Differential Lateral Septal Vasopressin Innervation in Aggressive and Nonaggressive Male Mice

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COMPAAN, J. C., R. M. BUIJS, C. W. POOL, A. J. H. DE RUITER AND J. M. KOOLHAAS. Differential lateral septal vasopressin innervation in aggressive and nonaggressive male mice. BRAIN RES BULL 30(1/2) 1–6, 1993.—The vasopressinergic (VP) projection from the bed nucleus of the stria terminalis (BNST) to the lateral septum (LS) is sexually dimorphic and dependent of androgens at adult and neonatal age. We studied the relation between testosterone (T) and VP in male mice, which were genetically selected for their differences in aggression level. Aggressive males, characterized by a short attack latency (SAL), have a higher production capacity of T at adult age compared to males with a long attack latency (LAL). Neonatally, however, a higher T production occurs in the nonaggressive LAL males than in SAL males. In the present study we showed a more dense VP-immunoreactive (VP-ir) innervation in the LS and a higher VP-ir neuron density in the BNST of LAL males as compared to SAL males. The described differences may be the consequence of a differential neonatal androgen effect on the organization of the forebrain vasopressinergic network.

Vasopressin Lateral septum Aggression Testosterone Bed nucleus of the stria terminalis

THE vasopressin (VP) innervation of the brain can be subdivided both on the basis of neuronal origin and on differential susceptibility to gonadal steroid hormones. In particular, the VP-immunoreactive (VP-ir) projection from the bed nucleus of the stria terminalis (BNST) to the lateral septum (LS) is sexually dimorphic (9,13,30).

The VP system in the BNST-LS is affected by the level of circulating androgens both neonatally and at adulthood. Neonatal treatment of female rat pups with testosterone results in a more dense VP fiber network in the LS in later life (10). This effect of testosterone seems to be the basis of the sex differences observed in the BNST-LS VP system, in which males have a larger number (30) of VP neurons in the BNST and a higher fiber density in the LS (13). Males also have a larger biosynthetic capacity of VP neurons in the BNST at adulthood (25). This biosynthetic capacity appears to depend upon the adult levels of circulating androgens. Following castration at adult age of male rats (11,12,30) and mice (7), the VP-ir completely disappears in the LS and BNST, due to a reduction of VP synthesis (24). After replacement with testosterone (T) (12,30) or administration of estradiol (E2) combined with 5α-dihydrotestosterone (14), the VP-ir can be restored to the level of the intact male. Thus, the density of the BNST-LS vasopressinergic system is already organized neonatally, whereas the VP synthesis depends upon adult androgens.

Several behaviors, including intraspecific aggressive behavior, are known to be sexually dimorphic and dependent upon both neonatal (6,17) and adult (1,32) availability of androgens as well. In two selection lines of mice, genetically selected for their level of aggression (31), we found that adult males of the aggressive line with a short attack latency (SAL) have a higher plasma T level and T sensitivity, a higher seminal vesicle weight (32), and a larger testicular T production capacity (8) in comparison to mice of the nonaggressive line with a long attack latency (LAL). Neonatally however, the LAL males produce more T than the SAL mice (8). Functionally, this high level of neonatal T in the genetically nonaggressive male pups is involved in a further reduction in aggressive behavior at adult age (6). Preliminary results show that in the currently investigated strains of mice the vasopressin content of the lateral septum is testosterone dependent as well (7).

In the following experiments we investigated the involvement of the VP system in the lateral septum and BNST in the above-mentioned differentiation within the male sex. Therefore, we measured the VP content of the LS and the distribution of VP-ir fibers in the LS and VP-neurons in the BNST employing biochemical and quantitative immunocytochemical procedures. Adult plasma-T levels were determined with radioimmunoassay. On the basis of the positive relationship between T and VP as outlined above, we expect to find individual differences in the

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BNST-LS vasopressinergic system in these selection lines of mice.

