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Deficits in social behavior and reversal learning are more prevalent in male offspring of VIP deficient female mice

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Abstract

Blockage of vasoactive intestinal peptide (VIP) receptors during early embryogenesis in the mouse has been shown to result in developmental delays in neonates, and social behavior deficits selectively in adult male offspring. Offspring of VIP deficient mothers (VIP +/–) also exhibited developmental delays, and reductions in maternal affiliation and play behavior. In the current study, comparisons among the offspring of VIP deficient mothers (VIP +/–) mated to VIP +/– males with the offspring of wild type (WT) mothers mated to VIP +/– males allowed assessment of the contributions of both maternal and offspring VIP genotype to general health measures, social behavior, fear conditioning, and spatial learning and memory in the water maze. These comparisons revealed few differences in general health among offspring of WT and VIP deficient mothers, and all offspring exhibited normal responses in fear conditioning and in the acquisition phase of spatial discrimination in the water maze. WT mothers produced offspring that were normal in all tests; the reduced VIP in their VIP +/– offspring apparently did not contribute to any defects in the measures under study. However, regardless of their own VIP genotype, all male offspring of VIP deficient mothers exhibited severe deficits in social approach behavior and reversal learning. The deficits in these behaviors in the female offspring of VIP deficient mothers were less severe than in their male littermates, and the extent of their impairment was related to their own VIP genotype. This study has shown that intrauterine conditions had a greater influence on behavioral outcome than did genetic inheritance. In addition, the greater prevalence of deficits in social behavior and the resistance to change seen in reversal learning in the male offspring of VIP deficient mothers indicate a potential usefulness of the VIP knockout mouse in furthering the understanding of neurodevelopmental disorders such as autism.

Keywords

Autism; Morris water maze; Mouse model; Neurodevelopmental disorder; Neuropeptide; Sex differences; Sociability; Social approach; Spatial reversal learning; Vasoactive intestinal peptide

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Introduction

Recent studies have indicated that VIP has an important role in the regulation of embryonic growth and development in the mouse. During the early postimplantation period of embryogenesis, characterized by neural tube closure and the initiation of neurogenesis, treatment of whole cultured embryonic day 9 (E9) mouse embryos with VIP resulted in dose-dependent and coordinated growth of the embryo brain and body (Gressens et al., 1993; Hill et al., 1999; Glazner et al., 1999; Servoss et al., 2001). Treatment of pregnant mice with a VIP antagonist during, but not after, the period of E9-E11, produced microcephaly and growth restriction of the fetus (Gressens et al., 1994). Mice that had been exposed to a VIP antagonist during this period of embryogenesis exhibited developmental delays and growth restriction as neonates (Wu et al., 1997; Hill, 2007), and neuronal damage (Hill et al., 1994), and regionally specific changes in brain VIP chemistry as adults (Hill et al., 1994; Hill et al., 2007b). In addition, learning deficits and reduced and abnormal social behaviors were seen selectively in the adult male mice (Hill et al., 2007a, 2007b). VIP knockout mice have recently become available (Colwel et al., 2003), and although the VIP null female mice have exhibited some infertility, the fertile heterozygous VIP deficient females (VIP +/-) mated with VIP +/- males produced all three genotypes of offspring, VIP +/+, VIP +/- and VIP -/- (Girard et al., 2006). Previous studies have shown that the VIP +/- mice had 50% of the VIP of WT mice (Girard et al., 2006) and that VIP null mice (VIP -/-) experienced disrupted circadian rhythms (Colwell et al., 2003; Aton et al., 2005), airway hyperresponsiveness (Szema et al., 2005), increased open field activity, and muscle weakness (Girard et al., 2006). Importantly, VIP null mice did not have compensatory increases in the expression of pituitary adenylate cyclase-activating polypeptide (PACAP) (Girard et al., 2006), a peptide which shows much homology with VIP and which shares several receptors with VIP. These results indicate that VIP possesses biological functions distinct from PACAP and that the deficits seen in VIP null mice were due to the deficiency of VIP, not due to compensatory changes in PACAP. Recent studies have shown that all genotypes of offspring of VIP deficient (VIP +/-) females exhibited growth restriction and developmental delays similar to those of offspring exposed to a VIP antagonist during early embryogenesis. Moreover, offspring born to VIP deficient mothers exhibited reductions in maternal affiliation and play behavior, regardless of their own VIP genotype (Lim et al., 2008). Maternal behavior did not differ between wild type (WT) and VIP deficient mothers; and cross fostering of litters did not influence offspring development (Lim et al., 2008). These results indicated that the delays and deficits in the offspring of VIP deficient mothers were due to prenatal events and that, perhaps like the VIP antagonist treated mice; the deficits were the result of insufficient VIP during embryogenesis.

Receptors for VIP appear early during embryogenesis in the rodent (Waschek et al., 1996), and are abundant on the floor plate of the neural tube (Hill et al., 1999; Spong et al., 1999), an important neural organizational site (Jessell and Dodd, 1992; Klar et al., 1992). Although VIP is present in mouse embryos and VIP receptors are active during embryogenesis, VIP mRNA is not expressed the embryo until later in development (Hill et al., 1996; Waschek et al., 1996; Hill et al., 1999; Spong et al., 1999). The source of VIP acting on embryonic receptors appears to be the maternal uterine tissue in which VIP mRNA, VIP peptide and VIP binding sites are enriched in the decidua. The embryo is in intimate contact with these maternal tissues during early placentation (Spong et al., 1999), and VIP has been shown to cross the developing placenta during early embryogenesis (Hill et al., 1996). The action of maternal uterine VIP on embryonic receptors may be the mechanism through which embryonic growth and development is regulated, and the route whereby VIP deficient mothers induce developmental and behavioral deficits on all their offspring, regardless of offspring VIP genotype. Together these results indicate that deficits in behavior expression may result from experiencing VIP deficiency during intrauterine development and that the deficits may be greater among male offspring. In the current study, both WT and VIP +/- females were mated with VIP +/- males.

These matings made it possible to examine both the damage to offspring caused by their own VIP deficiencies, and the damage caused by exposure to maternal VIP deficiency during prenatal development. In addition to examining the general health, olfactory, and motor capabilities of these mice as adults, we have examined their anxiety-like behavior, social behavior, and learning and memory. The new social approach task that was created to test potential mouse models of autism (Crawley, 2004; Moy et al., 2004; Nadler et al., 2004; Crawley et al., 2007) was used to evaluate social behaviors. Learning and memory were assessed with cued and contextual fear conditioning, and acquisition and reversal of spatial discrimination the Morris water maze.

Materials and methods

Animals

VIP KO mice were generated as previously described (Colwell et al., 2003) and backcrossed for at least 6 generations with C57BL/6. Three groups of mice were bred from the founder population from UCLA. 1) VIP +/- (VIP deficient) females were mated with VIP +/- males and produced 17 litters from which 15 male and 19 female wild type offspring (+/+), 27 male and 22 female heterozygote offspring (+/-), and 12 male and 17 female null offspring (-/-) were studied. Some VIP +/- females were used exclusively for breeding. 2) WT (VIP +/+) females were mated with VIP +/- males and produced 11 litters from which 15 male and 14 female +/+ offspring, and 11 male and 12 female +/- offspring were studied. 3) WT pairs were also bred producing 12 litters of +/+ offspring that were used to maintain the colony. Since VIP null females did not produce viable young, we were unable to evaluate the offspring of female mice who did not express VIP. The total number of litters of each kind was made up of several cohorts of mating over a period of several months. Females were mated at 3-5 months of age. Mice were housed under controlled temperature and humidity and a 12/12 h light/dark cycle and testing occurred under normal lighting conditions during the light period of the light cycle. Food and water was provided *ad libitum*. The developmental milestones of cliff aversion, negative geotaxis, rooting, forelimb grasping, surface righting, air righting, activity, auditory startle, ear twitch, and eye opening were examined in the offspring, as were homing behavior, maternal preference, and play behavior. The offspring were observed from birth to 21 days of age and the results of these experiments are reported elsewhere (Lim et al., 2008). Following the implanting of ID chips (Bio Medic Data Systems, Seaford, DE) at 2 months of age, animal testing occurred in the order in which the tests are listed below. During testing the mice were identified by their ID number and sex, the investigators were blind to both the VIP genotype of the mouse and of its mother. Prior to testing, cages of test mice were habituated to the room in which testing was to take place for a minimum of 1 h.

All experimental procedures for this study were approved by the National Institute of Mental Health Animal Care and Use Committee and followed the NIH Guide for the Care and Use of Laboratory Animals. The use of animals was not excessive and no animal suffering occurred.

Elevated plus maze

The elevated plus maze was the first test administered to the mice and it examined anxiety-like behavior before the mice had experienced much handling as adults. It was performed as previously described (Holmes et al., 2003). The Plexiglas maze (San Diego Instruments, San Diego, CA) was elevated 38 cm off the floor and was composed of two open arms (2 in wide and 12 in long) and two closed arms (2 in wide and 12 in long) extending from a central platform. An observer scored the number of entries the mouse made into the two open and into the two closed arms, as well as the time spent in each arm, during a 5 min observation period.

