether sleep loss is forced or voluntary, and suggest that c-Fos induction after SD depends not only on the time of day and duration of SD [32, 33], but also on the context of arousal that animals experience [18, 34-36]. These results complement those of a recent study [36] reporting that voluntary and forced SD produced different levels of arousal, sleep rebound, and arousal-related phosphoproteins markers in mice. Collectively, these results suggest that the SD method should be taken into consideration when interpreting the effects of sleep loss in animals.

### 4.1 The Pattern of Activation in Arousal, Limbic, and Autonomic Regions Following Sleep Deprivation Depends on the Deprivation Method

All the 3 SD methods were effective in increasing c-Fos immunoreactivity in most of the brain areas and neuronal populations studied that have arousal and limbic/autonomic functions, but the extent of activation varied depending on the SD method. The extents of c-Fos induction among the 3 sleep-deprived groups are likely related to the nature of behaviours and associated arousal levels that ensued during SD. The ST rats showed more behavioral immobility and more attempts to initiate sleep than the EI and ES rats, which continued to exhibit exploratory behavior and made few attempts to initiate sleep particularly during the second hour of SD.

Gentle sensory stimulation tended to increase c-Fos immunoreactivity only moderately in most of the brain areas, and the increase was significant only in the locus coeruleus. This is somewhat discrepant from previous studies using 3 h of gentle handling that reported significant increases in c-Fos labeling in many brain regions [6, 32, 33, 37]. It is possible that the shorter SD in the current study (2 h *vs*. 3 h) resulted in lower c-Fos labeling. However, it is also possible that the increases after gentle stimulation would have reached statistical significance had there been only two conditions (i.e., ST and HC groups). The activation of the locus coeruleus after SD with sensory stimulation is consistent with the report that the locus coeruleus is particularly responsive to sensory stimuli that elicit arousal [38].

Three main profiles of c-Fos response were observed across the brain areas examined (Supplementary Table 5). First, exploration resulted in the greatest increase in c-Fos immunoreactivity in many regions, regardless of whether or not the exploration was accompanied by social interaction with a familiar rat. These areas included the prelimbic and anterior cingulate cortices, medial septum, paraventricular thalamic nucleus, supramammillary nucleus, ventral tegmental area, dorsal raphe nucleus, and locus coeruleus; similar trends were seen in the shell of the accumbens nucleus and in

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the perifornical hypothalamic area. Previous studies have shown increases in c-Fos labeling in these as well as other areas after 0.5-5 h of exploration of a novel environment with or without social opportunities [8, 9, 39-41].

The similarity in c-Fos patterns observed in the aforementioned brain regions suggests that these regions might be part of a common neuronal circuit involved in attentive and motivational arousal. This possibility is supported by several lines of evidence. It has been suggested that reciprocal connections between the anterior cingulate cortex and locus coeruleus are critical for sustaining wakefulness in a novel environment, such as one with social interaction opportunities [9]. The paraventricular thalamic nucleus has been implicated in arousal and attention via its projections to limbic regions of the cerebral cortex [42]. The ventral tegmental area has reciprocal projections with the accumbens shell and the prelimbic cortex, as part of the reward circuit, and these projections might mediate the rewarding aspect of novelty exploration [43] and social interaction [44]. Recently, it has been suggested that reciprocal connections between the ventral tegmental area and accumbens shell may promote behavioural arousal driven by motivation [45]. The activation of the supramammillary nucleus is likely relevant to this type of arousal, given its projection to the medial septum and its role in the generation of the hippocampal theta rhythm [46], which is prominent during active exploratory behaviour in rodents [47].

Second, a number of other brain areas examined were activated preferentially by exploration with social interaction, as opposed to exploration under social isolation. These brain areas might be part of the circuit involved in arousal associated with social interaction, or social arousal, with an emotional component. These nuclei include the core of the accumbens nucleus, the paraventricular hypothalamic nucleus, and the basolateral and medial amygdala, with a similar trend in the central amygdala. Consistent with these results, previous studies have shown increases in c-Fos expression in the basolateral and medial amygdala after social interaction with conspecifics [48, 49]. The medial amygdala is suggested to be part of a 'social behaviour network' [50], and it receives strong projections from the accessory olfactory system, which relays information on social stimuli in rodents [51]. Both the basolateral and medial nuclei project to the central amygdala, which plays an important role in coordinating behavioural, autonomic, and endocrine responses elicited by emotional stimuli [52]. Notably, the central amygdala may activate the hypothalamic-pituitary-adrenal stress responses [53], probably through its indirect projections to the paraventricular hypothalamic nucleus [54]. The highest c-Fos levels observed in the latter nucleus after SD with exploration with social interaction may reflect stress and anxiety associated with exploration with social interaction.

A third pattern of neuronal activation was observed in unidentified rostral basal forebrain neurons (also see below, Section 4.2) and histaminergic neurons in the tuberomammillary nucleus. These neuronal groups were more responsive to SD induced by exploration without social interaction. In the absence of a conspecific, the novelty of the environment would be a dominant arousing stimulus in this condition. The finding with the tuberomammillary nucleus is consistent with a role of histaminergic neurons in sustaining high levels of arousal necessary for attending to changing environment [15, 16] and for certain motivated behaviours [55]. In addition, the number of histaminergic neurons expressing c-Fos was positively correlated with the amount of

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wakefulness during the preceding 1 h [25], and histamine levels in the rat basal forebrain were increased even after 1 h of SD induced by gentle handling [56].

