Published in final edited form as:
*J Neuroendocrinol*. 2011 October ; 23(10): 906–914. doi:10.1111/j.1365-2826.2011.02196.x.

# Effects of neonatal treatment with valproic acid on vasopressin immunoreactivity and olfactory behaviour in mice

**Elaine K. Murray**[*], **Megan M. Varnum**, **Jared L. Fernandez**, **Geert J. de Vries**, and **Nancy G. Forger**
Neuroscience and Behavior Program, Department of Psychology and Center for Neuroendocrine Studies, University of Massachusetts, Amherst MA, 01003

## Abstract

Recent findings demonstrate that epigenetic modifications are required for sexual differentiation of the brain. For example, neonatal administration of the histone deacetylase inhibitor, valproic acid, blocks masculinisation of cell number in the principal nucleus of the bed nucleus of the stria terminalis (BNST). Here we examined effects of valproic acid on neurochemistry and behaviour, focusing on traits that are sexually dimorphic and linked to the BNST. Newborn mice were treated with saline or valproic acid and the effect on vasopressin immunoreactivity and olfactory preference behavior examined in adulthood. As expected, males had more vasopressin immunoreactive fibers than females in the lateral septum and medial dorsal thalamus, two projection sites of BNST vasopressin neurons. Neonatal valproic acid increased vasopressin fiber density specifically in females in the lateral septum, thereby reducing the sex difference, and increased vasopressin fibers in both sexes in the medial dorsal thalamus. Effects were not specific to BNST vasopressin projections, however, as valproic acid also significantly increased vasopressin immunoreactivity in the anterior hypothalamic area in both sexes. Subtle sex-specific effects of neonatal valproic acid treatment were observed on olfactory behavior. As predicted, males showed a preference for investigating female-soiled bedding whereas females showed a preference for male-soiled bedding. Valproic acid did not significantly alter olfactory preference, per se, but increased the number of visits females made to female-soiled bedding and the overall time females spent investigating soiled versus clean bedding. Taken together, these results suggest that a transient disruption of histone deacetylation at birth does not have generalized effects on sexual differentiation, but does produce lasting effects on brain neurochemistry and behaviour.

## INTRODUCTION

Epigenetic modifications provide a mechanism for early life events to have long-lasting consequences. Neonatal exposure to endocrine-disrupting chemicals, endogenous hormones, or environmental stressors causes epigenetic changes (e.g., covalent modifications of histone tails or the methylation of cytosine residues of DNA) that may underlie relatively stable changes in gene expression (1–4). Recently, epigenetic modifications have been implicated in sexual differentiation of the brain (5). Many neural sex differences depend on testosterone exposure during early postnatal life (6) and steroid hormones are thought to work, at least in part, by orchestrating changes in the epigenome (7, 8).

We recently demonstrated that neonatal treatment with the histone deacetylase (HDAC) inhibitor, valproic acid, transiently increases histone acetylation in the mouse brain and

[*]Address of corresponding author: Center for Neuroendocrine Studies, 517 Tobin Hall, University of Massachusetts, Amherst, MA 01003, Phone: (413) 545-0554, ekmurray@cns.umass.edu.

**Disclosure statement**: EKM, MMV, JLF, GJD and NGF have nothing to declare.

blocks masculinisation of volume and cell number in the principal nucleus of the bed nucleus of the stria terminalis (BNST) in both males and testosterone-treated females (9). This suggested that a disruption of histone acetylation could interfere with sexual differentiation and raised the question of whether other neural sex differences, or behaviors that depend on neural sex differences, would be affected by valproic acid treatment. In the current study, we determined the effect of neonatal treatment with valproic acid on neurochemistry and behaviour, focusing on vasopressin innervation of the brain and olfactory preference behavior, because both are sexually dimorphic and related to the BNST.

The neuropeptide vasopressin plays a role in social behaviours (10) and is produced by several discrete nuclei in the forebrain and hypothalamus, some of which are sexually dimorphic (11). Males of many species have more vasopressin-expressing cells in the BNST and medial amygdala (MeA) than do females and the projections of these neurons to areas including the lateral septum, medial dorsal thalamus and lateral habenula are also greater in males (12–14). In rodents, the sex differences in vasopressin cell number in the BNST and MeA and vasopressin fibers in the lateral septum can be reversed by gonadal hormone exposure during early postnatal life (15–17). Here, we tested the hypothesis that a disruption in the balance of histone acetylation and deacetylation by neonatal valproic acid treatment would affect the sexual differentiation of vasopressin projections from the BNSTp and MeA. We also examined projections from the non-sexually dimorphic vasopressin populations in the suprachiasmatic nucleus (SCN) and paraventricular nucleus of the hypothalamus (PVN) in mice exposed to valproic acid at birth.