General health and neurological reflexes, sensory and motor abilities

Due to the reduced strength and increased motor activity reported for VIP $-/-$ mice compared with WT mice (Girard et al., 2006), and to reveal any additional physical limitations, particularly any limitations that would compromise behavioral testing, the general health, neurological reflexes, sensory reactivity, and motor abilities of all genotypes of offspring of both VIP deficient and WT mothers were examined. The following tests were performed as previously described (Crawley et al., 1997; Crawley and Paylor, 1997; Crawley, 2007; Hill et al., 2007b). On the day of testing, the home cage was moved into the test room one hour before testing to habituate the mice to the environment of test room. Following habituation, each mouse was removed individually, placed into an empty cage, and its behavior was observed by a pair of investigators for a 3 min period during which the presence of the following was noted: transfer freezing, wild running, stereotypical movements, amount of time spent in exploration, and the number of times the mouse groomed. The investigators then weighed the mouse, placed it on clean lab bench paper, and examined it as follows. Fur was examined for piloerection and bald spots, and fur condition was ranked on a scale of 1-3, with 3 being normal healthy fur. The presence and condition of the whiskers was examined. Body tone was ranked on a scale of 1-3, with 1 = flaccid, 2 = normal, and 3 = stiff, and was determined by gently squeezing the lower abdomen; limb tone by gently raising a finger placed under a hind limb. The presence or absence of the following reflexes was examined as follows. Suspending the mouse by the tail and observing the twisting of its trunk examined the neurological reflex of trunk curl. Righting reflex was determined by suspending the mouse by the tail, resting its back against the side of the hand and noting the ability of the mouse to turn and climb onto the back of the hand. The end of a cotton-tipped applicator (6 in. length, Solon Manufacturing Company, Solon, ME) was pulled out into a fine tip that was used to test the corneal blink reflex by approaching the cornea with the tip of the applicator. The pinna reflex was determined by gently brushing the ear with the tip of the applicator. The vibrissae reflex was determined by brushing the whiskers with the tip of the applicator. Toe pinch response was examined by gently squeezing a toe of the hind foot with a pair of tweezers. Vision was tested with forepaw reaching in which the mouse was suspended by the tail and gently lowered to the edge of the bench and noting if the forepaws are extended before the whiskers or paws touched the bench. Reactivity was assessed by noting the frequency of vocalizations and bouts of struggling during the testing period, the tendency to pull away while being petted (petting escape), and the tendency to bite at a wooden dowel gently placed in the mouth. Positional passivity was examined when the forepaws of the mouse were placed on a slightly elevated platform and it was noted if the animal moved or stayed stationary. Strength was assessed by the wire hang test in which the mouse was placed on the flat portion of a rat cage lid that was gently raised and lowered twice so that the mouse would grip the lid. The lid was then quickly, but gently, inverted over a mouse cage containing 1/2 inch of shavings. The latency to fall off of the inverted lid was timed with 60 seconds as the maximum time.

On subsequent days following the general health examination described above, one of the following tests was administered. Pain perception was measured by the latency to flick the tail away from the path of an infrared beam (Tail-Flick Analgesia Meter, Columbus Instruments, Columbus, OH) and the latency to shake or lick the paws when placed on a 55°C platform for a maximum of 1 min (Hot Plate Analgesia Meter, IITC Inc., Woodland Hills, CA), (Wrenn et al., 2004). Motor coordination and balance were measured on an accelerating rotarod (Ugo Basile, Stoelting, Wood Dale, IL) as described by Wrenn et al., (2004) where the latency to fall off a rotating dowel was measured as the rotational speed of the dowel accelerated from 4 to 40 rpm during a 5 min period. Auditory startle and prepulse inhibition were measured using the SR-Laboratory System (San Diego Instruments, San Diego, CA) as previously described (Paylor et al., 1998; Holmes et al., 2001). Auditory startle amplitude was measured following presentation of four, 40 ms 120 dB sound bursts. Prepulse inhibition was determined by

examining the amplitude of the startle response caused by the presentation of 42 randomized pairings of the acoustic startle stimulus of 40 ms 120 dB sound bursts, with prepulse tones of 20 ms at 74, 78, 82, 86 and 90 db, presented 100 ms before the startle stimulus. The interval between trials was 10-20 s. Spontaneous activity was measured in an open field as previously described (Holmes et al., 2001; Wrenn et al., 2004). The activity of an individual mouse in the 40×40×35 cm square arena with Plexiglas walls and floor was measured by a Digiscan optical animal activity system (RXYXCM, Omnitech Electronics, Accuscan, Columbus, OH). A 16×16 array of infrared photo beams was located along the sides of the walls of the enclosure and movement of the mouse was detected by beam breaks. Each mouse was placed in the center of the arena and the number of beam breaks that occurred during 6 consecutive 5 min periods was determined.

Olfactory habituation/dishabituation

Since social interactions in mice are largely dependent on olfaction, the ability of the mice to differentiate between the odors of unfamiliar mice was examined with the olfactory habituation/dishabituation method (Luo et al., 2002; Wrenn et al., 2003; Crawley et al., 2007). Test mice were habituated for 30 min to a clean test cage without food, water, or nesting materials. A cotton-tipped applicator (6 in. length, Solon Manufacturing Company, Solon, ME) that had been dipped into water was suspended from the lid of the cage at a level of approximately 4.4 cm from the bottom of the cage. After 2 min, the applicator was replaced with a second applicator that had been dipped into water, and after another 2 min, the second applicator was replaced with a third applicator that had been dipped into water. An observer with a stopwatch scored the number of seconds the test mouse sniffed each consecutive cotton-tipped applicator that had been dipped into water. Following the three presentations of water, the ability of the test mouse to distinguish between the odors of two unfamiliar mice was examined in the same manner. A cotton-tipped applicator was swiped across the floor of a dirty cage of an individually housed mouse of the same sex (Cage 1). The test mouse was presented consecutively with 3, 2 min exposures of the odors of the unfamiliar mouse in Cage 1 on freshly swiped cotton tipped applicators. Dishabituation occurred if the test mouse sniffed the first applicator containing the odors from Cage 1 more than it had sniffed the third applicator containing water and it indicated that the mouse could distinguish the odor on the applicator. Habituation occurred if the amount of time sniffing the applicators decreased with consecutive exposures. Following the third exposure to the applicator from Cage 1, the test mouse was presented with 3 consecutive 2 min exposures of applicators that had been swiped on the floor of a dirty cage housing a second unfamiliar mouse (Cage 2). Dishabituation occurred if the test mouse sniffed the first applicator containing the odor of Cage 2 significantly more than the mouse had sniffed the third presentation of the odor from Cage 1 and indicated that the test mouse could differentiate between the odors of the two unfamiliar mice.

Social Approach Task: Habituation, sociability phase and preference for social novelty phase

Social behavior was examined with the recently designed social approach task that was created to test potential mouse models of autism (Crawley, 2004; Moy et al., 2004; Nadler et al., 2004; Crawley et al., 2007). Sociability was assessed by quantifying the amount of time spent in the chamber with an unfamiliar mouse and the time spent sniffing an inverted wire cup containing an unfamiliar mouse. The automated apparatus consisted of a 24.75×16.75 in polycarbonate box that was subdivided into three, 8.5×16.75 in chambers by partitions (fabricated by George Dold, Research Services Branch, NIH, see Nadler et al., 2004 for specifications and photographs). Access between chambers was possible through an opening in each partition. The openings were equipped with doors that could slide down and prevent passage between chambers. In addition, each opening had two sets of infrared beams that, when broken by the passage of a mouse, permitted the automatic recording by a controller (NI cFP-2000 detector, National Instruments, Austin, TX) of the direction of movement of the

mouse, the number of transitions between chambers, and the amount of time spent in each chamber. Environmental cues around the apparatus were similar on both sides and lighting was uniform from above. Five male and 5 female WT offspring of WT mothers were tested in the empty apparatus and no side preferences were apparent. The cages of mice to be examined were brought into the experimental room a minimum of an hour before testing. During testing, an unfamiliar mouse was kept stationary by placing it in a cylindrical chrome wire cup (Galaxy Pencil/Utility Cup, Kitchen Plus, <http://www.kitchen-plus.com>). A disposable plastic cup was inverted over the top 20% of the wire cup to prevent the test mouse from climbing onto it. The unfamiliar mice that were used in the experiments were male and female C57BL/6 (The Jackson Laboratory, Bar Harbor, ME). Before use in the experiments, these mice were habituated to the test apparatus for a minimum of 30 min on each of three consecutive days to ensure that they would sit quietly in the wire cages during the experiments.

The social approach task was composed of three phases that were administered in the following order: 1) Habituation phase, where the mouse was habituated to the test apparatus; 2) Sociability phase, that compared the approach of the test mouse to an unfamiliar mouse (Mouse 1) with its approach to a novel object; and 3) Preference for Social Novelty phase which compared the approach of the test mouse to the now-familiar Mouse 1 with its approach to a novel mouse (Mouse 2).

During the 10 min habituation phase the test mouse was restricted to the central chamber of the apparatus. This was followed by the 10 min sociability phase in which the doors to the two adjacent side chambers were opened and the test mouse had free access to all three chambers. An unfamiliar mouse of the same sex (Mouse 1) was located in a wire cage in one of the side chambers; the other side chamber held a novel object, an empty wire cage. The amount of time the test mouse spent in each chamber and the number of transitions between chambers was automatically recorded for the 10 min phase. An investigator sitting quietly 4 ft from the test box observed the behavior of the test mouse and recorded, with stopwatches, the amount of time the test mouse sniffed the wire cage holding the unfamiliar Mouse 1 in one side chamber and the amount of time the test mouse sniffed the empty wire cage in the other side chamber. Following the sociability phase, the test mouse was placed in the center compartment with the doors between chambers closed. Mouse 1 remained undisturbed in the wire cage in the side chamber, but the empty cage in the other side chamber was replaced with a wire cage containing a novel mouse (Mouse 2). The 10 min preference for social novelty phase began when the doors between compartments were reopened and the test mouse again had free access to all three chambers. As above, the amount of time the test mouse spent in each of the three chambers and number of transitions between chambers was automatically recorded. The observer scored, with stopwatches, the amount of time the test mouse sniffed the wire cage containing the now-familiar Mouse 1 in one side chamber, and the wire cage containing the novel mouse, Mouse 2, in the other side chamber.