METHOD

Animals

Male mice (Mus musculus domesticus), genetically selected for either long attack latency (LAL) or short attack latency (SAL) (31), were used from generations 16–17 (LAL) and 38–39 (SAL). The mice were housed on a sawdust bedding in small Plexiglas cages (17 × 11 × 13 cm) in animal rooms at controlled light/dark cycle (12L:12D; lights off at 1230 h) and temperature (19–21°C). Standard lab chow and water was available ad lib. The litters were weaned at 3 weeks. At the age of sexual maturity (6–8 weeks) the individual males were paired with one female of the same selection line. At the age of 14 weeks, the males of both selection lines were tested for their attack latency score (ALS) according to the standard procedure described by Van Oortmerssen et al. (31) (Table 1). The ALS is the mean of the attack latencies scored on 3 consecutive days, determined between 1300 and 1500 h. Each experimental animal was confronted with a gonadally intact male albino mouse opponent (MAS-GRO). The test was terminated immediately after an attack occurred, or, in the absence of any attack, after 10 min. Thus, for those animals that did not fight at all, the ALS was set as 600 s.

After behavioral testing the animals were sacrificed and the brains investigated. Three different experiments were carried out. A radioimmunoassay (RIA) was performed to measure the total septal VP content (experiment I: SAL: n = 11; LAL: n = 7). In a second experiment, the detailed distribution and density of VP-containing fibers of the lateral septal area were studied in both selection lines using quantitative immunocytochemistry (ICC) (experiment II: SAL: n = 6; LAL: n = 6). In the third experiment, the VP-ir cells were counted in the BNST (experiment III: SAL: n = 6; LAL: n = 6). In experiments I and II blood samples were also taken in which plasma testosterone (T) levels were measured (RIA).

Blood Samples (Experiments I and II)

For blood sampling, the animals were mildly anaesthetized by ether and an intraperitoneal injection of sodium pentobarbital (4.0 mg/100 g b.w.t.). In Experiments I and II, prior to transcardial perfusion or decapitation, one blood sample was taken by cardiac puncture for measuring the plasma-T concentration. The samples were kept on melting ice in tubes, containing heparin (10 μl of 500 IU/ml). After centrifugation (5000 rpm, 10 min, 4°C) the plasma samples were stored at −20°C until assay performance.

Plasma concentrations of T were determined by a standard radioimmunoassay for human testosterone (Medgenix). The minimal detectable dose of T determined was 0.044 ng/ml and the intra- and interassay variation coefficients were 4.7% and 8.1%.

Experiment I

After blood sampling, SAL and LAL males of Experiment I were killed by decapitation. The brains were removed from the skull and the septum was dissected rapidly, using the optic chiasma as a landmark. The septal tissue was homogenized in 0.25 ml 0.1 N HCl, immediately frozen in liquid nitrogen, and were stored at −80°C. Just before the assay procedure the homogenates were sonified. Extraction of VP from the samples was performed according to Bevilacqua et al. (5) and the VP content was determined by radioimmunoassay. Standard curves ranged from 0.25 to 64 pg VP per 50 μl and consisted of 50 μl antibody solution, 50 μl sample or standard solution (both in assay buffer), and 10 μl (2000 cpm) tracer. Anti-VP (M160480) was raised in a rabbit and was used in a final dilution of 1: 100,000. Crossreactivity (ratio of pg VP/peptide at B/B0 = 50%) with oxytocin was 0.009%, with vasotocin 5.8% and with lypressin-vasopressin 22%. After 3 days of incubation at 4°C the free label was separated from the bound by adding 50 μl of second antibody coated cellulose suspension (SAC-CEL, IDS, USA) to the assay tube followed by centrifugation at 1700 × g for 5 min, after which the pellet was counted in a Packard-Cobra A5005 gamma counter. Detection limit defined as B/B0 of ±90% was 25 pg/sample. Intraassay coefficient of variation (cv) in the direct assay for 2, 4, 8, and 16 pg VP/tube is 15.6, 10.5, 6.8, 6.8, and 9.0%, respectively. Interassay cv in ten different assays for 2 pg VP/tube is 13.4%. Because the septal area contains a dense network of VP-ir fibers, whereas the surrounding tissue contains only scattered fibers, the data reflect the total amount of VP per septum.