Finally, the ventrolateral preoptic nucleus and the hippocampal dentate gyrus showed no significant differences in c-Fos levels across the experimental conditions. The ventrolateral preoptic nucleus contains GABAergic neurons that are sleep-active and express c-Fos during sleep [6, 57, 58]. The lack of SD effects in this nucleus is consistent with previous studies using c-Fos-immunoreactivity and 2 or 3 h of SD with gentle sensory stimulation [6, 59]; however, a decrease in c-Fos immunoreactivity has also been reported [57]. In the dentate gyrus, a trend of an increase was observed in the superior, but not inferior, blade under all SD conditions. Although statistical significance was not reached, this result is in line with previous studies showing increased c-Fos expression in the superior, but not inferior, blade of the dentate gyrus following 30 min of exploration of a novel environment (*e.g.*, [27, 28]).

### 4.2 The Pattern of Activation in the Basal Forebrain Depends on the Deprivation Method, Region, and Neurotransmitter Phenotype

Although unified by the presence of cholinergic projection neurons, the basal forebrain consists of several nuclei that extend rostrocaudally, and we analyzed them separately. We also conducted double labeling, which allowed us to compare c-Fos induction among different neurotransmitter phenotypes in the same nuclei. We found that the basal forebrain showed nucleus- and cell type-specific increases in c-Fos labeling in response to the 3 SD procedures, as discussed below.

We observed considerable regional differences in the c-Fos responses of basal forebrain neurons to SD, in that rostral basal forebrain neurons were fairly responsive, but middle and caudal basal forebrain neurons showed little or no response. The paucity of response in caudal basal forebrain neurons may not mean that these neurons do not respond to SD, and it is possible that the present SD conditions were not sufficient to induce significant c-Fos in them. Nonetheless, the present results indicate considerable differences in responsiveness among basal forebrain neurons, with rostral basal forebrain neurons being far more responsive to SD regardless of the method, and particularly when SD is induced with voluntary exploration of a novel environment.

In addition to the regional differences, we observed differences among neurochemically different neurons in their responsiveness to the 3 procedures of SD. Specifically, subsets of both cholinergic and GABAergic neurons in the medial septum were activated most strongly following exploration combined with social interaction. This is consistent with previously reported increase in c-Fos immunoreactivity in cholinergic neurons and parvalbumin-containing neurons (representing GABAergic neurons projecting to the hippocampus) in the medial septum/diagonal band following 1 h of exploration of a novel environment [60]; other basal forebrain nuclei were not analyzed in that study. Two hours of SD with gentle sensory stimulation also increased c-Fos labeling in cholinergic neurons in the medial septum, as well as other basal forebrain nuclei [5, 6]. The cholinergic and GABAergic neurons in the medial septum that project to the hippocampus have been shown to be involved in modulating arousal, attention, and sensory processing [61]. In the present study, the co-activation of cholinergic and GABAergic neurons in the medial septum during SD with exploration and social

interaction may have contributed to increased attentive arousal associated with this condition.

Unlike in the medial septum, in the diagonal band nuclei, which are located slightly more caudally, only a subset of GABAergic, and not cholinergic, neurons were responsive to SD, and these GABAergic neurons showed similar levels of increase in c-Fos immunoreactivity regardless of the SD method. This result is consistent with a previous report that a proportion of c-Fos-immunoreactive neurons in the intermediate level of the basal forebrain after 3 h of sensory stimulation was GABAergic [6]. The present findings suggest that the activation of GABAergic neurons in the diagonal band nuclei is associated with general wakefulness common to the 3 experimental conditions.

Importantly, the comparison of total c-Fos-immunoreactive neurons *vs.* c-Fos-positive cholinergic and GABAergic neurons indicates that most of the activated neurons in the rostral basal forebrain were neither cholinergic nor GABAergic, and therefore were presumably glutamatergic [31]. Thus, glutamatergic neurons in the rostral basal forebrain appear to be most responsive to SD with exploration but without social interaction. In support of this interpretation, about half of presumably glutamatergic (*i.e.*, non-cholinergic and non-GABAergic) neurons in the intermediate level of the basal forebrain discharged in association with muscle tone and cortical activation [23]. These neurons could activate neurons in the frontal cortex [62] and perifornical hypothalamic area, including orexin/hypocretin-containing neurons [63], to promote both cortical and behavioural activation.

### 4.3 Methodological Considerations

One possible confound with this study is that the housing condition during the last ~2 h before perfusion was different between the control *vs.* sleep-deprived groups. The control group remained in a familiar room (to minimize arousal), while the 3 sleep-deprived groups were transferred to a new room, which would increase arousal levels. In addition, although rats experienced regular cage exchanges as part of maintenance routine in the animal colony room, the cage exchange prior to the SD procedures could have acted as a mild psychological stressor/stimulant. However, although all the 3 SD groups experienced cage exchange and room novelty, significant increases in c-Fos labeling occurred mostly under the exploratory/social conditions, and not under the sensory stimulation condition. Thus, it is more likely that the patterns of c-Fos immunoreactivity we observed in the 4 groups of animals mainly reflected differences in SD procedures, rather than the exposure to novelty associated with these procedures.

Although c-Fos has been successfully used for mapping patterns of neuronal activation following periods of SD (*e.g.*, [32-34]), this technique has some limitations. For example, the functional significance for the association between c-Fos induction and behavioral response during SD cannot be ascertained conclusively, as c-Fos provides only correlative information. To study the role of brain areas that showed increased c-Fos staining following different SD methods , specific areas could be inactivated, for example, by using cell-specific lesions or pharmacological agents [64]. Another limitation of c-Fos is that the absence of c-Fos in some neurons does not necessarily imply the absence of activation, although greater c-Fos levels are indicative of greater activation [65].