Trace cued and contextual fear conditioning

The method used for trace cued and contextual fear conditioning as a measure of cognitive function is based on methods of the established literature and described by Wrenn et al., (2004). In the current experiments, a fully automated system (San Diego Instrument Freeze Monitor™, San Diego, CA) was used as previously described (Hill et al., 2007a). The movements of the test animal were detected by photo beam breaks and the amount of time spent in freezing (fear response periods in which no photo beams were broken) was recorded by the Freeze Monitor™ software. On the first day of testing, mice were individually placed in an approximately 10×10×12.5 in clear acrylic enclosure equipped with a 16×16 array of infrared photo beams. A 2 min exploration period (pre-conditioned stimulus) preceded the exposure of each mouse to 4 pairings of the conditioned stimulus (30 sec of 80 dB white noise)

followed 2.5 sec later (trace interval) by the unconditioned stimulus (1 sec of 0.5 mA AC current through the metal grid floor). A second 2 min exploration period followed the exposure to the stimuli. The number of seconds freezing during the 2 min exploration periods before and after the conditioned stimulus-unconditioned stimulus pairings was recorded. Twenty-four h after the training period, the mice were again individually placed in the training enclosure for a 5 min period (contextual testing), but without the conditioned stimulus or unconditioned stimulus. The number of seconds freezing during the 5 min period was recorded. Twenty-four h after contextual testing the mice were individually tested for novel-context cued fear. They were placed into a novel environment, an approximately 10×10×14×7 in (deep) triangular-shaped black acrylic enclosure equipped with a 16×16 array of infrared photo beams, in which one wall had been swiped with a cotton-tipped applicator dipped in a 1:100 dilution of vanilla extract (McCormick, Hunt Valley, MD). Following a 3 min exploration period, the animal was exposed to 3 min of the cue (conditioned stimulus tone used in training), but without foot shock. The number of seconds freezing was recorded for both the pre-cue and the cue periods.

Morris water maze – spatial discrimination training and reversal training

The Morris water maze assesses spatial learning and memory (Morris, 1984). Testing was performed in a circular pool, 1.4 m in diameter, filled with water made opaque with the addition of nontoxic white paint (Crayola, Forks Township, PA). Video tracking of mouse movements was conducted with a video camera focused on the full diameter of the pool. Navigational parameters were analyzed with the WaterMaze Software (Actimetrics, Inc. Evanston, IL).

The test was composed of three phases that were administered in the following order: 3 days of visible platform training, 7 days of acquisition of spatial discrimination training in which the platform was hidden, and 3 days of reversal training in which the hidden platform was moved to a new location. Probe tests were administered following the last day of acquisition training and the last day of reversal training.

The 3 days of visual platform training consisted of 4 trials per day separated by 1 min intervals in which the mouse was gently lowered into the pool, facing the wall of the pool. The visible platform was placed into a different quadrant of the pool for each successive trial. For each trial, the mouse was allowed a maximum of 60 s to locate the visible platform and was left on the platform for 30 s before being removed. If the platform was not reached in 60 s, the mouse was guided to the platform and allowed to sit on it for 30 s. Visible platform training allowed the mouse to become familiar with the pool and the platform.

The 7 days of acquisition training consisted of 4 trials per day separated by 1 min intervals in which the mouse was placed into the pool, facing the wall of the pool, in a new quadrant on each successive trial. For each mouse, the hidden platform remained in the same position for the 7 days of training. As above, the mouse was allowed a maximum of 60 s to locate the hidden platform and was left on the platform for 30 s before being removed. If the platform was not reached in 60 s, the mouse was guided to the platform and allowed to sit on it for 30 s. One hour following the 7th day of hidden training, a 60 s probe test was administered in which the platform was removed from the pool and the swim speed and the amount of time the mouse spent in each of the four quadrants of the pool were recorded.

In reversal training the platform was moved to a new position and training consisted of 4 trials per day separated by 1 min intervals in which the mouse was placed into the pool, facing the wall of the pool, in a new quadrant on each successive trial. For each mouse, the hidden platform remained in the same new position for the 3 days of training. As above, the mouse was allowed a maximum of 60 s to locate the hidden platform and was left on the platform for 30 s before being removed. If the platform was not reached in 60 s, the mouse was guided to the platform and allowed to sit on it for 30 s. Training on the new position took place for 3 days and 1 h

following the 3rd day of hidden training a 60s probe test was administered as described above. An average latency of 15 seconds or less to locate the platform across the block of 4 consecutive trials each day was the criterion for learning in the 1.4 meter pool.

Statistical Analysis

During behavioral testing, data sheets included the ID number and the sex of the mouse under study; the investigators were unaware of the VIP genotype of the test mouse or the genotype of its mother. The genotype of the mouse and its mother were entered into the data sheets prior to statistical analysis. Data analysis included examination of potential sex differences and where no significant sex difference occurred, data from male and female mice were pooled. Data were analyzed using Chi Square for categorical data, or Analysis of Variance, with repeated measures (RMANOVA) where appropriate, followed by contrasts between groups with Fisher's PLSD for within factor *post hoc* comparisons, and with Bonferroni/Dunn for multiple hypotheses *post hoc* analyses (Statview - Abacus Concepts, Berkeley, CA). Statistical significance was set at $p \leq 0.05$, except for Bonferroni *post hoc* analyses in which the significance level was specified for each test. All data are presented as mean $\pm$ one standard error of the mean.

Results

Elevated Plus maze, general health, neurological reflexes, strength, and motor capabilities

The results of the elevated plus maze, general health, weight, reflexes, strength, and motor performance tests of the mice are shown in Table 1. All groups behaved similarly in the elevated plus maze, which indicated that no group exhibited high levels of anxiety-like behavior. Except for weight, wire hang, and rotarod performance, as indicated, there were no sex differences in the measures. Because they weighed less, female mice were able to perform these tasks longer than male mice. Few differences were found among the treatment groups and the offspring of VIP deficient mothers appeared healthy and normal in almost all measures taken here. However, contrary to the offspring of WT mothers, the offspring of VIP deficient mothers did not vocalize during handling and showed no positional passivity. They also groomed significantly less than the offspring of WT mothers ($F_{4,120} = 30.6$, $p < 0.0001$; Fisher's PLSD *post hoc* comparison of offspring of VIP deficient mothers with WT offspring of WT mothers, $p < 0.0001$ for +/+; $p < 0.0001$ for +/-, $p < 0.0001$ for -/-). The offspring of VIP deficient mothers were significantly more active in the open field (RMANOVA, $F_{4,104} = 5.9$, $p = 0.0002$, Bonferroni *post hoc* comparison with WT offspring of WT mothers (significance level $p < 0.005$), $p = 0.0024$ for +/+, $p < 0.0001$ for +/-, $p = 0.02$ for -/-) and, in the rotarod test of strength and coordination, the -/- male offspring of VIP deficient mothers had shorter latencies, ($F_{4,83} = 1.7$, $p = 0.15$, Fisher's PLSD *post hoc* comparison to WT male offspring of WT mothers, $p = 0.029$ for -/-). Also, the -/- males had shorter latencies to release on the wire hang ($F_{4,45} = 1.6$, $p = 0.18$, Fisher's PLSD *post hoc* comparison to WT male offspring of WT mothers $p = 0.016$ for -/- males). These results are consistent with the greater activity and poorer muscle strength of male VIP -/- mice that have been reported previously (Girard et al., 2006).

Olfactory habituation/dishabituation

The results of the olfactory habituation/dishabituation test appear in Table 2. All groups of mice recognized the novel odor from Cage 1, as all groups sniffed the first presentation from Cage 1 significantly more than the third presentation of water ($F_{1,126} = 664.6$, $p < 0.0001$). Additionally, the offspring of VIP deficient mothers sniffed the first presentation of the odor from Cage 1 significantly more than did the offspring of the WT mothers ($F_{4,126} = 2.86$, $p = 0.026$, Fishers PLSD *post hoc* comparisons with WT offspring of WT mothers: $p = 0.007$ for +/+ offspring; $p = 0.03$ for +/- offspring; $p = 0.04$ for -/- offspring). All groups of mice

exhibited habituation to the odors of both Cage 1 and Cage 2, as the amount of sniffing decreased with succeeding presentations of the odors of these mice (Cage 1, $F_{2,252} = 542$, $p < 0.0001$; Cage 2, $F_{2,246} = 140.7$, $p < 0.0001$); there were no significant differences among genotypes (Cage 1, $F_{4,126} = 2.02$, $p = 0.096$; Cage 2, $F_{4,123} = 0.2$, $p = 0.93$). The significant increase in sniffing for all groups with the first presentation of the odor from Cage 2 illustrated that all groups had olfactory capabilities sufficient to differentiate between the odors of two unfamiliar mice ($F_{1,123} = 72.3$, $p < 0.0001$); there were no significant differences among genotypes ($F_{4,123} = 0.56$, $p = 0.68$).

