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Regardless of genotype, offspring of VIP deficient female mice exhibit developmental delays and deficits in social behavior

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Abstract

Pharmacological studies indicate that vasoactive intestinal peptide (VIP) may be necessary for normal embryonic development in the mouse. For example, VIP antagonist treatment before embryonic day 11 resulted in developmental delays, growth restriction, modified adult brain chemistry and reduced social behavior. Here, developmental milestones, growth, and social behaviors of neonates of VIP-deficient mothers (VIP +/-) mated to VIP +/- males were compared with the offspring of wild type mothers (VIP +/+) mated to VIP +/- and +/- males, to assess the contributions of both maternal and offspring VIP genotype. Regardless of their own genotype, all offspring of VIP deficient mothers exhibited developmental delays. No delays were seen in offspring of wild type mothers, regardless of their own genotype. Body weights were significantly reduced in offspring of VIP deficient mothers, with VIP null (-/-) the most affected. Regardless of genotype, all offspring of VIP deficient mothers expressed reduced maternal affiliation compared with wild type offspring of wild type mothers; +/- offspring of wild type mothers did not differ in maternal affiliation from their wild type littermates. Play behavior was significantly reduced in all offspring of VIP deficient mothers. Maternal behavior did not differ between wild type and VIP deficient mothers, and cross-fostering of litters did not change offspring development, indicating that offspring deficits were induced prenatally. This study illustrated that the VIP status of a pregnant mouse had a greater influence on the growth, development and behavior of her offspring than the VIP genotype of the offspring themselves. Deficiencies were apparent in +/+, +/- and -/- offspring born to VIP deficient mothers; no deficiencies were apparent in +/- offspring born to normal mothers. These results underscore the significant contribution of the uterine environment to normal development and indicate a potential usefulness of the VIP knockout mouse in furthering the understanding of neurodevelopmental disorders with social behavior deficits such as autism.

Keywords

Vasoactive intestinal peptide; Developmental milestones; Developmental delays; Social behavior; Neurodevelopmental disorders; Autism; Neuropeptide; Maternal attachment; Play behavior

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Recent studies have revealed that vasoactive intestinal peptide (VIP) regulates growth and development during the early postimplantation period of mouse embryogenesis characterized by neural tube closure and the initiation of neurogenesis. Treatment of embryonic day 9 (E9) whole cultured mouse embryos with a dose range of VIP showed a concentration dependent and dramatic increase in growth and development over a 4 h period (Gressens et al., 1993; Dibbern et al., 1997; Hill et al., 1999; Glazner et al., 1999; Servoss et al., 2001). Treatment with the same concentration range of the related peptide, pituitary adenylate cyclase polypeptide (PACAP) had no effect at low concentrations and an inhibitory effect on growth at higher concentrations (Hill et al., 1999), illustrating the different roles of these peptides during development. Dramatic growth of whole cultured mouse embryos was also seen with downstream effectors of VIP including activity dependent neurotrophic factor (ADNF) (Dibbern et al., 1997; Glazner et al., 1999) and insulin-like growth factor-1 (IGF-1) (Servoss et al., 2001), which was shown to be a mediator of VIP and ADNF actions during embryogenesis. VIP stimulated the expression of IGF-1 in mouse embryos (Servoss et al., 2001), suggesting that the VIP-induced increase in cell division during embryogenesis might be occurring through IGF-1, which is known to have potent mitogenic actions (Baker et al., 1993; Liu et al., 1993; Powell-Braxton et al., 1993). VIP is a known secretagogue of several trophic factors including chemokines and cytokines (Brenneman et al., 1995; Brenneman et al., 1999). In the mouse embryo, VIP also stimulates the secretion of nerve growth factor (Hill et al., 2002), a well-recognized regulator of neuronal differentiation and survival, and VIP is known to regulate the expression of activity dependent neuroprotective protein (ADNP) (Bassan et al., 1999; Pinhasov et al., 2003; Giladi et al., 2007), a necessary factor for embryonic neural tube closure.

