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Neural Connections of the Anterior Hypothalamus and Agonistic Behavior in Golden Hamsters

Yvon Delville[a] Geert J. De Vries[b] Craig F. Ferris[a]

[a]Program in Neuropsychiatric Sciences, Psychiatry Department, University of Massachusetts Medical Center, Worcester, Mass., and [b]Psychology Department, University of Massachusetts, Amherst, Mass., USA

Key Words

Aggression · Arginine-vasopressin · Anterograde tracing · Retrograde tracing · Neuronal activation · c-Fos-immunoreactivity

Abstract

In male golden hamsters, offensive aggression is regulated by an interaction between arginine-vasopressin and serotonin at the level of the anterior hypothalamus. The present studies were conducted to study a neural network underlying this interaction. The connections of the anterior hypothalamus were examined by retrograde and anterograde tracing in adult male hamsters. Several limbic areas were found to contain both types of tracing suggesting reciprocal connections with the anterior hypothalamus. Their functional significance relating to the consummation of aggression was tested by comparing neuronal activity (examined through quantification of c-Fos-immunolabeling) in two groups of animals. Experimental animals were sacrificed after attacking an intruder. Control animals were sacrificed after exposure to a woodblock carrying the odor of an intruder that elicited behaviors related to offensive aggression without its consummation. An increased density of Fos-immunoreactivity was found in experimental animals within the medial amygdaloid nucleus, ventrolateral hypothalamus, bed nucleus of the stria terminalis and dorsolateral part of the midbrain central gray. These data suggest that these areas are integrated in a neural network centered on the anterior hypothalamus and involved in the consummation of offensive aggression. Finally, c-Fos-immunoreactivity was combined with labeling of serotonin and vasopressin neurons to identify sub-populations particularly associated with offensive aggression. Vasopressin neurons in the nucleus circularis and medial division of the supraoptic nucleus showed increased neuronal activity in the fighters, supporting their role in the control of offensive aggression.

Introduction

Aggressive behavior can be classified as either offensive or defensive [Blanchard and Blanchard, 1988]. Offensive aggression is characterized by the initiation of attacks and bites by an aggressor, whereas defensive aggression lacks active approach. Each behavior is characterized by unique sequences of behaviors and activating stimuli [Blanchard and Blanchard, 1977; Brain, 1981]. In a laboratory setting, offensive aggression is easily observed in male golden hamsters. Resident males readily approach and attack male intruders placed in their home cage. In this species, offensive aggression includes a sequence of behaviors ranging from olfactory investigation to biting attacks as well as a stereotyped scent marking behavior [Seigel, 1985].

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Yvon Delville
Psychology Department, University of Texas
Mezes 330, Austin, TX 78712 (USA)
Tel. (512) 232-5731, Fax (512) 471-6175
E-Mail Delville@psy.utexas.edu

Abbreviations

III	third ventricle	DRP	posterior parts of the DR	oc/ot	optic chiasma/tract
A1	A1 noradrenergic cells	f	fornix	PMCo	posteromedial cortical amydaloid nucleus
ac	anterior commissure	fi	fimbria of the hippocampus		
AD	anterodorsal thalamic nucleus	FSt	fundus striati	PnR	pontine raphe nucleus
AH	anterior hypothalamus	HDB	horizontal part of the diagonal band	PP	peripeduncular nucleus
AHi	amygdalohippocampal area	Hip	hippocampus	PVA	anterior paraventricular thalamic nucleus
Aq	acqueduct	ic	internal capsule		
Amb	nucleus ambiguus	LC	locus coeruleus	PVN	paraventricular hypothalamic nucleus
AP	area postrema	lfp	longitudinal fasciculum of the pons		
Arc	arcuate nucleus	LH	lateral hypothalamus	PVP	posterior paraventricular thalamic nucleus
AV	anteroventral thalamic nucleus	LHb	lateral habenular nucleus		
BLA	basolateral amygdaloid nucleus	LiR	linear raphe nucleus	py	pyramidal tract
BST	bed nucleus of the stria terminalis	LPB	lateral parabrachial nucleus	Re	nucleus reuniens
BSTL	lateral division of the BST	LPO	lateral preoptic area	RMg	raphe magnus nucleus
BSTM	medial division of the BST	LS	lateral septum	ROb	raphe obscurus nucleus
Ca1	field CA1 of the hippocampus	lSON	lateral division of the supraoptic nucleus	RPA	raphe pallidus nucleus
cc	corpus callosum			S	subiculum
CeA	central amygdaloid nucleus	MCG	midbrain central gray	SCN	suprachiasmatic nucleus
CGdl	dorsolateral part of the midbrain central gray	MeA	medial amygdaloid nucleus	sm	stria medullaris
		MHb	medial habenular nucleus	Sol	nucleus of the solitary tract
CGvl	ventrolateral part of the midbrain central gray	ml	medial lemniscus	SON	suparoptic nucleus
		mlf	medial longitudinal fasciculus	sp5	spinal trigeminal tract
CLi	caudal linear raphe nucleus	MnPO	median preoptic nucleus	st	stria terminalis
CoA	cortical amygdaloid nucleus	MnR	median raphe nucleus	TgA	tegmental area
cp	cerebral peduncle	mSON	medial division of the supraoptic nucleus	VDB	vertical part of the diagonal band
CPu	caudate-putamen			VLH	ventrolateral hypothalamus
Den	dorsal endopiriform nucleus	MPA	medial preoptic area	VMN	ventromedial hypothalamic nucleus
DMN	dorsomedial hypothalamic nucleus	MPB	medial parabrachial nucleus	VTA	ventral tegmental area
DG	dentate girus	mt	mammillo-thalamic tract	xcsp	decussation of the superior cerebellar peduncle
DR	dorsal raphe nucleus	MTu	medial tuberal nucleus		
DRA	anterior parts of the DR	NC	nucleus circularis		

Offensive and defensive aggression appear to be controlled through separate neural sites in rodents [Albert and Walsh, 1984; Blanchard and Blanchard, 1988]. In hamsters, a number of areas have been implicated in the control of offensive aggression, primarily through lesions and electrical stimulation studies. These areas are distributed across the limbic system and include the septum, hypothalamus, amygdala and preoptic area [Bunnell et al., 1970; Sodetz and Bunnell, 1970; Hammond and Rowe, 1976; Shipley and Kolb, 1977; Potegal et al., 1981a, b, 1996b]. For instance, offensive aggression is inhibited by electrical stimulation of the septum [Potegal et al., 1981b], whereas the behavior is activated by stimulation of the corticomedial amygdala [Potegal et al., 1996b].

Neurochemical interactions controlling offensive aggression have been uncovered at the level of the anterior hypothalamus (AH). In this area, two neurotransmitters, arginine-vasopressin (AVP) and serotonin (5-HT), play a critical role in the modulation of offensive aggression in golden hamsters. In the presence of an intruder, micro-injections of low doses of AVP into the AH facilitate offensive aggression [Ferris et al., 1997]. This facilitation of aggressive behavior is mediated through activation of vasopressin V_{1A} receptors, as blockade of V_{1A} receptors within the AH inhibits the behavior [Ferris and Potegal, 1988; Potegal and Ferris, 1990]. In contrast, 5-HT inhibits aggressive behavior [Delville et al., 1996a; Ferris et al., 1997; Joppa et al., 1997]. Offensive aggression can be inhibited by peripheral administration of fluotexine, a 5-HT re-uptake inhibitor, as well as micro-injections of selective 5-HT receptor antagonists directed at the AH [Delville et al., 1996a; Ferris et al., 1997, 1999].

The present studies were conducted to establish a neural network involved in the consummation of offensive aggression in golden hamsters. Previous studies have reported changes in neuronal activity in relation with aggression in golden hamsters [Kollack-Walker and Newman, 1995]. However, these studies were not specifically related to either offensive or defensive aggression. Furthermore, these stud-

ies were not based on the anatomical connections of a specific area involved with the facilitation of offensive aggression. In addition, previous studies have described some of the connections of the AH in golden hamsters [Morin et al., 1994]. However, these studies were not extensive nor were they related to the consummation of offensive aggression. Therefore, the present study includes a description of the connections of the AH and an analysis of changes in neuronal activity specifically related to the consummation of offensive aggression and based on the connections of the AH. First, anterograde and retrograde tracing studies were performed to provide an extensive description of the connections of the AH. Second, the functional significance of these connections in the consummation of offensive aggression was tested by immunocytochemistry to c-Fos, a marker of cellular activity [Dragunow and Faull, 1989]. Finally, the proportion of AVP and 5-HT cells also containing c-Fos immunoreactivity was analyzed in an attempt to localize sub-populations particularly associated with aggressive behavior.

Materials and Methods

Animals and Treatment

Male golden hamsters (100–120 g, 8–9 weeks old) were purchased from Harlan Sprague Dawley (Indianapolis, Ind., USA). They received food and water ad libitum, and were housed individually in a reverse daylight cycle (14L,10D; lights on at 19:00) for at least two weeks before the experiments. The animals were housed according to NIH guidelines at the Department of Animal Medicine of the University of Massachusetts Medical Center, an AALAC accredited facility.

Retrograde Tracing

Retrograde tracing was performed using Fluoro-Gold (FG) [Schmued and Fallon, 1986]. Fluoro-Gold (2% in 0.9% saline, Fluorochrome, Inc., Englewood, Co., USA) was injected iontophoretically into the AH of a first set of male hamsters (n = 12) under pentobarbital anesthesia (10 mg; Nembutal; Abbott laboratories, North Chicago, Ill., USA). The stereotaxic coordinates were 1.2 mm anterior to bregma, 1.7 mm lateral (left) to the midline, and 7.6 mm down from the dura at a 8° angle from the sagittal plane. The incisor bar was placed at the level of interaural line. The injections were performed through a glass micropipette (tip diameter: ca. 50 µm) for 10 min (current: +5 µA, 7 s on, 7 s off).

One week later, the animals were deeply anesthetized with sodium pentobarbital, injected intracardially with heparin (5000 Units in 1 ml saline) and perfused transcardially with 0.9% saline containing 0.2% sodium nitrite (to dilate blood vessels) followed by a solution containing 4% paraformaldehyde and 2.5% acrolein in 0.1 *M* potassium phosphate buffered saline (KPBS, pH 7.4) for 15–20 min, and followed by saline for another 3 minutes. Then, the brains were removed from the skull, and placed in 20% sucrose in KPBS at 4 °C until they were sliced into 40 µm-thick coronal sections with a freezing microtome. The sections were saved in a cryoprotectant [Watson et al., 1986] until labeled by immunocytochemistry to FG [Chang et al., 1990].

Anterograde Tracing

Anterograde tracing was performed using *Phaseolus vulgaris* leucoagglutinin (Pha-L, Vector Laboratories, Inc., Burlingame, Calif., USA) as an anterograde tracer [Gerfen and Sawchenko, 1984]. A second and separate set of male hamsters (n = 19) was injected iontophoretically with Pha-L (0.5% in saline; Vector Laboratories, Burlingame, Calif., USA) into the AH under pentobarbital anesthesia (10 mg; Nembutal; Abbott laboratories, North Chicago, Ill., USA). The stereotaxic coordinates were similar to those used for the iontophoresis of FG. The injections were performed through a glass micropipette (tip diameter: ca. 15 µm, this smaller opening is optimal for Pha-L) for 15 min (current: +5 µA, 7 s on, 7 s off).

Ten days later, the brains were fixed with 4% paraformaldehyde and 2.5% acrolein and sectioned as explained above. The sections were placed in a cryoprotectant until labeled by immunocytochemistry to Pha-L [Gerfen and Sawchenko, 1984].

Behavioral Observations

Male golden hamsters (n = 17, 10 weeks old) were observed for agonistic behavior during a 10-min encounter with a slightly younger male intruder (9 weeks old) or small block of hard wood (5×9×1.5 cm) that had been left overnight in the home cage of one of the intruders. These groups were constituted of individuals originating from a third and separate set of hamsters (n = 4 per group) pooled with Pha-L-injected animals (n = 4 + 5). In each group, these animals behaved similarly during testing and in each group Pha-L injections had no effect on the immunocytochemical labeling of c-Fos. This experiment was designed to reveal neural differences related to the consummation of offensive aggression. The animals exposed to woodblocks (controls, n = 9) were used to control for neural activation associated with heightened arousal and display of behaviors related to offensive aggression, but without its consummation [Siegel, 1985]. These woodblocks were intended to approximate the size of a hamster and carry the scent of an intruder. During testing, the presence of the woodblock elicited olfactory investigation, increased locomotion and flank marking. The residents were also observed to nibble the woodblock (which clearly differed from an attack, or bite and chase sequence). All the animals exposed to intruders (fighters, n = 8) investigated, attacked, bit and chased the intruders several times. None of the intruders attacked or bit the residents, instead they attempted to avoid them. One hour later, all animals were perfused transcardially with 4% paraformaldehyde and 2.5% acrolein as described above.