The BNST is part of the accessory olfactory system, also known as the vomeronasal system, which is crucial for the detection of pheromones and influences several aspects of reproduction, including sex discrimination, attraction and mate recognition (18). Neural activation is increased in the BNST and other nodes of the vomeronasal circuitry when smelling sexually relevant cues, and this activation as well as the behavioural response to such cues are sexually dimorphic (19–23). Specifically, adult male mice spend significantly more time investigating bedding from female mice than bedding soiled by other males, whereas females show the opposite preference (22, 23). Because neonatal valproic acid treatment prevents the development of a sex difference in cell number in the BNSTp, we asked here whether olfactory preference and the response to socially relevant olfactory cues might also be affected.

## METHODS

### Animals

Wild-type C57BL/6 mice from our breeding colony at the University of Massachusetts were used for all experiments. Animals were housed under 14:10 light dark conditions at 22°C with food and water available ad libitum, unless otherwise stated. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Massachusetts, Amherst.

### Experiment 1: Effect of neonatal VPA treatment on vasopressin immunoreactivity

Male and female mice were administered subcutaneous injections of 50 mg/kg valproic acid or saline on P1 and P2 (day of birth = P1). We previously demonstrated that a single injection of valproic acid at this dose leads to increased histone acetylation in the neonatal mouse brain 24 hours after treatment, that returns to baseline by 96 hours post-treatment (9). Animals were sacrificed in adulthood and their brains processed for vasopressin immunoreactivity (n=9–11/group). Blood samples were collected via cardiac puncture prior to sacrifice, spun, and plasma kept at −20°C until assay. Vasopressin innervation was

measured in four brain regions: the lateral septum, medial dorsal thalamus (MDT), anterior hypothalamic area (AHA) immediately lateral to the PVN, and the paraventricular nucleus of the thalamus (PVA). The lateral septum and MDT receive sexually dimorphic vasopressin innervation from the MeA and BNST (24). The AHA contains lateral projections from the PVN, a non-sexually dimorphic vasopressin cell group, and the PVA receives innervation from vasopressin cells in the SCN, also not sexually dimorphic (25).

### Experiment 2: Effect of neonatal VPA treatment on olfactory behavior

Males and females were treated with valproic acid (50 mg/kg in 0.9% saline) or saline on P1 and P2. At four months of age, subjects were individually housed and behavior testing began one week later (n= 10–21/group). Olfactory preference and hidden cookie tests were performed for each subject as described below. Body weights and anogenital distance of all animals and testes weights in males were recorded at sacrifice.

### Immunohistochemistry

Brains were removed, post-fixed in 5% acrolein in 0.1M phosphate buffer (pH 7.6) overnight, and immersed in 30% sucrose until sectioning into three, 30 μm series. One series was stained for vasopressin. Free-floating sections were rinsed three times in Tris-buffered saline (TBS; 0.05 M Tris, 0.9% NaCl, pH 7.6), then incubated for 30 minutes in 0.05 M sodium citrate in TBS. After rinsing in TBS sections were placed for 30 minutes in 0.1 M glycine in TBS, rinsed again, and placed in blocking solution [20% normal goat serum (NGS), 0.3% Triton-X (Labchem, Inc., Pittsburgh, PA) and 1% $H_2O_2$ in TBS] for 30 min. Sections were then incubated overnight in primary antibody (1:8000; T-5048, Peninsula Laboratories, San Carlos, CA, USA) in TBS with 2% NGS, 1% BSA, and 0.3% Triton-X.

The next day, sections were rinsed 3 times in TBS containing 1% NGS and 0.02% Triton-X and incubated in secondary antiserum (1:250 biotinylated goat anti-guinea pig for vasopressin immunoreactivity; Vector Laboratories, Burlingame, CA, USA) in TBS with 2% NGS and 0.32% Triton-X for 1 hour. This was followed by rinses in TBS containing 0.2% Triton-X, washing in avidin–biotin complex in TBS (Vectastain Elite ABC Kit, Vector Laboratories) for 30 minutes, followed by another series of TBS rinses. Finally, the staining was visualized using diaminobenzidine (DAB peroxidase substrate kit, Vector Laboratories). Sections were mounted onto slides and coverslipped with Permount.

### Quantification of vasopressin immunoreactivity

All analyses were performed blind to treatment group. Photomicrographs were taken of each region and fiber density was quantified using Image J (NIH). Gray-level thresholding was used to determine the number of pixels covered by staining, as in (26). Vasopressin fiber density is expressed as number of labeled pixels in a counting frame (150,000 $\mu m^2$) placed in a consistent anatomical location for each brain region. Subjects for which the relevant section was damaged or unavailable were dropped from a given analysis; final number of animals included for each brain region is indicated below.

### BNSTp Volume Measurements

Following quantification of vasopressin immunoreactivity, coverslips were soaked off and slides counterstained with thionin to determine BNSTp volumes. Measurements were made on slides coded to conceal the sex and treatment of the animals and were performed as previously (9). The outline of the BNSTp was traced bilaterally in every section in which it appeared using StereoInvestigator software (MicroBrightfield, Williston, VT). Volume was calculated by multiplying the summed area by the sampling ratio (3) and section thickness (30μm).