Receptors for VIP are expressed early in mouse development (Waschek et al., 1996) and autoradiography revealed VIP binding sites as early as E9 where they were localized to the floor plate of the embryonic neural tube (Hill et al., 1999; Spong et al., 1999), a site with an important role in regulating neural migration during embryogenesis (Jessell and Dodd, 1992). Although the peptide has been identified in embryo tissues at the same time as the earliest expression of VIP receptors (Spong et al., 1999), VIP gene expression in the embryo occurred later in embryogenesis (Gozes et al., 1988). In the mouse embryo, VIP gene expression has not been identified until E12 (Waschek et al., 1996; Spong et al., 1999) – after the period of VIP regulation of embryonic growth (Gressens et al., 1994). Prior to E12 in the mouse, when the embryonic yolk sac circulation is in intimate contact with the maternal decidua of the pregnant uterus, both VIP and its mRNA are abundant in the decidua, where immunoreactive VIP has been localized to gamma delta T lymphocytes (Spong et al., 1999). The mRNA for VIP in the uterine decidua was most abundant from E7–E10, peaked at E8, and was no longer detectable by E11–E12 (Spong et al., 1999). VIP has been shown to cross the developing placenta during early embryogenesis (Hill et al., 1996), and the maternal uterine tissues are the likely source of the peptide acting on embryonic VIP receptors to regulate growth and development in the mouse embryo. The early appearance of VIP and PACAP and their shared receptors in development (Waschek et al., 1996; Shuto et al., 1996) is consistent with their actions on neuronal survival (Brenneman et al., 1990; Brenneman et al., 2002), proliferation (Brenneman et al., 1990; Pincus et al., 1990; Pincus et al., 1994; Lu et al., 1996; Nicot et al., 2002; Waschek 2002) and neurite outgrowth (Iwasaki et al., 2001; Falluel-Morel et al., 2005).

Blockade of VIP actions has been shown to influence post natal development in the rodent (Hill et al., 1991) and VIP antagonist treatment of pregnant mice during, but not after, the period of E9–E11 was shown to produce microcephaly, growth restriction and an upregulation of VIP binding sites in the fetus (Gressens et al., 1994). Similar VIP antagonist treatment prior to E11 has also been shown to result in developmental delays and growth restriction in neonatal mice (Wu et al., 1997; Hill, 2007), and in adults, neuronal damage (Hill et al., 1994), and regionally specific changes in VIP chemistry in the brain including increased VIP binding sites

and increased VIP-positive neurons (Hill et al., 1994; Hill et al., 2007b), increased neuronal survival, and evidence of increased synaptogenesis in cultured cortical cells (Hill et al., 2007b). Blockage of PACAP activity with the antagonist PACAP 6–38 did not delay the appearance of developmental milestones (Hill, 2007). Reduced VIP has been shown to have an impact on sexual social behaviors (Gozes et al., 1989, 1993) and recent studies have shown that, as adults, male mice that had experienced blockage of VIP during this early embryogenesis exhibited reduced and abnormal social behaviors (Hill et al., 2007a, 2007b), indicating that VIP reduction during embryogenesis may induce autism-like deficits in social behavior. The above studies describe long-term, deleterious, and specific consequences of interference with normal VIP actions during embryogenesis and may indicate a link between a dysregulation of VIP and the appearance of neurodevelopmental disorders.

VIP and PACAP knockout mice have recently been developed (Colwell et al., 2003; Colwell et al., 2004) and the null mutants of both peptides weighed less than age matched wild type (WT) mice and had corresponding reductions in size (Girard et al., 2006). Similar to the deficits in diurnal rhythms of rodents exposed to reduced VIP during development (Gozes et al., 1995), both VIP and PACAP null mice showed deficits in circadian rhythm generation (Colwell et al., 2003; Anton et al., 2005); additionally, adult VIP null exhibited muscle weakness (Girard et al., 2006) and airway hyperresponsiveness and inflammation (Szema et al., 2005). While VIP null females have shown some infertility, VIP +/- females are reproductively competent and have approximately 50% of the VIP as WT mice (Girard et al., 2006). Importantly, in the absence of the complementary peptide, neither VIP nor PACAP knockout mice showed increased expression of genes for the peptide or peptide receptors (Girard et al., 2006), illustrating that the phenotypic expression of VIP knockout mice was not due to compensatory actions of PACAP.

