

ORIGINAL RESEARCH ARTICLE

# Vasopressin V1b receptor knockout reduces aggressive behavior in male mice

SR Wersinger<sup>1</sup>, El Ginns<sup>2</sup>, A-M O'Carroll<sup>3</sup>, SJ Lolait<sup>3</sup> and WS Young III<sup>1</sup>

<sup>1</sup>Section on Neural Gene Expression, NIMH, Bethesda, MD, USA; <sup>2</sup>Brudnick Neuropsychiatric Research Institute, University of Massachusetts Medical School, Worcester, MA, USA; <sup>3</sup>University Research Centre For Neuroendocrinology, University of Bristol, UK

Increased aggression is commonly associated with many neurological and psychiatric disorders. Current treatments are largely empirical and are often accompanied by severe side effects, underscoring the need for a better understanding of the neural bases of aggression. Vasopressin, acting through its 1a receptor subtype, is known to affect aggressive behaviors. The vasopressin 1b receptor (V1bR) is also expressed in the brain, but has received much less attention due to a lack of specific drugs. Here we report that mice without the V1bR exhibit markedly reduced aggression and modestly impaired social recognition. By contrast, they perform normally in all the other behaviors that we have examined, such as sexual behavior, suggesting that reduced aggression and social memory are not simply the result of a global deficit in sensorimotor function or motivation. Fos-mapping within chemosensory responsive regions suggests that the behavioral deficits in V1bR knockout mice are not due to defects in detection and transmission of chemosensory signals to the brain. We suggest that V1bR antagonists could prove useful for treating aggressive behavior seen, for example, in dementias and traumatic brain injuries.

*Molecular Psychiatry* (2002) 7, 975–984. doi:10.1038/sj.mp.4001195

**Keywords:** homologous recombination; aggression; memory; vasopressin 1b receptor; knockout

## Introduction

Aggression is an evolutionarily ancient behavior. It is also a major health concern and is a symptom of various neurological and psychiatric disorders in both children and adults, including Alzheimer's and other dementias, schizophrenia, and traumatic brain injury.<sup>1–4</sup> Knowledge obtained to date on the genetic and neural mechanisms of aggression has not had much clinical impact on treatment which relies largely on the empirical use of antipsychotics.<sup>5</sup>

Vasopressin (VP) is implicated in agonistic behavior in taxonomically diverse groups including mammals (eg hamsters, voles) and fish (eg wrasse).<sup>6–11</sup> Even in humans, a life history of aggression against others correlates with increased CSF levels of VP.<sup>12</sup> Vasopressin also modulates social memory in rodents.<sup>13–16</sup> Administration of vasopressin V1a receptor (V1aR) agonists and antagonists affects both of these behaviors.<sup>8,14,17–19</sup>

Greater understanding of VP's and its receptors' functions in animal models may lead to improved clinical treatment for aggression. The recently cloned pituitary V1b receptor (V1bR; sometimes referred to as

V3R) is also expressed in the brain,<sup>20,21</sup> suggesting a potential role in aggression and other behaviors. This idea has remained untested because pharmacological agents specific for the V1bR have not been available. To bypass this limitation, we created V1bR knockout mice (V1bRKO) through targeted disruption of the V1bR gene. We report here an extensive evaluation of this line's phenotype, finding a marked reduction in aggression.

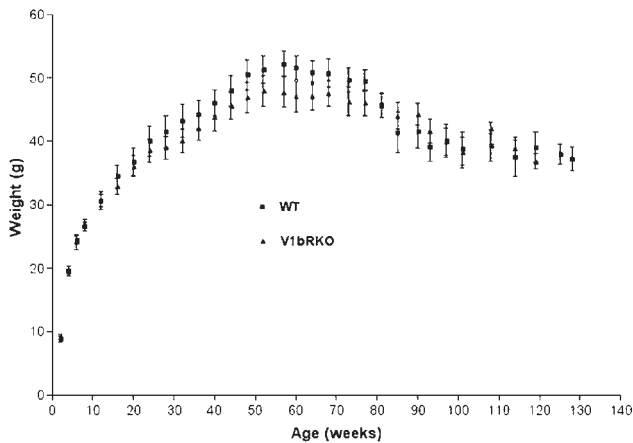
## Materials and methods

### *Targetted disruption of the V1b receptor gene*

A 1FIX II mouse 129/SvJ genomic library (Stratagene, La Jolla, CA, USA) was screened with a <sup>32</sup>P-labelled *Pvu*II fragment of a rat V1bR cDNA. Two independent clones were identified and the largest (~16.6 kb; GENBANK Accession Nos AF152533 and AF152534) was used to construct the targeting vector. A 1.2-kb *Pvu*II fragment 5' to the coding region for transmembrane regions I–VI was inserted in the targeting vector pPNT at the *Xho*I site. The 1.7-kb *Pst*I/*Sac*I piece containing the 3' end of the exon (TMVI) and most of the following introns were inserted at the *Hinc*II site of pPNT (this destroyed the thymidine kinase selection). The targeting construct thus eliminated the V1bR coding region from the initiating methionine just prior to TMVI. The construct was linearized with *Not*I and electroporated into embryonic stem cells for selection

Correspondence: WS Young III, NIMH, Bldg 36, Room 2A11, MSC 4068, Bethesda, MD 20892-4068, USA. E-mail: scott@codon.nih.gov

Received 7 January 2002; revised 12 April 2002; accepted 9 May 2002



**Figure 1** Growth curves of male WT (squares,  $n = 7$ ) and V1bRKO (triangles,  $n = 12$ ) mice. Weights (g, mean  $\pm$  SEM) were taken biweekly for 2 months then monthly until death. Both genotypes showed similar growth and longevity (Table 2).

as described previously.<sup>22</sup> Two embryonic stem cell clones were identified by PCR and confirmed by Southern analysis. Chimeric mice were generated from one of them and germ line transmission was observed. Genotyping was performed by PCR. The growth curves and longevity of the mice were not significantly different (Figure 1, Table 2)

### Subjects

All subjects were littermates of crosses using mice heterozygous for the mutation. The offspring were genotyped at weaning using PCR analysis of DNA isolated from tail clips. The parents of the subjects were a mix of the C57Bl/6 and 129/SvJ strains.

## Behavior tests

### Aggression

All mice ( $n = 8$  WT, heterozygote, and V1bRKO male mice) were socially naive and singly housed on a 12L:12D light cycle (lights on at 0600 h, EST) with food and water available *ad libitum* for at least 2 weeks prior to testing. The subjects were between 60–90 days of age. Weight-matched, adult, gonad- and olfactory bulb-intact male 129/Sv mice were used as stimulus animals because this strain of mouse is fairly docile, and these mice do not attack quickly. The stimulus mice were socially experienced and used up to three times per day, unless they were attacked (in which case they were not used any more that day). A subject was never tested again with the same stimulus animal. After several minutes, on occasion, a stimulus mouse would attack the subject. Only two stimulus animals ever initiated attacks against subjects. This did not allow for systematic analysis of defensive behavior. All testing was conducted during the dark phase of the light cycle.

