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Substance P neurotransmission and violent aggression: The role of tachykinin NK_1 receptors in the hypothalamic attack area

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ABSTRACT

Substance P and its tachykinin NK_1 receptors are highly expressed in brain regions involved in emotional control. We recently showed that NK_1-mediated substance P neurotransmission is deeply involved in the control of aggressiveness. To get further insights into the NK_1 receptor/aggression relationship, we studied the role of NK_1 receptor-expressing neurons of the hypothalamic attack area, the only brain region in rats from which biting attacks can reliably be elicited by both electrical and neurochemical stimulation. We show here that the hypothalamic attack area preferentially expresses the NK_1 type of tachykinin receptors. When such neurons were lesioned by substance P-conjugated saporin (SP-sap) infused into the hypothalamic attack area, violent attacks were dramatically reduced, whereas milder forms of aggression (soft bites and offensive threats) remained unaltered. The lesions were neuron type-specific as SP-sap lesions markedly reduced NK_1 staining without significantly affecting total cell counts. NK_1 staining in the neighboring lateral hypothalamus was not affected, which confirms the spatial specificity of the lesion. Surprisingly, the lesions also reduced anxiety-like behavior in the elevated plus-maze. This effect is likely explained by the extensive connections of the hypothalamic attack area with brain regions involved in the control of anxiety. The present findings suggest that violent and milder forms of attack are differentially controlled. NK_1 receptor-expressing neurons of the hypothalamic attack area are tightly and specifically involved in the former but not in the latter. Our data also raise the possibility of a coordinated control of violent attacks and anxiety by the same NK_1-expressing neurons.

1. Introduction

Anatomical studies have shown a high expression of tachykinin NK_1 receptors in brain regions that are important for the control of affective behaviors and stress responses (Tsuchida et al., 1990; Yip and Chahl, 2000; Saffroy et al., 2003; Czeh et al., 2006). Therefore, the substance P-NK_1 receptor pathways have been repeatedly and still implicated in the pathophysiology of psychiatric disorders, especially of anxiety and depression, despite some discouraging clinical trials (Kramer et al., 1998; File, 2000; Rupniak et al., 2001; Bilkei-Gorzo et al., 2002; Santarelli et al., 2002; Czeh et al., 2006). Importantly, the brain distribution of NK_1 receptors appears to be remarkably consistent across species (Rigby et al., 2005; species compared: rats, guinea-pigs, gerbils, and marmosets).

Our recent studies suggest that the tachykinin NK_1 receptor plays an important role also in the control of aggressive behavior (Halasz et al., 2008). In a double labeling study, we have shown that resident/intruder conflicts markedly activate NK_1 receptor-expressing neurons in the medial amygdala and hypothalamic attack area, two brain regions that are intimately involved in the control of attacks. The activation was behavior-specific, as psychosocial encounters — that involve sensory but not physical contacts between subjects — lead to a markedly lower activation. In addition, we showed that the systemic administration of the NK_1 blocker L-703,606 dramatically decreased hard bites without affecting soft bites. Other behaviors (e.g. exploration, social interactions, offensive and defensive postures as well as dominance-related behaviors) remained unchanged as well. Violent but not other forms of attack were also inhibited by L-703,606 in a recently developed model of abnormal aggression (Haller and Kruk, 2006). Taken together, these findings suggest that NK_1 receptor-expressing neurons are specifically involved in the control of violent attacks (Halasz et al., 2008).

We hypothesized that the effects of tachykinin NK_1 receptor blockade were primarily mediated by NK_1 receptors located in the hypothalamic attack area. The role of this brain region in attack behavior was described in early 20th century (Hess, 1928), and was amply confirmed later (Kruk, 1991; Siegel et al., 1999). This is the only brain region in rats from which attacks can rapidly, specifically and reliably elicited by electrical stimulation. Aggressive behaviors other than attack are not affected by stimulation. Notably, hypothalamic regions with similar roles were identified in all the species studied so

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far; moreover, the results of psychosurgery confirm that it exists in humans as well (Sano et al., 1966). The hypothalamic attack area largely corresponds to the intermediate hypothalamus (Geeraedts et al., 1990). Cell density within this region is approximately one third of that seen in the paraventricular nucleus of the hypothalamus; yet, synaptic density is about three time larger (Aalders and Meek, 1993). We have recently provided a three-dimensional reconstruction of this area in rats, and characterized it neurochemically (Hrabovszky et al., 2005).

There are two reasons to believe that NK_1 receptor-expressing neurons of the hypothalamic attack area play important roles in mediating the effects of NK_1 blockers: (i) these blockers selectively inhibited attacks, without affecting other forms of aggression (Halasz et al., 2008), (ii) substance P-ergic fibers originating from the medial amygdala innervate attack-related hypothalamic structures, and significantly contribute to the expression of attacks (Shaikh et al., 1993; Siegel et al., 1999; Yao et al., 2001).

The role of NK_1 receptor-expressing neurons was studied here by means of saporin-conjugated substance P (SP-sap) that was infused locally into the hypothalamic attack area. Saporin is a ribosome inactivating protein from *Saponaria officinalis*. When conjugated with neuropeptides, it can be used to selectively lesion neuron populations that express the receptors of these. The local infusion of SP-sap results in the specific lesioning of neurons that express NK receptors, while other cells remain unaffected (Mantyh et al., 1997; Wiley and Lappi, 1997; Wiley and Kline, 2000; Suzuki et al., 2002; McKay et al., 2005). The hypothalamus expresses both the NK_1 and NK_3 receptors the latter being densely expressed in various regions, including the paraventricular, arcuate, and perifornical nuclei (Tsuchida et al., 1990; Yip and Chahl, 2001; Saffroy et al., 2003). A close inspection of published photomicrographs shows that the hypothalamic attack area does not express NK_3 receptors (Langlois et al., 2001; Krajewski et al., 2005; Burke et al., 2006). Yet, the issue was not studied specifically.