Finally, the neurotransmitter phenotype of c-Fos-immunoreactive neurons was examined only in the basal forebrain and the tuberomammillary nucleus, and not in other brain areas. The results for the other brain areas, therefore, apply to the entire neuronal population in a given region, and it is possible that a subpopulation or subpopulations of neurons in that region responded differently from the majority to different SD procedures. For example, in the perifornical hypothalamic area, orexin/hypocretin-containing neurons are known to express c-Fos during active wakefulness (see Introduction) and thus would be likely activated by the SD methods used in the present study, whereas melanin-concentrating hormone-containing neurons are known to express c-Fos during sleep [66, 67] and thus would be unlikely to be activated by SD.

## 5. Conclusions

The 3 different paradigms of SD used in the present study (gentle sensory stimulation, exploration under social isolation, exploration with social interaction) elicited condition-specific patterns of c-Fos activation in select areas of wake-regulatory, limbic, and autonomic systems. These results suggest that voluntary *vs.* forced wakefulness mobilize different neuronal circuits that support different aspects of arousal, including sensory, attentive and motivational activation. In particular, several brain regions, including the paraventricular hypothalamic nucleus and certain amygdala nuclei, as well as a subset of cholinergic and GABAergic neurons in the medial septum, were activated mostly after exploration combined with social interaction, and thus may contribute to the increase in arousal that is essential for the expression of social behaviours. Additional studies are needed to determine the functions of the activated brain regions under different social and environmental contexts.

Although the SD methods used for the EI and ES groups in the present study are not entirely analogous to voluntary SD in humans, these SD methods and gentle sensory stimulation may be useful for examining the state of the brain undergoing voluntary *vs.* forced SD. Given that humans may be sleep-deprived not only passively by unwelcome sensory stimuli imposed on them (such as noise and pain), but also voluntarily as a result of work requirement or lifestyle choices, it is important that animal models of sleep loss should take these contexts into consideration. The present results clearly show that the brain responds to voluntary and passive SD differently.

**List of Abbreviations**

| ABC | Avidin-biotin-horseradish peroxidase complex |
|---|---|
| ADA | Adenosine deaminase |
| DAB | Diaminobenzidine |
| EI | Exploration under social isolation |
| ES | Exploration with social interaction |
| GAD | Glutamic acid decarboxylase |
| HC | Home cage control |
| SD | Sleep deprivation |
| ST | Gentle sensory stimulation |
| TBS | Tris buffered saline |
| VAChT | Vesicular acetylcholine transporter |

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## Figure legends

### Figure 1

Photomicrographs of several brain regions that showed increases in c-Fos immunoreactivity in response to 2 h of sleep deprivation induced by exploration of a novel environment, either under social isolation (A-D) or combined with social interaction (E-H). The sections shown in A-C, E and F are singly labeled for c-Fos (black neuronal nuclei), whereas those shown in D, G and H are doubly labeled for c-Fos (black nuclei) and transmitter markers (brown cytoplasm). In the double-labeled sections, black arrowheads indicate examples of double-labeled cells, whereas white arrowheads indicate examples of single-labeled cells for transmitter markers. (A-C) Intense c-Fos immunoreactivity was found in the anterior cingulate cortex (ACC; A), posterior region of the paraventricular thalamic nucleus (PVTp; B), and locus coereleus (LC; C) after exploration under social isolation (as well as in combination with social interaction, not illustrated). (D) In the ventral division of the tuberomammillary nucleus (TMNv), a subset of ADA+ (histaminergic) neurons is also immunostained for c-Fos in their nuclei following exploration under social isolation. (E, F) Strong c-Fos labeling is found in the parvicellular division of the paraventricular hypothalamic nucleus (PVHp; E), and in the medial amygdala (MeA; F) in response to exploration with social interaction. (G-H) In the basal forebrain, subsets of VAChT+ (cholinergic; G) and GAD+ (GABAergic; H) neurons in the medial septum (MS) are also immunostained for c-Fos following exploration with social interaction. Other abbreviations: 3V, 3rd ventricle; 4V, 4th ventricle; cc, corpus callosum; st, stria terminalis; D3V, dorsal 3rd ventricle; me5, mesencephalic 5 tract; opt, optic tract; PVHm, magnocellular PVH; scp, superior cerebellar peduncle; Scale bars: 200 µm (A-E) and 50 µm (F-H).

### Figure 2

Numbers (means + SEM) of c-Fos-immunoreactive (c-Fos+) neurons in the 7 brain regions in which there was a significant increase in response to exploration, irrespective of social context (EI and ES > HC). These regions include (in rostrocaudal order): A) Prelimbic cortex (PrL); B) Anterior cingulate cortex (ACC); C) Paraventricular thalamic nucleus, posterior region (PVTp); D) Supramammillary nucleus (SuM); E) Ventral tegmental area (VTA); F) Dorsal raphe nucleus (DR); and G) Locus coeruleus (LC). Rats were either left undisturbed in their home cage (HC, open bars) or underwent 2 h of sleep deprivation with gentle sensory stimulation (ST; horizontal hatches), exploration under social isolation (EI, oblique hatches), and exploration with social interaction (ES; black bars). Cell counts are expressed per section per side of the brain (n = 8 rats/group, except n = 5 or 6/group for PrL). *Different from the HC group; #Different from the ST group ($P < 0.05$, Tukey-Kramer post hoc tests).

### Figure 3

Numbers (means + SEM) of c-Fos+ neurons in the 4 brain regions in which there was a significant increase only in response to exploration combined with social interaction (ES > HC). These regions include (in rostrocaudal order): A) Core division of the accumbens nucleus (Acb); B) Paraventricular hypothalamic nucleus, parvicellular division (PVHp); C) Medial amygdala (MeA); and D) Basolateral amygdala (BLA). Cell counts are

expressed per section per side of the brain (n = 8/group). *Different from the HC group ($P < 0.05$, Tukey-Kramer post hoc tests).

