NEUROCHEMICAL CHARACTERIZATION OF HYPOTHALAMIC NEURONS INVOLVED IN ATTACK BEHAVIOR: GLUTAMATERIC DOMINANCE AND CO-EXPRESSION OF THYROTROPIN-RELEASING HORMONE IN A SUBSET OF GLUTAMATERIC NEURONS

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Abstract—The electrical stimulation of a specific hypothalamic area rapidly evokes attacks in rats. Noteworthy, attack-related hypothalamic structures were identified in all species studied so far. The area has been extensively mapped in rats, and its anatomical connections have been studied in detail. However, technical difficulties precluded earlier the precise identification of the neural elements mediating the aggressive effects of stimulation. It now appears that a dense and distinct group of glutamatergic cells expressing vesicular glutamate transporter 2 mRNA extends over the entire hypothalamic attack area. Rostral parts overwhelmingly contained glutamatergic neurons. In more caudal parts, glutamatergic and fewer GABAergic neurons were found. The remarkable similarity in the distribution of hypothalamic attack area and glutamatergic cell groups suggests that these cells mediate the aggressive effects of stimulation. Surprisingly, thyrotropin releasing hormone mRNA was co-localized in a subset of glutamatergic neurons. Such neurons were present at all rostro-caudal levels of the hypothalamic attack area, except for that part of the hypothalamic attack area extending into the ventro-lateral part of the ventromedial hypothalamic nucleus. Earlier data on the projections of hypothalamic thyrotropin releasing hormone neurons suggest that this subpopulation plays a specific role in attack behavior. Thus, we identified three neuronal phenotypes in the hypothalamic structure that is involved in the induction of attacks: glutamatergic neurons co-expressing thyrotropin releasing hormone, glutamatergic neurons without thyrotropin releasing hormone, and GABAergic neurons dispersed among the glutamatergic cells. Assessing the specific roles and connec-

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Abbreviations: GAD 65, glumatic acid decarboxylase, 65 kDa isoform; GAD 67, glumatic acid decarboxylase, 67 kDa isoform; HAA, hypothalamic attack area; POD, horseradish peroxidase; TRH, thyrotropin-releasing hormone; VGLUT, vesicular glutamate transporter; VGLUT2, type 2 vesicular glutamate transporter.

1 The term “hypothalamic attack area” was adopted from our earlier studies (e.g. Kruk, 1990; Siegel et al., 1999). The term “intermediate hypothalamus” derives from the work of Geeraerts et al. (1990a,b). The nomenclature of different hypothalamic structures are similar to that used by Paxinos and Watson (1998).

Hypothalamic structures involved in the execution of various behaviors including attack were identified already in early 20th century by electrophysiological techniques (Hess, 1928). Later research with thinner electrodes and lower current intensities revealed that attack could be specifically elicited from a distinct area of the hypothalamus in all species investigated so far (Kruk, 1991; Siegel et al., 1999). Species differences in brain structure preclude a precise match in the anatomical localization and the size of attack-related hypothalamic structures, but the available data clearly show that the hypothalamus contains an attack controlling structure in probably all mammalian species. The surgical destruction of the postero-medial hypothalamus abolishes excessive forms of human aggression and violence (Sano et al., 1966; Ramamurthi, 1988), demonstrating that a hypothalamic structure involved in attack exists in humans as well.