In the present experiments, we (i) investigated the distribution of tachykinin NK_1 and NK_3 receptors in the area surrounding the hypothalamic attack area; (ii) locally infused SP-sap into this brain area to lesion tachykinin receptor-expressing neurons, and (iii) evaluated the effects of the lesion in the resident/intruder test of aggression. The efficacy, cell type and spatial specificity of SP-sap lesions were checked. The hypothalamic attack area is highly interconnected with brain nuclei that control anxiety e.g. the prefrontal cortex, amygdala, and aminergic nuclei (Roeling et al., 1994; Delville et al., 2000; Petrovich et al., 2001; Hur and Zaborszky, 2005). Therefore, we also investigated the effects of the lesions in the elevated plus-maze test of anxiety.

2. Materials and methods

2.1. Animals

Subjects were 2–3 months old male Wistar rats (Charles River Laboratories; Hungary) weighing 400–450 g. Groups of 4 rats were housed before experimentation in 1354G Eurostandard Type 4 cages ($59.5 \times 38 \times 22$ cm). After surgery, they were transferred to individual cages of $60 \times 40 \times 50$ cm. Cage walls were opaque except for the front wall, which was transparent. Standard laboratory rat food (Charles River Laboratories, Hungary) and tap water were available *ad libitum*, while temperature and relative humidity were kept at 22 ± 2 °C and 60 ± 10%, respectively. Rats were maintained in a reversed light/dark cycle of 12 h with lights off at 10:00 h. Acclimatization to the day/night schedule lasted 2 weeks.

Male Wistar rats weighing approximately 300 g were used as opponents in aggressive encounters. These rats were group housed but otherwise maintained under similar conditions. Each intruder was used only once.

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.

2.2. Experimental design

Experiment 1 investigated the distribution of tachykinin NK_1 and NK_3 receptors in the hypothalamic attack area. We performed this experiment to evaluate the involvement of neurons bearing these receptors in mediating the effects of SP-sap lesions studied in Experiment 2. Earlier experiments suggested that the hypothalamic attack area expresses NK_1 but not NK_3 receptors (Langlois et al., 2001; Krajewski et al., 2005; Burke et al., 2006; Halasz et al., 2008). Yet, this brain area has never been directly investigated in this respect. We stained in 8 rats hypothalamic sections anterior to, coincident with, or posterior to the hypothalamic attack area for either the NK_1 ($N = 4$) or the NK_3 receptor ($N = 4$).

In *Experiment 2*, the hypothalamic attack area of rats was bilaterally infused with either vehicle or SP-sap. After 1 week recovery, rats were submitted to the resident/intruder test in the early hours of the dark period. Behavior was video recorded through the transparent front wall of the cage and was later analyzed by means of a computer-based event recorder by an experimenter blind to the treatments. One week later, rats were studied in the elevated plus-maze test in the early hours of the dark phase. Behavior was video recorded by an experimenter blind to the treatments. The order of testing was randomized in both tests. After the completion of the experiments, the efficacy and selectivity of SP-sap treatments was assessed by immunocytochemical methods. In particular, we stained the hypothalamic attack area for both the NK_1 receptors and the neuronal marker NeuN (Wolf et al., 1996) to evaluate the efficacy and specificity SP-sap lesions, respectively. The spatial extension of the lesions was evaluated by staining NK_1 receptors in the lateral hypothalamic area, a brain structure adjacent to the hypothalamic attack area, which contains a significant population of NK_1-positive neurons.

Rats showing extensive reductions in NeuN staining — i.e. extensive non-specific neuronal damage — were excluded from the analysis. The resulting sample size was 10 for controls and 6 for the SP-sap-lesioned rats.

2.3. Brain surgery

Rats were anaesthetized with a mixture of ketamine, xylazine, and promethazine (50–10–5 mg/kg i.p.), and their hypothalamic attack area was bilaterally infused with 6.25 ng SP-sap dissolved in 0.5 µl saline. Controls received 0.5 µl saline. The coordinates of the infusions were as follows: antero-posterior from Bregma: −2.3 mm, dorso-ventral from dura: 9.8 mm, medio-lateral from Bregma: 1.3 mm. These coordinates were based on the atlas of Paxinos and Watson (1998). The infusions were administered *via* fused-silica capillaries (outer diameter: 100 µm), and lasted 3 min. The capillaries were kept in place for 2 min after the completion of the infusion.

Dose-choice was based on preliminary experiments in which 2.5, 3.75 or 6.25 ng SP-sap was infused into the hypothalamic attack area of rats. We evaluated the extent of the lesion by NK_1 receptor immunocytochemistry. With the largest dose (6.25 ng) the radius of the lesion was 450 µm, which approximated the size of the hypothalamic attack area well (Hrabovszky et al., 2005).