**Figure 4**
Numbers (means + SEM) of c-Fos+ neurons in 6 nuclei of the basal forebrain, including the medial septum (MS; A), vertical diagonal band (VDB; B), horizontal diagonal band (HDB; C), magnocellular preoptic nucleus (MCPO; D), substantia innominata (SI; E), and magnocellular basal nucleus (MBN; F), in the 4 treatment groups (n = 8/group). c-Fos+ neurons were counted on sections double-immunostained for c-Fos and VAChT. Cell counts include neurons single-labeled for c-Fos and those double-labeled for c-Fos and VAChT, and are expressed per section per side of the brain. *Different from the HC group; #Different from the ST group ($P < 0.05$, Tukey-Kramer post hoc tests).

**Figure 5**
Percentages (means + SEM) of neurons double-labeled for c-Fos and either VAChT (A) or GAD (B) across basal forebrain nuclei in the 4 treatment groups (n = 8/group). Percentages were calculated by dividing the number of VAChT+/Fos+ or GAD+/Fos+ neurons by the total number of VAChT+ or GAD+ neurons (regardless of the presence of c-Fos), respectively, in each animal. *Different from the HC group; #Different from the ST group ($P < 0.05$, Tukey-Kramer post hoc tests).

**Figure 6**
Percentages (means + SEM) of neurons double-labeled for c-Fos and ADA in the dorsal (A) and ventral (B) divisions of the tuberomammillary nucleus (TMNd and TMNv, respectively) in the 4 treatment groups (n = 8/group). Percentages were calculated by dividing the number of ADA+/Fos+ neurons by the total number of ADA+ neurons (regardless of the presence of c-Fos) in each animal. *Different from the HC group ($P < 0.05$, Tukey-Kramer post hoc tests).

## Supplementary data

**Supplementary Table 1.** Numbers of c-Fos-immunoreactive neurons in brain regions that did not show statistically significant differences ($P > 0.05$) between the 4 treatment groups.

| Brain regions | Treatment group | | | | ANOVA |
|---|---|---|---|---|---|
| | HC | ST | EI | ES | |
| Accumbens nucleus, shell | 84.0 ± 28.3 | 154.5 ± 27.3 | 193.9 ± 39.2 | 196.8 ± 31.7 | $F_{3,28} =$ 2.69 $P = 0.065$ |
| Ventrolateral preoptic nucleus | 17.2 ± 2.7 | 21.9 ± 1.2 | 23.3 ± 2.2 | 23.0 ± 2.8 | $F_{3,26} =$ 1.47 $P = 0.24$ |
| Dentate gyrus, inferior blade | 8.8 ±2.1 | 6.1 ± 1.9 | 4.8 ± 1.1 | 5.1 ± 1.6 | $F_{3,28} =$ 0.32 $P = 0.81$ |
| Dentate gyrus, superior blade | 10.8 ± 2.5 | 24.6 ± 5.7 | 23.7 ± 4.4 | 23.6 ± 6.1 | $F_{3,28} =$ 1.86 $P = 0.16$ |
| Amygdala, central nucleus | 12.6 ± 3.8 | 24.0 ± 4.4 | 18.3 ± 4.5 | 29.7 ± 5.1 | $F_{3,28} =$ 2.67 $P = 0.067$ |
| Perifornical hypothalamic area | 49.3 ± 12.8 | 82.1 ± 13.0 | 99.2 ± 17.4 | 99.3 ± 18.6 | $F_{3,28} =$ 2.68 $P = 0.067$ |

All values represent means ± SEM (n= 8/group). Rats were either left undisturbed in their home cage (HC) or underwent 2 h of sleep deprivation with gentle sensory stimulation (ST), exploration under social isolation (EI), and exploration with social interaction (ES) during the light phase (starting 3 h after lights on). c-Fos immunoreactive neurons were counted on sections single-immunostained for c-Fos. The cell counts are expressed per section per side of the brain.

**Supplementary Table 2.** Numbers of neurons double-labeled for vesicular acetylcholine transporter and c-Fos (VAChT+/Fos+), single-labeled for c-Fos (VAChT−/Fos+), and total numbers of neurons labeled for VAChT (regardless of the presence of c-Fos) across basal forebrain nuclei in the 4 treatment groups.