**Resident-Intruder:** The home cages were not changed for at least 3 days prior to testing. A stimulus male was added to the mouse's home cage. If no aggression was observed in the first 5 min, a latency of 300 s was recorded and the test ended. Otherwise, the test was allowed to continue for 2 min after aggression was first observed. All interactions were videotaped using a Sony night shot digital video camera (Sony model DRV120) for analysis since many of the behaviors occur very quickly. Each mouse was given three tests with 3 days between each test. The latency to attack, number of attacks, number of bites, and number of tail rattles were recorded. An attack was scored whenever the subject approached the stimulus animal and engaged in any aggressive behavior. Thus, the number of attacks includes the number of bites. In addition,

**Table 1** Vasopressin 1b receptor knockout (V1bRKO) mice show reduced aggression compared to their wild-type (WT) littermates

	WT	V1bRKO
Resident-Intruder Paradigm:		
Attack latency (seconds $\pm$ SEM)	298 $\pm$ 19	702 $\pm$ 13
Number of attacks/test	14 $\pm$ 3	2.2 $\pm$ 1.1
Number of bites/test	9.4 $\pm$ 2.7	0.8 $\pm$ 0.7
Number of tail rattles/test	5.0 $\pm$ 1.8	1.4 $\pm$ 1.1
Percentage attacking by test 1	62.5%	12.5%
Percentage attacking by test 2	100%	25%
Percentage attacking by test 3	100%	62.5%
Neutral Arena Paradigm:		
Attack latency (seconds $\pm$ SEM)	442 $\pm$ 15	709 $\pm$ 16
Number of attacks/test	15.4 $\pm$ 2.7	3.3 $\pm$ 1.4
Number of bites/test	8.4 $\pm$ 2.0	1.7 $\pm$ 1.2
Number of tail rattles/test	6.3 $\pm$ 1.9	0.3 $\pm$ 0.8
Percentage attacking by test 1	37.5%	12.5%
Percentage attacking by test 2	87.5%	37.5%
Percentage attacking by test 3	100%	37.5%

behaviors associated with defensive posturing were recorded. *Neutral Test Cage:* The subjects used in this paradigm had not been previously used, and were also socially naïve. The experimental male was placed in a clean cage and given 30 s to acclimate. The stimulus male was then added to the cage. Testing proceeded as described in the resident-intruder paradigm.

#### *Social recognition*

All stimulus animals were ovariectomized 129/Sv females. None of the subjects had been used for testing and began socially naïve. Mice were pre-exposed to arbitrarily chosen ovariectomized females for four to seven 30-min sessions to extinguish sexual behavior, no more than once per day. There was no genotypic effect in the number of sessions required to extinguish the display of sexual behavior. All testing was conducted during the dark phase of the light cycle.

*Task 1* Mice ( $n = 8$  of each genotype) were observed over a series of eleven 1-min tests. In the first ten tests, the mice were exposed to the same stimulus female. There were 5 min between tests. On the eleventh test, a novel female was placed in the cage. The times the mouse spent engaged in both anogenital and full body sniffing were quantified.

*Task 2* Mice ( $n = 8$  of each genotype) were also observed over a series of two 5-min tests with 30 min between tests. Again, the time the mouse spent engaged in both anogenital and full body sniffing of the same stimulus female was quantified.

#### *Basile Rotor-rod and hanging wire cage*

The methods of many of the following behavior tests are based on methodology previously described.<sup>23</sup> The Basile accelerating Rotor-rod adapted for mice (San Diego Instruments, San Diego, CA, USA) was used to assess gross locomotor function in the light phase of the light cycle. Briefly, male mice ( $n = 8$  of each genotype) were placed on a rod that was accelerated from 0 rpm to 60 rpm over 120 s. Photo beam sensors detected the mice when they fell off the rod 30 cm onto a foam-padded base. The latency to fall was recorded. In the strength test, mice of both sexes ( $n = 8$  of each genotype) were placed on the underside of a wire grid approximately 20 cm above soft wood-chip bedding. If the mouse had not fallen within 60 s, the test was ended. The latency to release was recorded.

#### *Vision test*

Male mice ( $n = 8$  of each genotype) were held by their tails about 12 inches above and about 2 inches out from the surface of a table in the light phase of the light cycle. They were slowly lowered so that they would pass close to the edge of the table, but far enough away so that their whiskers would not touch the table surface. Mice with vision reach for the table as they are lowered past the surface of the table.

#### *Food consumption*

Male mice ( $n = 14$  per genotype) were food-deprived overnight. The next day, small food dishes (Dyets, Inc, Bethlehem, PA, USA) were filled with powdered food (Dyets, Inc) and weighed. Each mouse was placed individually into a clean cage containing the dish in the light phase of the light cycle. After 120 min the mouse was removed, the dishes weighed again, and the amount of food consumed recorded.

#### *Body temperature*

Adult (120–140 days of age) mice of both sexes ( $n = 5$  of each genotype) were anesthetized using isoflurane administered using a Surgivet anesthesia machine. An Omega microprocessor thermometer (model HH23) was used to measure rectal temperature in the light during the dark phase of the light cycle. The paraffin-coated probe was lubricated and inserted into the animals's rectum. The temperature was recorded within 30 s after beginning anesthesia.

#### *Hidden-cookie test*

Male mice ( $n = 8$  of each genotype) were food-deprived overnight. A small cube (five mm on each side) of Nutter Butter cookie (Nabisco™) was buried beneath about 4 cm of clean woodchip bedding in a random location. The mouse was placed in the cage, again in a random location, in the light phase of the light cycle. The latency to locate the cookie was recorded. We defined finding the cookie as when the mouse held it in both paws.

#### *Sex behavior test*

Adult, socially naïve male mice ( $n = 10$  each of each genotype) were placed into a Plexiglas testing cage (30 cm × 10 cm × 10 cm) and allowed to acclimate for 30 min. Gonadectomized, steroid-primed 129/Sv females (implanted with a capsule containing 50 µg estradiol benzoate in 25 µl sesame oil, followed by a subcutaneous injection of 0.5 mg progesterone in 25 µl sesame oil 4–6 h before use) were used as stimulus animals. Each female was screened with a highly experienced male to ensure receptivity. Briefly, the female was added to the testing cage and behavior was scored for 60 min in the dark phase of the light cycle under red-light illumination. Each mouse was tested until an ejaculation was observed or he had been given three tests. The latency to and number of mounts, mounts with thrusts, intromissions, and ejaculations were recorded.

#### *Elevated-plus maze*

The subjects for the elevated plus maze had previously been used (at least 2 weeks before) to assess sensorimotor function. Male mice ( $n = 8$  each of each genotype) were placed onto the center of an elevated plus maze adapted for mice (San Diego Instruments). The test was conducted in a soft white light background during the dark phase of the light cycle. The time the animal spent in the center and in each arm

was recorded, as well as the number of entrances into each arm. The test lasted 5 min.