Immunocytochemistry (Experiments II and III)

Under deep pentobarbital anaesthesia, the males in Experiment II were transcardially perfused with a premix of 50 ml 0.9% saline containing 3.5% heparin-500 IU/ml, followed by a fixative (150 ml 3–4% paraformaldehyde + 0.05% glutaraldehyde). During this perfusion the descending aorta was clamped. The brains were dissected, postfixed (only in Experiment II: 4% paraformaldehyde, 24 h and 5% glutaraldehyde, 72 h), and stored in 0.1 M phosphate buffer (PB), pH = 7.2, with 0.1% sodium azide at 4°C. Subsequently, the brains were cut on a Vibratome (30 μm; Experiment II) or a cryostat microtome (20 μm; Experiment III). The free-floating sections were preincubated in subsequent steps of 0.1% H2O2 and 5% normal goat serum

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<th>TABLE 1</th>
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<td>MEAN ATTACK LATENCIES (ALL EXPERIMENTS), PLASMA-T LEVELS (EXPERIMENTS I + II), VP CONTENT OF THE SEPTAL AREA (EXPERIMENT I), BNST (EXPERIMENT III) OF SAL AND LAL MALES (±SEM)</td>
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<tr>
<th>ALS (s)</th>
<th>T (ng/ml)</th>
<th>LS VP (pg/septum)</th>
<th>BNST VP-ir Cell Bodies</th>
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<tr>
<td>SAL</td>
<td>33.6 ± 6.9</td>
<td>1.57 ± 0.49</td>
<td>289.05 ± 19.8</td>
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<tr>
<td>LAL</td>
<td>581.1 ± 13.8</td>
<td>1.23 ± 0.53</td>
<td>382.89 ± 15.5*</td>
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*† Significant difference: *p < 0.01; †p < 0.05 (Mann–Whitney U-test).
(NGS), for 20 min each at room temperature. Thereafter the sections were exposed by successive incubations to rabbit anti-VP ("Truus" from the Netherlands Institute for Brain Research, Amsterdam; 1:1000), one overnight at 4°C, goat-antirabbit IgG (Zymed; 1:100; 75 min at room temperature), and Rh-peroxidase antiperoxidase (Dakopatts; 1:400; 75 min at room temperature). The peroxidase complex was reacted with 3,3′-diaminobenzidine (Sigma; 30 mg/100 ml Tris) and hydrogen peroxide (0.006%) for visualization. Thereafter, the sections were mounted on glass slides, air dried, and coverslipped.

In Experiment III, the same procedure was followed as in Experiment II, except that 28 h before perfusion colchicine (8 μg/2 μl 0.1 M phosphate buffer; pH = 7.2) was administered into the right lateral ventricle (3.5 mm anterior to lambda, dept: 1.7 mm and 1.1 mm lateral) to block axonal transport. SAL and LAL males were anaesthetized with a combination of Ketanest (Parke-Davis, 0.25 mg/10 g b.w.t) and Rompun (Bayer, 0.20 mg/10 g b.w.t.). Thereafter, the animals were mounted in a stereotaxic apparatus according to Gerkena et al. (18), fitting the upper jaw in an anatomical mold and using a nose clamp to avoid the use of ear bars. The dorsal skull surface was positioned horizontally by adjusting the midsagittal line, connecting the lambda and bregma landmarks, to coincide with the horizontal plane.

Quantification of Immunoreactivity

Per animal every fourth section of the lateral septal area was measured, using an automatic image analyzing system (IBAS). The lateral septum was defined between the levels F 1.0 and F 0.0 (27), which means from 1000 μm anterior to bregma until bregma level. In each section both left and right LS were measured in a total of 12 field areas (objective 20×). The density of VP-ir fibers (AREA), defined as the percentage VP-ir area compared to the field area, and the amount of VP/ fiber (optical density: OD) were determined to obtain a semiquantitative measure of the total amount of VP per section (integrated optical density: IOD = AREA × OD). In every third section of the BNST the VP-ir cells were counted in both hemispheres.