Immunocytochemical Procedures

Immunocytochemistry to c-Fos was performed as follows [Berghorn et al., 1994]. After washing in 0.05 *M* KPBS buffer, the sections were treated for 10 min with 1% sodium borohydrite to remove residual aldehydes. After several washes, the sections were pre-incubated in 20% normal goat serum (NGS), 1% hydrogen peroxide, and 0.3% Triton X-100, respectively, to prevent non-specific labeling, eliminate unreacted peroxidase, and to make the sections permeable. Then the sections were incubated for 48 h at 4 °C in the primary antibody (rabbit polyclonal antibody to c-Fos, 0.05 µg/ml, sc-52, Santa Cruz Biotechnology, Inc., Santa Cruz, Calif., USA) in the presence of 2% NGS and 0.3% Triton X-100. After several rinses, the sections were incubated in the secondary antibody (biotinylated goat anti-rabbit IgG, 2.5 µg/ml, Vector Laboratories, Inc.). After another series of rinses, the sections were processed through a tertiary incubation in an avidin-peroxidase complex (Vectastain® ABC Elite Kit, Vector Laboratories, Inc.). After several more rinses, the sections were labeled in the presence of hydro-

gen peroxide with Nickel-conjugated diaminobenzidine (nickel sulfate: 2.5%; diaminobenzidine: 0.02%; hydrogen peroxyde: 0.0025%) diluted in 0.175 *M* sodium acetate buffer (pH 6.0), which formed a blue-black precipitate over the cell nuclei. Sections were mounted on gelatin-coated slides and coverslipped with permount. The labeling was specific as omission of the primary antibody and preincubation with purified control peptide for the sc-52 antibody prevented all immunoreactive labeling.

Immunocytochemistry to FG was performed with a slightly modified protocol using a rabbit polyclonal antibody to FG (AB153, 1/1000, Chemicon International, Inc., Temecula, Calif., USA). All solutions were prepared with 0.05 *M* TBS buffer. The sections were incubated in the primary antibody for 1 h at 37 °C. The secondary antibody (biotinylated goat anti-rabbit IgG, Vector Laboratories, Inc.) concentration used was 7.5 µg/ml. The sections were labeled by incubation in diaminobenzidine (Sigma Chemical Co., St Louis, Mo., USA; diaminobenzidine: 0.066%, hydrogen peroxide: 0.024%) which resulted in a dark brown precipitate over cell bodies and some fibers. This labeling only occurred within areas that have fluorescent staining by observation of the FG under UV excitation light. Furthermore, omission of the primary antibody prevented all labeling. Retrogradely labeled cells were easily visible under light microscopy and mapped with a camera lucida.

Immunocytochemistry to Pha-L was performed with a goat polyclonal antibody to Pha-L (AS-2224, 2 µg/ml, Vector Laboratories, Inc.) using a similar procedure. All solutions were prepared with 0.05 *M* KPBS buffer. The secondary antibody used was a biotinylated horse anti-goat IgG (7.5 µg/ml, Vector Laboratories, Inc.). Normal horse serum was used during the procedure. The immunoreactivity was labeled with diaminobenzidine, which formed a precipitate over fibers. Anterogradely labeled fibers were easily visible under light microscopy and mapped with a camera lucida.

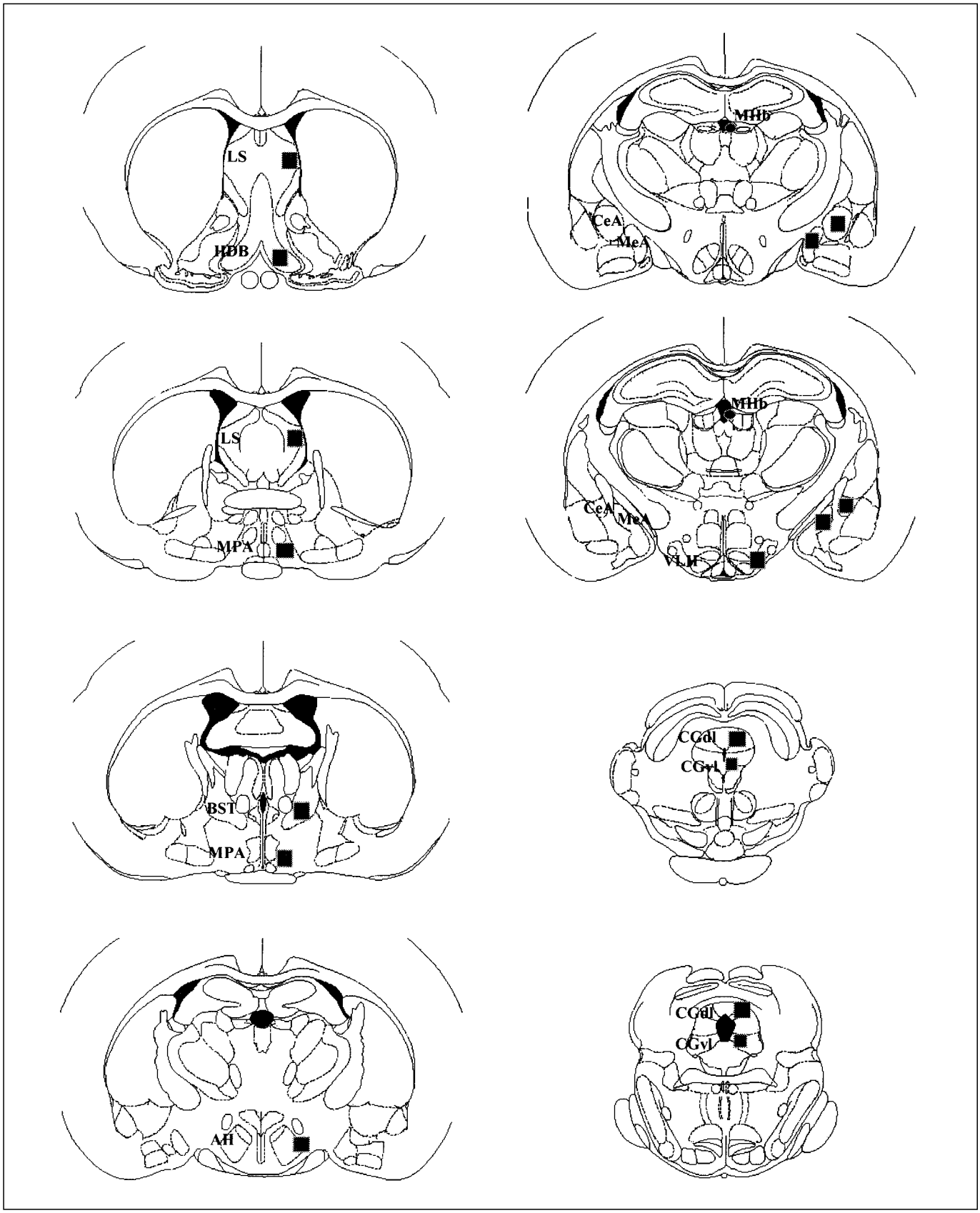
Immunocytochemistry to c-Fos was also combined with immunocytochemistry to AVP or tryptophan hydroxylase (TH, to identify 5-HT neurons). After labeling c-Fos as explained above, the sections were transferred in 0.05 *M* TBS and pre-incubated in 20% NGS or 2% normal donkey serum containing 1% hydrogen peroxide and 0.3% Triton X-100. Afterwards, the sections were placed in the primary antibody which was either a mouse monoclonal anti-AVP (BER-312, 1/4000, generously donated by Dr. F. Robert, INSERM, France) or a sheep polyclonal anti-TH (AB1541, 0.75 µg/ml, Chemicon International Inc.). The secondary antibodies used in these procedures were either a biotinylated goat anti-mouse IgG (7.5 µg/ml, Vector Laboratories, Inc.) or a biotinylated donkey anti-sheep IgG (9 µg/ml, Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa., USA). After a tertiary incubation, the sections were labeled with diaminobenzidine which formed a brown precipitate over the cell bodies. Double-labeled cells were easily recognized as having a brown cytoplasm and a black nucleus. The specificity of the AVP antibody has been previously established [Robert et al., 1985]. In our laboratory, either omission of the primary antibody or preincubation in excess AVP prevented immunoreactive labeling. This antibody has also been used for combined labeling of AVP and c-Fos in rats [Fénelon et al., 1994]. Furthermore, the distribution of TH-ir cells in the raphe nuclei matched previous observations of 5-HT-ir cells in these nuclei using a rabbit polyclonal antibody to 5-HT (NT102, 1/5000, Eugene Tech International, Inc., Ridgefield Park, N.J., USA).

C-Fos Quantification

The number of c-Fos-ir nuclei was determined within specific areas using the Image software (v. 1.56, NIH, Bethesda). The areas analyzed were selected on the basis of the distribution of the anterograde and retrograde tracing maps rather than on the basis of neuroanatomical structures as used in previous studies [Kollack-Walker and Newman, 1995]. Only areas shown to have reciprocal connections with the anterior hypothalamus were selected for quantification. These areas (fig. 1) included the ventral part of the lateral septal nucleus (LS), the nucleus of the horizontal limb of the diagonal band (HDB), the posterior part of the medial division of the bed nucleus of the stria terminalis (BST), the medial preoptic area (MPA), the AH just ventrolateral to the nucleus circularis, the VLH which included the medial aspects of the medial tuberal nucleus and the ventrolateral part of the ventromedial hypothalamic nucleus, the medial habenular nucleus (MHb), the dorsal part of the medial amygdaloid nucleus (MeA), the central amygdaloid nucleus (CeA), the dorsolateral part of the midbrain central gray (CGdl), and the ventrolateral part of the midbrain central gray (CGvl). The sections containing the selected areas were observed (using a 10× objective) as digitized images captured with a video camera (Pulnix TM 745, Motion Analysis, Inc., Eugene, Oreg., USA) mounted on a Zeiss microscope. The images were imported on a Macintosh computer through a frame grabber board (LG-3, Scion Corp., Walkersville, Md., USA). The illumination was kept constant for all measurements. The camera settings (gain) were adjusted for each sampled area to match each density histogram to a standard (and optimal) peak value to minimize background variations. Cell counts were performed automatically by the Image software after standardized (based on the shape of each density histogram) gray-level thresholding [Shipley et al., 1989]. Each count was verified for clustered cells counted as a single unit. Counts were taken from standard samples (a square of 300×300 µm) placed over the areas of interest shown in the digitized images. However, c-Fos-ir cells in the MHb were counted within 200 µm diameter circles because of the small size of this nucleus. For each area, measures were taken from either side of the brain and over consecutive sections. These measures (4–12) were averaged for each individual. The individual averages were compared between groups with Student t-tests (two-tailed) for each area sampled. The results were presented as a cell density (i.e. average number of cells per standard sample) in each group.

The proportion of AVP- and TH-ir cells also containing c-Fos-IR was counted from camera lucida drawings of the hypothalamus and midbrain for the animals showing cells in these areas. In the hypothalamus, the cells counts were divided into four areas: paraventricular hypothalamic nucleus (PVN), nucleus circularis (NC), medial division of the supraoptic nucleus (mSON), and lateral division of the supraoptic nucleus (lSON), as previously described [Ferris et al., 1992; Delville et al., 1994]. In the midbrain, the cell counts were divided into four areas: anterior part of the dorsal raphe nucleus (DRA), the dorsal part of the caudal linear nucleus of the raphe (CLi), the posterodorsal part of the dorsal raphe nucleus (DRP) and the dorsal part of the median raphe nucleus (MnR). These subdivisions were selected as they were most likely to be retrogradely labeled from the AH [Ferris et al., 1999]. The percentage of AVP- and TH-ir cells also containing c-Fos-ir was compared between groups with Student t-tests (two-tailed) for each area sampled.