| Basal forebrain nuclei, cell phenotype | Treatment group | | | |
|---|---|---|---|---|
| | HC | ST | EI | ES |
| **MS** | | | | |
| VAChT+/Fos+ | 0.3 ± 0.1 | 0.9 ± 0.3 | 1.7 ± 0.3 | 2.9 ± 0.7*# |
| VAChT−/Fos+ | 20.1 ± 6.2 | 40.5 ± 6.4 | 62.4 ± 4.9*# | 48.8 ± 4.1* |
| Total VAChT+ | 56.1 ± 5.9 | 54.6 ± 6.9 | 72.7 ± 5.9 | 63.9 ± 6.6 |
| **VDB** | | | | |
| VAChT+/Fos+ | 0.3 ± 0.1 | 0.9 ± 0.3 | 1.2 ± 0.2 | 1.2 ± 0.3 |
| VAChT−/Fos+ | 11.7 ± 4.3 | 25.0 ± 5.5 | 36.6 ± 4.7* | 28.7 ± 4.4 |
| Total VAChT+ | 49.1 ± 5.4 | 57.7 ± 5.9 | 65.7 ± 4.9 | 58.9 ± 4.5 |
| **HDB** | | | | |
| VAChT+/Fos+ | 1.5 ± 0.5 | 2.0 ± 0.7 | 3.4 ± 0.4 | 3.1 ± 0.7 |
| VAChT−/Fos+ | 6.0 ± 1.9 | 12.1 ± 3.9 | 21.0 ± 2.2* | 11.5 ± 2.3 |
| Total VAChT+ | 46.5 ± 3.7 | 45.9 ± 2.6 | 48.8 ± 2.4 | 48.6 ± 3.7 |
| **MCPO** | | | | |
| VAChT+/Fos+ | 0.5 ± 0.2 | 0.8 ± 0.3 | 0.8 ± 0.2 | 0.9 ± 0.2 |
| VAChT−/Fos+ | 2.2 ± 0.8 | 6.3 ± 2.1 | 8.4 ± 1.6 | 4.7 ± 1.4 |
| Total VAChT+ | 42.7 ± 6.7 | 39.3 ± 5.1 | 48.0 ± 3.3 | 39.3 ± 4.9 |
| **SI** | | | | |
| VAChT+/Fos+ | 0.3 ± 0.1 | 0.2 ± 0.1 | 0.0 ± 0.0 | 0.4 ± 0.1 |
| VAChT−/Fos+ | 0.5 ± 0.4 | 1.0 ± 0.5 | 0.5 ± 0.2 | 2.1 ± 0.7 |
| Total VAChT+ | 24.5 ± 1.4 | 22.4 ± 1.8 | 23.4 ± 1.2 | 24.1 ± 1.1 |
| **MBN** | | | | |
| VAChT+/Fos+ | 0.1 ± 0.1 | 0.1 ± 0.0 | 0.0 ± 0.0 | 0.1 ± 0.1 |
| VAChT−/Fos+ | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.0 ± 0.0 | 0.1 ± 0.0 |
| Total VAChT+ | 21.2 ± 1.6 | 22.9 ± 2.4 | 21.6 ± 1.4 | 23.9 ± 1.2 |

. All values represent means ± SEM (n = 8

rats/group). HDB, horizontal diagonal band; MBN, magnocellular basal nucleus; MCPO, magnocellular preoptic nucleus; MS, medial septum; SI, substantia innominata; VDB, vertical diagonal band. Cell counts are expressed per section per side of the brain. *Different from the HC group; #Different from the ST group ($P < 0.05$, Tukey-Kramer post hoc tests).

**Supplementary Table 3.** Numbers of neurons double-labeled for c-Fos and glutamic acid decarboxylase (GAD+/Fos+), single-labeled for c-Fos (GAD−/Fos+), and total numbers of neurons labeled for GAD (regardless of the presence of c-Fos) across basal forebrain regions in the 4 treatment groups.

| Basal forebrain nuclei, cell phenotype | Treatment group | | | |
|---|---|---|---|---|
| | HC | ST | EI | ES |
| **MS** | | | | |
| GAD+/Fos+ | 1.6 ± 0.8 | 5.5 ± 0.7* | 6.2 ± 0.9* | 9.2 ± 1.2*# |
| GAD−/Fos+ | 7.3 ± 2.5 | 25.1 ± 4.2* | 42.4 ± 4.1*# | 28.9 ± 3.2* |
| Total GAD+ | 66.4 ± 2.8 | 79.3 ± 4.6 | 72.7 ± 4.7 | 75.0 ± 5.1 |
| **VDB** | | | | |
| GAD+/Fos+ | 0.8 ± 0.3 | 3.8 ± 0.6* | 3.2 ± 0.6* | 3.7 ± 0.6* |
| GAD−/Fos+ | 2.9 ± 1.1 | 14.6 ± 3.2 | 9.4 ± 1.7* | 12.5 ± 3.3 |
| Total GAD+ | 70.6 ± 3.4 | 84.6 ± 4.8 | 91.0 ± 4.9 | 77.5 ± 5.6 |
| **HDB** | | | | |
| GAD+/Fos+ | 0.7 ± 0.3 | 3.8 ± 0.7* | 3.6 ± 0.6* | 3.7 ± 0.7* |
| GAD−/Fos+ | 2.4 ± 1.2 | 11.4 ± 2.4* | 18.1 ± 2.9* | 10.6 ± 2.2 |
| Total GAD+ | 60.2 ± 2.5 | 67.1 ± 4.5 | 65.2 ± 4.7 | 65.5 ± 5.1 |
| **MCPO** | | | | |
| GAD+/Fos+ | 0.2 ± 0.1 | 0.9 ± 0.3 | 0.5 ± 0.2 | 1.1 ± 0.4 |
| GAD−/Fos+ | 0.7 ± 0.3 | 4.3 ± 1.0 | 3.0 ± 0.8 | 4.6 ± 1.4* |
| Total GAD+ | 51.4 ± 2.6 | 60.7 ± 2.3 | 46.2 ± 2.2 | 50.2 ± 5.0 |
| **SI** | | | | |
| GAD+/Fos+ | 0.1 ± 0.1 | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.5 ± 0.2 |
| GAD−/Fos+ | 0.9 ± 0.4 | 1.4 ± 0.6 | 1.1 ± 0.4 | 2.8 ± 1.0 |
| Total GAD+ | 42.4 ± 3.3 | 45.8 ± 2.4 | 33.8 ± 1.5 | 44.6 ± 3.9 |
| **MBN** | | | | |
| GAD+/Fos+ | 0.1 ± 0.1 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| GAD−/Fos+ | 0.1 ± 0.1 | 0.0 ± 0.0 | 0.1 ± 0.1 | 0.2 ± 0.1 |
| Total GAD+ | 36.4 ± 2.1 | 37.2 ± 2.4 | 30.7 ± 2.0 | 38.0 ± 2.9 |

All values represent means ± SEM (n = 8/group). Cell counts are expressed per section per side of the brain. *Different from the HC group; #Different from the ST group (P < 0.05, Tukey-Kramer post hoc tests).