Social Approach Task

Due to sex differences in the results obtained from the offspring of VIP deficient mothers (see below), the data of male and female mice are presented separately below.

Sociability Phase—All offspring of WT mothers exhibited normal social behavior in the sociability phase of the social approach task regardless of their genotype (+/+ or +/-) or sex (Fig. 1). They all spent significantly more time in the compartment housing Mouse 1 than in the compartment containing the novel object (Fig. 1 A and B) ($F_{1,40} = 68.9$, $p < 0.0001$, for the overall effect of chamber; $F_{1,40} = 1.89$, $p = 0.17$, for genotype; $F_{1,40} = 1.81$, $p = 0.18$ for sex; $F_{1,40} = 0.72$, $p = 0.40$ for genotype $\times$ chamber). Fisher's PLSD *post hoc* analysis comparing time spent within the two chambers showed that both sexes of both genotypes spent significantly more time in the chamber with Mouse 1 than in the chamber with the novel object ($p < 0.0001$ for +/+ males; $p < 0.0001$ for +/- males; $p = 0.003$, for +/+ females; $p = 0.0002$ for +/- females). In addition, regardless of sex or genotype, all offspring of WT mothers exhibited normal sociability as they sniffed Mouse 1 significantly more than they sniffed the novel object ($F_{1,40} = 0.206$, $p < 0.0001$ for the overall effect of stranger vs. novel object; $F_{1,40} = 0.0003$, $p = 0.98$ for genotype; $F_{1,40} = 1.17$, $p = 0.28$ for sex; $F_{1,40} = 0.61$, $p = 0.43$ for genotype $\times$ stranger vs. novel object). Fisher's PLSD *post hoc* analysis showed that all groups sniffed Mouse 1 significantly more than the novel object ($p < 0.0001$ for +/+ males; $p < 0.0001$ for +/- males; $p < 0.0001$ for +/+ females; $p < 0.0001$ for +/- females).

The offspring of VIP deficient mothers exhibited impaired sociability, with the male offspring exhibiting significantly greater impairment than the female offspring. None of the male offspring of VIP deficient mothers exhibited normal sociability as no genotype spent significantly more time in the compartment with Mouse 1 than they did in the compartment containing the novel object (Fig. 1 A) ($F_{1,27} = 3.29$, $p = 0.08$ for main effect of stranger vs. novel object; $F_{2,27} = 0.20$, $p = 0.81$ for genotype; $F_{2,27} = 0.19$, $p = 0.98$ for genotype $\times$ stranger vs. novel object). Fisher's PLSD showed that no male genotype spent significantly more time in the compartment with Mouse 1 than in the compartment with the novel object ($p = 0.26$ for +/+ males, $p = 0.17$ for +/- males; $p = 0.19$ for -/- males). Although as a group, the male offspring of VIP deficient mothers spent significantly more time sniffing Mouse 1 than the novel object ($F_{2,27} = 8.8$, $p = 0.006$ for main effect of time sniffing; $F_{2,27} = 1.69$, $p = 0.20$ for genotype; $F_{2,27} = 1.61$, $p = 0.22$ for genotype $\times$ sniff time), Fisher's PLSD *post hoc* analysis showed that no genotype of the male offspring sniffed Mouse 1 significantly more than they sniffed the novel object (Fig. 1 C) ($p = 0.11$ for +/+ males; $p = 0.24$ for +/- males; $p = 0.49$ for -/- males). Additionally, all genotypes of male offspring of VIP deficient mothers sniffed Mouse 1 significantly less than did WT male offspring of WT mothers (Fig. 1 C) ($F_{4,49} = 20.7$, $p < 0.0001$), Fisher's PLSD *post hoc* analysis of genotype vs. WT males: +/+ males, $p < 0.005$ for +/+ males; $p < 0.0001$ for +/- males; $p = 0.0004$ for -/- males).

The female offspring of VIP deficient mothers exhibited less severe deficits than their male siblings and the extent of their deficits was related to their own genotype. Female offspring of VIP deficient mothers spent more time in the chamber with Mouse 1 than in the chamber with

the novel object (Fig. 1 B) ($F_{4,50} = 33.1$, $p < 0.0001$, for main effect of time, $F_{4,50} = 1.3$, $p = 0.25$ for genotype, $F_{4,50} = 0.42$, $p < 0.80$ for time $\times$ genotype). *Post hoc* analysis with Fisher's PLSD showed that the female $+/+$ and $+/-$ offspring of VIP deficient mothers spent significantly more time in the compartment with Mouse 1 than with the novel object ($p < 0.0001$ for $+/+$ females; $p = 0.03$ for $+/-$ females). The $-/-$ females of VIP deficient mothers approached significance at $p = 0.055$.

All female offspring of VIP deficient mothers sniffed Mouse 1 significantly more than the novel object (Fig. 1 D) ($F_{1,30} = 64.45$, $p < 0.0001$ for main effect of time sniffing) and Fisher's PLSD *post hoc* showed that all genotypes sniffed Mouse 1 more than the novel object ($p < 0.0001$ for $+/+$ females; $p = 0.009$ for $+/-$ females; $p = 0.0004$ for $-/-$ females). However, there was a significant main effect of genotype ($F_{2,30} = 4.41$, $p = 0.02$) and a significant interaction of sniff time $\times$ genotype ($F_{2,30} = 6.06$, $p = 0.006$). Fisher's PLSD *post hoc* analysis showed that although $+/+$ female offspring of VIP deficient mothers sniffed Mouse 1 the same amount of time as wild type females ($p = 0.94$), the $+/-$ and $-/-$ female offspring of VIP deficient mothers sniffed Mouse 1 significantly less than did WT female offspring of WT mothers ($p = 0.03$ for $+/-$ females; $p = 0.002$ for $-/-$ females).

Preference for Social Novelty Phase—All male and female offspring of WT mothers exhibited normal behavior in the preference for social novelty phase of the social approach test regardless of genotype ($+/+$ or $+/-$). They all spent significantly more time in the compartment with Mouse 2 than in the compartment of the now-familiar Mouse 1 (Fig. 2 A and B) ($F_{1,44} = 39.4$, $p < 0.0001$ for main effect of time; $F_{1,44} = 0.43$, $p = 0.51$ for main effect of genotype; $F_{1,44} = 0.57$, $p = 0.45$ for main effect of sex). Fisher's PLSD *post hoc* analysis showed that both sexes of both genotypes of offspring of WT mothers spent significantly more time in the chamber with Mouse 2 than with Mouse 1 ($p = 0.008$ for $+/+$ males; $p = 0.003$ for $-/-$ males; $p < 0.0001$ for $+/+$ females; $p < 0.0001$ for $+/-$ females). In addition, all offspring of WT mothers, regardless of genotype ($+/+$ or $+/-$), or sex, sniffed the novel Mouse 2 significantly more than they sniffed Mouse 1 (Fig. 2 C and D) ($F_{1,43} = 134.4$, $p < 0.0001$ for main effect of sniff time; $F_{1,43} = 3.3$, $p = 0.07$ for genotype; $F_{1,43} = 2.5$, $p = 0.12$ for sniff time $\times$ genotype). Fisher's PLSD *post hoc* analysis demonstrated that both sexes of both genotypes sniffed Mouse 2 significantly more than they sniffed Mouse 1 ($p = 0.008$ for $+/+$ males; $p = 0.003$ for $+/-$ males; $p = 0.0001$ for $+/+$ females; $p = 0.0001$ for $+/-$ females).

The offspring of VIP deficient mothers exhibited deficits in the preference for social novelty phase of the social approach test. Although as a group the offspring of VIP deficient mothers spent more time in the compartment with Mouse 2 than in the compartment with Mouse 1 (Fig. 2 A and B) ($F_{1,60} = 7.3$, $p = 0.008$, for main effect of time; $F_{2,60} = 2.5$, $p = 0.08$ for genotype; $F_{2,60} = 0.17$, $p = 0.83$ for time $\times$ genotype), Fisher's PLSD *post hoc* analysis demonstrated that no genotype of either sex spent significantly more time in the compartment with Mouse 2 than in the compartment with Mouse 1 (Fig. 2 A and B) ($p = 0.054$ for $+/+$ males; $p = 0.19$ for $+/-$ males, $p = 0.078$ for $-/-$ males; $p = 0.57$ for $+/+$ females; $p = 0.20$ for $+/-$ females; $p = 0.11$ for $-/-$ females). Also, as a group, the offspring of VIP deficient females sniffed Mouse 2 significantly more than they sniffed Mouse 1 (Fig. 2 C and D) ($F_{1,60} = 49.4$, $p < 0.0001$ for main effect of sniffing); however, there was a sex difference, where females sniffed Mouse 2 significantly more than Mouse 1 ($F_{1,61} = 15.8$, $p = 0.0002$), and a sniff time $\times$ sex interaction ($F_{1,61} = 11.7$, $p = 0.001$). Fisher's PLSD *post hoc* analysis showed that the female mice were the primary contributors to the significantly greater sniffing of Mouse 2 compared with Mouse 1 for the offspring of VIP deficient mothers (Fig. 2 C and D) ($p = 0.20$ for $+/+$ males; $p = 0.054$ for $+/-$ males; $p = 0.10$ for $-/-$ males; $p = 0.0003$ for $+/+$ females; $p = 0.0003$ for $+/-$ females; $p = 0.0002$ for $-/-$ females). However, all female offspring of VIP deficient mothers, including the WT female offspring, sniffed Mouse 2 significantly less than did the WT female offspring of WT mothers, regardless of genotype or sex ($F_{4,103} = 24.8$, $p < 0.0001$; Fisher's PLSD *post*

hoc analysis comparison with WT ($p < 0.0001$ for $+/+$ offspring; $p < 0.0001$ for $+/-$ offspring; $p < 0.0001$ for $-/-$ offspring).