### Testosterone and Estradiol Measurements

Assays for testosterone in both sexes and estradiol in females were performed at the University of Virginia Center for Research in Reproduction Ligand and Assay Core Lab, which is supported by NICHD (SCCPRR) grant U54-HD28934. Testosterone levels were measured by radioimmunoassay with a sensitivity level of 0.07 ng/ml. Serum estradiol was assayed by ELISA using a commercially available kit with a sensitivity level of 3.0 pg/ml. Published intra-assay variability was 3.6% and 3.1% for testosterone and estradiol assays, respectively.

### Olfactory Preference Test

Animals were singly housed prior to testing and all behavioural tests were conducted during the animals' dark phase. Olfactory preference for bedding from conspecifics was conducted as previously described (22) with minor modifications. Briefly, the test consisted of a habituation trial followed by an experimental trial (10 min each), both conducted in 47 cm × 25.4 cm opaque cages with 1 cm of sani-fresh chips. Three ceramic containers (2.54 cm high, 6.3 cm diameter) were placed in the left, right, or center third of the cage. In the habituation trial, all containers were filled with clean bedding. In the experimental trial, the containers were filled with male-soiled bedding, female-soiled bedding, and clean bedding, respectively, with the position of each type of bedding randomly assigned for each animal. A clean cage and new bedding was used for each trial.

'Male-soiled bedding' consisted of shavings collected from the cages of four gonadally intact, sexually-experienced males following four days of use. Female-soiled bedding was collected from the cages of four stimulus females that were ovariectomised and provided with a 5 mm silastic implant containing 17-β-oestradiol benzoate. These females were injected with 1 mg progesterone in sesame oil on the day prior to bedding collection to induce estrous. Shavings were stored at −20°C until use.

All experimental trials were videotaped and later scored by an investigator blind to treatment. JWatcher software was used to determine the number of visits to each container and the time spent sniffing each container. The olfactory preference for each animal was calculated by subtracting time spent sniffing male bedding from time spent sniffing female bedding, as in (22).

### Hidden Cookie Test

The hidden cookie test was used to evaluate the ability to detect volatile odors (27). Animals were placed into clean cages and food-deprived overnight. The test consisted of a habituation trial followed by an experimental trial separated by 2–3 hours. In the habituation trial, a 5×5 mm piece of Nutter Butter (Nabisco) cookie was placed in clear view on top of a 2cm layer of sani-fresh chips; the animal was then placed in the cage and allowed up to 5 min to locate the cookie. In the experimental trial, the cookie was buried 1 cm under the chips in a random location and time taken to locate the cookie was recorded.

### Statistical Analysis

The effects of sex and valproic acid treatment on vasopressin immunoreactivity, BNSTp volume, olfactory preference, frequency of visits to bedding containers, and ability to find a hidden cookie were analyzed with 2-way ANOVAs (treatment-by-sex). Planned comparisons using Fishers Least Significant Difference were conducted following significant main effects or interactions. Time spent investigating bedding in the olfactory preference test was also analyzed using mixed multifactorial ANOVA testing for main effects of treatment and sex and within subjects effects of olfactory stimulus (male, female, clean bedding) using a repeated measures design. Body weight and anogenital distance were

compared by 2-way ANOVAs and testes weights and serum testosterone were compared in saline and valproic acid-treated males by 2-tailed t-tests. Serum estradiol and testosterone levels were also compared in saline and valproic acid-treated females by 2-tailed t-tests.

## RESULTS

### Effect of neonatal valproic acid treatment on vasopressin immunoreactivity and BNSTp volume in adulthood

Neonatal valproic acid treatment tended to increase vasopressin immunoreactivity in all projection fields examined, although significance level and sex specificity varied by region. The lateral septum and MDT receive vasopressin innervation from the BNST and MeA. In the lateral septum, males had significantly more vasopressin-immunoreactive fibers than females (Figure 1, $F_{1,32}$= 49.26, $p < 0.001$). There was no significant main effect of valproic acid treatment on vasopressin immunoreactivity, but there was a significant sex by treatment interaction (Figure 1, $F_{1,32}$= 6.39; $p < 0.05$). Post-hoc analysis revealed that valproic acid treatment significantly increased vasopressin immunoreactivity only in females (Figure 1, $p < 0.05$).

In the MDT, we again found a significant main effect of sex on vasopressin immunoreactivity (Figure 2, $F_{1,27} = 32.4$, $p < 0.001$). There was also a main effect of valproic acid treatment ($F_{1, 27} = 11.71$, $p < 0.005$) with no significant sex-by-treatment interaction (Figure 2); valproic acid treatment increased vasopressin immunoreactivity in the MDT of both males and females.