2.4. Resident/intruder test

Subjects were faced with smaller opponents for 20 min in their home-cage. The encounter was performed under dim red illumination that was provided by two 25 W red lamps. Behavior was video recorded and scored later by an experimenter blind to the treatments,

by means of a computer-based event recorder (H77, Budapest, Hungary). The duration of the following behavioral variables was recorded: *resting* (no obvious actions), *exploration* (walking and/or sniffing directed towards the environment), *social investigation* (sniffing directed towards the opponent's flank, nasal or anogenital region), *self care* (self-grooming with forepaws and scratching with hind legs), *offense* (aggressive grooming, lateral threat, offensive upright posture, mounting and chasing taken together), *defense* (defensive upright, defensive kick, fleeing and freezing taken together), *dominant posture* (keeping down the opponent while he is laying on his back), *subordinate posture* (laying on back while kept down by the opponent). We mention that submission was very low in subjects and the few cases when it occurred were when the resident lost balance due to a push from the intruder.

Attack episodes were analyzed in detail at low speed (frame-by-frame when necessary) for identifying the type of attacks. An attack was identified as *hard bite* when it involved kicking (clinch fights) or induced a strong startle response in the intruder (large jumps or immediate submission). *Soft bites* were not associated with kicking and induced no response or mild quivering only. Similar approaches were employed earlier (Lammers et al., 1988; Ogawa et al., 2000; Halasz et al., 2008).

2.5. The elevated plus-maze test

The apparatus was made of dark grey painted wood (arm length 50 cm, arm width 17 cm, wall height 30 cm and platform height 80 cm). The plus-maze was illuminated by a red lamp of 40 W (~1 lx). Subjects were placed in the central area of the apparatus with head facing a closed arm. Exposure lasted 5 min. Closed-arm entries were considered indicators of locomotor activity whereas open arm exploration was used as a measure of anxiety (Pellow et al., 1985). Open arm exploration was characterized by two variables: percentage time spent in the open arm, and percentage open arm entries (100 × open arm entries/total arm entries). In addition to these 'classical' measures, we analyzed risk assessment behaviors (stretch attend posture and head dipping), which are considered to be ethological measures of anxiety in the elevated plus-maze (Cole and Rodgers, 1993; Rodgers and Dalvi, 1997). Stretch attend posture was defined as exploratory posture in which the body is stretched forward and then retracted to the original position without any forward locomotion. Head dipping consisted of exploratory movements of head/shoulders over the side of the maze. Head dipping and stretch attend postures were further differentiated as a function of their occurrence in different parts of the maze. Thus, the closed arms and

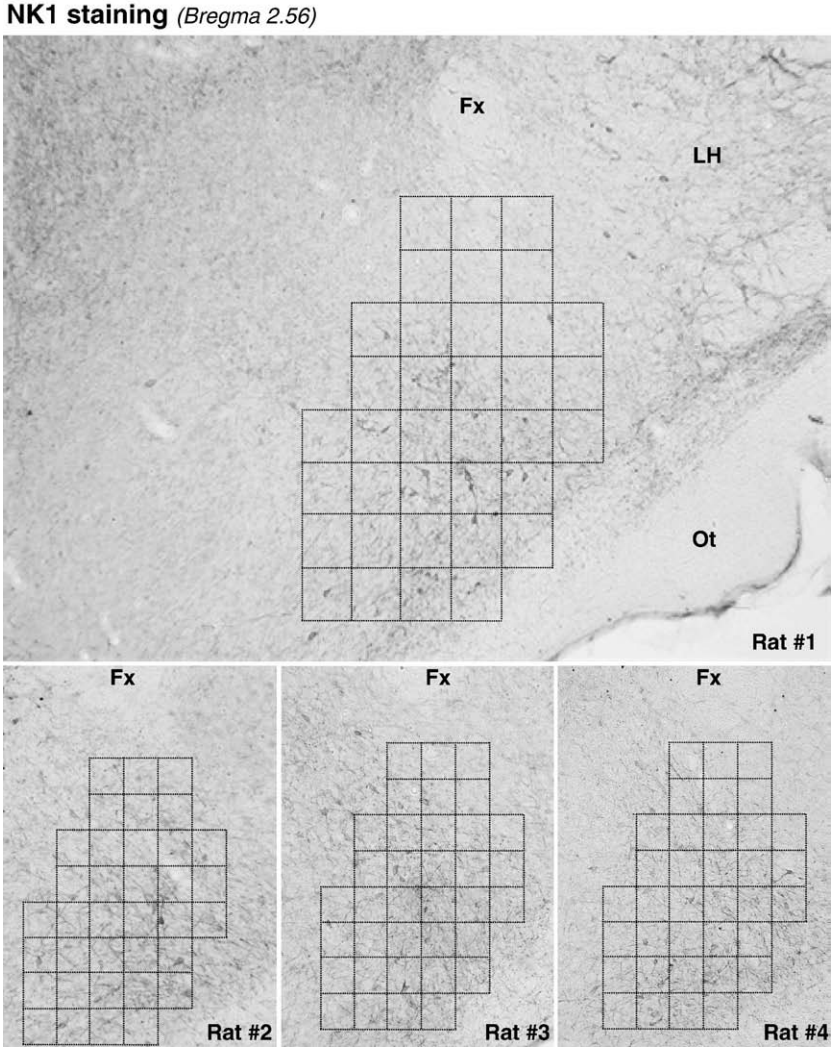

Fig. 1. The distribution of NK1 receptors in the basal hypothalamus. The whole area was shown for rat #1, whereas the relevant region was shown for rats #2–4. The grid superimposed over the microscopic image delineates the hypothalamic attack area. Its location and shape was established earlier by the use of movable electrodes (Lammers et al., 1988). The cells of the grid represent areas from which attacks could be elicited with 80% reliability. The same data set was used earlier for a three-dimensional reconstruction of the region (Hrabovszky et al., 2005). *Fx*, fornix; *LH*, lac; *Ot*, optic tract.

centre platform were together designated as "protected" areas (i.e., offering relative security), and "percent protected" scores for head dipping and stretched attend posture were calculated as the percentage of these behaviors displayed in or from the protected areas (e.g., % protected stretched attend posture = (protected stretched attend posture/total stretched attend posture) × 100). Decreased anxiety is indicated by a decrease in % protected stretched attend posture and % protected head dipping (Cole and Rodgers, 1993; Rodgers and Dalvi, 1997).