Entries—The number of entries to the two side compartments containing Mouse 1 and a novel object in the sociability phase, and Mouse 1 and Mouse 2 in the preference for social novelty phase did not differ significantly among groups, regardless of genotype of mother or genotype or sex of offspring (Fig. 3): Sociability phase ($F_{4,104} = 0.18$, $p = 0.94$ for main genotype effect); Preference for social novelty phase ($F_{4,103} = 2.0$, $p = 0.09$ for main genotype effect). Although the average number of entries into the side with Mouse 1 was greater than the average number of entries into the side with the novel object for all groups, only in the WT female offspring of VIP deficient mothers (Fig. 3 B) did the difference reach the level of significance ($p = 0.008$). No significant differences were found in any group in the number of entries into the side containing Mouse 1 compared with the number of entries into the compartment housing Mouse 2 in the preference for social novelty phase of the test. The similarities in number of transitions between compartments by all groups indicated that the mice in the study had similar activity levels in the social approach test arena.

Cued and contextual fear conditioning

As there were no sex differences in the performance of cued and contextual fear conditioning, the results for males and females are combined.

Training—All genotypes of both WT mothers and VIP deficient mothers performed equally during the training period and all groups froze significantly more following the conditioned stimulus (Fig. 4 A) ($F_{1,95} = 934.8$, $p < 0.0001$ for main effect of time freezing; $F_{4,95} = 1.3$, $p = 0.24$ for main effect of genotype; $F_{4,95} = 0.77$, $p = 0.54$ for time freezing $\times$ genotype interaction), a further indication that there were no differences in pain perception among groups.

Same context—All genotypes of offspring of both WT mothers and VIP deficient mothers froze equally when returned to the training chamber in which they had experienced the pairing of conditioned stimulus and unconditioned stimulus 24 h earlier (Fig. 4 B) ($F_{4,96} = 1.3$, $p = 0.24$ for comparisons of all genotypes to $+/+$ offspring of WT mothers). These data indicated that all groups recognized and remembered the chamber.

Novel context—All genotypes of offspring of both WT and VIP deficient mothers froze equally in the novel chamber upon hearing the conditioned stimulus tone that had been paired with the unconditioned stimulus 2 days earlier (Fig. 4 C) ($F_{1,94} = 629.6$, $p < 0.0001$ for main effect of time freezing; $F_{4,94} = 1.4$, $p = 0.24$ for main effect of genotype; $F_{1,94} = 1.8$, $p = 0.13$ for time freezing $\times$ genotype interaction). These data indicate that all mice in all groups recognized the tone and remembered that it had been paired with the unconditioned stimulus of foot shock.

As indicated by this test, the offspring of VIP deficient mothers exhibited no deficits in emotional learning and memory compared with the offspring of WT mothers.

Morris water maze

There were no sex differences in performance in the visible or acquisition phases of the Morris water maze; therefore, sexes are combined in the comparisons below. In the probe test following reversal training, sex differences were evident; therefore, the probe test data from males and females are shown separately.

Visible training—The three days of trials with the visible platform showed that all mice in all treatment groups could see the platform as their latency to reach the platform decreased each day until all groups had reached the platform on day 3 in less than the criterion of 15 sec (Fig. 5 A). However, the offspring of VIP deficient mothers exhibited longer latencies to reach the platform and the performance of all three genotypes differed significantly from the +/+ offspring of WT mothers (Fig. 5A) (RMANOVA $F_{4,114} = 7.6$, $p < 0.0001$; Bonferroni/Dunn *post hoc* comparison with WT offspring of WT mothers, (significance level, $p < 0.005$): $p = 0.0001$ for +/+ offspring; $p < 0.0001$ for +/- offspring; $p = 0.0006$ for -/- offspring). There were no differences in swim speeds among treatment groups (data not shown); therefore, the longer latencies were not due to slower swim speeds.

Acquisition training—There were no significant differences in swim speeds among treatment groups and all mice of all treatment groups had equivalent latencies in the acquisition training phase (Fig. 5 B) (RMANOVA $F_{4,113} = 1.9$, $p = 0.12$). Bonferroni/Dunn *post hoc* comparisons of offspring of VIP deficient mothers with WT offspring of WT mothers (significance level $p < 0.005$): $p = 0.02$ for +/+ offspring; $p = 0.02$ for +/- offspring; $p = 0.16$ for -/- offspring). All groups, regardless of sex, genotype of mother, or genotype of offspring, exhibited reduced latencies with time and by the fourth day of hidden trials, all groups reached the hidden platform by the criterion of 15 sec or less.

The normal acquisition of spatial memory of all groups was further demonstrated by their performance in the probe test (Fig. 5C), in which all groups spent significantly more time in the trained quadrant that had housed the platform (target quadrant) than in the other three quadrants. Offspring of WT mothers: +/+ offspring ($F_{3,98} = 19.7$ $p < 0.0001$; Fisher's PLSD *post hoc* comparisons with time spent in target quadrant: $p < 0.0001$ for left quadrant; $p < 0.0001$ for right quadrant; $p < 0.0001$ for opposite quadrant); +/- offspring ($F_{3,72} = 11.0$ $p < 0.0001$; Fisher's PLSD *post hoc* comparisons with time spent in target quadrant: $p < 0.0001$ for left quadrant; $p < 0.0001$ for right quadrant; $p < 0.0001$ for opposite quadrant). Offspring of VIP deficient mothers: +/+ offspring ($F_{3,80} = 27.7$ $p < 0.0001$; Fisher's PLSD *post hoc* comparisons with time spent in target quadrant: $p < 0.0001$ for left quadrant; $p < 0.0001$ for right quadrant; $p < 0.0001$ for opposite quadrant) +/- offspring ($F_{3,96} = 32.2$ $p < 0.0001$; Fisher's PLSD *post hoc* comparisons with time spent in target quadrant: $p < 0.0001$ for left quadrant; $p < 0.0001$ for right quadrant; $p < 0.0001$ for opposite quadrant) and -/- offspring ($F_{3,99} = 20.0$ $p < 0.0001$; Fisher's PLSD *post hoc* comparisons with time spent in target quadrant: $p < 0.0001$ for left quadrant; $p < 0.0001$ for right quadrant; $p < 0.0001$ for opposite quadrant).

Reversal training—The offspring of WT mothers learned the new position of the hidden platform by day 2 as they had reduced their latencies to reach the platform to less than 15 sec (Fig. 6 A). Although all genotypes of the offspring of VIP deficient mothers did exhibit reduced latencies to reach the platform with time, only the +/+ offspring reached criterion of 15 sec. In addition, only the performance of the -/- offspring of VIP deficient mothers reached the level of significance and had significantly longer latencies than the WT offspring of WT mothers (Fig. 6 C) (RMANOVA, $F_{4,109} = 5.9$, $p = 0.0002$; Bonferroni/Dunn *post hoc* comparisons with WT offspring of WT mothers (significance $p < 0.005$): $p = 0.27$ for +/+ offspring; $p = 0.009$ for +/- offspring; $p < 0.0001$ for -/- offspring).

Among the offspring of VIP deficient mothers, a sex difference was seen in the number of returns to the previous location of the platform during the first day of the 3 days of reversal training (Fig. 6 B and 6 C). Male offspring of VIP deficient mothers crossed over the previous platform location significantly more frequently than the male WT offspring of WT mothers in Day 1, (Fig. 6 B) ($F_{4,53} = 5.3$, $p = 0.0012$; Fisher's PLSD *post hoc* comparison with WT male offspring of WT mothers, $p = 0.007$ for +/+ male offspring; $p = 0.004$ for +/- male offspring;

$p = 0.001$ for $-/-$ male offspring. On days 2 and 3, there were no differences in among male groups (Fig. 6 B) (Day 2, $F_{4,53} = 0.92$, $p = 0.45$; Day 3 $F_{4,53} = 0.22$, $p = 0.92$). Overall, female offspring of VIP deficient mothers crossed over the original platform site less often than male offspring of VIP deficient mothers, but did so significantly more often than the female offspring of WT mothers (Fig. 6 C) ($F_{4,53} = 0.0094$). Whereas, *post hoc* analysis showed that the WT offspring of VIP deficient mothers did not differ from the WT offspring of WT mothers, the $+/-$ and $-/-$ female offspring of VIP deficient mothers crossed over the original platform site significantly more often on day 1 (Fig. 6 C) (Fisher's PLSD *post hoc* comparison with female WT offspring of WT mothers, $p = 0.69$ for WT female offspring; $p = 0.034$ for $+/-$ female offspring; $p = 0.011$ for $-/-$ female offspring). On days 2 and 3, there were no differences in among female groups (Fig. 6 C) (Day 2, $F_{4,53} = 1.48$, $p = 0.22$; Day 3 $F_{4,53} = 0.22$, $p = 0.93$).