The precise anatomical location of the hypothalamic attack area1 of the rat (HAA) was described in detail earlier (Kruk et al., 1983, 1984a,b; Lammer et al., 1988). The functional area from which attacks can be elicited extends over a relatively large hypothalamic region that includes the lateral hypothalamic area, the anterior hypothalamic nuclei, the retrochiasmatic area, the ventrolateral part of the ventromedial hypothalamic nucleus, and the tuber cinereum area (dorsolateral to the ventromedial nucleus). This area appears to be different from brain structures involved in defense against predators, which consists of the anterior hypothalamic nucleus, dorsomedial part of the ventromedial hypothalamic nucleus, and the dorsal premamillary nucleus, which are highly interconnected (Thompson and Swanson, 2003). Although conspecific attacks can be elicited from one of these nuclei (the anterior hypothalamic nucleus), no attacks against conspecifics occur when the other two areas are stimulated. The limited overlap (and the strong interconnections of defense-related nuclei) suggests that the mechanisms underlying anti-predator defense and attack against conspecifics are distinct. The distinctive features of defensive aggression (against both predators and conspecifics) support this as-
A number of studies have suggested that the role of the hypothalamic area (HAA) in aggressive behavior is crucial (Blanchard and Blanchard, 1989). There are several lines of evidence supporting the crucial role of the HAA in attack behavior: (1) this is the only brain region in rats from which attacks can reliably be elicited by stimulation (Kruk et al., 1983; Lammers et al., 1988); (2) lesions placed within this region reduced aggression evoked by an intruder in territorial settings (Adams, 1971; Olivier, 1977; Olivier et al., 1983), and (3) this area is strongly activated by territorial fights in various species (Delville et al., 2000; Kollack-Walker and Newman, 1995; Halasz et al., 2002a).

The unilateral electrical stimulation of the HAA induces c-Fos activation not only in the ipsilateral, but also in the contralateral HAA; moreover, attacks occurred only when the unilateral stimulation resulted in a bilateral activation of the HAA (Halasz et al., 2002b). Taken together, these data clearly demonstrate the crucial role played by the HAA in the induction of attacks. Recently we have demonstrated that the adrenal stress response and the attacks induced by HAA stimulation are linked by a positive feedback loop, suggesting that the HAA plays a role in mediating the impact of stress on aggressiveness and violence (Kruk et al., 2004).

Despite the important role played by the HAA in aggressiveness and violence, little is known about the neural processes controlled by this region. In rats, attacks could be elicited when the HAA was locally treated with GABA antagonists, glutamate agonists or both (Adams et al., 1993; Roeling et al., 1993; Haller et al., 1998). This suggests that both glutamatergic and GABAergic mechanisms are operational within this region. Nevertheless, the functional type of neurons located within the HAA, per se, is unknown. From a functional point of view, glutamatergic and GABAergic neurons are of particular interest, as these constitute the major excitatory and inhibitory systems, respectively, in the brain. Reliable histochemical markers to identify the glutamatergic neuronal phenotype have only become available recently with the discovery of the vesicular glutamate transporters (VGLUT) that define a glutamatergic neuronal chemotype (Esclapez et al., 1993). The aim of the present work was to provide a background for understanding the control of attack behavior by identifying the chemotype of HAA neurons, with special reference to glutamatergic and GABAergic neurons. We assessed the expression of VGLUT2 and GAD 67 mRNAs by in situ hybridization in hypothalamic sections of adult male rats. The distribution patterns of marker mRNAs were compared with the images of the HAA represented at three different rostro-caudal levels, corresponding to Plate 24 (bregma −1.40), 26 (bregma −1.80), and 29 (bregma −2.30), of the rat brain atlas of Paxinos and Watson (1998). Earlier studies showed that the tripeptide thyrotropin-releasing hormone (TRH) and VGLUT2 are similarly distributed in the anterior hypothalamus (Hrabovszky et al., 2005). Based on these observations, we have performed co-localization studies of VGLUT2 and TRH mRNAs in the HAA using dual-label in situ hybridization.

**EXPERIMENTAL PROCEDURES**

**Animals**

Adult male Wistar rats (N=4; 220–240 g body weight) were purchased from the local breeding colony of the Medical Gene Technology Unit of the Institute of Experimental Medicine (Budapest, Hungary). They were kept under a 12-h light/dark schedule (lights off at 07:00 h, lights on at 19:00 h), in a temperature (22±2 °C) and humidity (60±10%) controlled environment with free access to laboratory rat food (Sniff Spezialdiaten GmbH, Soest, Germany) and tap water. Experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were reviewed and approved by the Animal Welfare Committee of the Institute of Experimental Medicine. All efforts were made to minimize animal suffering and the number of animals used.