#### *Open-field test*

Male WT and V1bRKO mice ( $n = 10$  per genotype) were tested during the dark phase of the light cycle using a Digiscan automated testing apparatus (San Diego Instruments). The box is 40 cm  $\times$  40 cm with eight photobeams in the  $x$  and  $y$  axes. Another set of eight photobeams 4 cm off the floor measure movement in the  $z$ -plane. Each animal was given a 30-min test. The apparatus measures both horizontal and vertical movement by counting the number of times various infrared beams are broken. The number of beam breaks were compared between the genotypes.

#### *The Morris water maze*

Male WT and V1bRKO mice ( $n = 10$  per genotype) were tested in a plastic pool one meter in diameter. A clear platform (12 cm in diameter) was used. The water was dyed green using Crayola™ non-toxic paint so the mouse could not see the submerged platform. The protocol is based on that described in Crawley.<sup>23</sup> The subjects were initially pre-trained. For this phase, a small flag was attached to the platform and the platform was placed above the level of the water. The mouse was placed on the platform in the dark phase of the light cycle under red-light illumination. All the mice were placed into the water within 15 s of being placed on the platform. The mice were then guided to the platform. After two such trials, the animals was placed in the middle of the pool and the latency to find the platform recorded. For this test, the platform remained in the same position in the pool and the mice were released from the same point in the pool. Next, the subjects were trained for the task. We used an intensive training paradigm with 12 trials per day, in three blocks of four trials each, for 3 days. Ten animals (five per genotype) were tested in two batches. All subjects were tested during the dark phase of the light cycle each day. For each mouse, the platform was placed under the surface of the water in a consistent but randomly determined (using a random number table) quadrant. The mouse was placed in the pool in a randomly determined quadrant in the pool facing the wall. The subject was released and the latency to reach the platform measured. If the subject had not found the platform within 60 s, the test ended. We defined our criterion as 10 s to reach the platform. After the end of the testing, we conducted a probe trial. In this trial, the platform was removed. The animal was placed in the pool in one 60 s test. The amount of time the subject spent in the quadrant where the platform should have been was recorded.

### **Hormone assays**

#### *Testosterone*

Two groups of ten WT, ten HET and ten V1bRKO males were removed from their cages during the first hour of the dark phase of the light cycle. The first group ranged

in age from 60–90 days. The second group was comprised of older males, ranging in age from 120–160 days. Mice were anesthetized with isoflurane and 0.3 cc of blood taken via cardiac puncture within 2 min of the initial cage disturbance. The blood was spun and the plasma stored at  $-70^{\circ}\text{C}$  until assayed using an EIA (Diagnostic Systems Laboratories, Webster, TX, USA). Each group was run in a separate assay. The intra-assay coefficient of variation (CV) ranged from 3.1% to 11.9% and the inter-assay CV was 8.2%.

#### *Corticosterone*

**Basal** To measure basal corticosterone levels, WT, HET and V1bRKO males ( $n = 10$  per genotype) were removed from their cages during the first hour of the dark phase of the light cycle and immediately anesthetized with isoflurane. Approximately 0.3 cc of blood was taken via cardiac puncture within 2 min of the initial cage disturbance and the plasma stored at  $-70^{\circ}\text{C}$  until assayed.

**Stress-induced** To measure the level of corticosterone experienced by our subjects in the resident-intruder aggression tests, a new group of socially naïve males was exposed to an adult, gonad-intact 129/Sv male ( $n = 8$  per genotype of WT, HET, and V1bRKO). All the subjects for this experiment were singly housed for at least a week and testing occurred in the dark phase of the light cycle. Aggression was observed in the WT and HET cages, but not in the V1bRKO cages. Unless the subject or stimulus animal was injured, the test was continued and the data included. Thirty minutes later, the mice were anesthetized with isoflurane and 0.3 cc of blood taken via cardiac puncture. As a control, we opened the home cage of some of the mice ( $n = 6$  per genotype) but did not add a male. Thirty minutes after this disturbance, the mice were anesthetized with isoflurane and 0.3 cc of blood taken via cardiac puncture. Again, blood was stored at  $-70^{\circ}\text{C}$  until assayed by RIA (Diagnostic Systems Laboratories). All samples were run in the same assay, and the intra-assay correlation of variation CV ranged from 8.1–10.3%.

#### *Fos-immunocytochemistry*

An adult, gonad-intact 129/Sv male was added to the home cages of singly housed males ( $n = 8$  of each genotype) for 5 min during the dark phase of the light cycle. Since fighting was rarely observed in V1bRKO mice, we only included WT and V1bRKO subjects that did not fight on initial exposure to the 129/Sv stimulus mice. We did this to control for activation associated with fighting itself. Thus, if we observed differences in Fos expression, we could be more confident that they reflect differences in the detection and/or processing of social cues rather than the performance of agonistic behavior *per se*. Another group of animals ( $n = 3$  per genotype) experienced the same treatment as the exposed group except an intruder was not added to the cage. The stimulus animal was then removed, and the mouse left undisturbed for 60 min. The subject was

then deeply anesthetized with isoflurane and decapitated. The brain was removed, blocked (into three sections) and immersion-fixed in 4% paraformaldehyde. After fixation, the brains were cryoprotected in 30% sucrose. The brains were cut into 40- $\mu$ m sections. The free-floating sections were processed for Fos-like immunoreactivity (Fos-ir) using a rabbit polyclonal antibody directed against c-Fos (Santa Cruz Biochemicals, Santa Cruz, CA, USA) and a Vector ABC Elite kit (Vector Laboratories, Burlingame, CA, USA). Fos-ir was visualized using diaminobenzidine tablets (Sigma, St Louis, MO, USA). The sections were mounted and coverslipped using Permount. The number of cells expressing Fos-ir in one field of view under the 40 $\times$  objective was counted using standard templates for the following brain regions: the accessory olfactory bulb, the main olfactory bulb, the bed nucleus of the stria terminalis, the medial preoptic area, the medial amygdala, the paraventricular nucleus, and the midbrain central gray. The number of neurons expressing Fos-IR in the unexposed groups was low and equivalent in the WT and V1bRKO animals. Therefore, these subjects were collapsed into one group, which we used as the control.

#### Statistics

Group means were compared using a one-way ANOVA followed by Student Newman-Keuls post-hoc analyses except for the analysis of the social recognition data. One-way ANOVA of repeated measures was used to analyze this data set. Differences in the percentage of animals showing aggressive behavior were compared using Fisher's Exact Test of Probability.