Deficits in the performance of the offspring of VIP deficient mothers were also found in the probe test following reversal training, which, furthermore, exhibited a sex difference. The male offspring of WT mothers spent significantly more time in the new target quadrant than in the other three quadrants (Fig. 6 D) ($+/+$ offspring $F_{3,40} = 9.3$, $p < 0.0001$, Fisher's PLSD *post hoc* comparison with new target quadrant, $p < 0.0001$ for left quadrant, $p = 0.0002$ for right quadrant; $p = 0.0002$ for opposite quadrant) ($+/-$ offspring $F_{3,36} = 5.4$, $p = 0.0034$, Fisher's PLSD *post hoc* comparison with new target quadrant, $p = 0.0006$ for left quadrant, $p = 0.005$ for right quadrant; $p = 0.009$ for opposite quadrant). However, no male offspring of VIP deficient mothers, regardless of their own genotype, spent significantly more time in the new target quadrant than all the other three quadrants. Among the male offspring of VIP deficient mothers, regardless of genotype, the time spent in the new target quadrant did not differ significantly from the time spent in the previous target quadrant (Fig. 6 D) ($+/+$ offspring of VIP deficient mothers ($F_{3,36} = 10.4$, $p < 0.0001$: Fisher's PLSD *post hoc* comparison with new target quadrant; $p = 0.0009$ for left quadrant; $p < 0.0001$ for right quadrant; $p = 0.06$ for previous target quadrant) ($+/-$ offspring of VIP deficient mothers ($F_{3,47} = 4.6$, $p = 0.007$: Fisher's PLSD *post hoc* comparison with new target quadrant: $p = 0.003$ for left quadrant; $p < 0.003$ for right quadrant; $p = 0.09$ for previous target quadrant) ($-/-$ offspring of VIP deficient mothers ($F_{3,52} = 4.7$, $p = 0.0055$: Fisher's PLSD *post hoc* comparison with new target quadrant; $p = 0.0036$ for left quadrant; $p < 0.0014$ for right quadrant; $p = 0.09$ for previous target quadrant).

Female offspring of WT mothers exhibited no deficits in the probe test following reversal training. Both $+/+$ and $+/-$ female offspring of WT mothers spent significantly more time in the new target quadrant than the other three quadrants (Fig. 6 E) ($+/+$ offspring $F_{3,48} = 4.1$, $p = 0.01$, Fisher's PLSD *post hoc* comparison with new target quadrant, $p = 0.005$ for left quadrant, $p = 0.007$ for right quadrant; $p = 0.008$ for previous target quadrant) ($+/-$ offspring $F_{3,28} = 4.3$, $p = 0.03$, Fisher's PLSD *post hoc* comparison with new target quadrant, $p = 0.02$ for left quadrant, $p = 0.01$ for right quadrant; $p = 0.01$ for previous target quadrant). Contrary to the $+/+$ male offspring of VIP deficient mothers, which exhibited deficits, the $+/+$ female offspring of VIP deficient mothers performed as well as the offspring of WT mothers and spent significantly more time in the new target quadrant than in the other three quadrants (Fig. 6 E) ($F_{3,40} = 5.4$, $p = 0.0033$: Fisher's PLSD *post hoc* comparisons with new target quadrant, $p = 0.005$ for left quadrant; $p = 0.0009$ for right quadrant; $p = 0.003$ for previous target quadrant). However, deficits in performance were seen in the $+/-$ and $-/-$ female offspring of VIP deficient mothers. The $+/-$ female offspring did not spend significantly more time in the new target quadrant compared with any of the other three (Fig. 6 E) ($F_{3,40} = 4.0$, $p = 0.01$: Fisher's PLSD *post hoc* comparisons with new target quadrant, $p = 0.12$ for left quadrant; $p = 0.68$ for right quadrant; $p = 0.07$ for previous target quadrant). The $-/-$ female offspring of VIP deficient mothers spent significantly more time in the new target quadrant than in the left and right quadrants; however, the time spent in the old target quadrant did not differ from the time in the new target quadrant (Fig. 6 E) ($F_{3,52} = 4.7$, $p = 0.0055$, Fisher's PLSD *post hoc* comparison

with new target quadrant, $p = 0.0036$ for left quadrant; $p = 0.0014$ for right quadrant; $p = 0.09$ for the previous target quadrant).

Discussion

The current study assessed the contributions of both the maternal VIP genotype, and the VIP genotype of offspring, to general health measures, social behavior and cognitive function. Adult WT and VIP deficient offspring of both WT and VIP deficient mothers were compared, the most salient comparison being between the VIP +/- offspring of WT and VIP deficient mothers. The most important discoveries were: 1) the VIP genotype of the mother was a greater predictor of deficiencies in her offspring than their own VIP genotype, and 2) the offspring of VIP deficient mothers exhibited deficiencies in social behavior and reversal learning that were more prevalent in male offspring.

The VIP deficient (+/-) offspring of WT mothers did not differ in a single measure from their WT littermates. The level of intrauterine VIP maintained by a WT mother during the period before the fetuses began making their own VIP apparently initiated the normal patterns of growth and development such that the subsequent deficiency in her VIP deficient offspring had no impact on the measures evaluated here. The results were more complex among the offspring of VIP deficient mothers. In some measures, for example, open field activity, all offspring of VIP deficient mothers showed the same increased activity, regardless of their own sex or genotype. However, in social approach and reversal training in the Morris water maze, sex differences were revealed. All male offspring of VIP deficient mothers had equal deficits in these measures, regardless of their own genotype. The female offspring of VIP deficient mothers had lesser deficits in these behaviors than their male littermates, with the greatest deficiencies seen in the VIP null females. Moderate deficiencies were seen in VIP +/- females, and the behaviors of WT female offspring of VIP deficient mothers were normal in most measures. Nonetheless, all VIP +/- offspring of VIP deficient mothers had deficiencies; none of the VIP +/- offspring of WT mothers had deficiencies.

While this study did not reveal major defects in general health measures in the offspring of VIP deficient mothers, these mice were more active in the open field, and the male null mutants (VIP -/-) appeared to have significant muscle weakness, confirming the previous findings of Girard et al. (2006). The significantly reduced grooming and lack of positional passivity of the offspring of VIP deficient mothers may be linked to the motor hyperactivity of these mice. Although none of the offspring of VIP deficient mothers vocalized during the general health examination, vocalization was not common among the offspring of WT mothers under these conditions. The general health and reflex data presented here are consistent with the attainment of developmental milestones of the VIP knockout mice (Lim et al., 2008) in which all offspring of VIP deficient mothers exhibited only delays in the appearance of developmental milestones and were eventually able to perform all tasks.

Mice are a highly social species, and in the automated three chamber social approach apparatus used here, normal C57BL/6 mice exhibit high levels of sociability as measured by frequent approaches and interactions with an unfamiliar mouse (Moy et al., 2004; Nadler et al., 2004). Compared with WT mice, the male offspring of VIP deficient mothers showed severe deficits in sociability, regardless of their own genotype, in all measures of the social approach test. The deficits were less severe in the female offspring of VIP deficient mothers where all genotypes expressed some sociability in both the sociability and preference for social novelty phases of the study. However, the degree of deficit in social behavior appeared to be related to the genotype of the female mouse with the VIP null females exhibiting the greatest reductions in all phases of the test and the WT females expressing normal behavior in the of sociability phase of the test. These results were similar to those obtained from mice that had been exposed to

VIP antagonist treatment during the period of E8-E10 (Hill et al., 2007a). While male adults in these experiments exhibited significantly reduced sociability in the sociability phase of the social approach task, and no preference for social novelty, female littermates did not show these deficiencies (Hill et al., 2007a). Furthermore, similarly treated male mice have been shown to have abnormal reactions in the social recognition task in which, despite having the capacity to differentiate between unfamiliar mice, they did not react to the introduction of a novel mouse by increased sniffing (Hill et al., 2007b). The results of these and the current experiments support the suggestion that there was critical period in the establishment of normal social responses, and that in the male mouse it was related to the action of VIP during embryogenesis. The social responses of female mice were less affected by maternal VIP deficits and were influenced by their own VIP deficiency. Since the olfactory habituation/dishabituation test showed that the mice in the previous (Hill et al., 2007a) and current experiments were capable of distinguishing between individual mice, their deficits appeared to be in their motivation to interact with members of their own species.

The offspring of VIP deficient mothers expressed normal learning in cued and contextual fear conditioning. And, although the offspring of VIP deficient mothers had longer latencies than the offspring of WT mothers during visible platform training in the Morris water maze, perhaps reflecting a different response to swimming or the use of a different strategy, these mice expressed no deficits in learning in the acquisition phase of spatial discrimination training, nor in memory in the probe test following acquisition training. However, the offspring of VIP deficient mothers exhibited impaired performance in the reversal phase of the Morris water maze, in which the position of the hidden platform was changed. During the first, but not subsequent days of reversal training, all male offspring of VIP deficient mothers swam over the previously correct platform position significantly more than WT males of WT mothers. On subsequent days of reversal training, the male mice did not swim significantly more often over the original platform position and appeared able to learn the new position. However, in the probe test following reversal training, male offspring of VIP deficient mothers did not prefer the new platform location over the old platform location, indicating an impaired memory for the new platform location. Among the female offspring of VIP deficient mothers the WT females were normal and showed normal reversal training and spent more time in the new target quadrant than in any of the other quadrants in the probe test. However, like their male littermates, the VIP deficient (+/–, and –/–) female offspring of VIP deficient mothers exhibited continued preference for the original platform position on day 1 of reversal training and in the probe test they did not spend more time in the new platform location than in the old platform location. The errors in performance by offspring of VIP deficient mothers in reversal learning, which examines cognitive flexibility (Coldren and Halloran, 2003; McAlonan and Brown, 2000; White, 2004), may have been due to a slower learning process or the use of a different strategy in this situation. However, they appear to be a perseveration of the previously learned strategy (perseveration errors) rather than the inability to learn a new strategy (regressive errors) and indicate a cognitive inflexibility, or a resistance to change, in these mice (Ragozzini et al., 2002a; Ragozzini et al., 2002b; Palencia and Ragozzini, 2004; Chadman et al., 2006).