Fig. 1. Diagrams showing the location of the areas selected to quantify c-Fos-ir (shaded areas). Diagrams were modified from the atlas of Paxinos and Watson [1997].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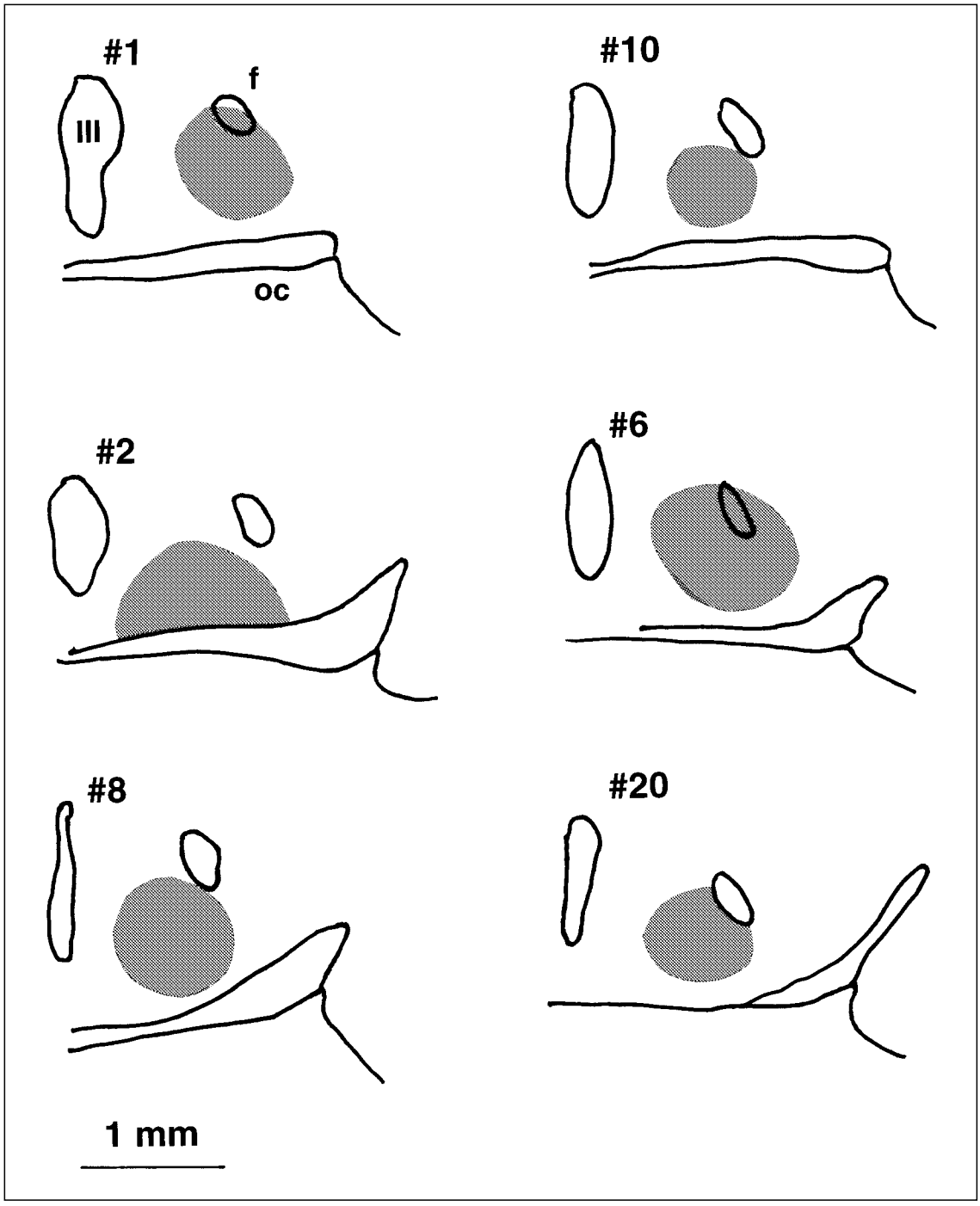

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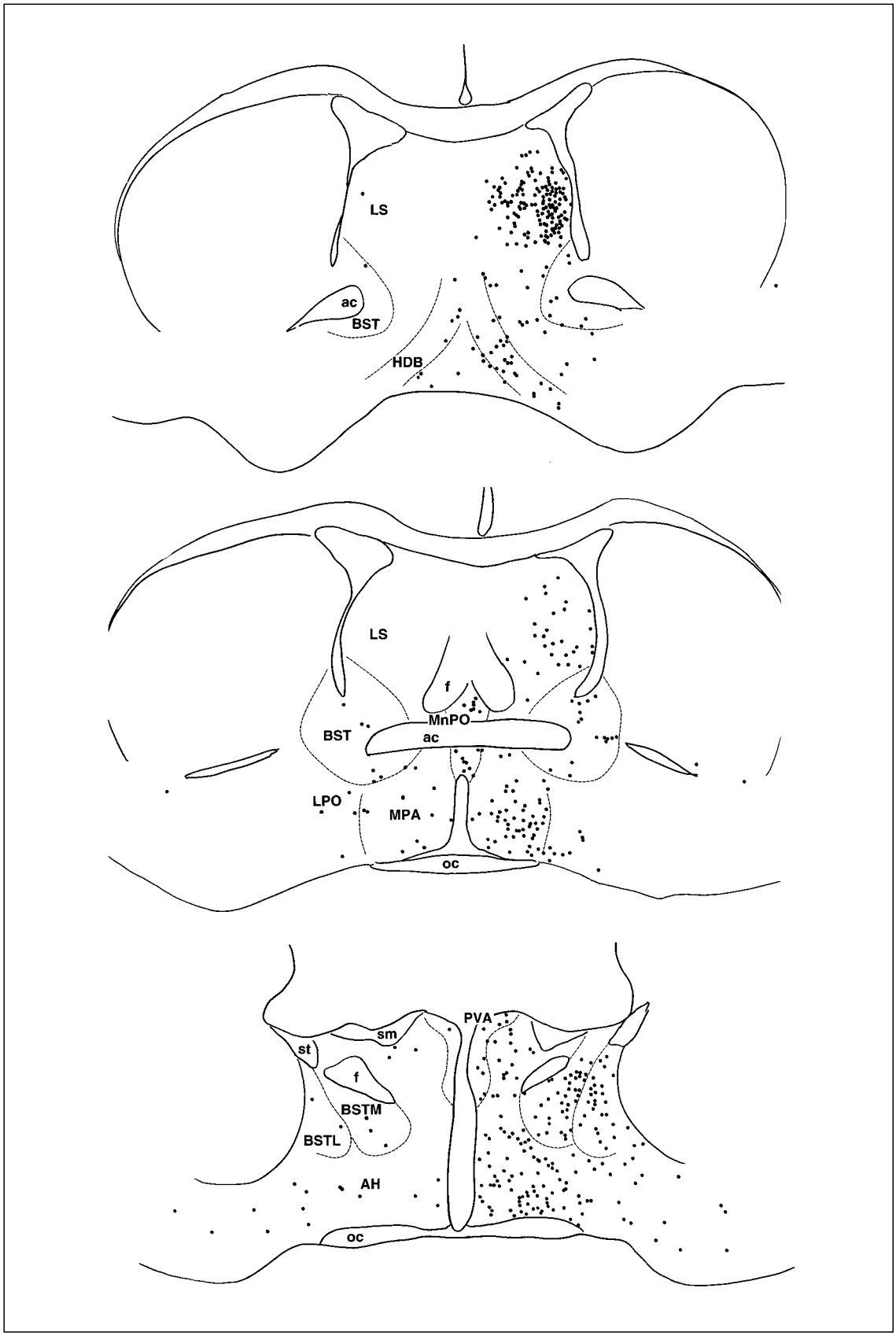
Fig. 2. Camera lucida drawings showing the location of FG injection sites (shaded areas) within the anterior hypothalamus in six different animals.

Fig. 3–6. Camera lucida drawings showing the distribution of FG-ir neurons (dots) in the brain of a male golden hamster (animal No. 8 in fig. 2) after an iontophoretic injection within the anterior hypothalamus. The shaded area represents the extent of the injection site.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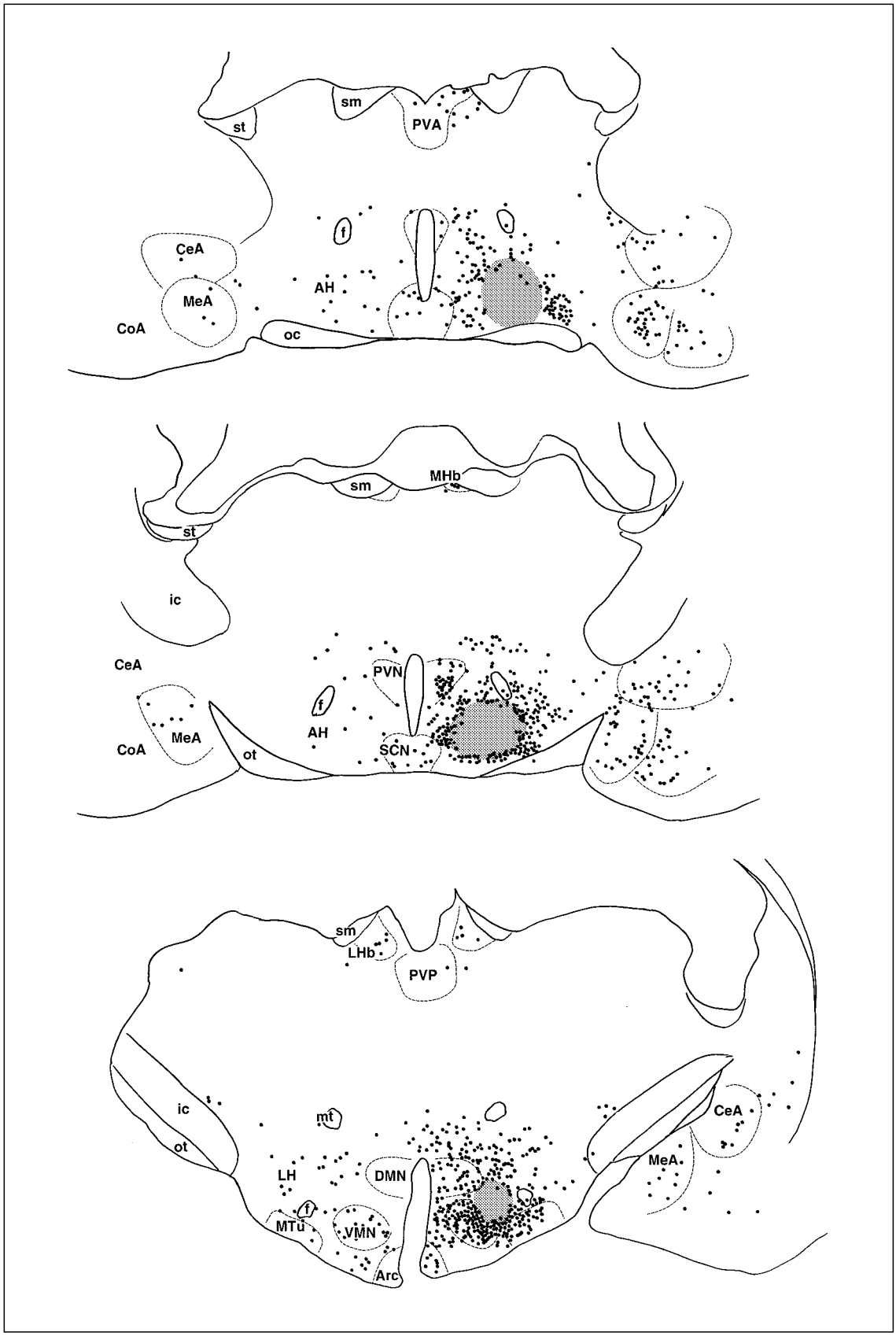

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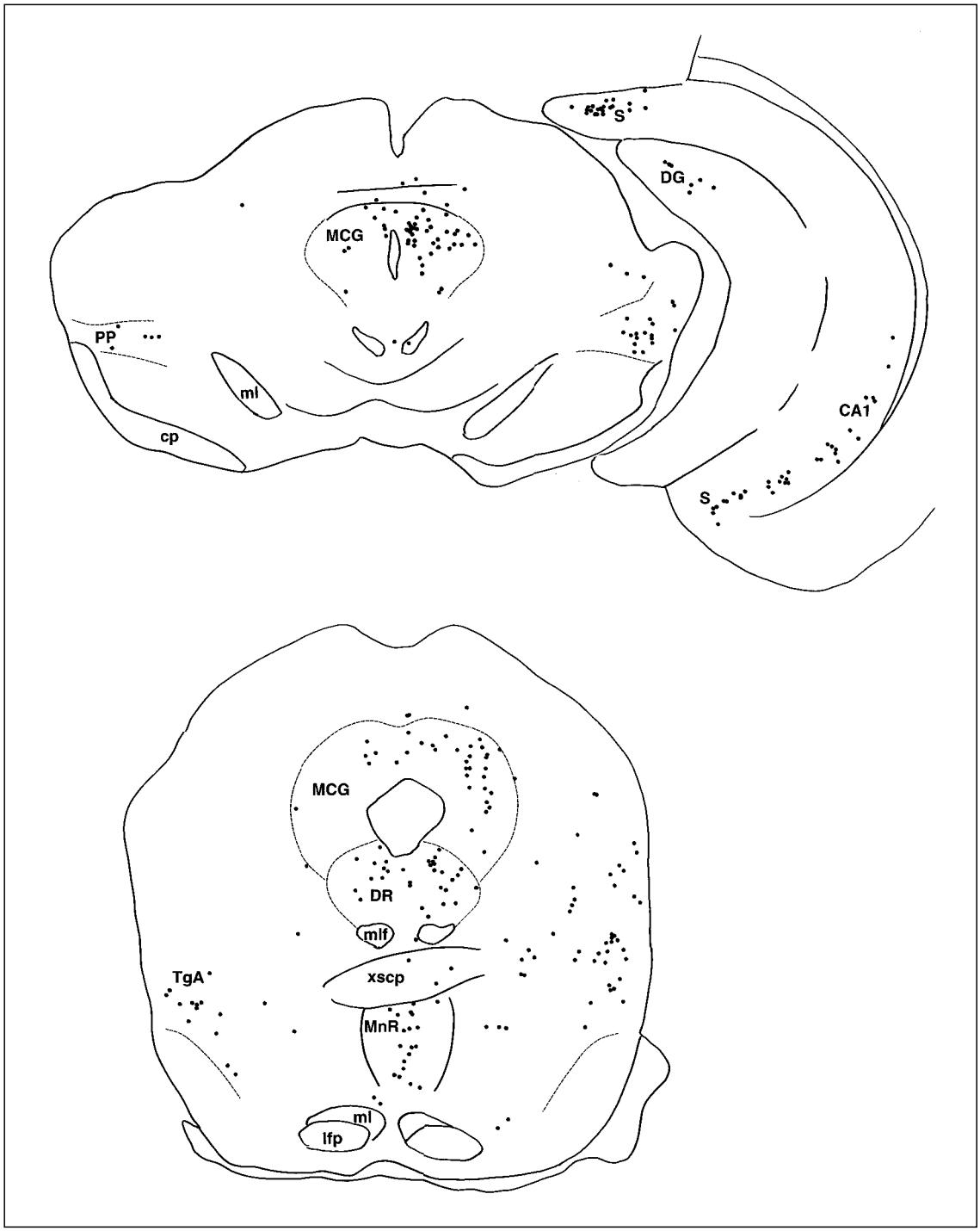