**Schematic reconstruction of the HAA**

The HAA was delimited earlier by stimulating electrically different regions of the hypothalamus (Kruk et al., 1983; Lammers et al., 1988). Altogether, these studies evaluated the behavioral effects of more than 1000 electrode placements. Placements triggering no response, attacks and/or other behaviors (e.g. social grooming, teeth chattering) were plotted against a stereotaxic atlas of the hypothalamus (van der Poel et al., 1983). From these studies, a distinct area emerged from which attacks could be elicited with high probability (>80%). In the present study, the coordinates of the outlines of the aggressive area (as determined earlier by non-parametric discriminant analysis) were carefully transferred to fit the atlas of Paxinos and Watson (1998). To facilitate visualizing the extent and orientation of the HAA with respect to major, classical anatomical landmarks of the hypothalamus, a computer-based 3-D reconstruction method was used that rendered the data points delimiting the outlines of the HAA into a smooth semi-transparent shape. Following the same procedure, the bottom of the brain, the ventricle, the fornix, the paraventricular nucleus of the hypothalamus and the ventromedial hypothalamic nucleus were also plotted to facilitate orientation. The characteristics of the HAA will be described in the Results section. We assessed the distribution of glutamatergic and GABAergic neurons at three levels of this shape: an anterior level (bregma −2.3), a posterior level (bregma −1.4) and a more caudal level (bregma −1.8). At these levels, the cross-section of the shape outlined by the above procedure was overlapped with single- and double-labeled tissue sections as shown below. The correspondence of original observations (Kruk et al., 1983; Lammers et al., 1988) and the HAA outlines used in this study was again carefully checked based on the location and shape of hypothalamic structures (such as different nuclei, the base of the brain, the fornix, etc.).

**Single-label in situ hybridization studies**

**Tissue preparation.** Four rats were decapitated and their brain snap-frozen on powdered dry ice. Twelve-micrometer-thick coronal sections were cut through the HAA with a Leica CM 3050 S cryostat (Leica Microsystems Nussloch GmbH, Nussloch, Germany) and collected serially on gelatin-coated microscope slides. Alternate sections were processed for in situ hybridization detec-
tion of VGLUT2 and GAD 67 mRNAs, respectively, using methods adapted from recent studies (Hrabovszky et al., 2004a, 2005).

Preparation of hybridization probes. The preparation of the “VGLUT2-879” cDNA construct used in the present studies (targeting bases 522-1400 of rat VGLUT2 mRNA; GenBank Acc. no. NM_053427) has been detailed elsewhere (Hrabovszky et al., 2004b). To label GABAergic neurons, a 538-bp cDNA template corresponding to bases 232-769 of GAD 67 mRNA Genbank Acc. no. M 76177 was kindly made available by Dr. S. L. Petersen (University of Massachusetts, Amherst, MA, USA). The procedures used to transcribe the 35S-labeled antisense and sense probes have been detailed elsewhere (Hrabovszky et al., 2004a).

In situ hybridization and posthybridization treatments. Sections were fixed with 4% paraformaldehyde (30 min), acetylated with 0.25% acetic anhydride (10 min), then dehydrated with ethanol and diethylated with chloroform (all these materials are from Sigma Aldrich Co., Budapest, Hungary), as previously described (Hrabovszky et al., 2004a). To explore recent methodological advancements, we applied high radioisotopic probe (80,000 cpm/μl), dextran sulfate (25%) and dithiothreitol (1000 mM, both form Sigma-Aldrich Co., Budapest, Hungary) concentrations in the hybridization solution and extended the hybridization time from 16 to 40 h. Following posthybridization which included an RNase A (50 μg/ml, 30 min) digestion of probe excess, the sections were dehydrated, air-dried and coated with NTB-3 autoradiographic emulsion (Kodak; Rochester, NY, USA), deluted 1:1 with MQ water. After 2 weeks of exposure, the slide autoradiographs were developed with D19 developer (Kodak; diluted 1.1 with MQ water; 2 min), rinsed with MQ water (30 s), and fixed with Kodak fixer (5 min). Then, the slides were rinsed in several changes of chilled MQ water, air-dried on slide trays, dehydrated in ethanol (95%, 5 min; 100%, 2×5 min), transferred briefly into xylene (30 s) and coverslipped with DPX mounting medium (Fluka Chemie; Buchs, Switzerland). Digital photomicrographs of dark-field images were prepared with an RT Spot digital camera (Diagnostic Instrument, Sterling Heights, MI, USA) on a Zeiss Axioshot microscope (Zeiss; Göttingen, Germany).