## Results

### *V1bRKO males exhibited less aggression than WT littermates*

Gonad-intact males were tested for aggression using the resident-intruder (Figure 2a, b) and the neutral arena (Figure 2c, d) paradigms. In both behavioral tests, the percentage of V1bRKO mice showing aggression was significantly reduced compared to their wild-type littermates (Table 1; Figure 2a, c;  $P < 0.01$ ). The V1bRKO mice showed a longer attack latency (Table 1) and those V1bRKO that did display offensive behavior showed significantly less agonistic behavior than their wild-type littermates (Table 1; Figure 2b, d;  $P < 0.01$ ). This effect has proven to be both robust and stable. Reduced aggression was observed in adult males of various ages, across two generations, and by males generated from heterozygous crosses in different animal facilities (data not shown). The percentage of V1bRKO males exhibiting aggressive behavior in a standard resident-intruder testing paradigm was 62.5%. All of their WT littermates were consistently aggressive by the end of the third resident intruder test. All the aggression data reflect that shown in Figure 2 and are summarized in Table 1. A video typifying the different levels of aggression is shown at <http://intramural.nimh.nih.gov/lcmr/snge/V1bRKOvid.html> (note: this video shows

pairs of male mice of the same genotype). The level of aggression we observed in our WT mice is high compared to typical levels observed in C57Bl or 1296J. Such robust aggression may be due to the genetic background (mixed background of C57Bl/6/129/SvJ), the stimulus animal (129/Sv), and/or the housing conditions (singly housed in micro-isolator caging). It seems to us that the high level of aggression in our WT mice makes it even more surprising that targeted disruption of the V1bR results in such reduced aggression.

### *Social recognition was impaired in V1bRKO males*

V1bRKO males did not show the same pattern of chemoinvestigatory behavior as their WT littermates in either test of social recognition (Figure 3a and b). Although the animals spent more time on body than anogenital sniffing, the patterns were consistent. Therefore, we report only the body sniffing data for both paradigms. When the mice were placed in a cage with the same stimulus female over the series of ten exposures, V1bRKO sniffed the stimulus animal significantly more than their WT littermates only during the 4th exposure (Figure 3a). Both WT and V1bRKO mice showed renewed interest in the new stimulus female presented at the 11th exposure. Similar results were obtained with a different group of animals (data not shown). These results showed a minimal effect, so we tested the mice for this behavior using a more challenging test. When given two 5-min exposures to the same stimulus female separated by 30 min, V1bRKO sniffed the female mouse for a similar time in both tests, whereas their WT littermates spent significantly less time sniffing the female mouse in the second test (Figure 3b).

### *Performance on other behavioral tasks were equivalent in V1bRKO and WT littermates*

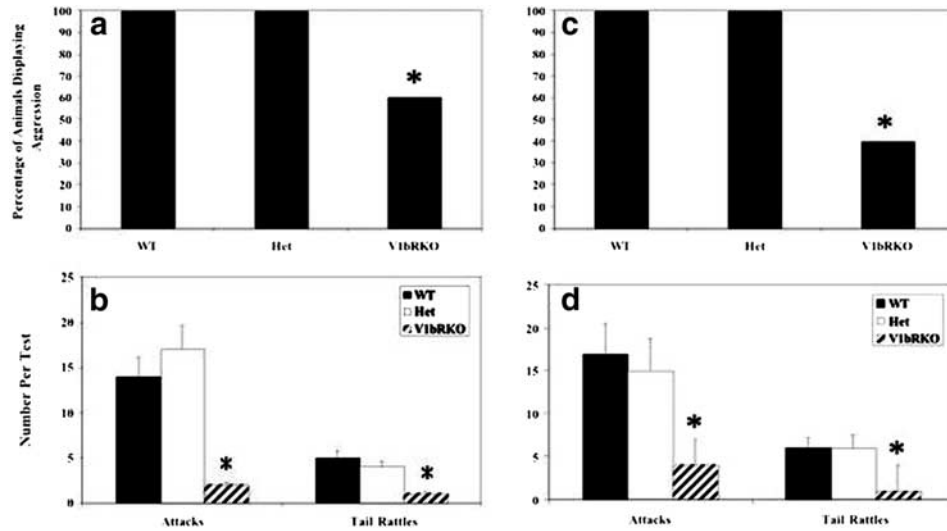
There were no other differences between WT and V1bRKO males on any of the other behavioral tests performed, including tests of olfaction, vision and other sensorimotor functions. Body temperature did not differ between the genotypes. These data are summarized in Table 2.

### *The Morris water maze*

There were no genotypic differences in the escape latency on any of the testing days (Day 1:  $38.1 \pm 9.4$  s for WTs,  $32.3 \pm 10.1$  s for V1bRKO; Day 2:  $14.9 \pm 5.2$  s for WTs,  $13.9 \pm 7.1$  s for V1bRKO; Day 3:  $8.7 \pm 3.4$  s for WTs,  $8.2 \pm 2.9$  s for V1bRKO;  $P > 0.10$  for all testing days) or in the percentage of time spent in the correct quadrant on the probe trial ( $41.5 \pm 3.6\%$  for WTs,  $43.0 \pm 3.9\%$  for V1bRKO;  $P > 0.10$ ). Both genotypes attained criterion by the third day.

### *The elevated plus maze*

There were no genotypic differences in the percentage of time spent in the open arms ( $7.2 \pm 1.7\%$  for WTs,  $7.0 \pm 1.4\%$  for V1bRKO;  $P > 0.10$ ), the percentage of entries to the open arms ( $45.9 \pm 1.8\%$  for WTs,  $44.7 \pm 2.5\%$  for V1bRKO;  $P > 0.10$ ), or the total number of



**Figure 2** Male V1bRKO mice are less aggressive than their wild-type (WT) or heterozygous (Het) littermates in a resident-intruder paradigm (a, b) and a neutral-cage paradigm (c, d). (a) A significantly lower percentage of V1bRKO males display aggressive behavior than of WT or Het males in the resident-intruder paradigm after three test sessions. (b) Of those mice displaying aggressive behavior, V1bRKO mice display significantly fewer events per test than WT or Het males in a resident intruder paradigm. The means include data only from tests in which aggression was observed. (c) A significantly lower percentage of V1bRKO males display aggressive behavior than of WT or Het males in a neutral-cage paradigm after three test sessions. (d) Of those mice displaying aggressive behavior, V1bRKO mice display significantly fewer events per test than WT or Het males in a neutral-cage paradigm. The means include data only from tests in which aggression was observed. (Data are expressed as mean  $\pm$  SEM; \* = significantly lower than other groups,  $P < 0.01$ .) More data are provided in Table 1.

entries into the arms ( $7.3 \pm 1.7$  for WT,  $7.0 \pm 1.6$  for V1bRKO;  $P > 0.10$ ).

#### The open field test

There were no genotypic differences in the number of total beam breaks ( $3740 \pm 71$  for WT,  $3613 \pm 70$  for V1bRKO;  $P > 0.10$ ) or vertical beam breaks ( $107.7 \pm 7.0$  for WT,  $94.0 \pm 7.1$  for V1bRKO;  $P > 0.10$ ). Also, the number of consecutive beam crossings were not significantly different ( $1947 \pm 200$  for WT,  $1767 \pm 172$  for V1bRKO).