2.6. Immunocytochemistry and histological analysis

2.6.1. Brain processing

Animals were anesthetized with sodium pentobarbital (Nembutal, Sanofi, 50 mg/kg, i.p.) and perfused through the ascending aorta with 150 ml ice-cold 0.1 M phosphate-buffered saline followed by 300 ml 4% paraformaldehyde (in 0.1 M phosphate-buffered saline). The brains were removed, post-fixed in the same solution for 3 h and cryoprotected overnight by 20% sucrose in phosphate-buffered saline at 4 °C. Six series of 30 μm frozen sections were cut in the frontal plane on a sliding microtome. Section planes were standardized according to the atlas of Paxinos and Watson (Paxinos and Watson, 1998).

Immunocytochemical analysis was performed at Bregma −2.56 mm, i.e. at the widest extension of the hypothalamic attack area (Hrabovszky et al., 2005). Due to interferences between NK_1 and NeuN staining, these were performed on different but adjacent sections.

Tachykinin NK_1-positive neurons were labeled as described earlier (Halasz et al., 2008). Briefly, the sections were labeled with specific rabbit polyclonal antibodies raised against a 23 amino acid synthetic peptide corresponding to the C-terminus (amino acids 385–407) of the rat NK_1/substance P receptor (1:5000, AB 5060, Chemicon, Temecula, California, US). The primary antibodies were detected by biotinylated donkey anti-rabbit IgG (1:1000) and streptavidin conjugated HRP (1:1000) (Jackson Laboratories, Bar Harbor, Maine, USA). The peroxidase reaction was developed in the presence of diaminobenzidine tetrahydrochloride (0.5 mg/ml), and hydrogen peroxide (0.005%) dissolved in Tris buffer (pH = 7.6). NK_1 labeling was evaluated in both the hypothalamic attack area and the lateral hypothalamic area, two adjacent structures that contain significant populations of NK_1 positive neurons.

Single labeling of NK_3 positive neurons was performed as described in Mileusnic et al. (1999). The method was slightly modified as described by Halasz et al. (2008). Briefly, the sections were labeled with specific rabbit polyclonal antibodies raised against internal sequence of amino acids of the proNKB protein (1:4000, NB300-201, Novus Biologicals). The primary antibodies were detected by biotinylated donkey anti-rabbit IgG (1:1000; Jackson Laboratories,