Specificity control experiments. To confirm VGLUT2 hybridization specificity via positive control experiments, the “VGLUT2-879” probe was replaced with the “VGLUT2-734” probe (gift from Dr. J. P. Herman; used earlier in Ziegler et al., 2002; Hrabovszky et al., 2004b, 2005), targeting a non-overlapping segment (bases 1704-2437) of VGLUT2 mRNA. The distribution pattern of VGLUT2 hybridization signals was identical using these two probes. Similarly, a second probe for GAD 67 recognizing bases 522-1400 of rat VGLUT2 mRNA; GenBank Acc. no. M 76177 was kindly made available by Dr. S. L. Petersen (University of Massachusetts, Amherst, MA, USA) chromogen, following a tyramid signal amplification procedure detailed elsewhere (Hrabovszky et al., 2005). Then, VGLUT2 mRNA was detected on autoradiographic emulsion, as described above for single-labeling experiments.

Regional analysis of GABAergic, glutamatergic and TRH neurons within the HAA

To facilitate the identification of anatomical structures expressing hybridization signals, sections adjacent to those hybridized for GAD 67 and VGLUT2 mRNAs were counterstained with Cresyl Violet. Additional confirmation for the correct rostro-caudal localization of sections within the brain was provided by anatomical structures that showed prominent GABAergic or glutamatergic properties. Following single-labeling experiments, low-power dark-field photomicrographs were prepared from those hypothalamic sections that best resembled plates 24 (bregma −1.4), 26 (bregma −1.8), and 29 (bregma −2.3) of the brain atlas of Paxinos and Watson (1998). Digital photomicrographs were prepared from these sections, stored as Adobe Photoshop (version 5.5) files and processed at 300 dpi resolution. Corresponding atlas schemas of equal magnifications at each of the three rostro-caudal levels were placed in separate layers of these files. In addition, grids outlining the HAA were overlaid with these images. The cells of the grids (150 by 150 μm; also used previously for the illustration of the HAA; Lammers et al., 1988) approximated the area affected by stimulation (van der Poel et al., 1983). To obtain an estimate on the relative ratio of GABA vs. glutamate neurons in single subdivisions of the HAA, autoradiographic clusters were counted within the HAA (areas covered by the red shape on Fig. 1). The analysis of TRH and VGLUT2 mRNAs in dual-labeled specimens was performed similarly, except for using bright-field illumination to prepare photomicrographs. Printed photographs that also contained the grid outlining the HAA were prepared to serve as reference images. Constant monitoring of the HAA in these prints, while analyzing single- and dual-labeled neurons at high-power, ensured that only cells within the HAA were considered. Cell counting used one section per region from each of the four rats and the percent ratio of dual-labeled neurons was expressed as mean±S.E.M.

RESULTS

The HAA

At certain hypothalamic locations, electrical stimulation activates the behavioral program for attack, resulting in a well-coordinated behavioral response (Kruk et al., 1983; Lammers et al., 1988; van der Poel et al., 1983). The response is not stereotyped, but continuously influenced by external and internal sensory information (e.g. no attack-like behavior is triggered in the absence of a partner, rats follow the partner and overcome its resistance, etc.). Attacks induced by HAA stimulation were two times more likely to be aimed at vulnerable targets (e.g. head) than territoriality-related attacks (Kruk et al., 1990). It was suggested earlier that attack targeting is different in offensive and defensive aggression (Blanchard and Blanchard, 2003). Nevertheless, the share of vulnerable targets was far from the levels seen in defensive aggression, suggesting that HAA stimulation-induced attacks were targeted randomly (vs. a clear-cut targeting of non-vulnerable body
parts in territorial aggression). The response area for attack is completely surrounded by non-attack zones from where either no behavioral response or entirely different responses could be induced. The response area for attack was located in the antero-posterior midsection of the hypothalamus, ventral to the fornix. It was not limited to a conventional nucleus or area, and encompassed: (1) part of the anterior hypothalamic nucleus rostral to the ventromedial nucleus; (2) the ventromedial hypothalamus at the level of the dorsal supraoptic commissure; (3) the ventrolateral part of the ventromedial nucleus; (4) the subfornical hypothalamus; (5) the area between the ventromedial hypothalamus and the medial forebrain bundle (the latter structure is outside the HAA) (Fig. 1). The HAA does not fall apart into segments but constitutes one continuous area. Social grooming (another type of social behavior) can also be induced from the hypothalamus, but the attack and social grooming areas show very limited overlap. Overlap with hypothalamic regions involved in defensive aggression was also limited (see introduction). In contrast, there was a considerable overlap with areas from which teeth chattering (another conspecific aggression-related