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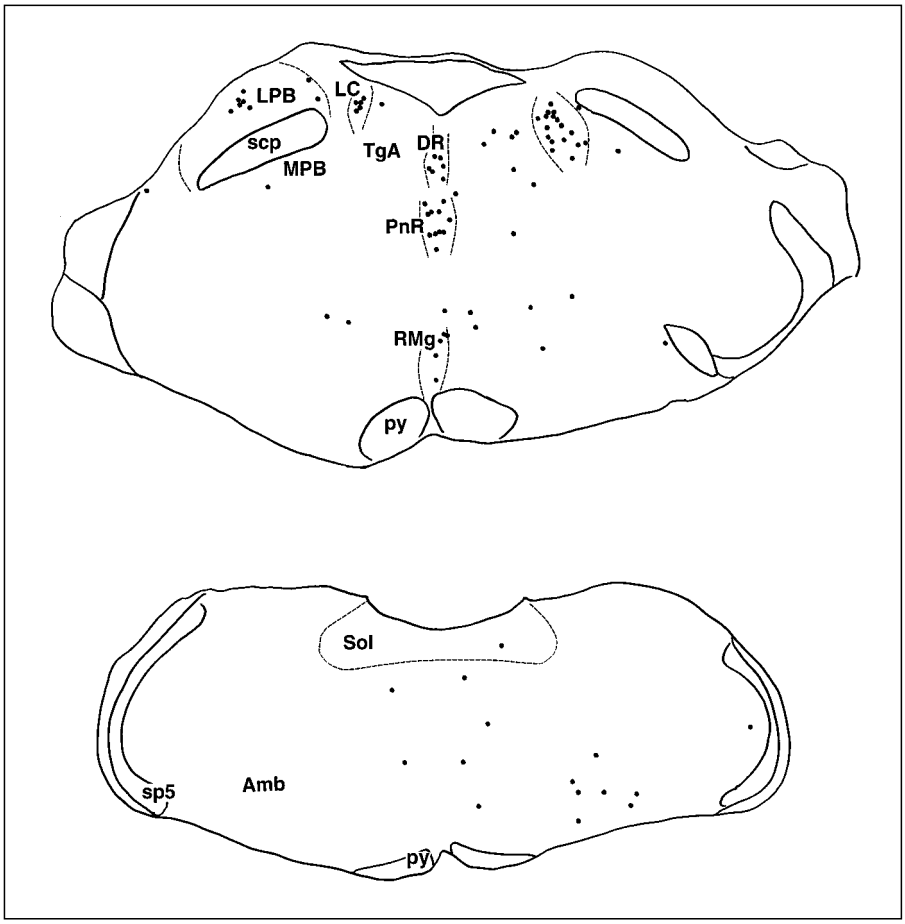

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(For legend, see p. 58.)

Results

Retrograde Tracing

Six out of 12 animals were injected with FG in the AH between the fornix and the optic chiasma (fig. 2). The other animals were injected either dorsal to the fornix, or at the level of the optic chiasm. The distribution of retrogradely labeled cells was similar in all animals injected within the AH between the fornix and the optic chiasma. The distribution of the retrogradely labeled neurons was mapped throughout the brain for one such animal (fig. 3–6). The majority of the retrograde labeling was found ipsilateral to the injection site, although some FG-ir cells were visible contralaterally, particularly within the lateral habenula, midbrain and lateral parabrachial nucleus.

Rostral to the injection site, FG-ir cells were found within the LS and HDB (fig. 3, 7). Between these areas, isolated cells were found in the anterior parts of the BST and the ventral pallidum. More caudally, retrogradely labeled cells were found in the MPA and extending into the lateral preoptic area, medial preoptic nucleus, median preoptic nucleus, and the posterior parts of the BST. At the level of the hypothalamus, cells were found extending laterally from the injection site into the amygdala, particularly the MeA, CeA, and cortical amygdaloid nucleus (fig. 4, 7). Furthermore, labeled cells were found extending further into the amygdalohippocampal area, the subiculum and the CA1 field of the hippocampus. Dorsally from the injection site, cells were found within the anterior paraventricular thalamic nucleus and habenula. Contralaterally from the injection site, cells were found in the ventromedial hypothalamic nucleus and extending towards the medial tuberal nucleus, and lateral hypothalamus (fig. 4, 7). Caudally, retrogradely labeled cells were found in three main zones extending along a rostro-caudal axis at the level of the midbrain. Labeled cells were found in the midbrain central gray, particularly its dorsolateral aspects (fig. 5, 7). Labeled cells were also found within the peripeduncular nucleus and extending into the lateral aspects of the tegmental area. Finally, cells were also found along the midline within the

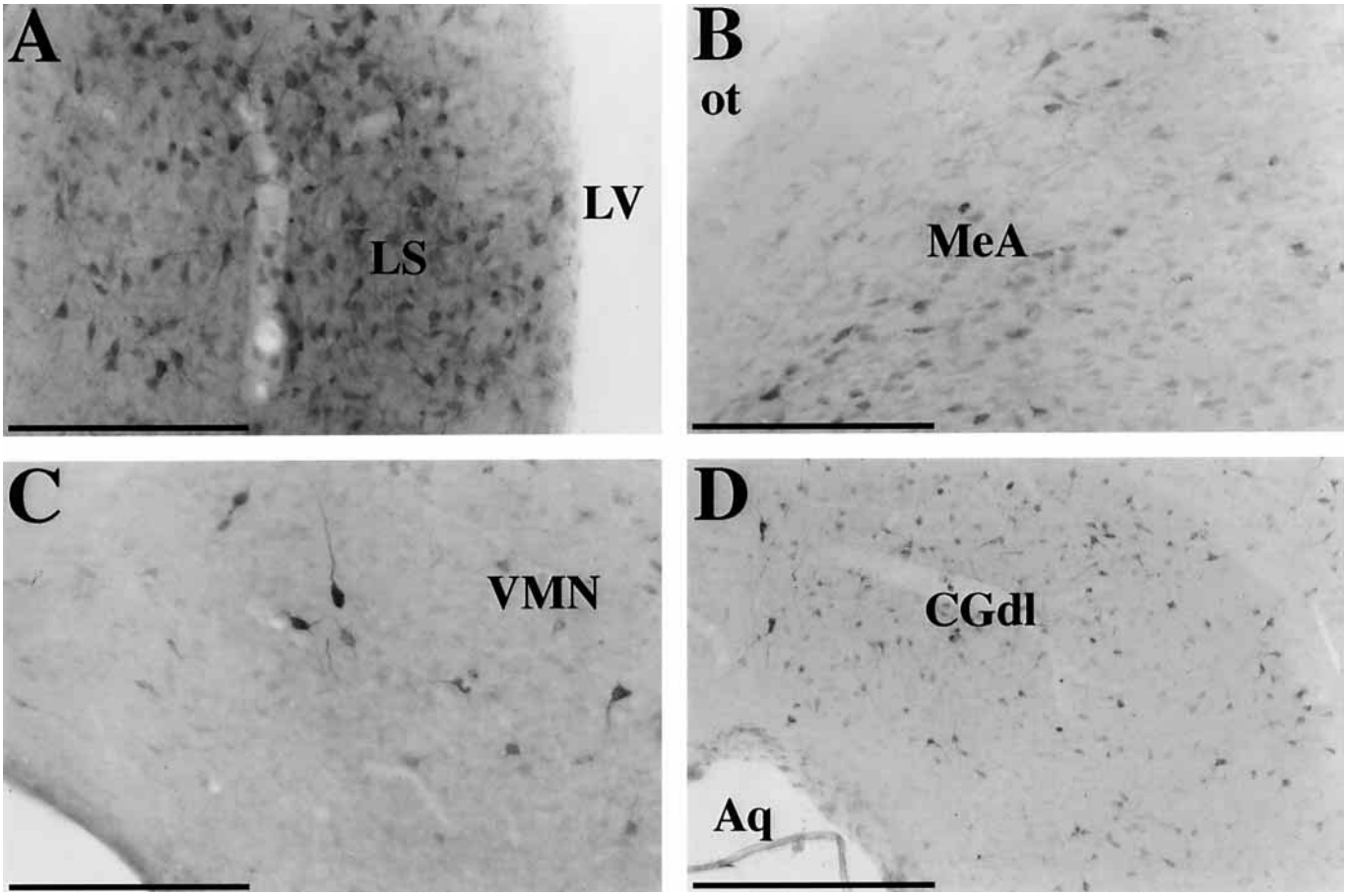


Fig. 7. Photomicrographs showing FG-ir neurons within the lateral septum (**A**), medial amygdala (**B**), ventrolateral hypothalamus (**C**), and midbrain central gray (**D**). Scale bars = 200 µm (**A**–**C**), 400 µm (**D**).

dorsal and median raphe nuclei. In the hindbrain, retrogradely labeled cells were found along the midline within the raphe nuclei. Laterally, labeled cells were found within and around the locus coeruleus and extending laterally into the lateral parabrachial nucleus. At the caudal levels of the hindbrain, FG-ir neurons were found within and around the nucleus ambiguus and the nucleus solitarius (fig. 6).

Anterograde Tracing

Anterogradely labeled fibers were observed in 4 hamsters injected within the AH (fig. 8). The distribution of Pha-L-ir fibers was observed and mapped in one animal injected within the AH between the fornix and the optic chiasma (fig. 9–11). The distribution of Pha-L-ir fibers was similar in all four animals.

In all animals, the observation of adjacent slides suggests that Pha-L-ir fibers appeared to be organized into several pathways leaving the injection sites. A first set of Pha-L-ir fibers appeared to project from the AH rostrally towards the preoptic area and BST. Some of these fibers appeared to project further rostrally towards the nucleus of the diagonal band and claustrum, and branched dorsally towards the LS (fig. 9, 12). A second set of Pha-L-ir fibers appeared to leave the AH laterally towards the fundus striati and amygdala, including the CeA and MeA (fig. 10, 12). Some of these fibers appeared to extend further as Pha-L-ir fibers were seen within the amygdalohippocampal area and hippocampus. Two other sets of Pha-L-ir fibers appeared to be directed medially passing just above and below the third ventricle forming two loops over the contralateral side: one medial to the fornix (covering the anterior hypothalamic area, ventromedial hypothalamic nucleus, dorsomedial hypothalamic nucleus) and one lateral to the fornix (covering the lateral hypothalamic area, VLH, medial tuberal nucleus) (fig. 10, 12). The second loop appeared to extend further to the contralateral amygdala. A fifth set of Pha-L-ir