**Supplementary Table 4.** Numbers of neurons double-labeled for adenosine deaminase and c-Fos (ADA+/Fos+), and total numbers of neurons labeled for ADA (regardless of the presence of c-Fos) in the dorsal and ventral divisions of the tuberomammillary nucleus (TMNd and TMNv, respectively) in the 4 treatment groups.

| TMN division, cell phenotype | Treatment group | | | | All values represent means ± SEM (n = 8/group). Cell counts are expressed per section per side of the |
|---|---|---|---|---|---|
| | HC | ST | EI | ES | |
| TMNd | | | | | |
| ADA+/Fos+ | 0.2 ± 0.1 | 0.9 ± 0.4 | 2.3 ± 0.4* | 1.3 ± 0.5 | |
| Total ADA+ | 19.1 ± 3.5 | 16.8 ± 3.0 | 24.6 ± 1.9 | 18.3 ± 2.3 | |
| TMNv | | | | | |
| ADA+/Fos+ | 1.1 ± 0.4 | 2.1 ± 0.7 | 4.9 ± 1.3* | 1.7 ± 0.5 | |
| Total ADA+ | 22.7 ± 2.5 | 25.7 ± 1.5 | 34.9 ± 2.8* | 22.6 ± 3.3$^{\dagger}$ | |

brain. *Different from the HC group; $^{\dagger}$Different from the EI group (P < 0.05, Tukey-Kramer post hoc tests).

**Supplementary Table 5.** Summary of the response profiles of select brain areas and neuronal groups to 3 methods of sleep deprivation.

| Response profile | Nuclei or neuronal groups |
|---|---|
| Maximal response to:<br>Exploration with/without social interaction (EI and ES) | Prelimbic cortex<br>Anterior cingulate cortex<br>Paraventricular thalamic nucleus<br>Supramammillary nucleus<br>Ventral tegmental area<br>Dorsal raphe nucleus<br>Locus coeruleus |
| Maximal response to:<br>Exploration with social interaction (ES) | Accumbens nucleus core<br>Rostral-middle basal forebrain (cholinergic and GABAergic neurons)<br>Paraventricular hypothalamic nucleus<br>Medial amygdala<br>Basolateral amygdala |
| Maximal response to:<br>Exploration with social isolation (EI) | Rostral-middle basal forebrain (possible glutamatergic neurons)<br>Tuberomammillary nucleus (histaminergic neurons) |
| Little or no response | Accumbens nucleus shell<br>Ventrolateral preoptic nucleus<br>Caudal basal forebrain (all neurons)<br>Central amygdala<br>Perifornical hypothalamic area<br>Dentate gyrus (inferior and superior blades) |