We have shown that maternal VIP deficiency had more severe outcomes in social behavior and spatial reversal learning in the male offspring than in their female littermates. The results indicate that for the normal expression of these adult behaviors, the male mouse required normal maternal levels of VIP during embryogenesis, whereas the female mouse was more dependent upon normal fetal expression of VIP. The reasons for the sex difference in the behaviors examined here are not clear. However, in the developing rodent testes, testosterone secretion is stimulated by VIP (Romanelli et al., 1997; El-Gehani et al., 1998a; El-Gehani et al., 1998b), and reduced maternal VIP during embryogenesis might have influenced levels of circulating testosterone, the rate of sexual differentiation, and the organization of the fetal male brain.

The mechanisms through which reduced VIP might influence prenatal development may occur through direct VIP actions on neurogenesis, (Brenneman et al., 1990; Pincus et al., 1990; Pincus et al., 1994; Okumura et al., 1994; Waschek, 1995; Lu et al., 1996; Iwasaki et al., 2001); however, the indirect actions of VIP provide a mechanism for its mediation of diverse developmental processes (Brenneman et al., 1990). VIP is known to regulate numerous neurotrophic and neuroprotective factors including several chemokines (Brenneman et al., 1999), cytokines (Brenneman et al., 1995; Brenneman et al., 2003), insulin-like growth factor 1 (IGF-1) (Servoss et al., 2001), nerve growth factor (Hill et al., 2002), activity dependent neurotrophic factor (Brenneman and Gozes, 1996; Glazner et al., 1999), and activity dependant neuroprotective protein (ADNP) (Bassan et al., 1999; Furman 2004). ADNP is necessary for embryonic neural tube closure (Bassan et al., 1999; Pinhasov et al., 2003), and recent evidence from the VIP knockout mouse indicated that the maternal VIP genotype influenced the ADNP expression in the brains of her offspring (Giladi et al., 2007). Although the mechanisms underlying the abnormalities are not fully understood, the behavior deficits of the offspring of VIP deficient mothers appear to be the result of insufficient VIP to accomplish the normal actions of this neuropeptide on neurogenesis, or that insufficient VIP interfered with the expression, secretion or timing of developmentally important downstream effectors.

The current study has shown that intrauterine events had a greater influence on behavioral outcome than did genetic inheritance. These results are consistent with an increasing awareness that suboptimal intrauterine conditions maybe related to an increased susceptibility to a wide range of chronic diseases (Gluckman and Hanson, 2004), including the evidence that damage caused by infections early in gestation may be linked to the incidence of psychiatric disorders with apparent developmental origins, such as schizophrenia and autism (Meyer et al., 2007). Higher than normal concentrations of VIP were found in the blood of newborns, that were later shown to have Down syndrome and autism (Nelson et al., 2001), and evidence indicates that autism has its origins during the neural tube closure period of early embryogenesis (see review, Arndt et al., 2005), the period during which VIP regulates embryogenesis in the mouse (Gressens et al., 1993; Gressens et al., 1994; Hill et al., 1994; Hill et al., 1996; Hill et al., 1999; Spong et al., 1999). Autism has a male to female ratio of about 4:1 and is characterized by deficits in social behavior and communication, and restricted and repetitive behaviors, sometimes characterized as a resistance to change (Kanner, 1943; American Psychiatric Association, 1994, Lord et al., 2000). In the current study the deficits in social behavior and resistance to change were more prevalent in male mice. In previous studies, reduced and abnormal social behaviors were apparent only in the male mice that had experienced VIP blockage during embryogenesis (Hill et al., 2007a, 2007b). Although it is not known whether intrauterine VIP is involved in the phenotype expressed by autistic persons, the deficits in social behavior, a resistance to change, and an increased prevalence of these deficits in male mice born to VIP deficient mothers indicate a potential usefulness of the VIP knockout mouse as a mouse model for some aspects of autism.

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Sociability Phase

Fig. 1. Sociability phase of the social approach task

A. Normal sociability was shown by all male offspring of WT mothers as they spent significantly more time in the chamber with Mouse 1 than in the chamber with the novel object. All male offspring of VIP deficient mothers exhibited abnormal sociability as they spent the same amount of time in the chamber with Mouse 1 as they did in the chamber with the novel object. B. The female offspring of WT mothers exhibited normal sociability by this measure, as did the +/+ and +/- female offspring of VIP deficient mothers. Only -/- female offspring were deficient by this measure. C. All male offspring of WT mothers exhibited normal sociability as they sniffed Mouse 1 significantly more than they sniffed the novel object. The male offspring of VIP deficient mothers did not exhibit sociability as they sniffed Mouse 1 as

much as they sniffed the novel object. They also sniffed Mouse 1 significantly less than did the $+/+$ males of WT mothers. D. All female offspring of WT mothers were normal as they sniffed Mouse 1 significantly more than they sniffed the novel object. All female offspring of VIP deficient mothers similarly exhibited normal sociability; however, the $+/-$ and $-/-$ genotypes were somewhat deficient as they sniffed Mouse 1 significantly less than did the $+/+$ females of WT mothers. * = significantly different from novel object, $p < 0.05$. # = significantly different from $+/+$ offspring of WT mothers, $p < 0.05$. Data presented as mean $\pm$ SEM. WT mothers: $+/+$ offspring, 11 males, 11 females; $+/-$ offspring 13 males, 10 females. VIP deficient mothers: $+/+$ offspring; 10 males, 11 females; $+/-$ offspring, 10 males, 11 females; $-/-$ offspring, 10 males, 11 females.

Preference for Social Novelty Phase

Fig. 2. Preference for social novelty phase in the social approach task

A. The male offspring of WT mothers showed normal preference for social novelty and spent significantly more time in the chamber with the novel Mouse 2 than in the chamber with the now-familiar Mouse 1. None of the male offspring of VIP deficient mothers showed normal preference for social novelty. B. The female offspring of WT mothers similarly showed normal preference for social novelty and spent significantly more time in the chamber with the novel Mouse 2 than in the chamber with the now familiar Mouse 1. None of the female offspring of VIP deficient mothers showed normal preference for social novelty by this measure. C. The male offspring of WT mothers showed preference for Mouse 2 by sniffing Mouse 2 significantly more than Mouse 1. None of the male offspring of VIP deficient mothers showed

preference by this measure. D. The female offspring of WT mothers also showed preference for Mouse 2 as they sniffed Mouse 2 significantly more than Mouse 1. While all of the female offspring of VIP deficient mothers showed preference by this measure, all genotypes were deficient as they sniffed Mouse 2 significantly less than did the $+/+$ females of WT mothers. * = significantly different from Mouse 1, $p < 0.05$. # = significantly different from $+/+$ offspring of WT mothers, $p < 0.05$. Data presented as mean $\pm$ SEM. WT mothers: $+/+$ offspring, 11 males, 11 females; $+/-$ offspring 13 males, 10 females. VIP deficient mothers: $+/+$ offspring; 10 males, 11 females; $+/-$ offspring, 10 males, 11 females; $-/-$ offspring, 10 males, 11 females.

Entries

Sociability Phase

Preference for Social Novelty Phase

Fig. 3. Number of entries between chambers during the sociability phase and the preference for social novelty phase

A. Compared with +/+ males of WT mothers, no group of males, regardless of mother or genotype, differed in the number of entries made into the side compartments in the sociability phase. B. Compared with +/+ females of WT mothers, no group of females, regardless of mother or genotype, differed in the number of entries made into the side compartments in the sociability phase. C. Compared with +/+ males of WT mothers, no group of males, regardless of mother or genotype, differed in the number of entries made into the side compartments in the preference for social novelty phase. D. Compared with +/+ females of WT mothers, only the +/+ offspring of VIP deficient mothers differed in the number of entries made into the side

compartments in the preference for social novelty phase. Data presented as mean $\pm$ SEM. * = significantly different, $p < 0.05$, compared with novel object side. Data presented as mean $\pm$ SEM. WT mothers: +/+ offspring, 11 males, 11 females; +/- offspring 13 males, 10 females. VIP deficient mothers: +/+ offspring; 10 males, 11 females; +/- offspring, 10 males, 11 females; -/- offspring, 10 males, 11 females.

Fig. 4. Trace cued and contextual fear conditioning of offspring of WT mothers and offspring of VIP deficient mothers

A. Day 1, percent of time freezing was detected during training prior to (Pre-CS) and following the conditioned stimulus – unconditioned stimulus pairings (Post-CS) and all groups froze equally and significantly more following the CS. B. Day 2, percent of time freezing was detected during testing of contextual conditioning and all groups froze equally demonstrating that they recognized the context of the CS from the previous day. C. Day 3, percent of time freezing was detected before (Pre-Cue) and during (Cue) the presentation of the auditory cue in a novel context and all groups, regardless of mother or genotype recognized and remembered the cue associated with the CS from day 1. Data presented as mean $\pm$ SEM. * = significantly

different, $p < 0.05$, compared with Pre-CS (Training) or Pre-Cue (Novel Context). WT mothers: +/+ offspring $n = 9$ males and 10 females; +/- offspring $n = 9$ males and 9 females. VIP deficient mothers: +/+ offspring $n = 10$ males and 10 females; +/- offspring $n = 10$ males and 12 females; -/- offspring $n = 12$ males and 10 females.