We also examined vasopressin immunoreactivity in projection fields from the PVN and SCN (the AHA and PVA, respectively) to determine whether valproic acid treatment altered innervation from other, non-sexually dimorphic vasopressin cell groups. Valproic acid increased vasopressin immunoreactivity in the AHA (Figure 3, $F_{1,28} = 12.2$, $p < 0.005$), with no effect of sex and no sex-by-treatment interaction. In the PVA, we did not find a significant effect of sex, valproic acid treatment or a sex-by-treatment interaction (Figure 3). Vasopressin immunoreactivity tended to be higher in the PVA of valproic acid-treated animals but this did not reach statistical significance.

The increase in vasopressin immunoreactivity in valproic acid-treated animals, particularly that in the lateral septum of females, was surprising given our previous observation that neonatal valproic acid feminized BNSTp cell number and volume (9). To examine effects of valproic acid on BNSTp size in the current study, we therefore counterstained the sections that had been processed for vasopressin immunoreactivity and performed volume analyses. Although this material is not optimally suited for morphological analyses, the pattern of findings was similar to that described previously: BNSTp volume was larger in control males than in females (Table 1, $p = 0.03$), whereas valproic acid-treated males did not differ from control or valproic acid-treated females ($p = 0.84$ and 0.33, respectively). The difference between control and VPA males was marginally significant in a posthoc test ($p = 0.056$) and significantly different in a two-tailed t-test ($p = 0.04$). The fact that the overall sex difference, as well as magnitude of the VPA effect, was smaller than observed previously (9, 28, 29) might be related to the difficulty of assessing morphology following immunocytochemistry and/or to the fact that the previous valproic acid effect was measured on postnatal day 21 (9), as opposed to in adulthood. Nonetheless, this analysis demonstrates that valproic acid can have markedly different effects on different sexually dimorphic traits within the same animal.

Gonadal steroid hormone levels of animals at sacrifice are presented in Table 2. Neonatal valproic acid treatment did not affect serum testosterone levels in males or in females or

serum estradiol in females. Testosterone levels in the majority of females were below the detection level of the radioimmunoassay; data reported in Table 2 represent only those animals with levels within the reportable range (4 saline-treated and 3 valproic acid-treated females).

### Experiment 2 - Effect of neonatal VPA treatment on olfactory behavior

As expected, ANOVA revealed a significant effect of sex on olfactory preference ($F_{1,52}$ = 11.7, $p < 0.01$): saline-treated males spent more time investigating female-soiled bedding and females spent more time investigating male-soiled bedding (Figure 4). There was no main effect of valproic acid treatment on olfactory preference ($F_{1, 52} = 0.9$, $p > 0.1$) and no interaction between sex and treatment (Figure 4; $F_{1, 52} = 0.4$, $p > 0.1$).

We noted, however, that valproic acid treatment increased the time spent investigating soiled bedding, especially in females (Figure 5). Using a mixed-design ANOVA with sex and treatment as between subjects factors and stimulus (male-soiled, female-soiled, clean bedding) as a within subjects factor, a main effect of stimulus ($F_{2, 106} = 45.3$,$p < 0.001$) as well as stimulus-by-sex ($F_{2,106} = 5.1$, $p < 0.05$) and stimulus by treatment interactions ($F_{2,106} = 4.0$, $p < 0.05$) were observed. As expected, both sexes spent much more time investigating soiled bedding versus clean. The stimulus-by-treatment interaction was due to the fact that valproic acid increased time spent investigating soiled bedding in females but not in males. Valproic acid-treated females investigated female-soiled bedding more than saline-treated females ($p < 0.015$). A similar trend was observed for male-soiled bedding but this did not reach statistical significance ($p = 0.061$). In addition, valproic acid treatment also increased the frequency of visits to the female-soiled bedding container by females ($p < 0.001$). Total frequency of visits to all three containers did not differ between any of the groups (data not shown), suggesting that overall activity was not affected by sex or treatment.

There was no main effect of valproic acid in the hidden cookie test (data not shown), indicating that valproic acid did not disrupt basic olfactory ability. We did, however, find that saline-treated males were slower than all other groups to find the cookie ($p < 0.05$; for sex-by-treatment interaction, data not shown). Valproic acid did not affect body weight, anogenital distance or testes weights in adulthood (all p-values > 0.05; data not shown), consistent with the absence of a valproic acid effect on body weight at weaning seen previously (9).

## DISCUSSION

We find that females treated with valproic acid neonatally had more vasopressin-immunoreactive fibres in the lateral septum than their saline counterparts and spent more time investigating female-soiled bedding. These effects are consistent with subtle disruptions of sexual differentiation by neonatal valproic acid exposure. The most striking finding of the current study, however, is that vasopressin immunoreactivity was higher in valproic acid-treated animals in all projection fields examined, reaching statistical significance in the MDT and AHA of both sexes and in the lateral septum of females. These results suggest that regulation of histone acetylation early in development plays a role in programming adult levels of immunoreactive vasopressin, independent of its effects on sexual differentiation.