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**Abbreviations used in the figures**

- AHA anterior hypothalamic nucleus, anterior part
- AHC anterior hypothalamic nucleus, central part
- ARC arcuate nucleus
- BST bed nucleus stria terminalis
- F fornix
- LA lateroanterior hypothalamic nucleus
- LH lateral hypothalamic area
- MPO medial preoptic nucleus
- OX optic chiasm
- Pa paraventricular nucleus
- Pe periventricular nucleus
- PVN paraventricular nucleus of the hypothalamus
- RCh retrochiasmatic area
- Sch suprachiasmatic nucleus
- SO supraoptic nucleus
- TC tuber cinereum area
- IIIV third ventricle
- VMH ventromedial nucleus
- VMHVL ventromedial nucleus, ventrolateral part
behavioral response) could be elicited. Later experiments confirmed early information on the localization of the HAA: electrode placements that induced attacks were always within the HAA (see e.g. Kruk et al., 2004).

Distribution of GABAergic and glutamatergic neurons in the HAA

The distribution of GABAergic and glutamatergic neurons was very similar in the four rats examined. To illustrate the findings, photomicrographs from rat 545 were used (Fig. 2A–H). Cell density within the HAA (within the areas covered by grids in Fig. 2) was around 850/mm² (920, 783, and 897 per mm² in the rostral, middle and caudal sections, respectively). As shown below, the relative distribution of glutamatergic and GABAergic neurons varied along the levels investigated.

**Bregma -1.40.** Most rostrally, the HAA overlapped with the lateroanterior hypothalamic nucleus and the area between this nucleus and the optic commissure (Fig. 2A). Both structures were predominantly occupied by glutamatergic neurons (79.8% of counted neurons were glutamatergic, whereas 20.2% were GABAergic; Fig. 2C). The strongest VGLUT2 signal was noticed in the central aspect of the HAA at this level. The medial aspect of the HAA also contained scattered GABAergic neurons. In addition, a narrow mantle zone exhibiting GABAergic dominance occurred above the optic chiasm (Fig. 2B). Medial to the HAA, a large GABAergic area was found, which corresponded to the medial preoptic and suprachiasmatic nuclei (Fig. 2B). A robust group of VGLUT2 mRNA expressing neurons, distinct from those in the HAA, was identified in the lateral hypothalamic...
area (Fig. 2C). This second excitatory cell mass was separated from the smaller glutamatergic cell group of the supraoptic nucleus by a perinuclear GABAergic neuronal population (Fig. 2B and 2C).

Bregma –1.80. At this level, the HAA overlapped with the latero-ventral subdivision of the central anterior hypothalamic nucleus and an area corresponding to the retrochiasmatic area (Fig. 2D). Glutamatergic neurons covered the whole HAA at this level as well. Overall, 58.4% of counted neurons were glutamatergic, whereas 41.6% were GABAergic. Neurons most strongly expressing VGLUT2 mRNA formed a laterally convex arch, which connected to the perifornical region and approached the lateral pole of the paraventricular nucleus (Fig. 2F). The central aspect of this arch included a dominantly (almost exclusively) glutamatergic cell population (compare Fig. 2E and 2F), whereas both GABAergic and glutamatergic neurons occurred in the medial and ventral aspects of the HAA. In this subdivision, GABAergic cells outnumbered glutamatergic ones (Fig. 2E and 2F). Reminiscent to the findings at bregma −1.40, a second large glutamatergic cell group was located lateral to the HAA, within the lateral hypothalamic area (Fig. 2F). Medial and dorsal to the HAA, the periventricular and paraventricular hypothalamic nuclei, respectively, could be distinguished. These nuclei were overwhelmingly glutamatergic.