Statistics

Mann–Whitney U-test was used to determine significant differences between LAL and SAL selection lines in plasma-T levels (Experiments I and II), VP content of the septal area (Experiment I), and number of VP-ir cells in BNST (Experiment III). The relation between individual plasma-T level and the corresponding VP content of the septal area was analyzed using the Spearman rank correlation test. The data on vasopressinergic innervation of the LS were evaluated for significance using multivariate analysis of variance with repeated measures (Pillais test in SPSS/PC+ MANOVA (19)). Significant effects between SAL and LAL selection lines on section level were determined with post hoc Student’s t-test.

RESULTS

Vasopressin

The RIA for VP (Experiment I) revealed that the septal VP content of LAL males is significantly higher (p = 0.0075) compared to the SAL males (Table 1). However, no correlation was found between the individual plasma-T levels and septal VP content, neither within each selection line nor in the total group of animals, irrespective the selection line. The quantitative immunocytochemical approach in Experiment II using the integrated optical density (IOD) of VP-ir fibers within the LS, reveals a significant effect for selection line, F(1, 10) = 12.22, p = 0.006, section level, F(7, 4) = 58.64, p = 0.001, and a significant interaction between selection line and rostro-caudal section level, F(7, 4) = 11.30, p = 0.017. This means that VP-ir in the LS of LAL males is higher compared to SAL males (Fig. 1). In both selection lines VP-ir IOD increases in the caudal part of the LS, in which LAL males have a higher VP-ir IOD compared to SAL males (Figs. 2 and 3).

The VP-ir fiber density (AREA) throughout the entire LS is higher in LAL males compared to the SAL males, F(1, 10) = 4.97, p = 0.05 (Fig. 1). In both selection lines the density of VP-ir fibers gradually increases from rostral to caudal, F(7, 4) = 97.18, p = 0.000, although in the caudal parts of the LS the
FIG. 2. Mean integrated optical density (IOD + SEM) of vasopressin immunoreactivity in every fourth section of the lateral septum in brains of aggressive SAL (○) and nonaggressive LAL (●) males. (⁎ p < 0.05; **p < 0.01).

LAL line differs significantly from the SAL animals, $F(7, 4) = 12.74, p = 0.014$.

The analysis of the optical density (OD) per fiber reveals that LAL VP-ir fibers in the LS contain more VP (OD) compared to the SAL selection line, $F(1, 10) = 11.17, p = 0.007$ (Fig. 1). In both selection lines the fibers in the more caudal part of the LS contain more VP, $F(7, 4) = 19.97, p = 0.006$. Finally, in Experiment III, the number of VP-ir cells in seven sections of the BNST (Table I) was determined. There is a difference in cell number between left and right parts of the brain. The difference in VP-ir cell number between left and right hemisphere is most likely due to the unilateral colchicine infusions. This VP-neuron number is significantly higher in LAL males compared to SAL males in the left ($p = 0.007$) and right ($p = 0.02$) side of the brain, respectively. Most cell bodies are observed around the most posterior part of the anterior commissure in ventral and dorsal parts of the BNST, and are usually of the multipolar type (Fig. 3).

Testosterone

The mean plasma-T levels of adult SAL and LAL males are presented in Table I. The males of the SAL selection line have a significantly shorter attack latency score (ALS = 33.6 ± 6.9 s) than the nonaggressive LAL males (ALS = 581.1 ± 13.8 s). However, no significant differences exist between plasma-T levels of both selection lines, although the mean plasma-T level of the aggressive SAL males (1.57 ± 0.49 ng/ml plasma) is somewhat higher compared to the nonaggressive LAL animals (1.23 ± 0.53 ng/ml plasma).

DISCUSSION

Both the biochemical and immunocytochemical experimental data in the present study reveal that the vasopressinergic projection from the BNST to the lateral septum is more dense in nonaggressive LAL males than in the aggressive SAL males. As measured in Experiment I, the total amount of septal VP is higher in the LAL selection line compared to the SAL line. The higher septal VP amount is consistent with both the higher density of VP-ir fibers and their higher VP content in the posterior part of the LS in LAL mice. This is reflected in a higher integrated optical density for VP-ir fibers. There is a theoretical possibility that our staining procedure was insufficient to detect VP-ir fibers in the LS of the SAL males, due to a lower VP content per fiber. Because of the possibility that a more active VP system may also have a high release resulting in a low fiber content of VP, colchicine was used to accumulate VP in the cell bodies. This procedure revealed a larger number of VP synthesizing cells in the BNST of the LAL males. Thus, the VP projection from the BNST to the LS is more robust in the males of the nonaggressive selection line, both in terms of number of VP cells in the BNST and the fiber density in the LS. These findings are consistent with the hypothesis that the differentiation in the T-dependent VP system between the two selection lines is due to the high neonatal T levels in the LAL males (8).