The purpose of the current study was to determine how VIP deficiencies of the mother, and of the offspring, might each contribute to the early postnatal growth and development of the offspring and to the expression of their social behaviors through examination of maternal affiliation and play behavior in pre-weaned offspring. VIP +/+, VIP +/- and VIP -/- offspring were obtained from mating VIP deficient mothers (VIP +/-) with VIP +/- males. These offspring were compared with the VIP +/+ and VIP +/- offspring from WT mothers that were obtained by mating VIP +/+ females with VIP +/- males.

Experimental procedures

Animals and testing procedure

VIP knockout animals were generated as previously described (Colwell et al., 2003). Three groups of mice were compared. Group 1: VIP deficient (VIP +/-) females were mated with VIP +/- males and produced 17 litters with 34 wild type (+/+), 46 heterozygote (+/-), and 29 null (-/-) offspring. Group 2: WT, (VIP +/+) females were bred with +/- males and produced 18 litters with 56 wild type (+/+) and 58 heterozygote (+/-) offspring. Male and female VIP +/+ mice were also bred and produced 12 litters from which 20 wild type (+/+) offspring served as additional controls.

To control for potential post-natal maternal effects on behavior and the achievement of developmental milestones, a second group of mice was generated and 6 litters of VIP deficient (VIP +/-) mothers mated with VIP +/- males (12 +/+, 18 +/-, and 6 -/- offspring), and 7 litters of WT (VIP +/+) mothers mated with VIP +/+ males (24 +/+ offspring), were cross-fostered on post natal day (P) 2. From P2 until P21, VIP deficient mothers raised the offspring of WT mothers and WT mothers raised the offspring of VIP deficient mothers. The pups to be cross-fostered were removed from the home nest, gently cleaned with alcohol swabs to remove the odor of their mother, and placed into a small dish containing nesting material of the foster

mother for 10 min before being introduced to the foster mother's nest. Each litter was composed of between 5 and 8 pups. The cross-fostered mice were examined daily for the acquisition of developmental milestones and weight gain until P21.

All mice were housed under controlled temperature and humidity under a 12 h light/dark cycle and were provided food and water *ad libitum*. All females were mated at 3–5 months of age. Behavioral testing of offspring began on postnatal day P1 and ended on P21. To allow for the identification of each mouse in a litter, they were tattooed on P1 according to the AIMS Pup Tattoo Identification System, Budd Lake, NJ with tattoo ink from Ketchum Tattoo, Inc., Lake Luzerne, NY. On the day of testing, the home cage was moved into the testing room and left to habituate for at least 30 minutes. During testing and the later analysis of taped observations, observers identified each mouse by its tattoo number and were blind to the genotype of either the mother or the offspring. On P10 tail snips were taken from all offspring and sent to UCLA for genotyping. The genotype of each pup was added to data sheets prior to statistical analysis. 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.

Developmental milestones and weight gain

Neonatal behavior development was evaluated as previously described (Wu et al., 1997; Hill et al., 2008). In short, beginning on P1, newborn mice were weighed and examined daily for the acquisition of the following developmental milestones requiring strength and/or coordination: 1) cliff aversion, P1–P14 – first day that pups positioned with forepaws and snout over the edge of a shelf to turned and begin to crawl away from the edge; 2) negative geotaxis, P1–P14 – first day that pups placed head down on a 45° incline turned 180° and began to crawl up the slope; 3) rooting, P1–P12 – first day that the head turned toward the side of the face being stroked with the tip of a cotton swab; 4) forelimb grasping, P4–P14 – first day pups could remain suspended for at least 1 second grasping a thin rod with their forepaws; 5) surface righting, P1–P13 – first day that pups placed on their back, turned over with all four paws touching the table surface, in less than one second; 6) air righting, P8–P21 – first day that pups released upside-down from a height of approximately 60 cm, turned right side-up and landed on all four paws on a bed of shavings; 7) activity, P8–P21 – first day that pups move outside of a circle 13 cm in diameter. In addition, the following developmental markers were examined: 1) auditory startle, P7–P18 – first day that pups responded with a quick involuntary jump after a small metal object was dropped on a lab bench 10 inches from the pup; 2) ear twitch, P7–P15 – first day that the ear twitched after stimulation with the tip of a cotton swab; 3) eye opening, P8–P21 – first day that both eyes are open. The battery of tests provided an assessment of development throughout the neonatal period since the behaviors measured were each expressed at differing periods throughout the first 21 days of life. Not all behaviors were tested daily, only those which were appropriate for the age of the pup. Testing of late appearing behaviors began 3 days before and continued 3 days after the behavior normally appeared in the offspring of WT mice. Responses were considered positive only after they had been observed for two consecutive days. Investigators working in pairs performed the behavioral testing.