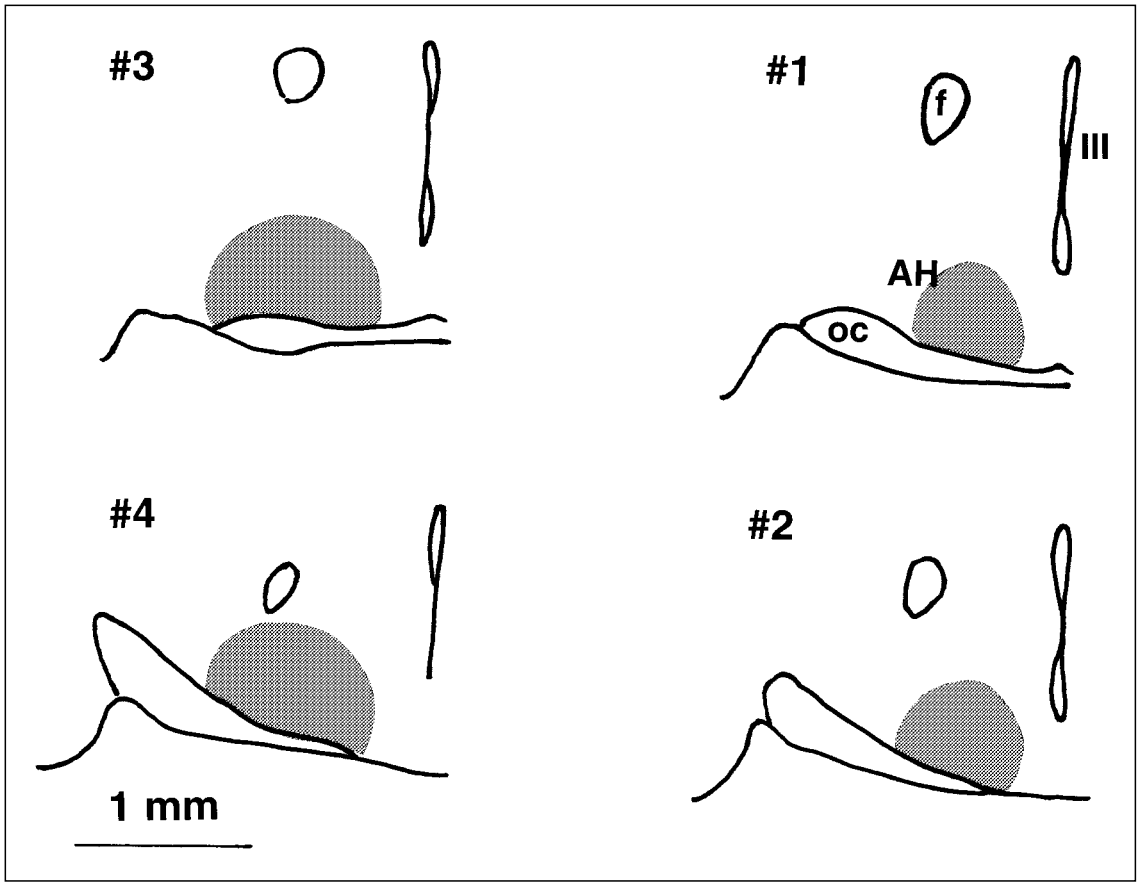


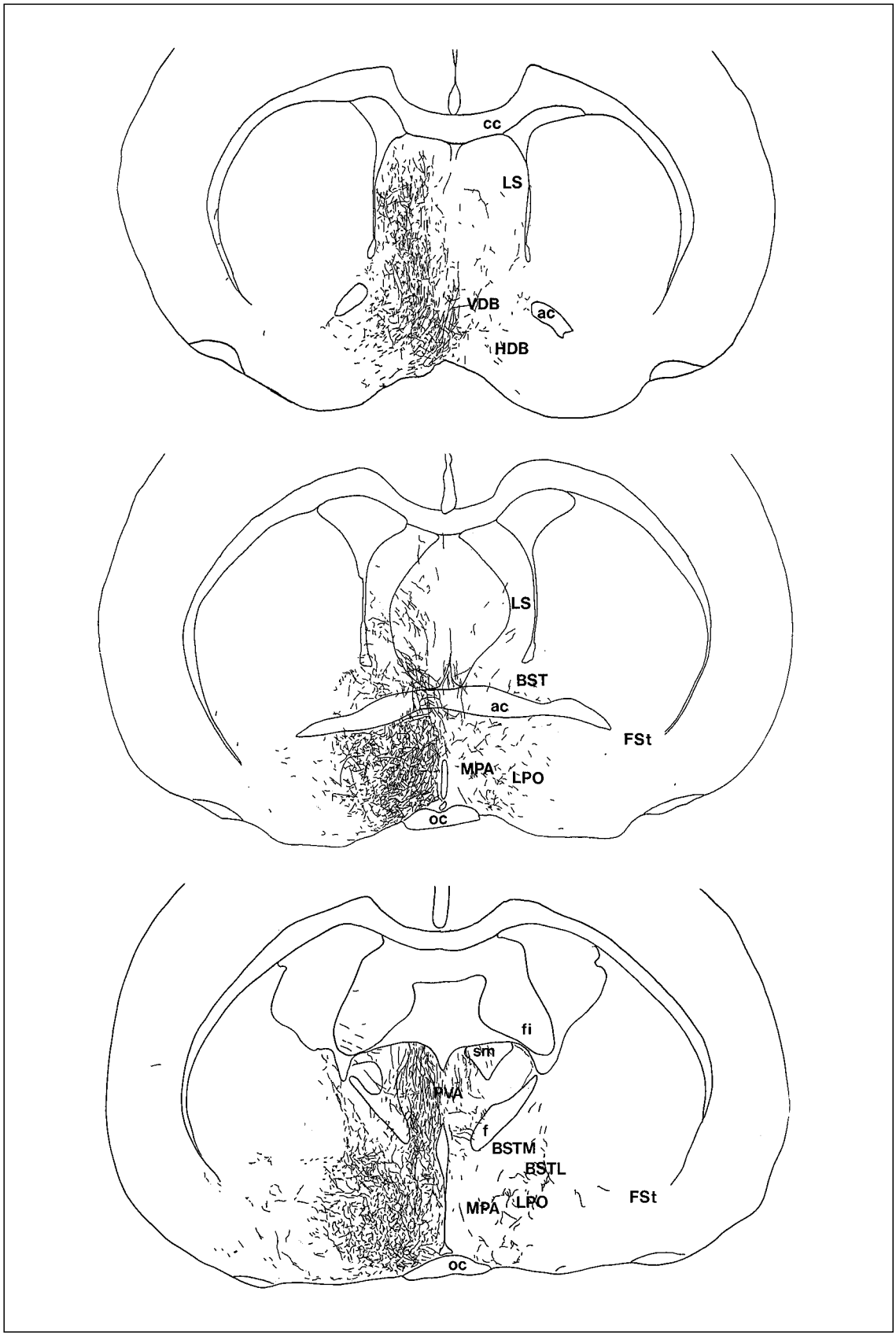
Fig. 8. Camera lucida drawings showing the location of Pha-L injection sites (shaded areas) within the anterior hypothalamus in four different animals.

fibers appeared to leave the AH dorsally to the midline, anterodorsal and anteroventral thalamic nuclei, then proceeded caudally along the medio-dorsal edge of the thalamus towards the midbrain central gray. Finally, a set of Pha-L-ir fibers appeared to leave the AH caudally towards the posterior hypothalamus and ventral tegmental area. The same set of fibers appeared to continue dorsally along the linear raphe nucleus towards the midbrain central gray (fig. 11–12). A branch of this fiber bundle arched along the zona incerta and the dorsal edge of the substantia nigra towards the peripeduncular nucleus and the parabrachial nuclei. Furthermore, Pha-L-ir fibers were seen further caudally within the hindbrain, particularly within serotonergic and noradrenergic cell groups (fig. 11).

Interestingly, Pha-L-ir fibers were also seen coursing in specific fiber tracts, such as the stria terminalis and the fimbria hippocampus.

C-Fos Quantifications

The density of c-Fos-ir cells was quantified in animals from both groups (i.e. controls vs. fighters). The areas selected for this quantification originated from the previous mapping of retrograde and anterograde tracing (fig. 3–6, 9–11). In particular, the selected areas were presumed to have reciprocal connections with the AH. Data analysis led to the following observations. In some areas, no statistical difference was observed between the groups (fig. 13). These areas included the AH [$t(13) = 1.11$, $p > 0.1$], HDB [$t(13) = 0.73$, $p > 0.1$], LS [$t(13) = 0.40$, $p > 0.1$], MHb [$t(12) = 0.66$, $p > 0.1$], MPA [$t(13) = 2.11$, $p = 0.057$], CGvl [$t(8) = 0.23$, $p > 0.1$], and CeA [$t(11) = 0.02$, $p > 0.1$]. In other areas, an increased density of c-Fos-ir cells was observed in the fighters as compared to the controls. Such areas included the BST [$t(13) = 2.94$, $p < 0.05$], CGdl [$t(6) = 9.8$, $p < 0.001$], MeA [$t(12) = 7.29$, $p < 0.001$], and VLH [$t(11) = 7.47$, $p < 0.01$] (fig. 13–14). In the MeA, VLH and CGdl,



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(For legend, see p. 66.)

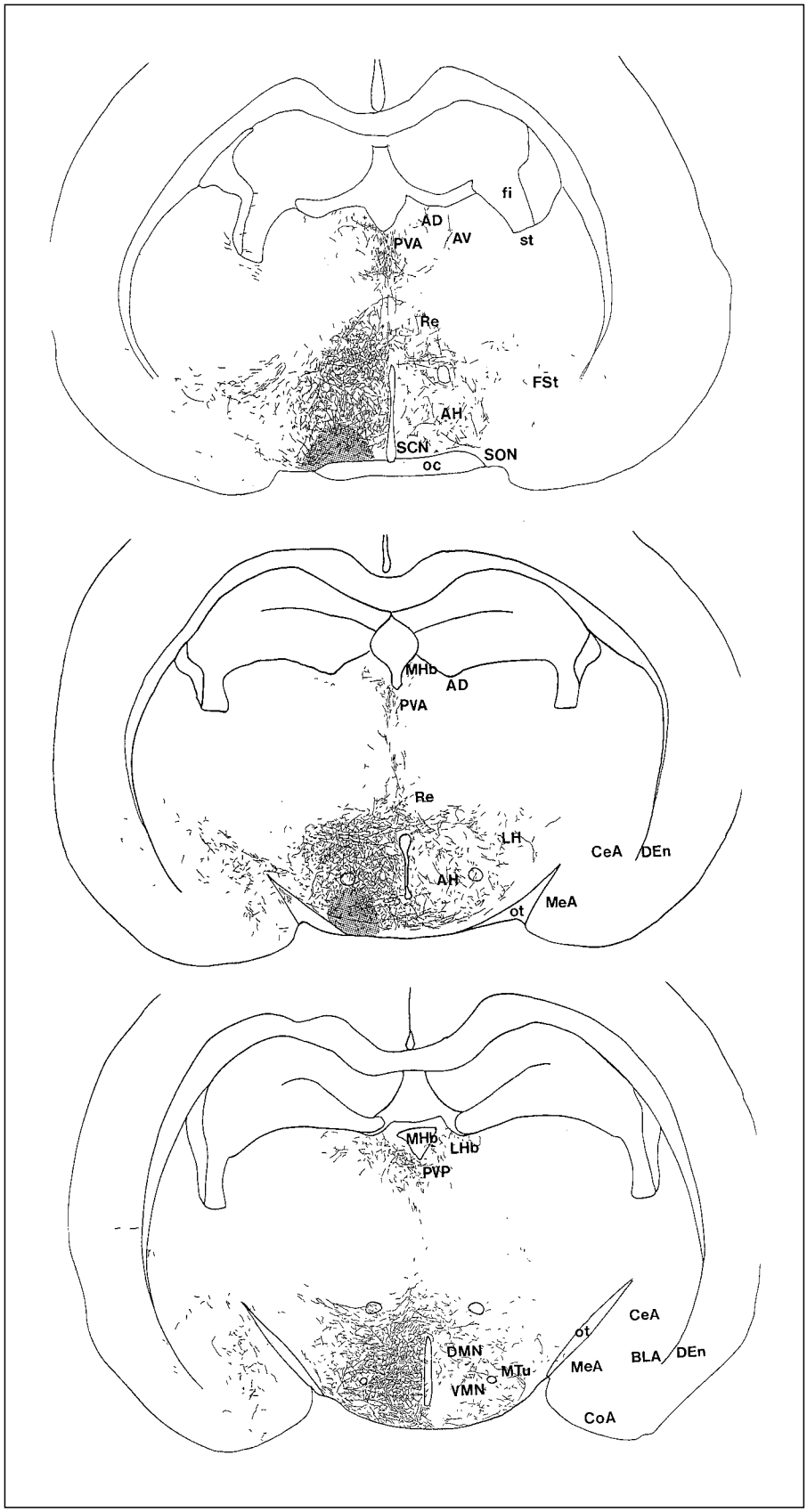

Fig. 9–11. Camera lucida drawings showing the distribution of Pha-L-ir fibers in an animal injected within the AH (animal No. 3 in fig. 8). The shaded area represents the extent of the injection site.