Fig. 5. Morris water maze

A. Visible platform training. Horizontal line at 15 seconds represents criterion of successful learning. Although all groups had reduced latency with time, the offspring of VIP deficient mothers took significantly longer than the offspring WT mothers to reach criterion. * = $p < 0.05$ compared with +/+ offspring of WT mothers. B. Hidden platform training. All groups learned the position of the hidden platform and reached criterion by day 5. C. Probe test in which the platform was removed and the percent time spent in each quadrant was recorded. All groups exhibited memory equally as they all spent significantly more time in the target quadrant than the other three quadrants. * = $p < 0.05$, target quadrant compared with other three quadrants. Data presented as mean $\pm$ SEM. WT mothers: +/+ offspring $n = 11$ males and 14

females; +/- offspring n = 10 males and 9 females. VIP deficient mothers: +/+ offspring n = 10 males and 12 females; +/- offspring n = 14 males and 11 females; -/- offspring n = 15 males and 12 females.

Reversal training

Fig. 6. Reversal training in the Morris water maze

Following the hidden platform training, the position of the platform was changed (reversal training) and the mice were tested for 3 days of hidden trials to locate the new position. A. Hidden platform, new position training. The offspring of VIP deficient mothers had longer latencies to reach the new platform position than did the offspring of WT mothers; however, only the $-/-$ genotype reached the level of significance. B. Number of times crossing over the previous platform location for each of the three days of hidden trials of male mice. Male offspring of VIP deficient mothers crossed over the previous platform location significantly more on day 1 than the male offspring of WT mothers. C. Number of times crossing over the previous platform location for each of the three days of hidden trials of female mice. Female

WT offspring of VIP deficient mothers crossed over the previous platform location as often as the WT offspring of WT mothers; however, the female $+/-$ and female $-/-$ offspring of VIP deficient mothers crossed over the previous platform location significantly often on day 1 than the female WT offspring of WT mothers. D. Probe test of male mice. The platform was removed and the percent time spent in each quadrant was measured. None of the male offspring of VIP deficient mothers showed a preference for the new target quadrant over the previous target quadrant. E. Probe test of female mice. The female offspring of VIP deficient mothers exhibited a different pattern than the males and the $+/+$ females performed normally and spent significantly more time in the new target quadrant than the previous target. The $+/-$ and $-/-$ females did not show a preference for the new target quadrant over the previous target quadrant. * = $p < 0.05$, target quadrant compared with other three quadrants. Data presented as mean $\pm$ SEM. WT mothers: $+/+$ offspring 11 males and 14 females; $+/-$ offspring, 10 males and 8 females. VIP deficient mothers: $+/+$ offspring 10 males and 11 females; $+/-$ offspring 13 males and 11 females; $-/-$ offspring 14 males and 12 females.

Table 1

Anxiety-like behavior, general health, reflexes, reactivity, nociception, strength, and motor ability of offspring of WT mothers (VIP +/+) mated with VIP +/- males, and VIP deficient mothers (VIP +/-) mated with VIP +/- males.

Genotype of offspring	VIP +/+ (n=23)	WT mother VIP +/- (n=20)	VIP +/+ (n=23)	VIP +/- (n=30)	VIP +/- (n=23)
<i>Elevated plus maze – anxiety-like behavior</i>					
Open entries	7.1±0.5	7.7±1.4	6.9±0.7	6.9±0.5	7.8±0.6
Closed entries	7.4±0.6	6.6±0.9	9.4±0.9	7.1±0.6	6.9±0.7
Open time (seconds)	114±10	113±14	105±12	115±9.3	112±14
Closed time (seconds)	122±9	140±12	129±11	112±7	107±10
<i>General Health</i>					
Weight (g)(7-9 months) males	32.7±1.3	32.0±1.4	32.8±1.8	34.1±1.0	31.7±0.9
Weight (g)(7-9 months) females	24.9±0.8	25.3±0.7	25.8±0.9	25.9±0.5	24.1±0.3
Fur condition (3 point scale)	3	3	3	3	3
Piloerection (%)	0	0	0	0	0
Missing whiskers (%)	0	0	0	6	0
Bald patches (%)	0	0	0	0	0
Body tone (3 point scale)	2	2	2	2	2
Limb tone (3 point scale)	2	2	2	2	2
Physical abnormalities (%)	0	0	0	0	0
<i>Reflexes</i>					
Trunk curl (%)	100	100	100	100	100
Forepaw reaching (%)	100	100	100	100	100
Righting (%)	100	100	100	100	100
Corneal (%)	100	100	100	100	100
Pinna (%)	100	100	100	100	100
Vibrissae (%)	100	100	100	100	100
Toe pinch (%)	100	100	100	100	100
Acoustic startle amplitude (120dB)	391.9±51.7	341.9±55.2	416.1±38.5	427.6±37.4	481.7±40.4
Prepulse inhibition of acoustic startle (% normal)	100	100	100	100	100
<i>Behavioral reactivity</i>					
Vocalizations (%)	12	6	0	0	0
Struggle (%)	100	80	100	100	100
Petting escape (%)	90	90	100	93	100
<i>Empty cage behavior</i>					
Transfer freezing (%)	0	0	0	0	0
Wild running (%)	0	0	0	0	0
Circling stereotypy (%)	0	0	0	0	0
Exploration (3 point scale)	2.0±0.1	2.1±0.1	2.0	1.9±0.05	2.0±0.04
Grooming (3 point scale)	2.4±0.2	2.1±0.3	0.0*	0.1±0.05*	0.1±0.1*
<i>Nociception</i>					
Tail flick (seconds)	2.2±0.1	2.4±0.1	2.3±0.2	2.3±0.1	2.5±0.1
Hot plate (seconds)	9.6±0.3	9.1±0.3	8.8±0.5	9.4±0.3	8.6±0.6
<i>Motor and strength abilities</i>					
Open Field (5 min) (beam breaks)	844.6±31.7	935.6±46.7	1161.0±53.4*	1241.2±52.6*	1184.3±46.9*
Positional passivity (%)	23	17	0	0	0
Wire hang (seconds) males	42.7±4.2	42.1±6.5	45.6±8.3	53.4±2.7	34.6±10.6
Wire hang (seconds) females	51.4±3.5	46.7±5.1	55.2±4.7	55.8±4.1	50.9±6.3
Rotarod (seconds) males	157.8±11.4	132.5±18.7	137.7±19.9	119.8±13.2	106.1±18.5*
Rotarod (seconds) females	165.8±11.8	159.0±19.5	186.5±12.3	177.2±12.1	148.1±16.8

Table 2

Olfactory habituation/dishabituation of offspring of WT mothers (VIP +/+) mated to VIP +/- males and VIP deficient mothers (VIP +/-) mated with VIP +/- males. The number of seconds spent sniffing $\pm$ SEM of 3, 2 min presentations each of water, odor from Cage 1, and odor from Cage 2 was recorded.

Offspring genotype	VIP +/+ (n=15)	WT mother VIP +/- (n=13)	VIP +/+ (n=26)	VIP deficient mother VIP +/- (n=49)	VIP +/- (n=18)
Sequence in which applicators were presented:					
1st Water	1.9 $\pm$ 0.3	2.06 $\pm$ 0.4	1.5 $\pm$ 0.2	1.8 $\pm$ 0.2	1.5 $\pm$ 0.2
2nd Water	1.6 $\pm$ 0.2	1.3 $\pm$ 0.2	1.1 $\pm$ 0.1	1.2 $\pm$ 0.1	1.2 $\pm$ 0.2
3rd Water	0.9 $\pm$ 0.1	1.3 $\pm$ 0.1	0.7 $\pm$ 0.1	0.9 $\pm$ 0.1	0.8 $\pm$ 0.1
1st Cage 1	7.0 $\pm$ 0.7 *	7.3 $\pm$ 0.9 *	10.3 $\pm$ 0.8 #	9.5 $\pm$ 0.5 #	9.4 $\pm$ 0.7 #
2nd Cage 1	2.4 $\pm$ 0.5	3.2 $\pm$ 0.9	3.8 $\pm$ 0.4	3.5 $\pm$ 0.3	3.3 $\pm$ 0.4
3rd Cage 1	1.5 $\pm$ 0.3	2.0 $\pm$ 0.4	2.4 $\pm$ 0.4	2.5 $\pm$ 0.3	2.2 $\pm$ 0.3
1st Cage 2	3.7 $\pm$ 0.7 *	3.8 $\pm$ 0.7 *	4.5 $\pm$ 0.4 *	3.9 $\pm$ 0.4 *	3.5 $\pm$ 0.4 *
2nd Cage 2	1.6 $\pm$ 0.4	1.9 $\pm$ 0.3	1.8 $\pm$ 0.3	1.98 $\pm$ 0.2	2.0 $\pm$ 0.3
3rd Cage 2	0.9 $\pm$ 0.2	1.1 $\pm$ 0.3	1.1 $\pm$ 0.2	1.5 $\pm$ 0.2	1.3 $\pm$ 0.2

* = significantly different from time spent sniffing previous applicator, $p < 0.05$.

= significantly different from time VIP +/- offspring of WT mother spent sniffing 1st applicator with scent from Cage 1, $p < 0.05$.