#### Basal testosterone was elevated in young V1bRKO males, but normal in older V1bRKO males

Plasma testosterone was elevated in V1bRKO males ranging in age from 60–90 days ( $4.1 \pm 1.1$  ng ml<sup>-1</sup> in WT,  $6.1 \pm 1.3$  ng ml<sup>-1</sup> in Hets and  $8.2 \pm 1.2$  ng ml<sup>-1</sup> in V1bRKO;  $P < 0.05$  between WT and V1bRKO). Older males (>120 days) showed no significant differences in plasma testosterone levels among the genotypes ( $5.1 \pm 1.6$  ng ml<sup>-1</sup> in WT,  $5.7 \pm 1.8$  ng ml<sup>-1</sup> in Hets, and  $5.4 \pm 1.7$  ng ml<sup>-1</sup> in V1bRKO;  $P > 0.10$ ).

#### V1bRKO had normal basal levels of corticosterone, and showed an acute stress-induced rise in corticosterone that was equivalent to WT littermates

As plasma corticosterone is a highly sensitive indicator of acute stress, we measured this steroid in male WT and V1bRKO mice, both under basal conditions and after exposure to a gonad-intact male (as in the resident-intruder paradigm). Basal corticosterone was normal in the V1bRKO males (WT =  $2.9 \pm 0.9$  ng ml<sup>-1</sup>, Het =  $3.1 \pm 1.0$  ng ml<sup>-1</sup>, V1bRKO =  $3.4 \pm 0.8$  ng ml<sup>-1</sup>).

Exposure to an intruder male significantly increased plasma corticosterone levels in all males, including the V1bRKO (WT =  $20.1 \pm 3.9$  ng ml<sup>-1</sup>, Het =  $19.0 \pm 4.2$  ng ml<sup>-1</sup>, V1bRKO =  $23.3 \pm 6.8$  ng ml<sup>-1</sup>,  $P < 0.05$  compared to basal conditions).

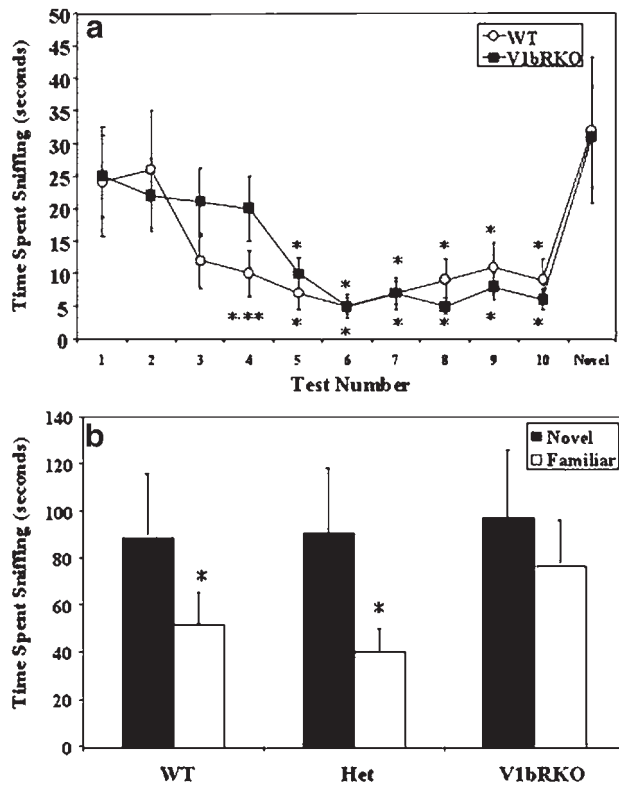
#### The number of neurons expressing fos-like immunoreactivity is increased in the brains of both V1bRKO and WT males following exposure to another male mouse

Exposure to a stimulus male increased the numbers of neurons expressing Fos-like immunoreactivity (Fos-ir) in the main and accessory olfactory bulbs (Figure 4a). The numbers of neurons expressing Fos-ir were also increased in the medial amygdala and bed nucleus of the stria terminalis after exposure to a social stimulus (Figure 4b). There were no genotypic effects. Fos-ir was not induced by a social stimulus in the preoptic area, paraventricular nucleus or the midbrain central gray in males of either genotype.

#### Discussion

The data presented in this paper show that the V1bR is likely to play an important, and previously undescribed, role in aggression, as well as a less critical role in social recognition as the impairment is mild when the task is simple and more pronounced when the task is more challenging. By contrast, all the other behaviors examined were normal, suggesting that the effect of the V1bRKO is relatively specific.

Previous work has shown VP increases agonistic behavior in several rodent species, including hamsters



**Figure 3** Social recognition is impaired in V1bRKO males as compared with their wild-type (WT) and heterozygous (data not shown; data for heterozygotes are not different from WT males and are not shown for simplicity) littermates. (a) In a series of 10-min exposures with 5 min between trials, WT males recognize the stimulus animal more quickly than V1bRKO males at trial 4. (Data are expressed as mean  $\pm$  SEM; \* = significantly different from trial 2  $P < 0.05$ ; \*\* = significantly different from V1bRKO group in the same test,  $P < 0.05$ .) (b) In a more challenging paradigm with a 30-min interval between trials, V1bRKO males do not appear to recognize a familiar female animal, suggesting a role for the V1bR in social memory. (Data are expressed as mean  $\pm$  SEM. \* = significantly less than first exposure,  $P < 0.05$ .)

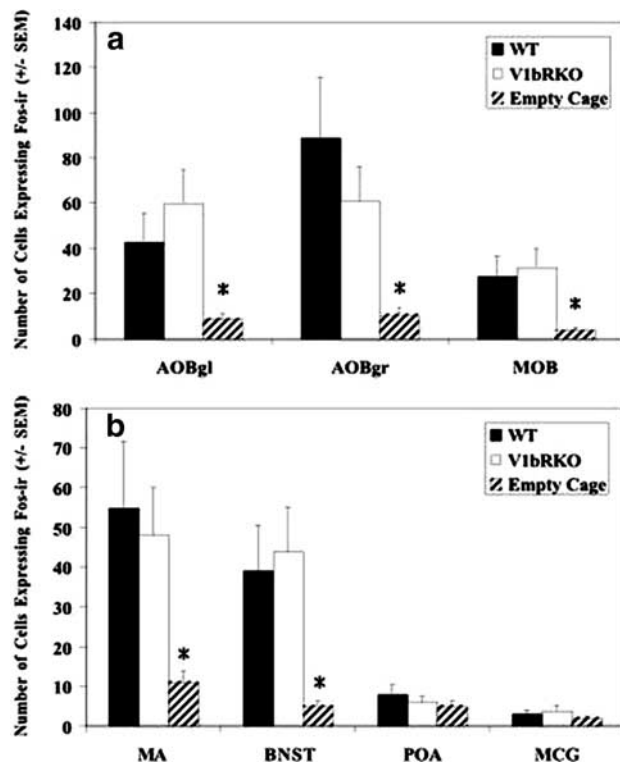
and voles.<sup>8–11</sup> Furthermore, specific V1aR agonists and antagonists also affect aggression,<sup>9,18,19</sup> suggesting that the V1aR mediates some of these effects. We have now shown that V1b receptors must play a role in mediating VP's effects on aggression. The underlying circuits and mechanisms remain to be deciphered. It is possible that other neurotransmitters such as serotonin, GABA, and nitric oxide will ultimately be involved in the mechanism by which the V1bR affects aggression.<sup>24</sup> Indeed, an interaction of the serotonin and vasopressin systems has been demonstrated in the hamster, for example.<sup>25,26</sup> Current research in our lab is focusing on identifying substances affected by the absence of the V1bR.

