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NEUROCHEMICAL CHARACTERIZATION OF HYPOTHALAMIC NEURONS INVOLVED IN ATTACK BEHAVIOR: GLUTAMATERGIC DOMINANCE AND CO-EXPRESSION OF THYROTROPIN-RELEASING HORMONE IN A SUBSET OF GLUTAMATERGIC 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 connections of these neuron subpopulations would contribute to our understanding of the mechanisms underlying attack behavior and aggression.

Key words: aggressive behavior, hypothalamus, glutamate, GABA, thyrotropin releasing hormone, VGLUT2.

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 area[1] of the rat (HAA) was described in detail earlier (Kruk et al., 1983, 1984a,b Lammers et al., 1988). The functional area from which attacks can be elicited extends over a relatively large hypothalamic region that includes the lateroanterior hypothalamic and the anterior hypothalamic nuclei, the retrochiasmatic area, the ventrolateral part of the vetromedial 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-

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Abbreviations: GAD 65, glutamic acid decarboxylase, 65 kDa isoform; GAD 67, glutamic 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 Geeraedts et al. (1990a,b). The nomenclature of different hypothalamic structures are similar to that used by Paxinos and Watson (1998).

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sumption (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 phenotype in neurons (Bellocchio et al., 2000; Takamori et al., 2000). Out of the three VGLUT proteins identified so far, the hypothalamus selectively expresses the VGLUT2 isoform (Ziegler et al., 2002; Lin et al., 2003). Consequently, the glutamatergic neuronal phenotype within the HAA can be identified via the *in situ* hybridization of VGLUT2 mRNA. The hypothalamus is also known to host a large number of inhibitory GABAergic neurons, which can be identified by the *in situ* hybridization of the mRNAs encoding the glutamic acid decarboxylase isoforms (GAD 65 and GAD 67) (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 Spezialdiaeten 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 −1.4) a posterior level (bregma −2.3), and midway between the two (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-