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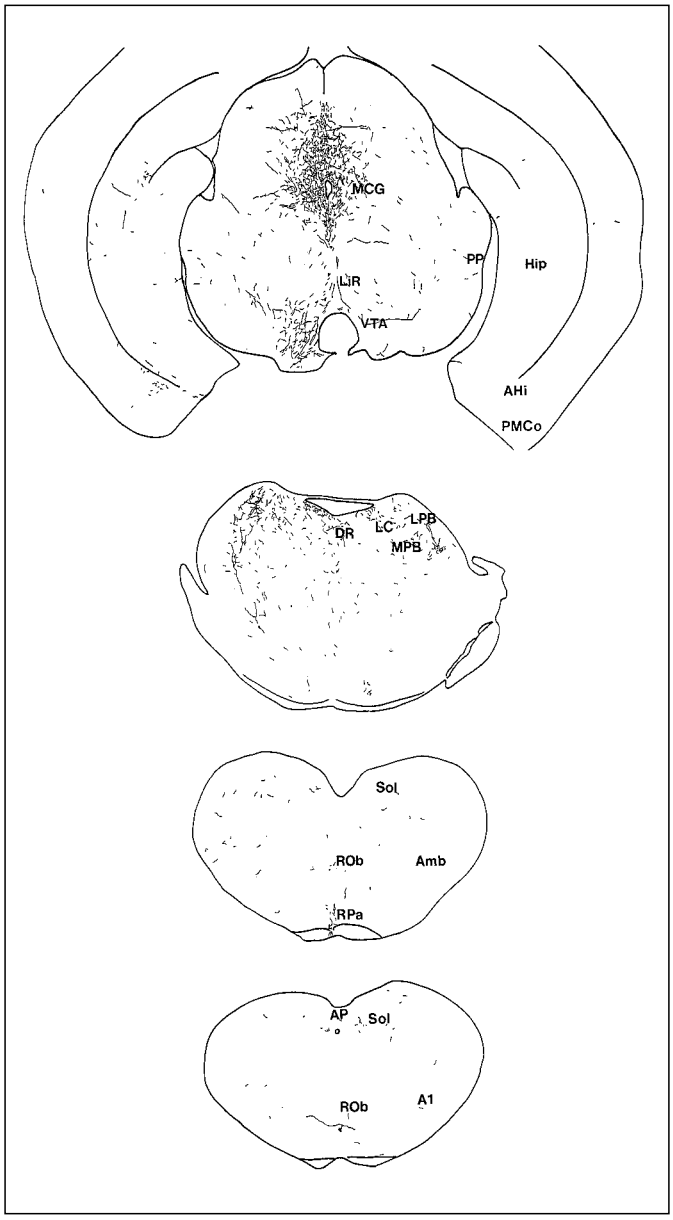

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the density of c-Fos labeling was about twice as high in fighters than in controls.

In addition to these observations, c-Fos-ir was observed within specific cell types such as AVP-ir neurons within the hypothalamus (PVN, NC, mSON and lSON) and TH-ir neurons within the raphe nuclei (fig. 15). The data were analyzed as the proportion of AVP-ir or TH-ir cells also containing c-Fos-ir. In the hypothalamus, the highest proportion of double-labeled cells was found in fighters within the NC and mSON (fig. 16). Compared to the controls, a 40–50% increase in the proportion of double-labeled cells was found in these areas. This increase was statistically significant in the NC and mSON [respectively: $t(12) = 2.83$, $p < 0.05$; $t(12) = 2.92$, $p < 0.05$]. The differences were not statistically significant in the other AVP cell groups [PVN: $t(12) = 1.56$, $p > 0.1$; lSON: $t(12) = 0.06$, $p > 0.1$]. In the raphe nuclei, few TH-ir cells were found to contain c-Fos-ir (fig. 16). The proportion of double-labeled neurons ranged from 1–4% in the DRP to 2–10% in the MnR. The proportion of double-labeled cells within all areas was higher in fighters when compared to controls. The differences between the groups were statistically significant in the MnR [$t(6) = 2.47$, $p < 0.05$].

Discussion

The present report includes a description of afferent and efferent connections of the AH. These data show that the AH has reciprocal connections with several areas. The significance of these connections to the consummation of offensive aggression was tested through c-Fos immunolabeling. These tests indicated an enhanced neuronal activity within the MeA, BST, VLH and CGdl in animals after attacking an intruder. These data suggest that these sites participate in a neural network controlling offensive aggression.

As tested in the present experiment, the AH is connected with several forebrain, midbrain and hindbrain areas. The present distribution of the afferent connections to the AH is consistent with previous descriptions in hamsters. Indeed, the AH of golden hamsters is known to receive afferents from the LS [Ferris et al., 1990a] and amygdala [Kevetter and Winans, 1981; Gomez and Newman, 1992; Coolen and Wood, 1998]. Furthermore, the present description of areas projecting to the AH of hamsters is also consistent with previous reports in rats or guinea pigs [Meibach and Siegel, 1977; Krettek and Price, 1978; Swanson and Cowan, 1979; Berk and Finkelstein, 1981; Sakanaka et al., 1981; Kita and Oomura, 1982; Tribollet et al., 1985; Staiger and Nürnberger, 1991; Thellier et al., 1994; Cameron et al., 1995; Ricciardi et al., 1996]. In addition, the present description of the efferent projections of the AH is also consistent with previous observations in golden hamsters [Morin et al., 1994]. Finally, the present description of the efferent projections of the AH in golden hamsters is also consistent with previous reports in rats or guinea pigs [Conrad and Pfaff, 1976; Saper et al., 1976; Swanson, 1976; Luiten et al., 1985; Tribollet et al., 1985; Veening et al., 1987; Villalobos and Ferssiwi, 1987; Semenenko and Lumb, 1992; Delville and Blaustein, 1993; Roeling et al., 1994; Risold et al., 1994].

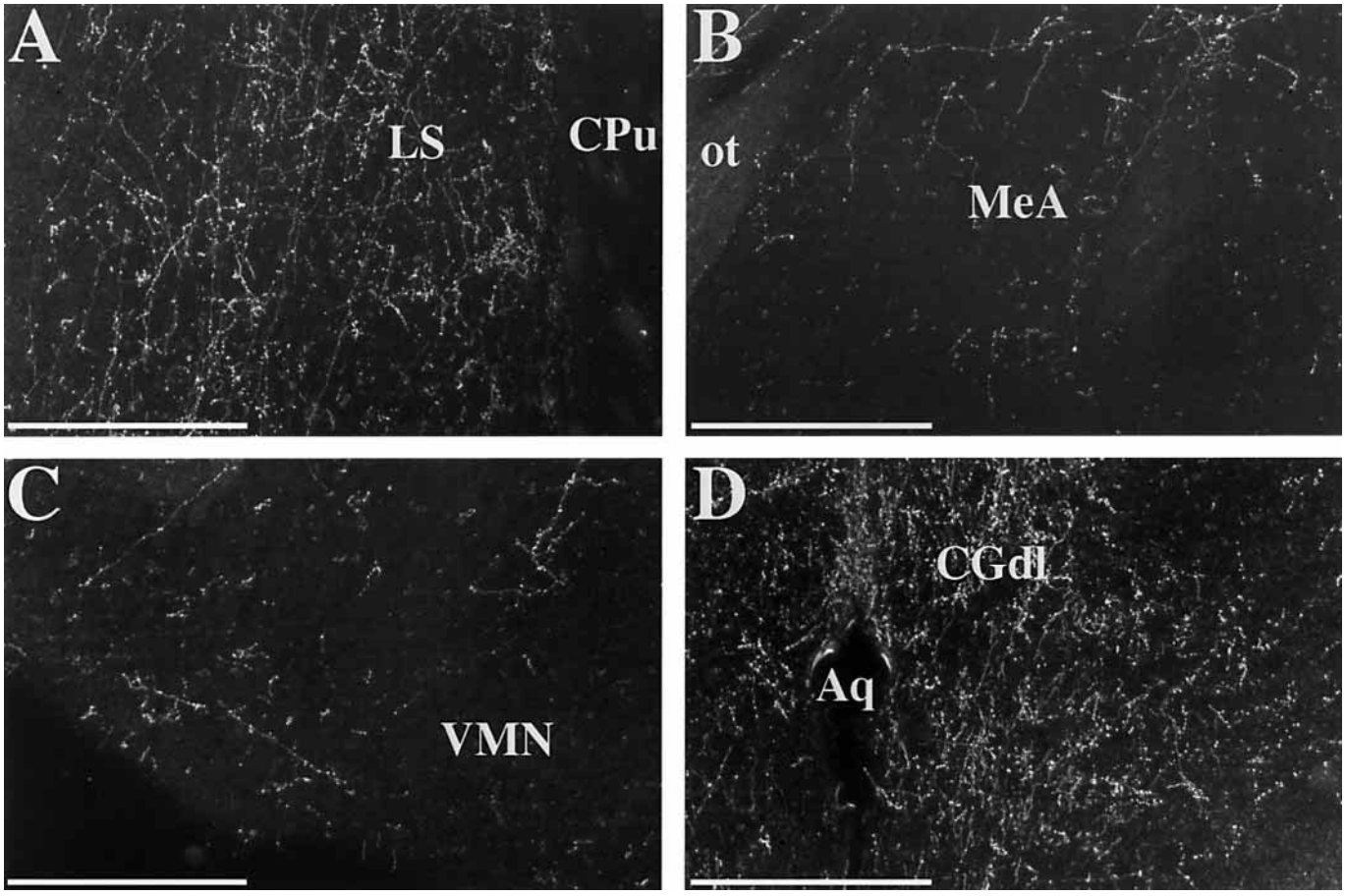

Fig. 12. Darkfield photomicrographs showing Pha-L-ir fibers in various areas (**A** lateral septum; **B** medial amygdala; **C** ventrolateral hypothalamus; **D** midbrain central gray) in the brain of an animal injected within the AH. Scale bars = 400 μm.

Interestingly, many areas found to receive projections from the AH also appeared to project to this area (fig. 17). Thus, the above mentioned pathways appear to be reciprocal, suggesting that the AH is at the center of a network of reciprocal connections involving several parts of the brain. The areas that appear to have reciprocal connections with the AH include principally the HDB, LS, MPA, CeA, MeA, VLH (as observed in the contralateral side), the posterior aspects of the BST, the habenula, the midbrain central gray, raphe complex, parabrachial nuclei and noradrenergic cells groups. The present data extend earlier reports of reciprocal connections between the AH and the LS [Ferris et al., 1990a] or the MeA [Kevetter and Winans, 1981; Gomez and Newman, 1992; Coolen and Wood, 1998] in golden hamsters. Similar observations of reciprocal connections between the AH and the midbrain central gray have been reported in rats [Veening et al., 1987; Semenenko and Lumb, 1992; Roeling et al., 1994; Risold et al., 1994].

Is neuronal activity within the areas interconnected with the AH related to the consummation of offensive aggression? This question was addressed through c-Fos immunocytochemistry and subsequent quantification. This method has the advantage of providing a clear anatomical resolution. In a previous study, an increase in c-Fos expression was reported within several parts of the brain in animals after winning a fight as compared to unstimulated controls [Kollack-Walker and Newman, 1995]. However, no substantial difference was noted between the winners and losers of the fights in that study. This observation prompted the authors to suggest that neuronal activation within these areas is not necessarily associated with the occurrence of aggressive or subordinate responses. In addition, several of

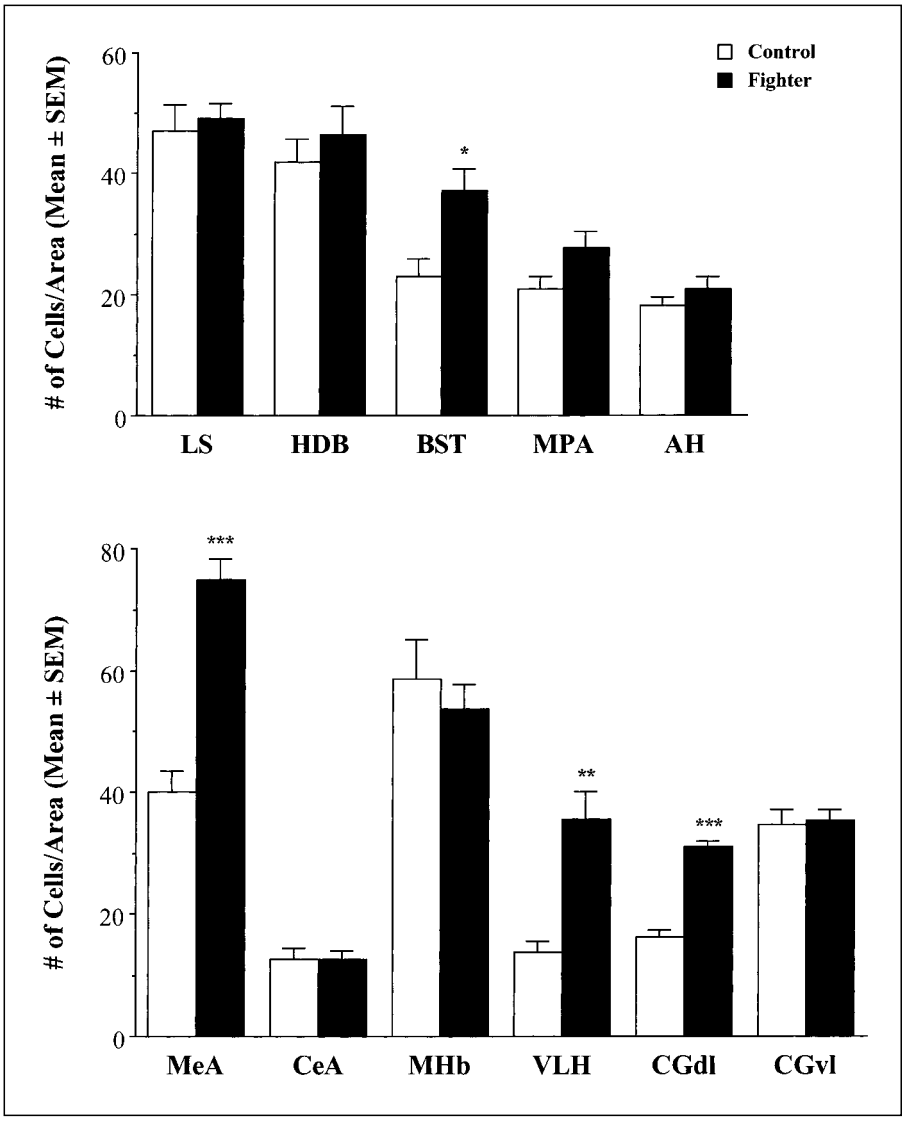

Fig. 13. Comparison of the density (number of c-Fos-ir nuclei per sample in each area analyzed) between control animals (control, exposed to a woodblock) and experimental animals (fighter, that attacked an intruder). Exposure to the woodblock or intruder lasted 10 min. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Student t-test, two-tailed, comparison with controls.