The higher optical density per fiber in the LAL males suggests a higher VP content per fiber. It is a matter of further research to find out to what extent this reflects a high VP synthesis or a low VP release. However, the lack of a significant correlation between the plasma-T levels and the various parameters of the VP system indicates that the adult T is not very important in this respect. The differences in adult plasma T between SAL and LAL males as reported in the literature (32) was not observed in this experiment. However, it has been reported in several species, including rat, that the plasma-T level may substantially fluctuate over 24 h in the adult male (2). This may explain the large interindividual variation in plasma-T levels and the absence of significant differences between the two selection lines. Moreover, De Vries et al. (9) also showed that VP projections from the BNST are more abundant in males than in females even after similar hormone treatment at adult age. Thus, the early organization of the VP fiber network seems to be the main factor in both the sexual differentiation and the differentiation within the male sex.

Vasopressin is known to be involved in regulatory mechanisms of several behavioral functions, among which is aggressive behavior. Infusions of VP in the LS enhances aggression of castrated male rats (23). Also, microinjections of VP into the BNST-LS stimulates flank-marking behavior, whereas VP antagonists reduces this behavior in male Golden hamsters (20). Flank-marking behavior normally occurs most frequently during aggressive encounters.

The present results seem in contrast with the above-mentioned reports, suggesting an enhancement of aggressive behavior after VP infusions in the BNST-LS VP system, although the currently investigated aggressive SAL males have a less abundant VP system compared to nonaggressive LAL males. A species difference may be one explanation. Although, the distribution and shape of vasopressin cells in the BNST of male mice is comparable to those of rats (29), the distribution of vasopressin fibers in the lateral septum differs between these species. In mice, the VP innervation is mainly present in the ventral LS; in rats (13) the medial LS is more innervated. In Golden hamsters no VP-ir could be detected in the LS at all (15,16).

An explanation in terms of a compensation by a more sensitive VP receptor system in the septal area of SAL males is unlikely. In rats, the distribution and amount of VP binding sites is not sexually dimorphic and gonadectomy at adult age, or T/E2 replacement does not affect the density of VP binding sites or affinity for VP (26,28).

Hence, it is difficult to speculate on the function of VP in aggressive behavior. However, these selection lines not only differ in adult aggressive behavior. A number of behavioral studies on SAL and LAL males show that the individual variation in aggressive behavior reflects a more fundamental differentiation in active or passive coping strategy based upon the degree in which
FIG. 3. Vasopressin immunoreactivity (VP-ir) in the lateral septum (LS) (upper part: A and B) and bed nucleus of the stria terminalis (BNST) (lower part: C and D) in nonaggressive LAL (left: A and C) and aggressive SAL (right: B and D) male mice. The level of the LS in photomicrographs A and B correspond to the LS level 7.5 (* 100 μm) in Fig. 2. Notice the thicker and higher density of VP-ir fibers in the LS of LAL males. VP-ir cells in the BNST were visualized after colchicine treatment. The number of VP-ir neurons in the BNST is significantly higher in the LAL mice. Bars in A and B: 100 μm; C and D: 50 μm.
behavior is guided by environmental stimuli (3,4). LAL males rely more on spatial cues than SAL males, suggesting a differential involvement of hippocampal function in the organization of behavior. Morphological (21) and electrophysiological (22) evidence support the view that hippocampal function may be modulated by the LS-VP via the medial septum. Hence, the functional significance of LS VP may be found in the modulation of the function of the hippocampus in coping behavior.

In summary, males of the nonaggressive selection line show a more abundant VP projection from the BNST to the LS, which may be due to higher levels of neonatal circulating androgens. Future experiments will focus on the involvement of neonatal T in the individual differentiation in LS VP within the male sex and the relation to the behavioral differentiation.

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