Because animals in the current study were gonadally intact, and vasopressin projections from the BNST and MeA are increased by gonadal steroids in adulthood (30) differences in circulating hormones could have contributed to the results seen here. However, we found no significant effect of valproic acid treatment on testosterone or estradiol levels at sacrifice. If

anything, both hormones were (nonsignificantly) lower in valproic acid-treated than in control animals, which is opposite to what would be predicted. In addition, vasopressin projections from the PVN are unaffected by circulating steroids (30), yet we saw increased vasopressin immunoreactivity in the AHA, a projection site of the PVN, in valproic acid-treated animals of both sexes.

Valproic acid is a widely prescribed drug used for the treatment of epilepsy and bipolar disorder (31, 32). Previous investigations have examined the effect of adult administration of valproic acid on a number of neural measures in rodents (33–35) or have examined effects of administration of high, teratogenic, doses to pregnant dams during mid-gestation (36, 37). As far as we are aware, there have been no previous reports on the effects valproic acid during perinatal life in rodents, other than our study examining sexual differentiation of the BNSTp (9). The current results suggest neonatal valproic acid exposure leads to long-lasting changes in adult neurochemistry and behaviour.

As an HDAC inhibitor, valproic acid likely leads to a global increase in the level of histone acetylation. Histone acetylation is most consistently associated with increased gene expression whereas histone deacetylation is associated with gene silencing (38, 39). Histone acetylation and DNA methylation are often linked, with increased acetylation leading to decreased DNA methylation and vice versa. Indeed, valproic acid treatment leads to decreases in DNA methylation at specific genes (40, 41). The widespread increased expression of vasopressin observed here is consistent with the possibility that valproic acid programmes an increase in gene expression by blocking the action of histone deacetylases.

Recent findings provide evidence that the vasopressin gene is in fact a target of epigenetic regulation. For example, the promoter region of the gene is hypermethylated in leucocytes of alcoholic patients, compared to controls (42). In the BNST of rats, adult gonadectomy increases methylation within the promoter region of the vasopressin gene, which correlates with decreased vasopressin expression in castrates (43). The early postnatal period is a critical time for determining life-long sexually dimorphic expression of vasopressin expression in the BNST and MeA of rats (15, 44) and it is likely that the vasopressin gene in other cell groups is also susceptible to epigenetic regulation during this time. Indeed, early life stress induces hypomethylation of an important enhancer region of the mouse vasopressin gene in the neurons of the PVN, leading to elevated expression of vasopressin for at least a year following the stressor (45).

Because we examined vasopressin immunocytochemically here, we do not know whether valproic acid acted on the vasopressin gene directly or on a downstream process. One direct test would be to examine the effect of valproic acid treatment on vasopressin mRNA and changes in histone acetylation or DNA methylation associated with the vasopressin gene. The observed increase in vasopressin fiber density following valproic acid treatment could also reflect other processes, such as an increase in axon outgrowth. This is a plausible, given recent evidence suggesting that HDAC inhibition promotes neurite outgrowth *in vivo* and *in vitro* (46, 47) and that selective inhibition of HDAC 6 promotes neurite regeneration following neuronal injury (48).

Like all HDAC inhibitors, valproic acid has actions other than histone deacetylase inhibition and it is also possible that the effects observed here are due to some of these other actions. Future studies could address this by determining the effects of neonatal treatment with other, chemically distinct, HDAC inhibitors. The use of valproic acid as an anti-seizure medication is based on its ability to increase GABA and/or inhibit the NMDA subtype of glutamate receptors following chronic administration (49, 50). It is not known whether short-term,

neonatal exposure to valproic acid alters GABA or glutamate signaling, but this might also be a fruitful hypothesis to test in future work.

Valproic acid increased the vasopressin immunoreactivity in the lateral septum of females, but did not fully masculinize this measure. Although the critical period for masculinization of septal vasopressin in mice is not known, work in rats suggests a sensitive period that lasts throughout the first postnatal week. The castration of male rat pups on either P1 or on P7 significantly decreased vasopressin-immunoreactive cells in the BNST and MeA and fibers in the lateral septum, and testosterone treatment as late as P7 fully restored vasopressin immunoreactivity in neonatally gonadectomized males (44). Treatment with an HDAC inhibitor for a longer period of time may therefore be required to completely disrupt sexual differentiation of vasopressin expression.

Olfactory behaviour was also subtly affected by valproic acid in this study. Treatment did not significantly affect overall preference score (time spent investigating female-soiled bedding minus time spent investigating male-soiled bedding) but did significantly increase the frequency of visits to female-soiled bedding and overall time spent investigating soiled bedding from both sexes specifically in females. We interpret this finding as an increased interest in, or attentiveness to, socially relevant cues. Although we are not aware of any demonstrated connection between vasopressin immunoreactivity and the specific olfactory behaviours measured here, vasopressin and its receptors are involved in the regulation of a number of social behaviors that have a clear olfactory component (51). It is therefore possible that the alterations in vasopressin expression in the lateral septum and changes in olfactory behavior may be causally related.