FIGURE 1
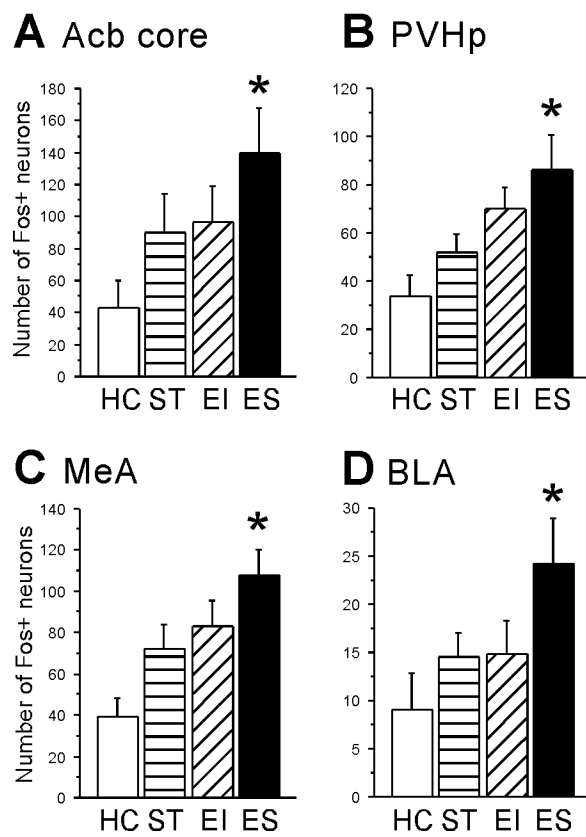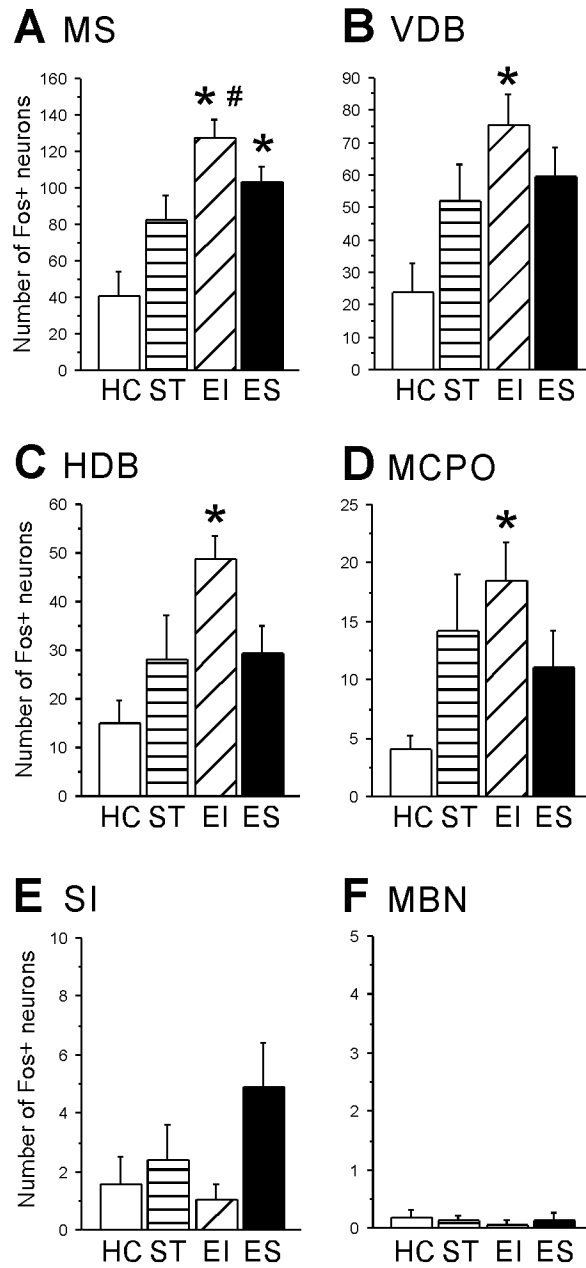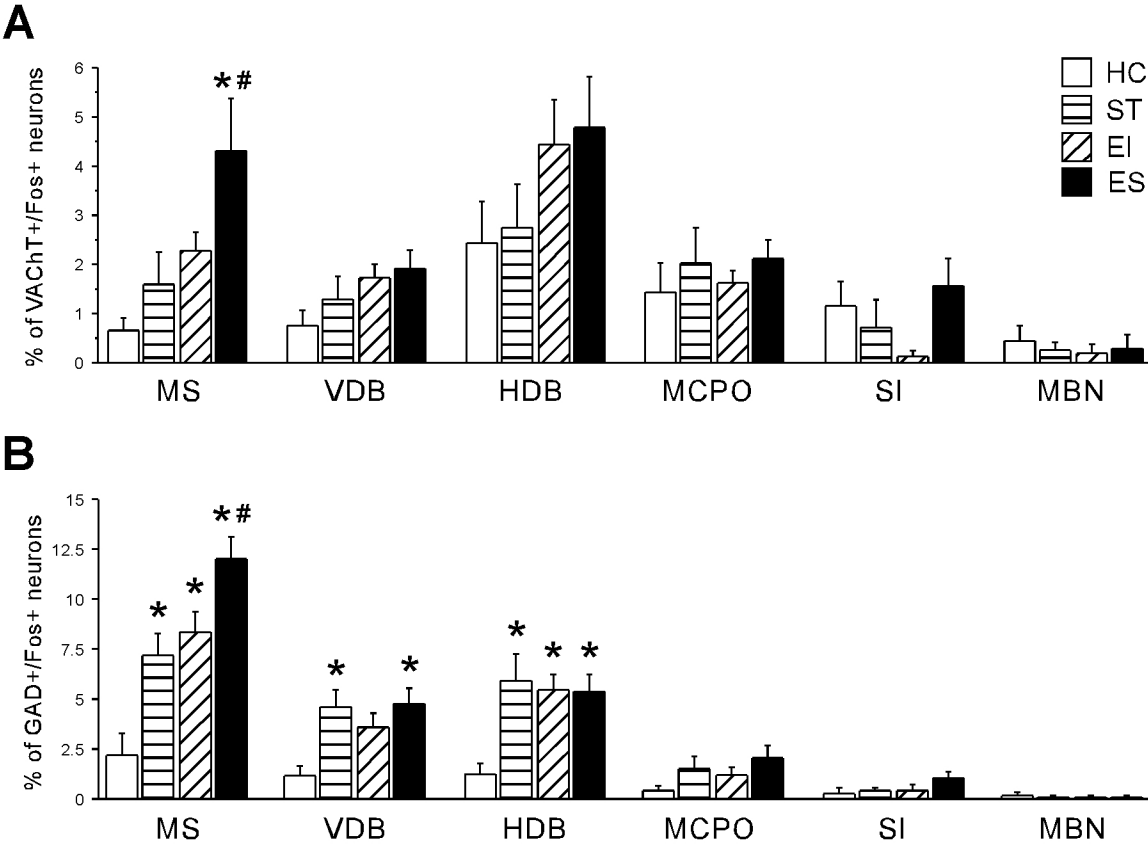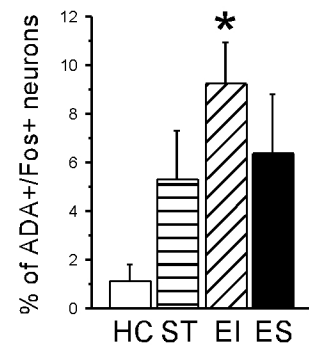
FIGURE 2