It is unlikely that aggression is reduced in V1bRKO mice because of a general deficit in sensorimotor function. The mice performed similarly to their WT littermates on every test but aggression and social recognition. Growth and sensorimotor function is normal (Table 2) in the V1bRKO mice. Depressed levels of testosterone that

**Table 2** Vasopressin 1b receptor knockout (V1bRKO mice) are normal, compared to their wild-type (WT) littermates, in most of the phenotypes we measured. Unless otherwise noted, results are for male mice and are not significantly different between genotypes. The methods, unless already cited, are in Crawley<sup>23</sup>

Phenotype	Status
Appearance	Normal
Body weight, growth	Normal (Figure 1)
Longevity	
WT	806 $\pm$ 52 days
KO	722 $\pm$ 108 days
Body temperature	
WT	37.9 $\pm$ 0.5°C
KO	38.3 $\pm$ 0.7°C
Fertility (male & female)	Normal
Vision	Normal
Main olfaction (time to find cookie)	
WT	21.3 $\pm$ 4.5 s
V1bRKO	22.7 $\pm$ 3.7 s
Accessory olfaction	Normal (Figure 4)
Motor coordination (Basile Rotor-rod, time to fall)	
WT	11.9 $\pm$ 3.2 s
V1bRKO	13.1 $\pm$ 2.8 s
Motor strength (hanging-wire test, time to fall)	
WT	57.5 $\pm$ 3.9 s
V1bRKO	56.9 $\pm$ 4.1 s
Hypothalamic-pituitary-adrenal axis	
Basal	Normal (see text)
Acute-stress	Normal (see text)
Plasma testosterone	
Young males (60–90 days)	Elevated 2-fold (see text)
Older males (>120 days)	Normal (see text)
Circadian rhythm (female)	Normal
Activity	Normal
Sex behaviour	Normal
Percent mounting	
WT	100%
V1bRKO	100%
Mount latency	
WT	28.9 $\pm$ 9.3 min
V1bRKO	32.2 $\pm$ 7.7 min
Aggression (Table 1)	
Resident-intruder	Reduced (Figure 2)
Neutral-arena	Reduced (Figure 2)
Social recognition	Impaired (Figure 3)
Food intake (after 12 h of food deprivation)	
WT	2.7 $\pm$ 0.8 g
V1bRKO	2.1 $\pm$ 0.4 g
Morris water maze	Normal
Elevated-plus maze	Normal

could produce decreased aggression in mice<sup>27</sup> were not found. The V1bRKO mice show motivation after food deprivation indicating that there is not a general deficit in motivated behavior in general. Sexual behavior, dependent on a complex array of perceptions and reactions, is also normal in our knockouts. Thus, it seems



**Figure 4** The number of neurons expressing Fos-like immunoreactivity (Fos-ir) in several forebrain regions after exposure to a stimulus male. (a) The glomerular layer of the accessory olfactory bulb (AOBgl), granular layer of the accessory olfactory bulb (AOBgr), and the main olfactory bulb (MOB) show elevated numbers of Fos-positive neurons in wild-type (WT) and vasopressin 1b receptor knockout (V1bRKO) mice littermates exposed to a stimulus male compared to animals placed alone in an empty cage ( $n = 3$  WT and  $n = 3$  V1bRKO, collapsed into one group). The number of neurons expressing Fos-ir was quantified at a consistent but arbitrary level of the AOB and MOB. (Data are expressed as mean  $\pm$  SEM; \* = significantly lower than exposed groups in the same brain region,  $P < 0.05$ .) (b) The number of neurons expressing Fos-like immunoreactivity was increased in the medial amygdala (MA) and bed nucleus of the stria terminalis (BNST) but not the preoptic area (POA) or midbrain central gray (MCG) in both WT and V1bRKO mice. The number of neurons expressing Fos-ir was quantified at a consistent but arbitrary level of each region. (Data are expressed as mean  $\pm$  SEM; \* = significantly lower than exposed groups in the same brain region,  $P < 0.05$ .)

likely that the V1bR plays an important and specific role in the generation of aggressive behaviors.

Anxiety may also affect aggression, although the mechanism by which it does so and the direction of the effect are complex.<sup>28</sup> In hamsters, fluoxetine reduces agonistic behavior, an effect mediated by the vasopressinergic system.<sup>26,29</sup> Therefore, if basal anxiety were reduced in V1bRKO, aggression could be reduced secondarily. Basal anxiety, as measured using the elevated plus maze, is normal in the knockouts. This test has been validated extensively as a marker of anxiety and performance on this test is highly sensitive to anxiolytic and anxiogenic drugs.<sup>30,31</sup>

It is possible that the mutation could affect how the animal perceives the threat of an intruding animal. V1bR is expressed in pituitary corticotrophs<sup>20,32,33</sup> and its absence might compromise the hypothalamic-pituitary-adrenal axis. The V1bRKO animals, despite a lack of aggression in their tests, clearly perceived the interaction with the intruder as stressful as evidenced by the increase in plasma corticosterone, a highly sensitive indicator of acute stress. These data also show that the V1bR is unnecessary for either the development or maintenance of basal regulation of the hypothalamic-pituitary-adrenal axis or for its activation by at least one acute stressor.

Social behavior in rodents is highly complex and relies heavily on olfaction.<sup>34–37</sup> The chemosensory responsive neural circuit of rodents has been well described and includes brain regions such as the main and accessory olfactory bulbs (MOB, AOB), the medial amygdala (MA), the bed nucleus of the stria terminalis (BNST), and the medial preoptic area (MPOA).<sup>37–41</sup> Chemosensory cues are transmitted from the main olfactory epithelium and vomeronasal epithelium to neurons in these brain regions where they are processed so that the appropriate behavioral response can be determined.<sup>37–41</sup> The MA and BNST both contain VP immunoreactivity<sup>42–44</sup> and V1aR binding and transcripts,<sup>45–47</sup> and V1bR immunoreactivity.<sup>33</sup> Aggressive behavior is correlated with VP immunoreactivity in fibers in the lateral septum (whose VP cell bodies are located in the BNST).<sup>48</sup> Thus, our functional mapping of this chemosensory responsive circuitry using Fos (Figure 4) is important because it confirms that chemosensory stimuli reach the chemosensory-responsive neural circuit. The increased Fos-like immunoreactivity induced by exposure to the intruder animal demonstrates that V1bRKO males detect and transmit chemosensory information as do WT littermates. In addition, two recent reports of the knockout of the *trp2* cation channel described male–male mounting and reduced aggression. This gene is expressed exclusively in the vomeronasal epithelium and its absence leads to defective olfaction.<sup>49,50</sup> We did not see male–male mounting (see video) or direct evidence of olfactory dysfunction. Our data are more consistent with a defect in the coupling of chemosensory cues to certain appropriate behavioral responses (see below).