Previously we found that neonatal valproic acid treatment blocked masculinisation of volume and cell number in the BNSTp of weanling male and androgenised female mice, and a similar effect was seen in adults of the current study. The present findings suggest that the same treatment also partially masculinises vasopressin expression and olfactory behavior in female mice. Although these outcomes may seem initially contradictory, it is unlikely that development of a masculine phenotype depends on global increases in histone acetylation (and, hence, gene expression) or that female development requires uniformly less acetylation. Rather, gonadal steroid hormones and their receptors, in conjunction with co-activators and co-repressors with histone acetyl transferase or histone deacetylase activity, respectively, likely orchestrate orderly patterns of acetylation of histones associated with specific genes. Depending on the specific genes affected, a disruption of this process may therefore have very different effects on different sexually dimorphic traits.

## Acknowledgments

The authors would like to thank Jill McCutcheon, Caroline Smith, and Lynn Bengston for technical assistance. This work was supported by National Institutes of Health Grant RO1-MH068482 (to N.G.F.)

**Supporting grants**: RO1-MH68482 (NGF)

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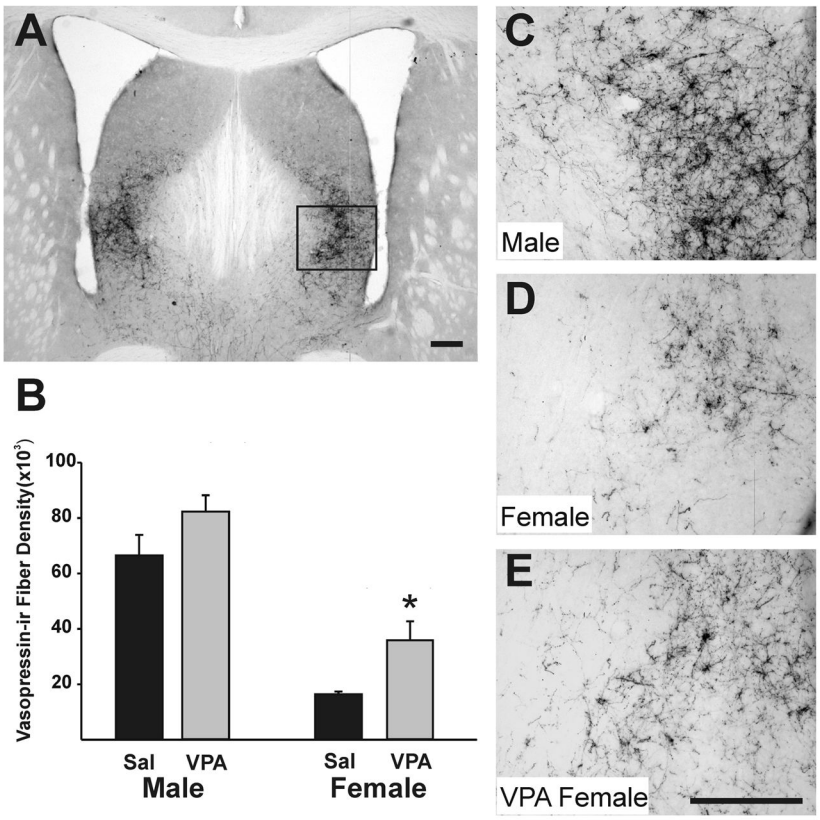


**Figure 1.**

Valproic acid increased vasopressin immunoreactivity in the lateral septum in females. (A) Low power photomicrograph illustrating vasopressin immunoreactivity in the lateral septum of a saline-treated male. The boxed area represents the region quantified in all animals. (B–D) High power photomicrographs of vasopressin-immunoreactive fibers in the lateral septum of a (B) saline-treated male, (C) saline-treated female, and (D) valproic acid-treated (VPA) female. (E) Mean (± SEM) vasopressin-immunoreactive fiber density in the lateral septum in males and females treated with saline (Sal) or VPA. Scale bars = 200 μm, * $p < 0.05$, n=8–10/group).

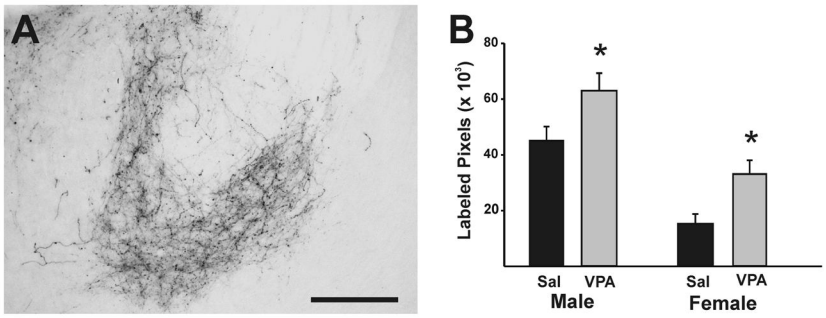


**Figure 2.**

Valproic acid increased vasopressin immunoreactivity in the medial dorsal thalamus (MDT) in both sexes (A) Photomicrograph of vasopressin fibers in the MDT of a saline-treated male (scale bar = 200 μm). (B) Mean (± SEM) vasopressin-iummunoreactive fiber density in the MDT in males and females treated neonatally with saline (Sal) or valproic acid (VPA) (* $p < 0.001$, n=7–9/group).