Bregma −2.30. The caudal portion of the HAA corresponded to the ventrolateral part of the ventromedial hypothalamic nucleus, and the tuber cinereum area, lateral and dorsal to the former (Fig. 2G). Glutamatergic neurons could be observed in the whole HAA at this level as well. Heavily labeled VGLUT2 mRNA expressing neurons were found to occupy the whole ventromedial nucleus, including its ventrolateral subnucleus from which attacks can be elicited (Fig. 2I). Within this subdivision, 88.3% of counted neurons were glutamatergic, and only 11.7% were GABAergic. The tuber cinereum area showed a slight dominance of GABAergic neurons (52.4%) but also contained intermingled glutamatergic cell bodies (47.6%; Fig. 2H). Next to the HAA, two major GABAergic neuronal populations were found dorsally and medially. The dorsal cell group appeared to correspond to the dorsomedial hypothalamic nucleus, whereas the medial cell group corresponded to the hypothalamic arcuate nucleus (Fig. 2H). Lateral to the HAA, a weak VGLUT2 staining was found in the lateral hypothalamic area (Fig. 2I).

Co-localization of TRH with VGLUT2 mRNA in the HAA

Neurons containing TRH mRNA were observed at all rostro-caudal levels of the hypothalamus that were included in the analysis. The distribution of TRH neurons in the anterior hypothalamic (Fig. 3A), retrochiasmatic, and tuber cinereum areas showed a marked overlap with that of glutamatergic cells, whereas they were absent from the ventromedial hypothalamic nucleus. Virtually all TRH neurons within the HAA were labeled for VGLUT2, showing also that glutamatergic but not GABAergic neurons co-expressed TRH. The analysis of a total of 4332 VGLUT2 expressing neurons in the HAA from four rats showed that the ratio of dual-labeled vs. single-labeled VGLUT2 neurons was 35.2 ± 7.1% at bregma −1.4, 27.6 ± 4.5% at bregma −1.8, and 33.2 ± 2.0% within the tuber cinereum area at bregma −2.3 (mean ± S.E.M.). The ventromedial hypothalamic nucleus was consistently devoid of the TRH hybridization signal; thus, it was clearly distinct from other subdivisions of the HAA.

Co-expression of TRH with VGLUT2 also occurred sporadically in other regions including the periventricular hypothalamic nucleus, and—in agreement with previous observations (Hrabovszky et al., 2005)—more abundantly in the paraventricular nucleus. These neurons were relatively distant from the HAA, and could be clearly distinguished from TRH/VGLUT2 neurons of the latter. In contrast with the HAA, the majority of glutamatergic cells in the lateral hypothalamic area lacked TRH mRNA (Fig. 2A).

DISCUSSION

The aim of the present study was to describe the distribution of the major excitatory (glutamatergic) and inhibitory (GABAergic) neurons within a hypothalamic structure that controls attack behavior. The whole HAA was densely populated by neurons. We have found that glutamatergic neurons were present in the whole extent of this area. Their abundance was larger than that of GABAergic cells except for the tuber cinereum area where GABAergic neurons slightly dominated. A large subpopulation of glutamatergic neurons was found to express TRH at all rostro-caudal levels of the HAA, except for those in the ventromedial hypothalamic nucleus, which were consistently devoid of TRH labeling. Thus, the neuron subtypes identified within the HAA include: glutamatergic neurons, glutamatergic neurons co-expressing TRH, and GABAergic neurons.