these areas were also activated after sexual interactions. Therefore, it is conceivable that the enhanced neuronal activation might be related to enhanced arousal rather than aggression. Consequently, the present study was designed to account for arousal through the use of a control group stimulated to perform elements of agonistic behavior short of overt aggression. Furthermore, our study focused on offensive aggression and did not include a group of animals sacrificed after losing a fight. Information from such animals involves too many confounding variables such as the stress of defeat and defensive aggression mixed with offensive aggression attempts. In the present experiment, neuronal activity as tested through c-Fos immunoreactive labeling was increased in several areas in animals after the consummation of offensive aggression. These areas included the MeA, BST, VLH and CGdl. As such, these data are consistent with the possibility that the AH is integrated within a neural network including these areas and involved in the regulation of offensive aggression.

The observation of increased c-Fos immunolabeling within the MeA in fighters is consistent with previous reports in male and female golden hamsters [Potegal et al., 1996a, b]. In males, electrical stimulation of the area is sufficient to elicit aggressive responding by residents towards intruders [Potegal et al., 1996b]. Interestingly, the effect of electrical stimulation was correlated with enhanced c-Fos expression within the anterior parts of the MeA. In females, the activation of aggressive responding is also associated

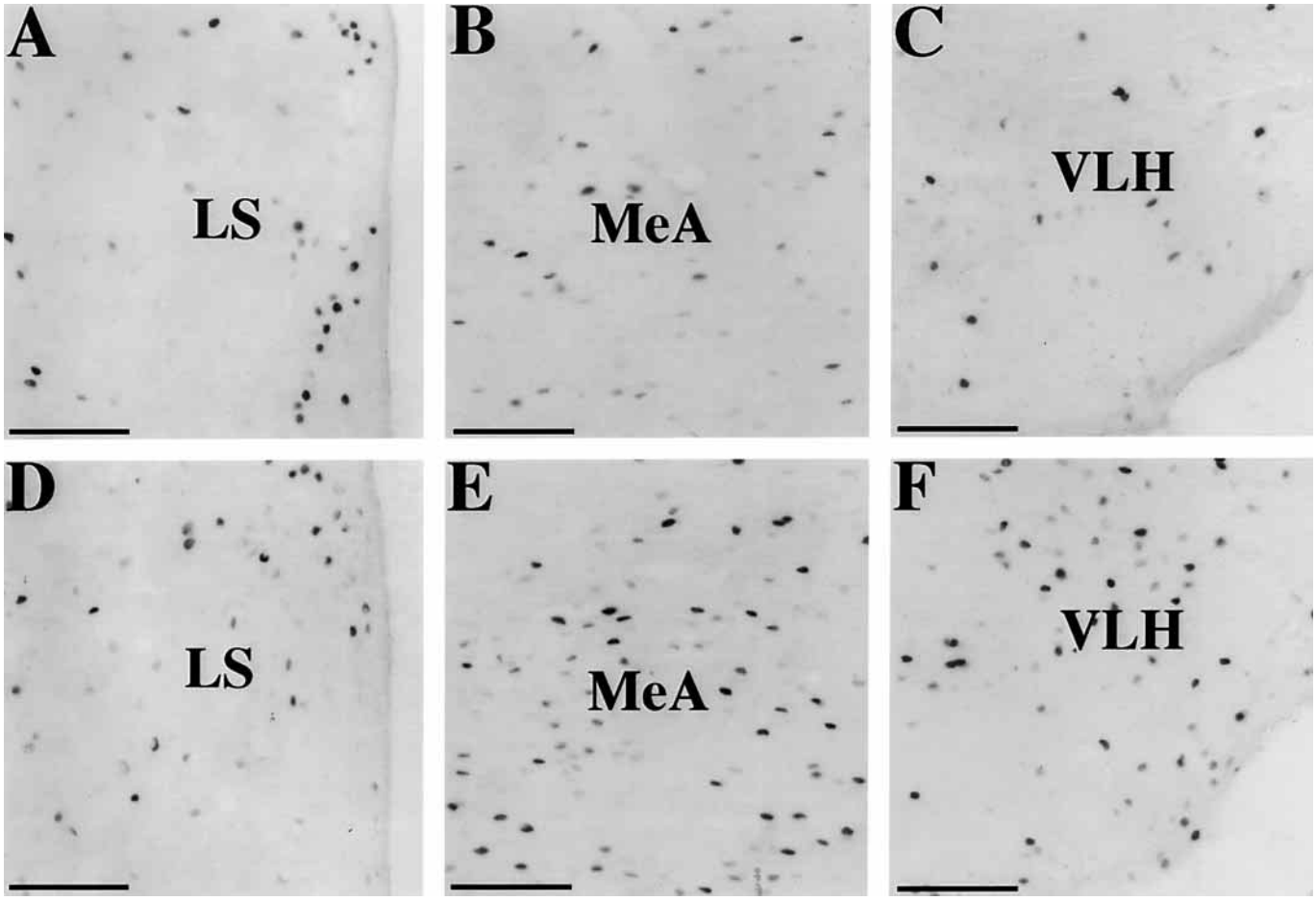

Fig. 14. Photomicrographs showing c-Fos-ir cells in the lateral septum (LS, **A**, **D**), medial amygdala (MeA, **B**, **E**), and ventrolateral hypothalamus (VLH, **C**, **F**) in sections from representative animals from the controls (**A**–**C**) and fighters (**D**–**F**). Scale bars = 100 μm.

with enhanced c-Fos expression within the MeA [Potegal et al., 1996a]. Our data support the hypothesis that the activation of the MeA is critical to the display of offensive aggression in golden hamsters. In addition, the observation of increased c-Fos immunolabeling within the BST of fighters in males might be related to the findings on the MeA. The quantification of c-Fos immunolabeling was focused on the posterior part of the medial division of the BST. This part of the BST is regarded as an extension of the MeA [de Olmos et al., 1985]. Therefore, the BST could also play a function similar to the MeA in the regulation of offensive aggression.

The observation of increased density of c-Fos immunolabeling within the VLH confirms previous observations about this area in the regulation of offensive aggression. In golden hamsters, micro-injections of AVP within the VLH are sufficient to facilitate offensive aggression [Delville et al., 1996b]. In rats, electrical stimulation of the VLH is sufficient to elicit aggressive responses [Bermond et al., 1982; Kruk et al., 1983; Lammers et al., 1988]. Testosterone implants located within the VLH are also sufficient to reinstate aggressive behavior in castrated male rats [Albert et al., 1987]. However, lesions of the area affect both offensive and defensive aggression in rats [Adams, 1971; Eclancher and Karli, 1971; Colpaert and Wiepkema, 1976; Olivier, 1977; Albert et al., 1991].

The present data show that the AH is capable of integrating inputs from several areas in the context of aggressive behavior. For instance, the output of the MeA might participate in the activation of territorial behaviors, such as offensive aggression, perhaps by an increased responsiveness of the AH to AVP release. Of course, activation of the MeA is not necessarily specifically related to aggression. Neuronal

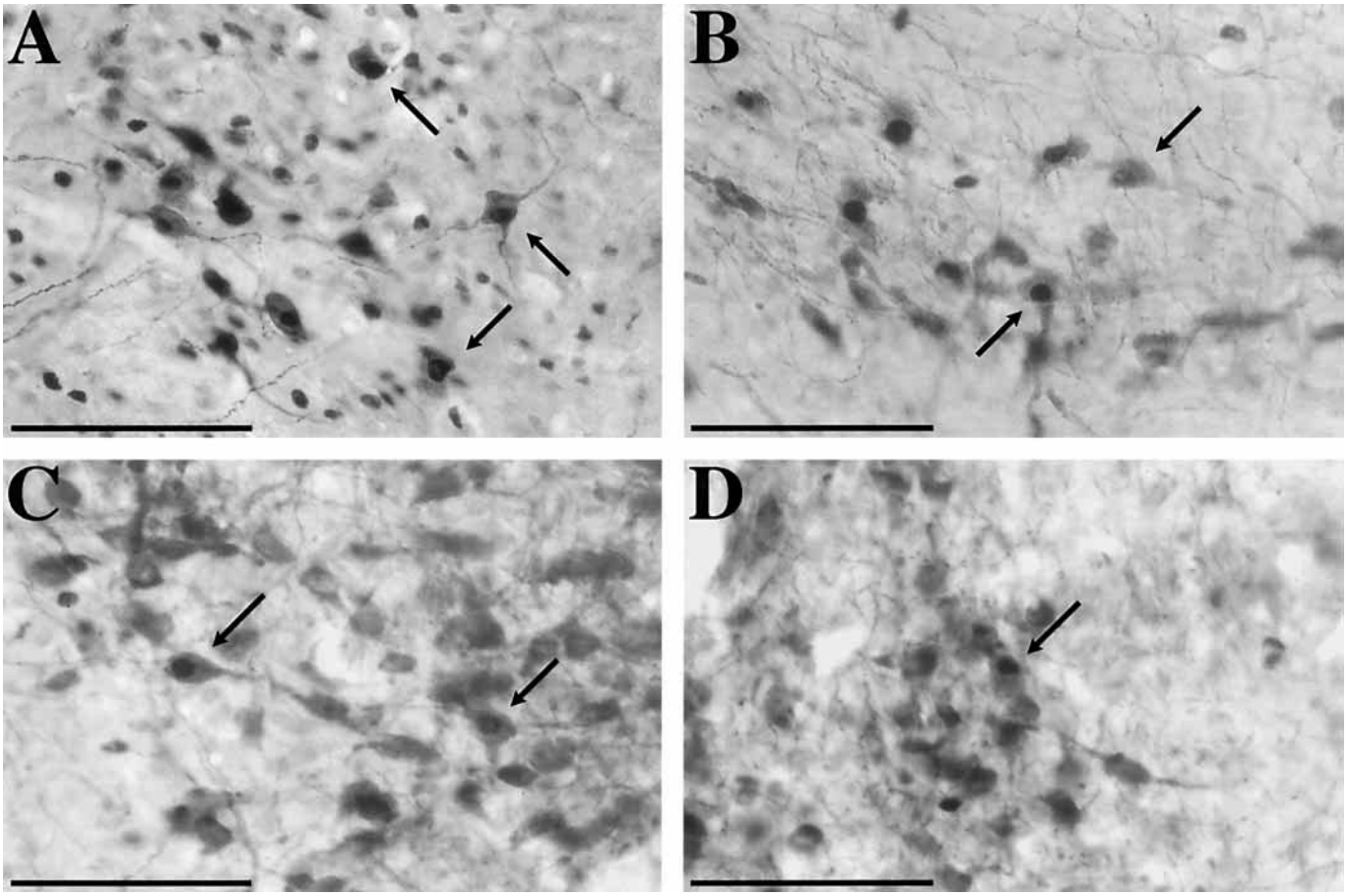

Fig. 15. Photomicrographs showing AVP- (**A**, **B**) and TH-ir (**C**, **D**) neurons within the hypothalamus (paraventricular hypothalamic nucleus: **A** medial division of the supraoptic nucleus; **B** and raphe nuclei (dorsal raphe nucleus); **C** median raphe nucleus; **D** also containing c-Fos-ir (dark nuclei visible inside the neurons). Scale bars = 100 μm.

activation within the MeA or any other area could also be observed under other behavioral circumstances associated with a high arousal. In previous studies, activation of the MeA has also been related to male sexual behavior [Kollack-Walker and Newman, 1995] and to exposure to acute social stress [Kollack-Walker et al., 1997]. Nevertheless, different subpopulations of intermixed cells could be activated under different behavioral conditions. For example, one might hypothesize that opiate neurons could be selectively activated under sexual situations and substance P neurons under aggressive situations. In addition, although the MeA might be activated under different testing conditions, it is likely that the behavioral output of the area depends on a variety of sensory and endocrine stimuli. In the case of offensive aggression these stimuli would be integrated within the neural network centered on the AH. The integration of sensory stimuli with this neural network might occur at several sites, such as the lateral parabrachial nucleus, midbrain central gray or amygdala. Indeed, the AH is connected with the lateral parabrachial nucleus and the nucleus solitarius, areas involved in the relay of autonomic inputs to the brain [Saper and Loewy, 1980; Holets et al., 1988; Herbert et al., 1990; Halsell, 1992]. Furthermore, the midbrain central gray is also involved in the relay of somatosensory inputs [Zemlan et al., 1978]. Finally, the AH is connected with the amygdala and the BST, and these areas are involved in the relay of olfactory and vomeronasal inputs to the brain [Winans and Scalia, 1970; de Olmos and Ingram, 1972; Raisman, 1972; Scalia and Winans, 1975; Krettek and Price, 1978; Kevetter and Winans, 1981; Luiten et al., 1985; Price et al., 1991; Canteras et al., 1992; Gomez and Newman, 1992].