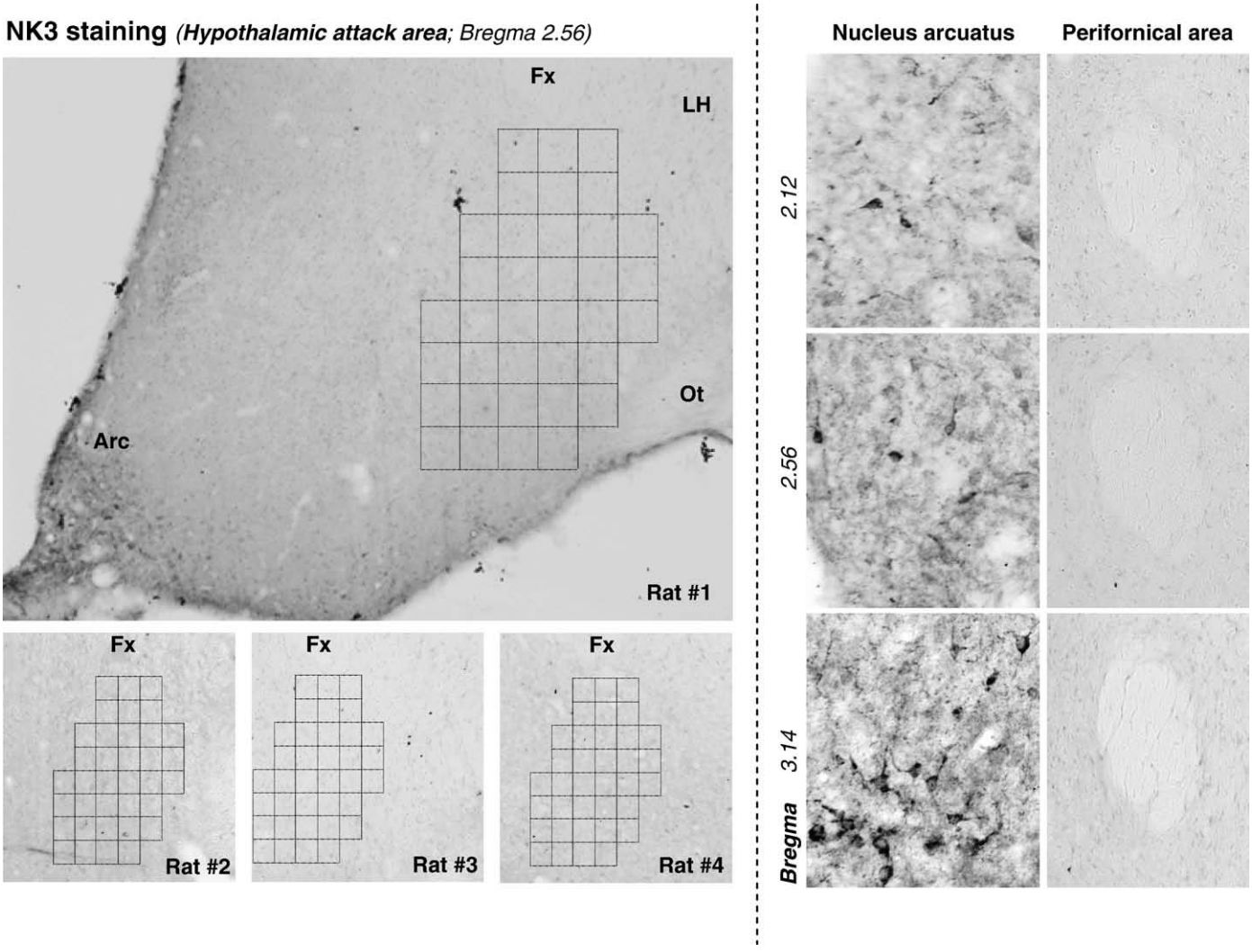

Fig. 2. The distribution of NK3 staining. Left hand panels show the whole area for rat #1, and the region containing the hypothalamic attack area for rats #2–4. This brain region was delimited by the grid that was superimposed over the photomicrographs (see the legend of Fig. 1 for further details). The right hand panels show representative photomicrographs of the arcuate nucleus and the fornix at levels −2.12, −2.56, and −3.14 from Bregma. *Fx*, fornix; *LH*, lateral hypothalamus; *Ot*, optic tract; *Arc*, arcuate nucleus.

USA) and Vectastain Elite ABC system. The peroxidase reaction was developed in the presence of diaminobenzidine tetrahydrochloride (0.5 mg/ml), nickel–ammonium sulphate (0.1%) and hydrogen peroxide (0.005%) dissolved in Tris buffer (pH = 7.6).

The neuronal marker NeuN was labeled with a mouse monoclonal anti-NeuN antibody (1:3000, MAB 377, Chemicon, US). The primary antibodies were detected by biotinylated anti-mouse donkey antibody (1:1000) and streptavidin conjugated HRP (1:1000) (Jackson Laboratories). The peroxidase reaction was developed in the presence of diaminobenzidine tetrahydrochloride (0.2 mg/ml), nickel–ammonium sulphate (0.1%), and hydrogen peroxide (0.003%) dissolved in Tris buffer.

2.6.2. Quantification of staining

The neurons labeled for tachykinin NK_1 receptors and the NeuN protein were clearly separable from the background, even the distal dendrites being identifiable with NK_1 staining. NK_1 receptor-expressing cells were counted by an investigator blind to treatments. Counts were made by viewing sections under a microscope at 500 fold magnification. NeuN protein expressing cells were counted at 250× magnification. Microscopic images were digitized by an Olympus DP70 camera and the number of positive profiles was counted by means of the ImageJ 1.34s (NIH, USA) software. Uniform thresholds were used and the minimum size of positive profiles was set at 25 pixels. The size of the scanned area was 0.318 mm^2 for both the hypothalamic attack area and the lateral hypothalamus (Halasz et al., 2002, 2008) and for both the NK_1 and NeuN labeling. Cells were counted bilaterally in two sections 180 μm apart.

2.7. Statistics

Data were shown as means ± S.E.M. Behavioral data were analyzed by Kruskal–Wallis ANOVA followed by Mann–Whitney *post-hoc* comparisons. Immunocytochemical data were analyzed by ANOVA followed by Newman–Keuls *post-hoc* comparisons. The level of significance was set at $P<0.05$.

3. Results

3.1. Experiment 1

The tachykinin NK_1 receptor was strongly expressed in the hypothalamic attack area, and the lateral hypothalamus (Fig. 1). The antibody labeled both cell bodies and a rich network of dendrites. Labeling was not uniform as strongly labeled clusters of staining were noticed on both cell bodies and dendrites (also see Fig. 3 for a higher magnification).

The NK_3 receptor was strongly expressed in the arcuate nucleus (Fig. 2). Staining was moderate anterior to, and at the level of, the hypothalamic attack area and strong in more posterior regions. As with the NK_1 receptor, strongly labeled clusters were seen on both the cell bodies and dendrites. No specific NK_3 staining was present in the hypothalamic attack area. The region surrounding the fornix was also devoid of staining. It occurs that the distribution of NK_1 and NK_3 receptors shows little overlap in the hypothalamic region investigated, the hypothalamic attack area showing strong NK_1 but not NK_3 staining.

3.2. Experiment 2

The location of infusion sites and representative photomicrographs of NK_1 staining were shown in Fig. 3. Tachykinin NK_1 receptors were uniformly dispersed throughout the hypothalamic attack area. Besides cell bodies, the dendrites also expressed NK_1 receptors, and formed a rich network within the area. Surprisingly, the number of NK_1 neurons was small, as only about 4% of hypothalamic attack area neurons expressed this receptor (total cell counts in controls was around 1550,