The HAA was amply characterized in a number of species (including humans) by electrophysiological and lesion techniques (Adams, 1971; Kruk, 1991; Olivier, 1977; Olivier et al., 1983; Siegel et al., 1999; Sano et al., 1966; Ramamurthi, 1988). The HAA appears distinct from the hypothalamic structures involved in defense, despite the vicinity of the structures involved. E.g., defense appears to be controlled (among other centers) by the dorsomedial VMH (Thompson and Swanson, 2003), whereas the HAA includes the ventrolateral VMH. Despite the fact that the spatial localization of the HAA was precisely described, two major issues remained unclear: the dispersed nature of the attack area (e.g. the neuron content of HAA subdivision that is outside the known nuclei) and the chemotype of neurons activated by electrical stimulation.

The HAA extends over several hypothalamic nuclei; moreover, attacks can be elicited only from sub-regions of these nuclei. For example, only electrode stimulation in the ventrolateral subnucleus of the ventromedial hypothalamic
nucleus can induce aggressive behavior. In addition, certain regions of the HAA are located outside the nuclei that are represented in brain atlases, ostensibly suggesting that attacks can be elicited by stimulating white matter. Thus, one could assume that passing fibers have a role in the induction of attacks. There are reasons showing, however, that this assumption is false. Firstly, the stimulation of efferent and afferent pathways of the HAA (identified earlier by Roeling et al., 1994) does not give rise to the expression of complete attack patterns. Secondly, attacks could be elicited by the local infusion of glutamate agonists and GABA antagonist into the HAA (Adams et al., 1993; Roeling et al., 1993; Haller et al., 1998). Such treatments are unlikely to affect passing fibers. There were some earlier attempts to describe anatomically the HAA regions that are situated outside the known nuclei. A relatively good match exists between nucleus-unrelated HAA subdivisions and a region defined (based on cytoarchitectonic criteria) as the intermediate hypothalamus (Geeraedts et al., 1990a,b). Cell density within the intermediate hypothalamus is approximately one third of that seen in the paraventricular nucleus of the hypothalamus (Aalders and Meek, 1993). Synaptic density, however, is large (about three times larger than in the paraventricular nucleus). The present findings further support the notion that the HAA does not cover areas occupied predominantly by “white matter.” VGLUT2, GABA or both staining were present over the whole extension of the HAA, and cell density was relatively high (approximately 850 neurons per mm² on average). Even if it is not limited to a conventional nucleus or area, the HAA appears to be a well-defined functional unit including a column of glutamatergic neurons associated with a population of GABAergic neurons mainly in its posterior subdivisions. Noteworthy, the distribution of glutamatergic cells rather followed the HAA than the nuclei outlined in brain atlases. E.g. at bregma —1.4, glutamatergic nuclei highly overlapped with the HAA, despite fact that this covered the lateroanterior hypothalamic nucleus.
and the area between this nucleus and the optic commissure. Similarly, many HAA-related glutamatergic cells were found outside the known nuclei at bregma – 1.8 and –2.3.

As attack can be elicited by stimulation, and stimulatory (glutamatergic) neurons (with one exception) dominate over GABAergic neurons in the HAA, our findings suggest that glutamatergic neurons play a major role in the induction of attacks. We found that there are relatively large areas within the HAA where the dominance of the glutamatergic over GABAergic neurons is almost total (see e.g. the central region of the anterior hypothalamic nucleus at bregma – 1.4 and –1.8, as well as the ventrolateral subdivision of the ventromedial nucleus at bregma –2.3). As glutamatergic domination was overwhelming in these subregions, glutamatergic neurons are very likely candidates to mediate the behavioral effects of stimulation. GABAergic neurons were slightly dominant over glutamatergic neurons only in the tuber cinereum area, from where attacks could also be elicited. It is worth of note, however, that the attack response gradually changed over a medio-lateral continuum in earlier experiments (Lammers et al., 1988).