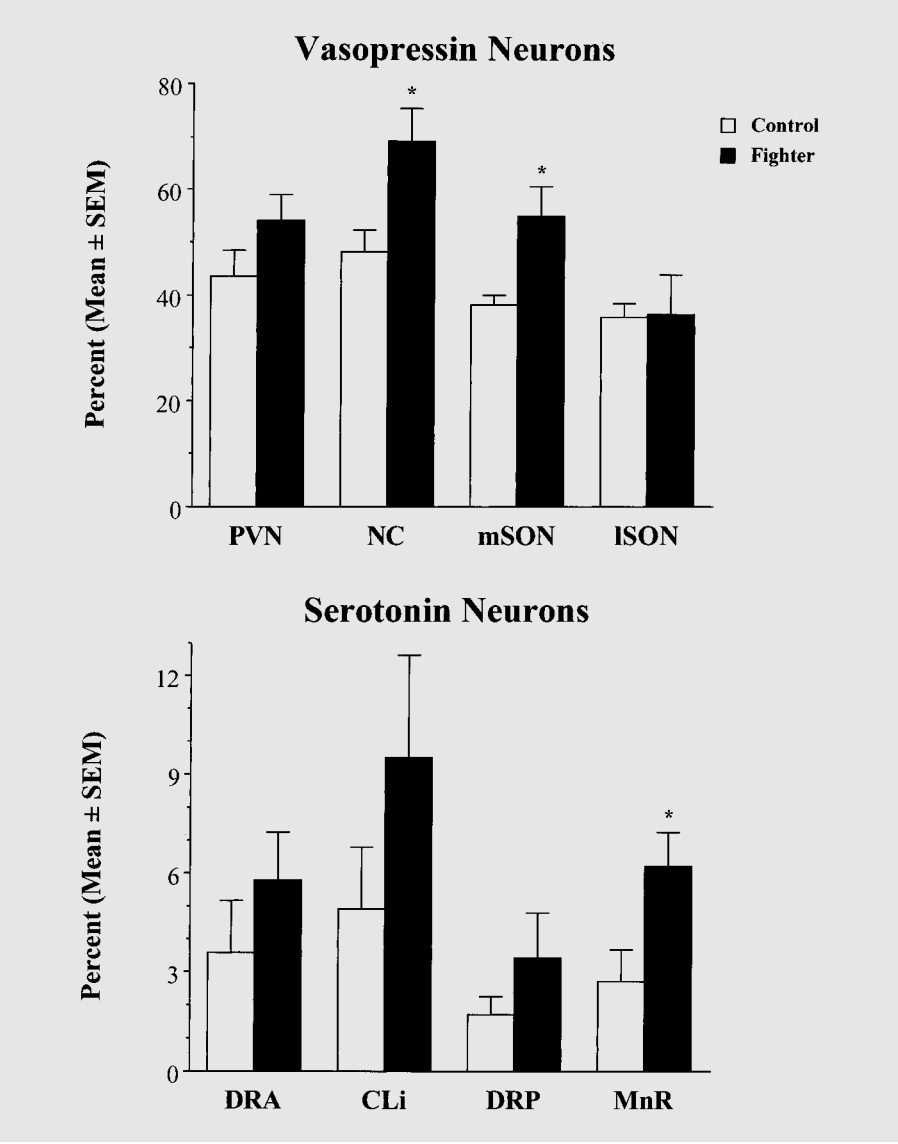

Fig. 16. Comparison of the proportion of vasopressin (AVP-ir) and serotonin (TH-ir) neurons also containing c-Fos-ir within the hypothalamus and midbrain between control animals (control, exposed to a woodblock) and experimental animals (fighter, that attacked an intruder). Exposure to the woodblock or intruder lasted 10 min. *$p < 0.05$, Student t-test, two-tailed as compared to the controls.

It is interesting to note that many of the areas connected to the AH contain AVP receptors in golden hamsters [Ferris et al., 1993]. This is certainly the case for the VLH, contralateral AH, LS, BST, amygdala, hippocampus, midline thalamic nuclei, ventral tegmental area, midbrain central gray, rostral telencephalon, and parabrachial nuclei [Ferris et al., 1993]. Several of these areas are involved in the control of agonistic behaviors by AVP [Ferris et al., 1984; Irvin et al., 1990; Hennessey et al., 1992; Delville et al., 1996b; Ferris et al., 1997]. This observation supports the concept of a network of AVP-sensitive sites involved in the regulation of agonistic behaviors [Ferris et al., 1994; Bamshad and Albers, 1996]. In the present study, it was predicted that several AVP neurons would also contain c-Fos-ir, particularly in the fighters. In the results of our experiment, a greater proportion of AVP neurons within the NC and mSON also contained c-Fos-ir. These neurons have been shown to be involved in the regulation of flank marking, another AVP-dependent agonistic behavior [Ferris et al., 1990b].

In the present study, increased c-Fos activity was observed within 5-HT neurons in the fighters. This observation was unexpected, as 5-HT inhibits offensive aggression in male golden hamsters [Delville et al., 1996a; Ferris et al., 1997; Joppa et al., 1997]. However, it is possible that this increased c-Fos activity was associated with enhanced physical activity rather than aggression specifically. This possi-

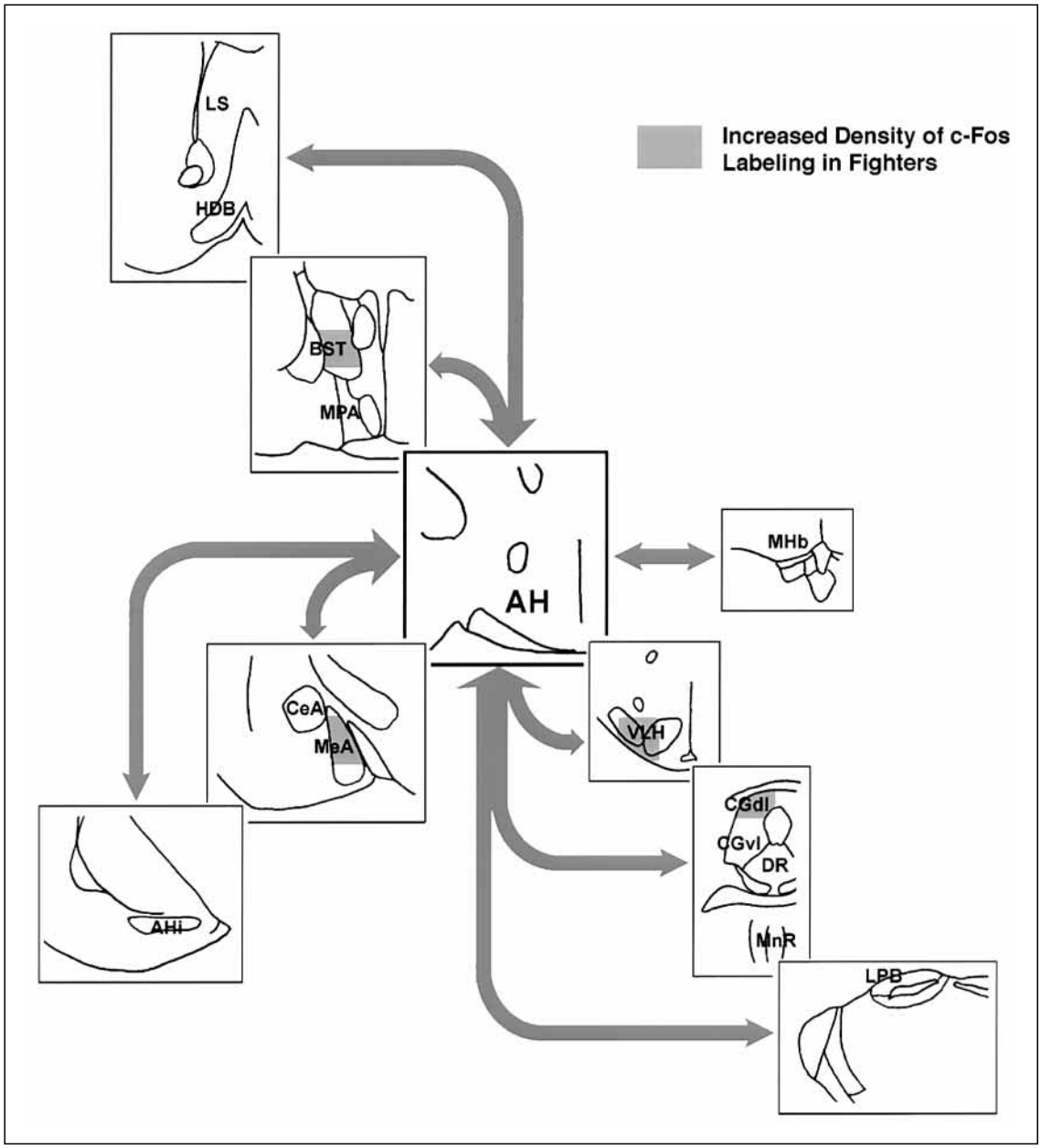

Fig. 17. Schematic drawing summarizing the reciprocal connections of the AH and the level of the neuronal activation within some of the areas shown as determined by their density of c-Fos-ir cells. Shaded areas represent increased density of c-Fos labeling in fighters.

bility is supported by results from previous studies performed in rats [Matsuo et al., 1996; Mendlin et al., 1996; Rueter and Jacobs, 1996]. Furthermore, it remains to be determined whether the experimental conditions in our study enhanced the release of 5-HT within the AH.

Finally, areas such as the AH did not appear to show any change in activity after an agonistic encounter, even though the area has been implicated in the regulation of offensive aggression [Ferris and Potegal, 1988; Ferris et al., 1997]. However, as c-Fos is the product of one of several immediate early genes, it is conceivable that other immediate early genes were activated within the AH in response to the consummation of aggression. Differential patterns of c-Fos and c-Jun expression have been observed in the paraventricular

hypothalamic nucleus in response to immobilization stress [Senba et al., 1994], as well as within luteinizing hormone-releasing hormone neurons in response to mating in ferrets [Wersinger and Baum, 1996]. Different patterns of c-Fos and Fos B expression were also found within the suprachiasmatic nucleus of rats across circadian rhythms [Peters et al., 1994] and in hamsters in response to light stimulation [Ebling et al., 1996]. In addition, although the AH is important for the facilitation of aggression by AVP, the area is also involved in the activation of flank marking behavior by AVP [Ferris et al., 1984; Ferris et al., 1990b]. In the present study, animals in the control group were observed to flank mark in the presence of a woodblock carrying the odor of a conspecific. Flank marking behavior might explain the lack of differences between the groups in the AH. Previous studies showed increased c-Fos immunolabeling within the AH after a fight [Kollack-Walker and Newman, 1995]. However, their control did not include exposure to an olfactory stimulus. As such, our present observation suggests that neuronal activation of the AH might not be specific to the consummation of offensive aggression but rather the onset of agonistic interactions.

In summary, the AH is at the center of a neural network of reciprocal connections including the LS, MeA and VLH. Elements of this network are activated during the consummation of offensive aggression toward an intruder. It is possible that this network integrates sensory inputs with neuroendocrine variables and mediates behavioral arousal. It is hypothesized that the BST, MeA, VLH and CGdl function to activate aggressive responding. The activity of the entire network is partially regulated by AVP and 5-HT release within the elements of the network. These neurotransmitters act as opposing neuromodulators through differential activation of the elements of the network.

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