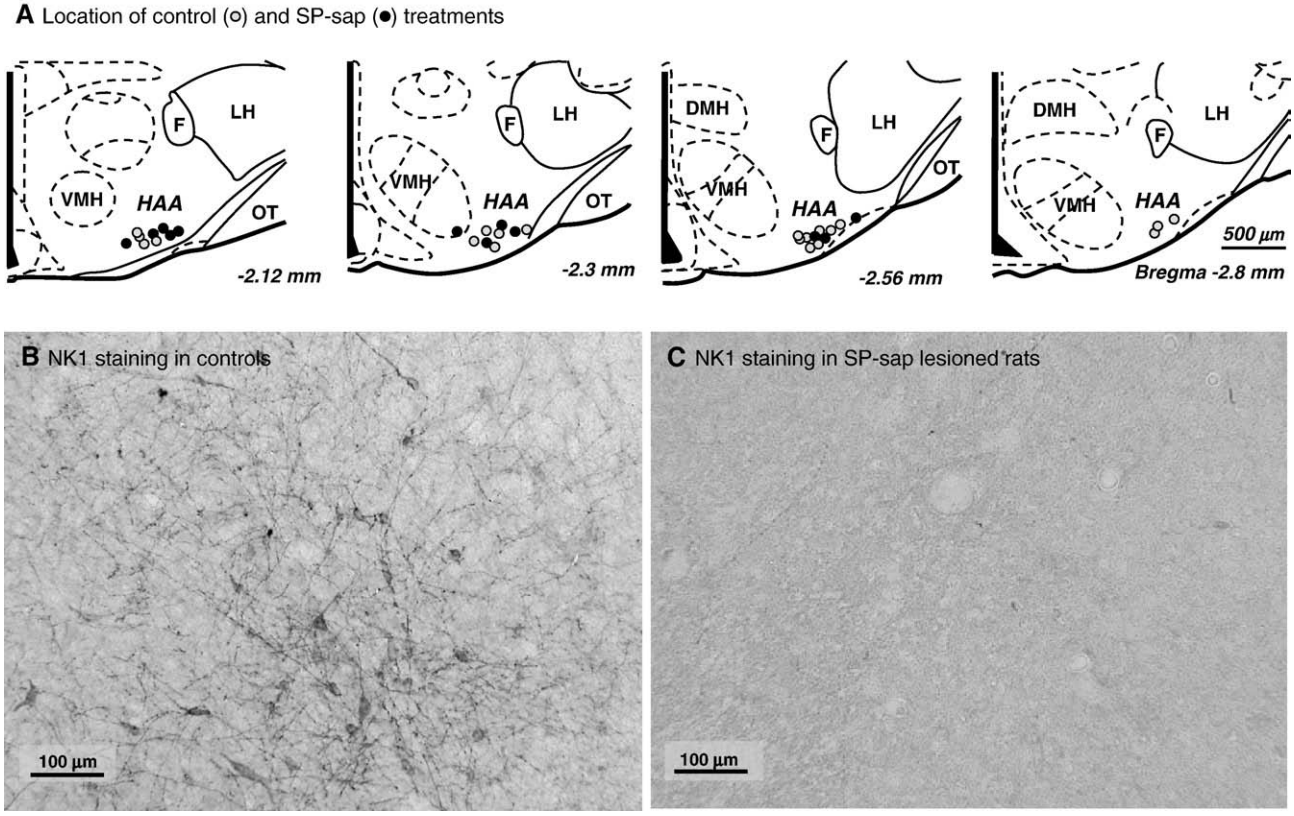

Fig. 3. The location of SP-sap infusions and representative photomicrographs showing NK1 staining. A, the placement of the tips of capillaries through which saporin-conjugated substance P (SP-sap) was infused into the hypothalamic attack area; B, NK1 staining in controls; C, NK1 staining in SP-sap lesioned rats.

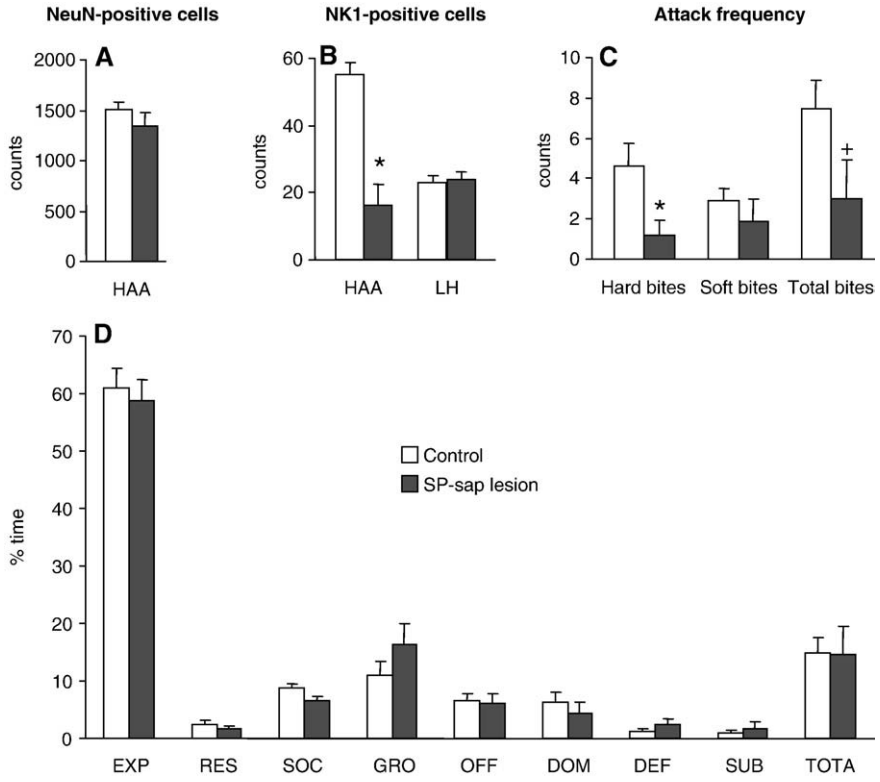

Fig. 4. The effects of SP-sap treatments on neuron counts (NeuN staining; A), NK1 staining (B), attack counts (C), and the duration of other behaviors shown in the resident–intruder test (D). HAA, hypothalamic attack area; LH, lateral hypothalamus; EXP, exploration; RES, resting; SOC, social interactions; GRO, grooming; OFF, offense; DOM, dominant posture; DEF, defense; SUB, submissive posture; +, marginally significant difference from control ($0.1>P>0.05$); *, significantly different from control ($P<0.01$ at least).

whereas the number of NK_1-stained cell bodies was around 55) (Fig. 4A,B).