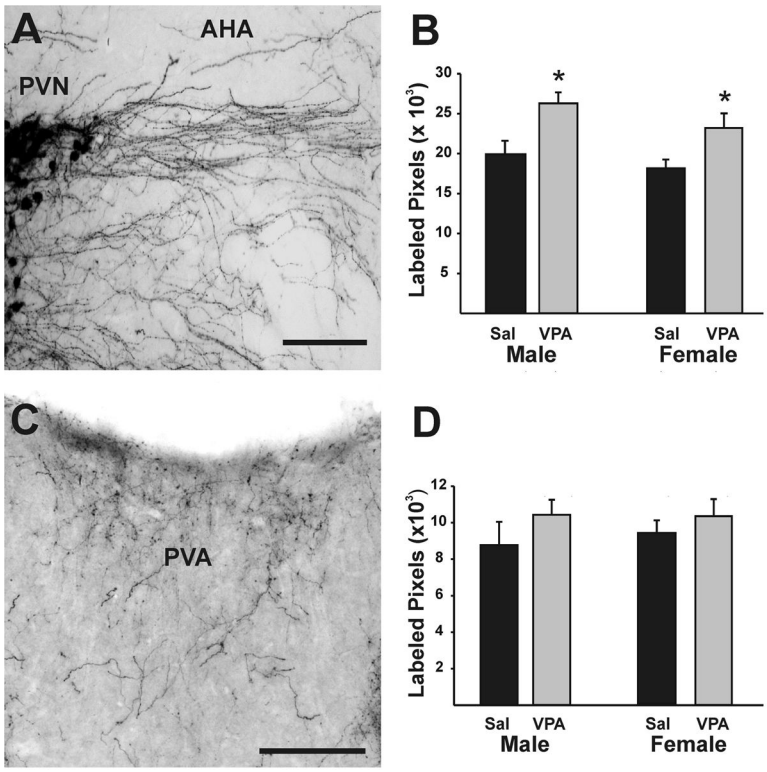


**Figure 3.**

Valproic acid increased vasopressin immunoreactivity in the anterior hypothalamic area (AHA) in both sexes. Photomicrographs illustrating vasopressin innervation in (A) the AHA, and (C) the paraventricular nucleus of the thalamus (PVA) of a saline-treated male. Mean (± SEM) vasopressin-ir fiber density in the AHA (B) and PVA (D) of males and females treated with saline (Sal) or valproic acid (VPA) (* $p < 0.005$, n=6–10/group).