A PrL

B ACC

C PVTp

D SuM

E VTA

F DR

G LC
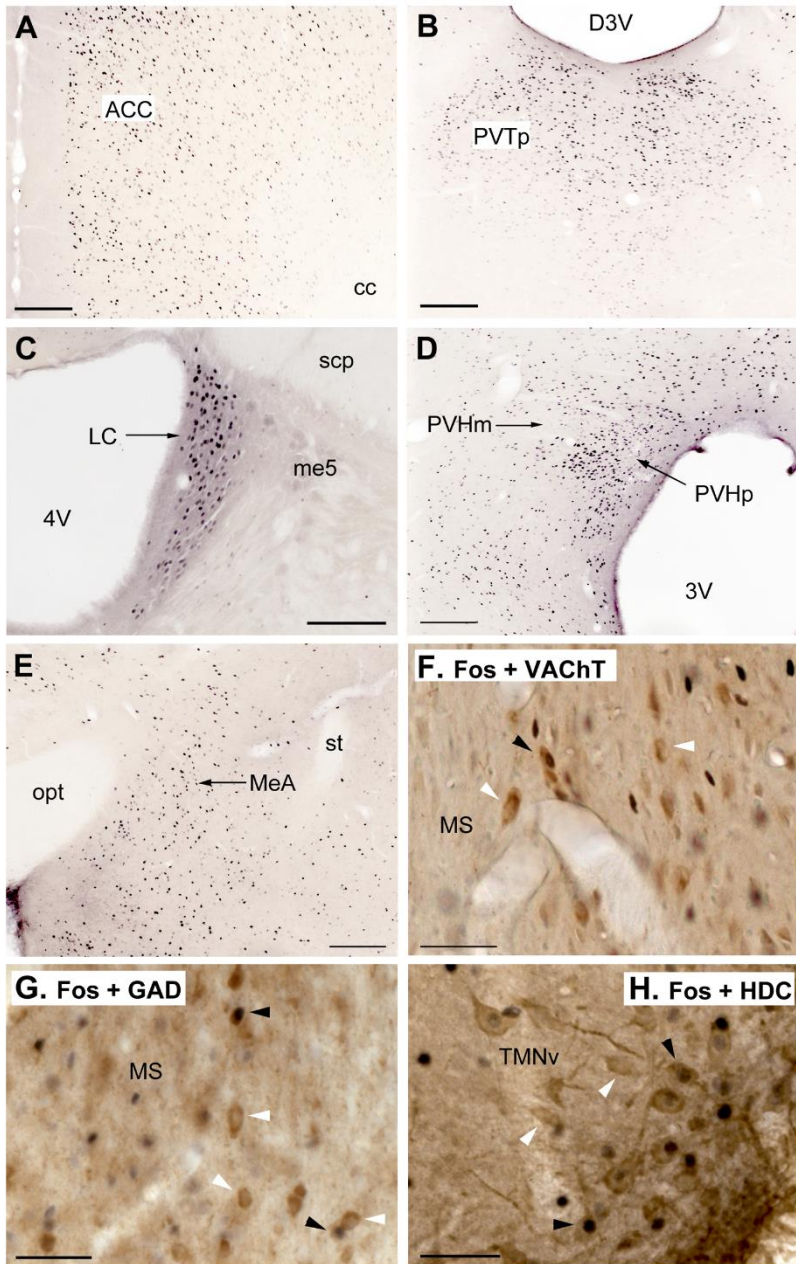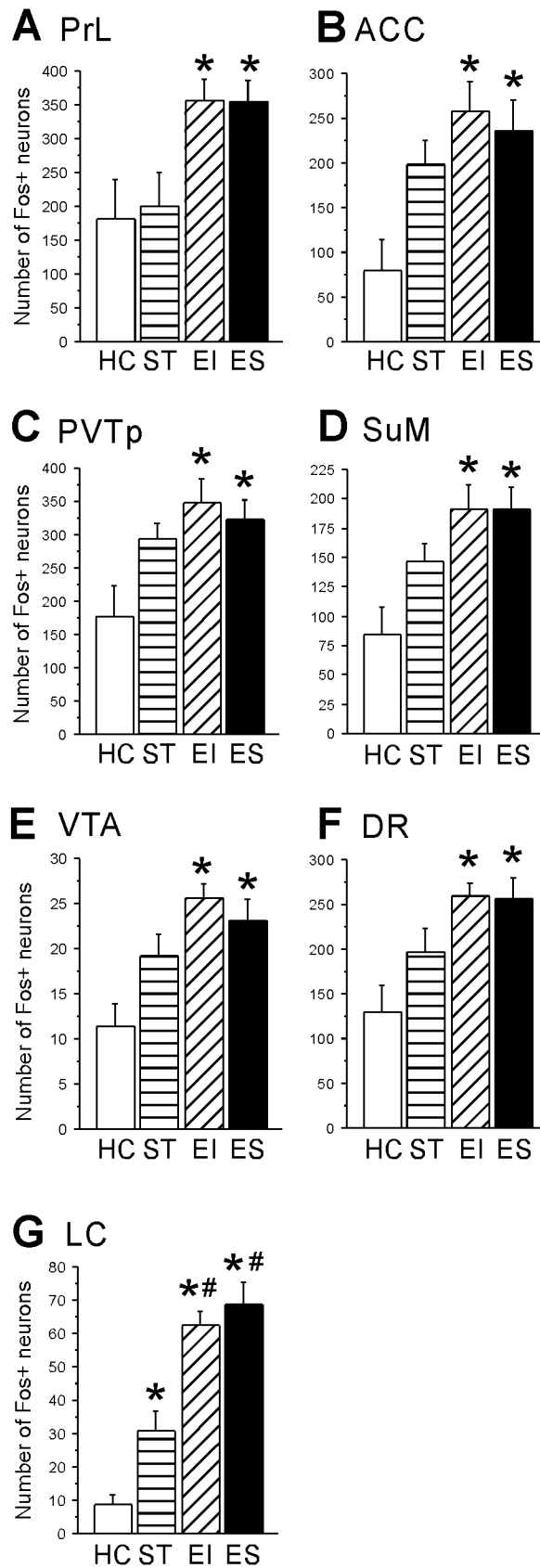
FIGURE 3

FIGURE 4

FIGURE 5

FIGURE 6



**A** TMNd

**B** TMNv