In the rats considered in this analysis, SP-sap treatment did not affect total cell counts in the hypothalamic attack area ($F(1,14)=1,34$; $P>0.3$) (Fig. 4A). In contrast, NK_1 staining was markedly reduced ($F(1,14)=32.31$; $P<0.0001$) (Fig. 4B). In the neighboring lateral hypothalamus, NK_1 staining remained unaffected ($F(1,14)=0.2$; $P>0.9$) (Fig. 4B).

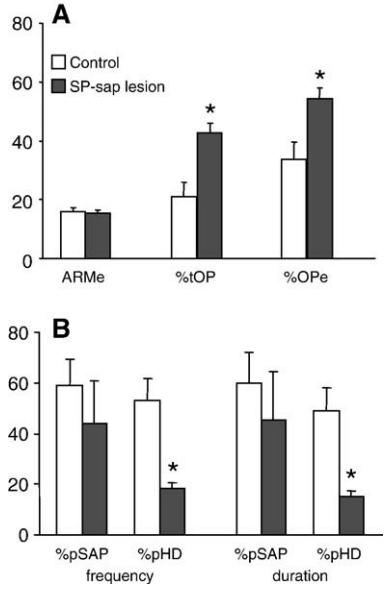

Fig. 5. The effects of SP-sap lesions on classical (A) and ethological (B) anxiety-related variables in the plus-maze. ARMe, total arm entries; %tOP, the duration of open arm visits expressed as % time; %OPe, % open entries (open arm entries/total arm entries*100); %pSAP, percent protected stretch attend posture; %pHD, percent protected head dipping; frequency, %pSAP and %pHD calculated for frequency; duration, %pSAP and %pHD calculated for duration (% time); *, significantly different from control.

SP-sap lesions markedly reduced the frequency of hard attacks ($H(1,14)=4.63$; $P<0.03$) without affecting the frequency of soft bites ($H(1,14)=1.60$; $P>0.3$) (Fig. 3C). The total frequency of biting attacks was marginally reduced ($H(1,14)=3.46$; $P<0.06$). Other behaviors showed non-significant variations (H(1,14 values were between 0.1 and 2,83, the corresponding P values being between 0.09 and 0.15) (Fig. 4D).

In the elevated plus-maze test, total arm entries were similar in the control and SP-sap lesioned rats (Fig. 5A). In contrast, open arm exploration was markedly increased by the lesion (%time in open arm: $(H1,14)=4.86$; $P<0.03$; % open arm entries: $H(1,14)=3.85$; $P<0.05$). Changes in protected head dipping were consistent with this finding, as this behavior decreased significantly (frequency: $H(1,13)=4.34$; $P<0.04$; duration: $H(1,13)=5.41$; $P<0.02$) (Fig. 5B). Stretch attend posture showed similar changes, but differences were not significant due to larger variation.

4. Discussion

4.1. Main findings

NK_3 receptors were strongly expressed in the arcuate nucleus but not in other regions. In contrast, NK_1 staining was strong in the hypothalamic attack area and the lateral hypothalamus. These findings confirm earlier studies on the hypothalamic distribution of tachykinin NK_1 and NK_3 receptors as reported in various species including rats, guinea-pigs, gerbils and humans (Tsuchida et al., 1990; Mileusnic et al., 1999; Langlois et al., 2001; Yip and Chahl, 2001;

Saffroy et al., 2003; Krajewski et al., 2005; Burke et al., 2006). The perifornical region (notably the perifornical nucleus) has been reported to express NK_3 receptors; however, this was seen in regions posterior to those investigated here.

In the hypothalamic attack area, SP-sap lesions markedly reduced NK_1 staining, without affecting NeuN staining. NK_1 staining remained unaffected in the lateral hypothalamus, a structure adjacent to the hypothalamic attack area. Thus, SP-sap treatments specifically lesioned NK_1 receptor-expressing neurons in the hypothalamic attack area.

The lesions dramatically reduced the frequency of hard bites, without affecting the frequency of soft bites. Other behavioral variables remained unchanged as well. Taken together, these findings suggest that the specific lesioning of NK_1 receptor-expressing neurons of the hypothalamic attack area markedly and specifically inhibits violent forms of attack in rats.

Surprisingly, SP-sap lesioned rats also showed a marked reduction of anxiety-like behavior in the elevated plus-maze, suggesting that NK_1-receptor-expressing neurons of the hypothalamic attack area are involved in the control of both violent attacks and anxiety.

4.2. Tachykinin NK_1 receptors in the hypothalamic attack area and aggression

The hypothalamic attack area is tightly and uniquely involved in the execution of attacks. Electrical stimuli, glutamate agonists and GABA antagonists delivered to this brain site rapidly elicited biting attacks in animals that did not show aggression under the experimental conditions employed (Adams et al., 1993; Haller et al., 1998; Kruk, 1991; Roeling et al., 1993). The role of the hypothalamic attack area appears crucial as attacks cannot be elicited by stimulation from any other brain region of the rat (Kruk, 1991; Siegel et al., 1999). Although the neurochemical identity of hypothalamic attack area neurons is known (Hrabovszky et al., 2005), the precise mechanisms regulating attack behavior are still poorly understood.