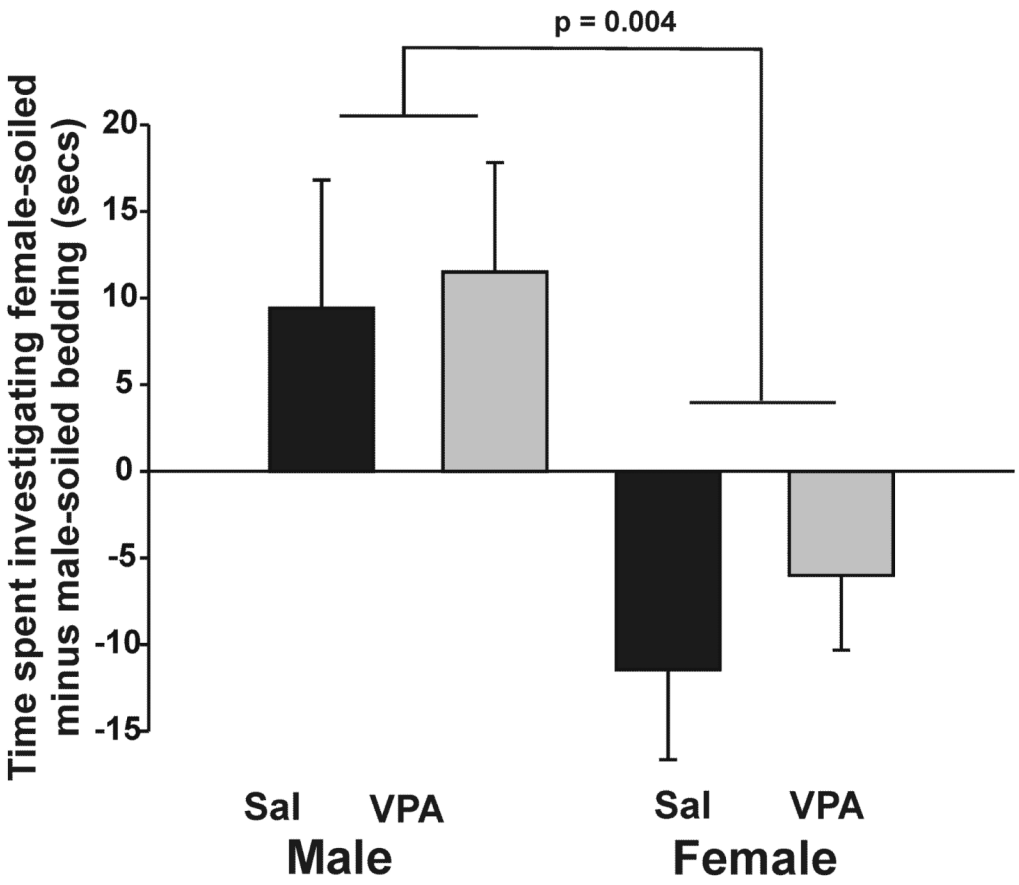


**Figure 4.**
Olfactory preference expressed as mean (± SEM) time spent sniffing female-soiled bedding minus time spent sniffing male-soiled bedding in males and females treated neonatally with saline (Sal) or valproic acid (VPA).

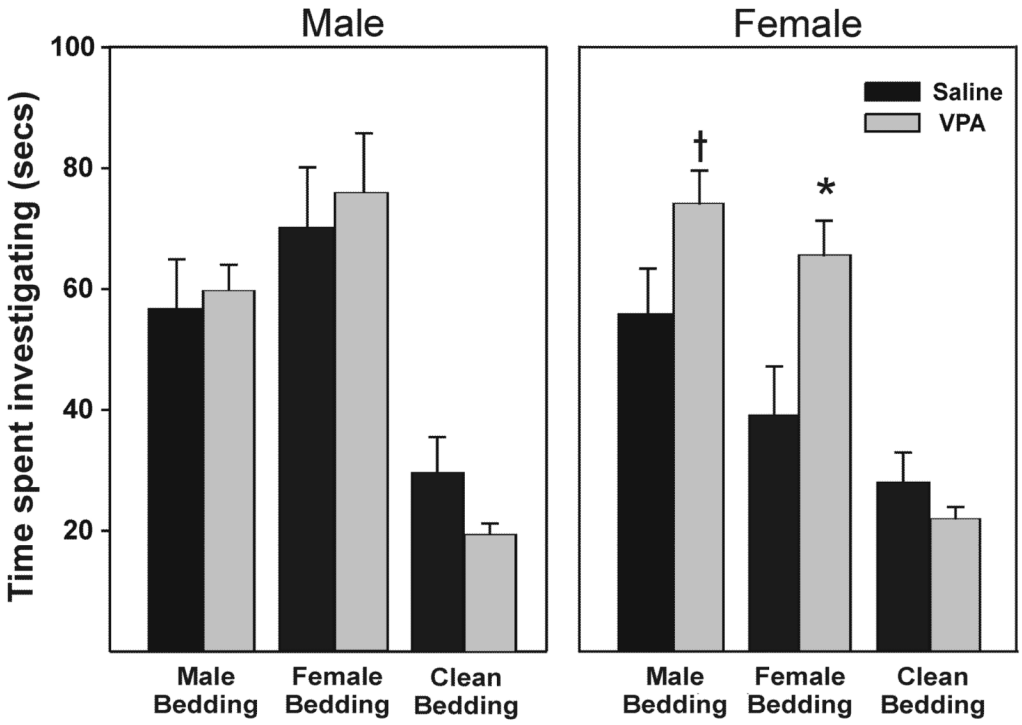


**Figure 5.**

Mean (± SEM) time spent sniffing male-soiled, female-soiled and clean bedding in males (left) and females (right) treated neonatally with saline (Sal) or valproic acid (VPA). Valproic acid significantly increased time spent investigating soiled-bedding in females (* $p < 0.05$, † $p = 0.06$).

## Table 1

Mean (± SEM) BNSTp volumes($\times 10^6$ $\mu m^3$) in males and females treated neonatally with saline or valproic acid (VPA). Saline-treated males had larger volumes than all other groups ($p < 0.05$), which did not differ significantly from each another.

| | |
|---|---|
| **Saline Male** | **88.6 ± 3.6*** |
| Saline Female | 76.5 ± 3.4 |
| VPA Male | 77.5 ± 3.3 |
| VPA Female | 71.9 ± 5.2 |

## Table 2

Mean ± SEM serum testosterone (ng/ml) and estradiol (pg/ml)in adult males and females treated with VPA neonatally. There was no significant effect of VPA on serum testosterone in males or in females, or on serum estradiol in females.

| | Male Saline | Male VPA | Female Saline | Female VPA |
|---|---|---|---|---|
| Testosterone | 1.44 ± 0.52 | 1.07 ± 0.52 | 0.09 ± 0.01 | 0.07 ± 0.01 |
| Estradiol | n.d. | n.d. | 4.6 ± 0.4 | 4.5 ± 0.3 |

n.d. = not done.