Vasopressin affects learning and memory, including social recognition, across many vertebrate classes.<sup>14–17</sup> Agonists and antagonists specific for the V1aR facilitate and inhibit, respectively, learning and memory in several paradigms, including social recognition.<sup>14–17</sup> We observed only a slight impairment in social recognition in the V1bRKO mice. Interestingly, oxytocin knockout mice are reported to show no social recognition (51). This apparently indicates that these evolutionarily related peptides also share aspects of this function and it will be worthwhile to compare oxytocin knockouts with either VP or V1aR/V1bR double knockouts in these tests.

A common mechanism by which the V1bR affects both aggression and social recognition is possible. Both

rely heavily on chemosensory cues being detected, processed, and integrated, and the animal making the appropriate behavioral responses. A function of the V1bR in the brain may be to couple those neurons that receive chemosensory information with those neural circuits that underlie the behavioral response to those cues. Since sexual behavior is normal, one would need to postulate that the V1bR couples only certain behaviors. This specificity is critically important from an evolutionary perspective. A male that encounters a receptive female but engages in agonistic behavior instead of sexual behavior will be reproductively (and therefore evolutionarily) unsuccessful. The chemosensory cues associated with the female must be processed and coupled with a sexual response. By contrast, chemosensory cues associated with a male may elicit aggression. One might predict that the coupling mechanism will necessarily be highly specific and under strict regulation. The V1bR, acting in concert with the V1aR and other neural systems, provides such an opportunity for control.

It is clear that the potential for pharmacological manipulation of the V1bR to affect aggression without major cognitive side-effects merits further research. Armed with this knowledge, we also can now more efficiently dissect the neural mechanism by which VP affects aggression. Studies are in progress to express the V1bR in different brain regions in an attempt to home in on the critical regions. Of course, the activation of the V1bR in the setting of a perceived threat will affect other systems that allow the mouse to respond. How and where numerous other neurotransmitters (eg serotonin, oxytocin, GABA, nitric oxide) and receptors fit into this response and whether their levels and distributions are changed will be the subject of intense scrutiny. Finally, although the acute stress response did not appear to be affected in the V1bRKO mice, further studies are in progress to assess the receptor's role in the chronic stress response where VP is known to play a key role.<sup>52,53</sup>

## Acknowledgements

The authors thank Jacki Crawley, Andrew Holmes, Jennifer Temple, GB Turgidson and Elka Skordalakes for helpful discussions and advice. We thank Joanna Sweigert for help with genotyping and various behavioral studies and, especially, Michael Brownstein and Howard Nash for critical reading of the manuscript. We also thank the animal care staff in Buildings 49 and 36 at NIH for honoring our numerous special requests. SJL and AMO'C were supported by the Wellcome Trust.

## References

- 1 Saver JL, Salloway SP, Devinsky O, Bear DM. Neuropsychiatry of aggression. In: Fogel BS, Schiffer RB, Rao SM. (eds). *Neuropsychiatry*. Williams & Wilkins: Baltimore, 1996, pp 523–548.
- 2 Rosenblatt A, Leroi I. Neuropsychiatry of Huntington's disease and other basal ganglia disorders. *Psychosomatics* 2000; **41**: 24–30.
- 3 Yeager CA, Lewis DO. Mental illness, neuropsychologic deficits, child abuse, and violence. *Child Adolesc Psychiatr Clin N Am* 2000; **9**: 793–813.
- 4 Filley CM, Price BH, Nell V, Antoinette T, Morgan AS, Bresnahan JF *et al*. Toward an understanding of violence: neurobehavioral aspects of unwarranted physical aggression: Aspen Neurobehavioral Conference consensus statement. *Neuropsychiatry Neuropsychol Behav Neurol* 2001; **14**: 1–14.
- 5 Tune LE. Risperidone for the treatment of behavioral and psychological symptoms of dementia. *J Clin Psychiatry* 2001; **62 Suppl 21**: 29–32.
- 6 Goodson JL, Bass AH. Social behavior functions and related anatomical characteristics of vasotocin/vasopressin systems in vertebrates. *Brain Res Rev* 2001; **35**: 246–265.
- 7 Moore FL, Lowry CA, Rose JD. Steroid-neuropeptide interactions that control reproductive behaviors in an amphibian. *Psychoneuroendocrinology* 1994; **19**: 581–592.
- 8 Ferris CF, Delville Y. Vasopressin and serotonin interactions in the control of agonistic behavior. *Psychoneuroendocrinology* 1994; **19**: 593–601.
- 9 Semsar K, Kandel FL, Godwin J. Manipulations of the AVT system shift social status and related courtship and aggressive behavior in the bluehead wrasse. *Horm Behav* 2001; **40**: 21–31.
- 10 Koolhaas JM, Everts H, de Ruiter AJ, de Boer SF, Bohus B. Coping with stress in rats and mice: differential peptidergic modulation of the amygdala-lateral septum complex. *Prog Brain Res* 1998; **119**: 437–448.
- 11 Bester-Meredith JK, Young LJ, Marler CA. Species differences in paternal behavior and aggression in peromyscus and their associations with vasopressin immunoreactivity and receptors. *Horm Behav* 1999; **36**: 25–38.
- 12 Coccaro EF, Kavoussi RJ, Hauger RL, Cooper TB, Ferris CF. Cerebrospinal fluid vasopressin levels: correlates with aggression and serotonin function in personality-disordered subjects. *Arch Gen Psychiatry* 1998; **55**: 708–714.
- 13 Popik PP, Vos PEP, Van Ree JMJ. Neurohypophysial hormone receptors in the septum are implicated in social recognition in the rat. *Behav Pharmacol* 1992; **3**: 351–358.
- 14 Dluzen DE, Muraoka S, Engelmann M, Landgraf R. The effects of infusion of arginine vasopressin, oxytocin, or their antagonists into the olfactory bulb upon social recognition responses in male rats. *Peptides* 1998; **19**: 999–1005.
- 15 Engelmann M, Wotjak CT, Ebner K, Landgraf R. Behavioural impact of intraseptally released vasopressin and oxytocin in rats. *Exp Physiol* 2000; **85**: 125S–130S.
- 16 Dantzer R. Vasopressin, gonadal steroids and social recognition. *Prog Brain Res* 1998; **119**: 409–414.
- 17 Alescio-Lautier B, Rao H, Paban V, Devigne C, Soumireu-Mourat B. Inhibition of the vasopressin-enhancing effect on memory retrieval and relearning by a vasopressin V1 receptor antagonist in mice. *Eur J Pharmacol* 1995; **294**: 763–770.
- 18 Ferris CF, Potegal M. Vasopressin receptor blockade in the anterior hypothalamus suppresses aggression in hamsters. *Physiol Behav* 1988; **44**: 235–239.
- 19 Everts HG, Koolhaas JM. Differential modulation of lateral septal vasopressin receptor blockade in spatial learning, social recognition, and anxiety-related behaviors in rats. *Behav Brain Res* 1999; **15**: 7–16.
- 20 Lolait SJ, O'Carroll AM, Mahan LC, Felder CC, Button D, Young WS III *et al*. Extrapituitary expression of the rat V1b vasopressin receptor gene. *Proc Natl Acad Sci* 1995; **92**: 6783–6787.
- 21 Hernando F, Schoots O, Lolait SJ, Burbach JP. Immunohistochemical localization of the vasopressin V1b receptor in the rat brain and pituitary gland: anatomical support for its involvement in the central effects of vasopressin. *Endocrinology* 2001; **142**: 1659–1668.
- 22 Tybulewicz VL, Crawford CE, Jackson PK, Bronson RT, Mulligan RC. Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-abl proto-oncogene. *Cell* 1991; **65**: 1153–1163.
- 23 Crawley JN. *What's Wrong With My Mouse: Behavioral Phenotyping of Transgenic and Knockout Mice*. Wiley-Liss: New York, 2000.
- 24 Nelson RJ, Chiavegatto S. Molecular basis of aggression. *Trends Neurosci* 2001; **24**: 713–719.
- 25 Ferris CF, Melloni RH Jr, Koppel G, Perry KW, Fuller RW, Delville Y. Vasopressin/serotonin interactions in the anterior hypothalamus control aggressive behavior in golden hamsters. *J Neurosci* 1997; **17**: 4331–4340.