The most vigorous forms of attacks (e.g. attack jumps to head and neck at low thresholds) were seen with electrodes located in the medial subdivisions of the HAA (i.e. from the ventromedial nucleus at bregma –2.3). One can hypothesize that attacks seen after the stimulation of more lateral subdivisions (e.g. the tuber cinereum area) are milder because the number of glutamatergic cells is lower in this area. The role of GABAergic neurons in attacks is unclear at this stage. These neurons may contribute to the induction of attacks e.g. by inhibiting brain centers that inhibit this behavior (i.e. by the “inhibition of inhibition”). Noteworthy, the efferent connections of the HAA include both brain centers that facilitate or inhibit aggressiveness (Roeling et al., 1994). This assumption is supported indirectly by the finding that aggressive rats treated with anabolic steroids showed increased hypothalamic levels of GAD 65 immunoreactivity (which suggests a positive relationship between hypothalamic GABA synthesis and aggressiveness; Grimes et al., 2003). Alternatively, GABAergic neurons may establish connections with neighboring hypothalamic regions. In the cat, hypothalamic regions from where conspecific (rage) and predatory (quiet) attacks can be induced reciprocally inhibit each other via GABAergic mechanisms (Siegel et al., 1999). Importantly, many projections of hypothalamic neurons remain within the hypothalamus in rats (Thompson and Swanson, 2003). Tentatively, GABAergic neurons may play a role in inhibiting other behavioral (or non-behavioral) responses controlled by the hypothalamus.

An interesting finding of the present study is the co-expression of the VGLUT2 and TRH signals in a large subpopulation of glutamatergic HAA neurons. The interpretation of this finding is difficult at present, but one can assume that glutamatergic neurons co-expressing TRH fulfill specific roles that are different from those fulfilled by glutamatergic neurons free of the TRH signal. Importantly in this respect, perifornical hypothalamic TRH neurons were shown to specifically target the lateral septum (Ishikawa et al., 1986), an area that is heavily involved in the control of aggression (Siegel et al., 1999). Our post hoc analysis of published figures in this report suggests that many retrogradely labeled TRH cells were located within the HAA. As the HAA in general establishes efferent connections with a large number of brain areas, whereas a subpopulation of HAA neurons (the TRH containing glutamatergic cells) appears to target a specific region (the lateral septum), these neurons might play a specific role in controlling attacks. The present studies raise the possibility that the TRH signal can be used to phenotypically mark this subpopulation of glutamatergic HAA neurons, including their efferent fibers. A few neurons of the periventricular hypothalamic nucleus and many neurons of the paraventricular nucleus did co-express VGLUT2 and TRH mRNAs, but these neurons were relatively distant from the HAA, and could easily be differentiated. Thus, the phenotypic identification of HAA TRH neurons (and their efferent connections) appears feasible, and may become useful for the elucidation of the role played by this neuronal subpopulation in attacks.

CONCLUSIONS

In summary, this report demonstrates that most subdivisions of the HAA are dominantly populated by glutamatergic neurons of the VGLUT2 phenotype. Glutamatergic neurons are intermingled with GABAergic neurons in certain subdivisions of the HAA, whereas the tuber cinereum region in the most caudal sections of the HAA is slightly dominated by GABAergic neurons (containing, however, many glutamatergic neurons). We found that a large subpopulation of glutamatergic HAA neurons contains TRH mRNA. Naturally, areas included in the HAA have various functions. E.g. the anterior hypothalamic nucleus controls blood pressure, whereas the ventromedial nucleus is involved in feeding and sexual behavior (King, 1991; Segovia and Guillamon, 1993; Wyss et al., 1999). However, the involvement of the HAA in attack is also clear, showing that at least part of the neuron types identified in this study are specifically involved in the control of this behavior. Our findings provide a background for understanding the organization of hypothalamic structures involved in the induction of attacks.

Acknowledgments—The support of Geert-Jan Kuip of Supernova Studios Delft the Netherlands in producing the 3-D outline of the HAA was invaluable. The authors are grateful to Drs. J. P. Herman, S. L. Petersen and R. M. Lechan for providing their VGLUT2, GAD 67 and TRH cDNA constructs, respectively, and to Gy. Kékesi for the excellent technical assistance. This research was supported by grants from the National Science Foundation of Hungary (T43407, T46574, T046785), the EU FP6 program (contract No. LSHM-CT-2003-503041), and the Harry Frank Guggenheim Foundation (grant to J. Haller). Note: This publication reflects the author’s views and not necessarily those of the EU. The information in this document is provided as is and no guarantee or
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(Accepted 17 March 2005)