Disparate earlier observations suggested that substance P and NK_1 receptors are involved in the control of attacks. It was shown that substance P-ergic fibers connecting the medial amygdala and attack-related hypothalamic structures play important roles in feline affective aggression (Shaikh et al., 1993; Yao et al., 2001). In addition, NK_1 knockout mice showed dramatically reduced attack counts as compared with wild types (De Felipe et al., 1998; Rupniak et al., 2001). We have recently shown that resident–intruder conflicts strongly activate NK_1 receptor-expressing neurons in the hypothalamic attack area of rats, and systemic NK_1 blockade substantially reduces the frequency of violent attacks (Halasz et al., 2008). Taken together, these earlier observations show that tachykinin NK_1 receptors are involved in the execution of attacks, and suggest that those present in the hypothalamic attack area may be especially important in this respect.

Here we found that the number of NK_1 receptor-expressing neurons in the hypothalamic attack area is low, but such neurons are dispersed throughout the whole area, and show an extensive network of NK_1 receptor-expressing dendrites. Despite their small number, these neurons appear crucial for the execution of attacks. Interestingly, however, the destruction of NK_1 neurons — similar to systemic NK_1 blockade — affected hard bites only, i.e. the most violent forms attack. As the number of soft bites was not changed by the lesion, these findings suggest that mild and violent forms of attacks are differentially controlled by the hypothalamic attack area. We suggest here that the differential regulation of mild and violent forms of aggression offers the possibility of specifically controlling violent attacks, and renders NK_1 antagonism a promising new approach to the treatment of excessive aggressiveness.

4.3. Tachykinin NK_1 receptors in the hypothalamic attack area and anxiety

Despite some discouraging human trials, NK_1 receptors are still considered important targets for the development of novel antidepressants and anxiolytics (McLean, 2005; Chahl, 2006; Czeh et al., 2006; Alvaro and Di Fabio, 2007). In laboratory animals, NK_1 blockade decreased anxiety in a variety of tests (File, 2000; Rupniak et al., 2001; Santarelli et al., 2001; Bilkei-Gorzo et al., 2002). Similar results were obtained with local treatments. NK_1 receptor blockers decreased anxiety when infused into the dorsal periaqueductal grey, lateral septum and medial amygdala (Aguiar and Brandao, 1996; Gavioli et al., 1999, 2002; De Araujo et al., 2001; Ebner et al., 2004). Here we show that a strong anxiolytic effect develops also when the NK_1 receptor-expressing neurons of the hypothalamic attack area are specifically lesioned. However, the hypothalamic attack area has not been implicated in anxiety earlier. Therefore, the strong anxiolytic effect of SP-sap lesions in this region is rather surprising. It is worth to mention, however, that the neurons of this region — the majority of which are glutamatergic; Hrabovszky et al., 2005) — send projections to a large number of brain centers, including those involved in the control of anxiety, e.g. the prefrontal cortex, amygdala, and aminergic nuclei (Roeling et al., 1994; Delville et al., 2000; Petrovich et al., 2001; Hur and Zaborszky, 2005). The strong anxiolytic effects noticed here were likely mediated by these connections.

It was reported earlier that anxiety and aggressiveness are positively correlated, i.e. high aggressiveness is associated with high anxiety, whereas low aggressiveness with low anxiety (*humans*: Eaves et al., 2004; Broman-Fulks et al., 2007; *mice*: Kikusui et al., 2004; Matsumoto et al., 2005; *rats*: Veenema et al., 2006). In line with these earlier findings, SP-sap lesions reduced both anxiety and aggression, suggesting that NK_1 neurotransmission increases both. Although the nature of this interaction needs further scrutiny (see Veenema and Neumann, 2007; Brunelli and Hofer, 2007), the present findings raise the possibility of a coordinated control of attacks and anxiety from the hypothalamic attack area.

The elevated plus-maze test was performed after testing the same animals for aggression 1 week earlier, which may be considered a limitation of the study. Indeed, a subset of behavioral paradigms was shown to be sensitive to the testing history of experimental subjects (McIlwain et al., 2001). Unfortunately, the elevated plus-maze test was not investigated in this respect. We note, however, that test batteries (i.e. tests performed in a fixed order in the same animals) often include the elevated plus-maze test (Stepanichev et al., 2006; Karl et al., 2008; Kalueff et al., 2007). In such studies, the interval between the various tests is usually much shorter than the one employed here. In addition, our subjects faced intruders of smaller size that did not retaliate, i.e. the subjects were not likely to be seriously affected by the aggressive encounter. Based on the above, we suggest that the resident–intruder test had no significant impact on the results of the plus-maze test that was performed 1 week later.

4.4. Concluding remarks

The hypothalamic attack area specifically expresses the NK_1 type of tachykinin receptors. Local SP-sap infusions dramatically and specifically reduced the number of NK_1 receptor-expressing neurons in the hypothalamic attack area. The lesion was both neurochemically and spatially specific as total cell counts showed minimal changes, and NK_1 staining was not affected in the neighboring lateral hypothalamus. The lesion dramatically reduced hard bites in resident–intruder conflicts without affecting other forms of aggression. The lesion also reduced anxiety-like behavior in the elevated plus-maze. The present findings confirm earlier reports on the involvement of NK_1 neurotransmission in the control of violent attacks, and identify the hypothalamic attack area as a mediator of this effect. The findings also suggest that violent and other forms of attack are differentially controlled, and raise the possibility of a coordinated control of violent attacks and anxiety by NK_1 receptor-expressing neurons of the hypothalamic attack area.

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