- 26 Ferris CF. Adolescent stress and neural plasticity in hamsters: a vasopressin-serotonin model of inappropriate aggressive behaviour. *Exp Physiol* 2000; **85**: 85S–90S.
- 27 Beeman EA. The effect of male hormone on aggressive behavior in mice. *Physiol Zool* 1947; **20**: 373–405.
- 28 Parmigiani S, Palanza P, Rogers J, Ferrari PF. Selection, evolution of behavior and animal models in behavioral neuroscience. *Neurosci Biobehav Rev* 1999; **23**: 957–970.
- 29 Delville Y, Mansour KM, Ferris CF. Serotonin blocks vasopressin-facilitated offensive aggression: interactions within the ventrolateral hypothalamus of golden hamsters. *Physiol Behav* 1996; **59**: 813–816.
- 30 Lister RG. The use of a plus-maze to measure anxiety in the mouse. *Psychopharmacology* 1987; **92**: 180–185.
- 31 Holmes A. Targeted gene mutation approaches to the study of anxiety-like behavior in mice. *Neurosci Biobehav Rev* 2001; **25**: 261–273.
- 32 Saito M, Sugimoto T, Tahara A, Kawashima H. Molecular cloning and characterization of rat V1b vasopressin receptor: evidence for its expression in extra-pituitary tissues. *Biochem Biophys Res Commun* 1995; **212**: 751–757.
- 33 Vaccari C, Lolait SJ, Ostrowski NL. Comparative distribution of vasopressin V1b and oxytocin receptor messenger ribonucleic acids in brain. *Endocrinology* 1998; **139**: 5015–5033.
- 34 Bronson FH. Rodent pheromones. *Biol Reprod* 1971; **4**: 344–357.
- 35 Schultz EF, Tapp JT. Olfactory control of behavior in rodents. *Psychol Bull* 1973; **79**: 21–44.
- 36 Edwards DA, Nahai FR, Wright P. Pathways linking the olfactory bulbs with the medial preoptic anterior hypothalamus are important for intermale aggression in mice. *Physiol Behav* 1993; **53**: 611–615.
- 37 DaVanzo JP, Sydow M, Garris DR. Influence of isolation and training on fighting in mice with olfactory bulb lesions. *Physiol Behav* 1983; **31**: 857–860.
- 38 Newman SW. The medial extended amygdala in male reproductive behavior. A node in the mammalian social behavior network. *Ann NY Acad Sci* 1999; **877**: 242–257.
- 39 Meredith M. Vomeronasal, olfactory, hormonal convergence in the brain. Cooperation or coincidence? *Ann NY Acad Sci* 1998; **855**: 349–361.
- 40 Wood RI. Thinking about networks in the control of male hamster sexual behavior. *Horm Behav* 1997; **32**: 40–45.
- 41 Keverne EB. The vomeronasal organ. *Science* 1999; **286**: 716–720.
- 42 De Vries GJ, Buijs RM, Swaab DF. Ontogeny of the vasopressinergic neurons of the suprachiasmatic nucleus and their extrahypothalamic projections in the rat brain—presence of a sex difference in the lateral septum. *Brain Res* 1981; **218**: 67–78.
- 43 De Vries GJ, Miller MA. Anatomy and function of extrahypothalamic vasopressin systems in the brain. *Prog Brain Res* 1998; **119**: 3–20.
- 44 Buijs RM, De Vries GJ, Van Leeuwen FW, Swaab DF. Vasopressin and oxytocin: distribution and putative functions in the brain. *Prog Brain Res* 1983; **60**: 115–122.
- 45 Burbach JPH, Adan RA, Lolait SJ, Van Leeuwen FW, Mezey E, Palkovits M *et al*. Molecular neurobiology and pharmacology of the vasopressin/oxytocin receptor family. *Cell Mol Neurobiol* 1995; **15**: 573–595.
- 46 Ostrowski NL, Lolait SJ, Young WS III. Cellular localization of vasopressin V1a receptor messenger ribonucleic acid in adult male rat brain, pineal, and brain vasculature. *Endocrinology* 1994; **135**: 1511–1528.
- 47 Szot P, Bale TL, Dorsa DM. Distribution of messenger RNA for the vasopressin V1a receptor in the CNS of male and female rats. *Mol Brain Res* 1994; **24**: 1–10.
- 48 Everts HG, De Ruiter AJ, Koolhaas JM. Differential lateral septal vasopressin in wild-type rats: correlation with aggression. *Horm Behav* 1997; **31**: 136–144.
- 49 Stowers L, Holy TE, Meister M, Dulac C, Koentges G. Loss of sex discrimination and male-male aggression in mice deficient for TRP2. *Science* 2002; **295**: 1493–1500.
- 50 Leybold BG, Yu CR, Leinders-Zufall T, Kim MM, Zufall F, Axel R. Altered sexual and social behaviors in trp2 mutant mice. *Proc Natl Acad Sci* 2002; **99**: 6378–6381.
- 51 Ferguson JN, Young LJ, Hearn EF, Matzuk MM, Insel TR, Winslow JT. Social amnesia in mice lacking the oxytocin gene. *Nat Genet* 2000; **25**: 284–288.
- 52 Aguilera G, Rabadan-Diehl C. Vasopressinergic regulation of the hypothalamic-pituitary-adrenal axis: implications for stress adaptation. *Regul Pept* 2000; **96**: 23–29.
- 53 Ma XM, Levy A, Lightman SL. Emergence of an isolated arginine vasopressin (AVP) response to stress after repeated restraint: a study of both AVP and corticotropin-releasing hormone messenger ribonucleic acid (RNA) and heteronuclear RNA. *Endocrinology* 1997; **138**: 4351–4357.