Homing

The homing test was performed as previously described (Venerosi et al., 2001). On P11, the mice were tested inside a standard mouse cage in which 1/3 of the cage was evenly filled with shavings from the home cage and the remaining 2/3 of the cage with clean shavings. Mouse pups were placed in the center of the clean shavings, facing away from the home cage shavings. The latency to turn and move onto the home cage shavings was assessed in three separate trials. The pup was considered to have successfully “homed” when all four of its paws had crossed

onto the home cage shavings and the pup remained there for at least 10 seconds. Each trial lasted a maximum of 150 seconds with an inter-trial interval of approximately 10 seconds. This test evaluates the locomotor and olfactory capability of the pups.

Maternal behavior

Maternal behaviors, as described by Brown et al., 1999 and Bartolomucci et al., 2004, were assessed in undisturbed cages of mothers with litters from P1 to P13. In addition, the nest quality was rated on a scale of 0–3: 0 for no nest, 1 for poor nest (flat, no to very low enclosures, scattered bedding in the periphery), 2 for incomplete nest (low enclosures and and/or scattered bedding in the periphery), and 3 for complete nest (high and tight enclosures), as previously described (Jin et al., 2005). Pup location (inside or outside the nest) and condition (grouped or scattered) was noted. The following maternal behaviors were recorded daily during 10, 30 s observation sessions, separated by 1 min intervals: 1) nursing (one or more pups suckling), 2) forced nursing (dam engaged in other behaviors, e.g. nest-building or self-grooming, while one or more pups suckle), 3) crouching over (arched-back nursing position over pups), 4) nest-building (carrying or pushing nest material towards nest or maintaining the current state while inside or outside the nest), and 5) licking and grooming pups. Non-maternal behaviors included: 1) self-grooming, 2) feeding and drinking, and 3) active (dam engaged in other activities outside the nest, such as rearing, digging, climbing, and exploration) were also scored. Frequency of pup vocalization was assessed.

Maternal preference

A test of maternal preference (Moles et al., 2004) was conducted on P14 in a standard mouse cage divided evenly into three sections: left, center, and right. The left 1/3 was filled with shavings from the mother's cage, the center 1/3 with clean shavings, and the right 1/3 was filled with shavings from the cage of a strange, lactating female mouse. For consecutive tests, the sides containing mother's cage shavings and stranger shavings were alternated to correct for any side preferences. Three 1 min trials, with inter-trial intervals of 10 seconds, were administered to each pup following a 30 min habituation to the test room. For the first trial, the pup was placed in the center of the fresh shavings with its snout facing the back wall of the test cage. During a 1 min interval, the time spent on the mother's shavings and time spent on the stranger's shavings was recorded. The pup was considered to be 'inside' the section when all 4 paws were touching the shavings within the specified region. For the second trial, the pup was placed in the center of the fresh shavings with its snout facing the section containing its mother's cage shavings and for the third trial the pup's snout faced the section containing the shavings of the strange, lactating female. The time spent on mother's shavings, and on stranger shavings, was averaged across the three trials. This test was used as a measure of the social behavior of maternal attachment (Moles et al., 2004).

Play behavior

The sociability period characterized by amicable and playful interactions of young mice extends from P15 to P25 (Williams and Scott, 1953; Dyer and Southwick, 1997) and play behavior of pre-weaned mice, as described by Grant and Mackintosh, 1963, Terranova et al., 1993 and Terranova et al., 1998; Terranova and Laviola, 2005, was examined in the early part of this period on P17. Mice were weighed prior to testing to ensure that the members of each pair of mice had similar body weights. To habituate to the test room and to promote play, mice were separated and individually housed in clean cages for 1 h. Pairs composed of the same sex, same genotype, and from the same litter were then reunited in the testing arena, a standard mouse cage filled with fresh shavings. Pairs were video taped for 30 min. Frame by frame analysis of the recorded video files for minutes 0 – 5, 10 – 15, and 20 – 25, was conducted using the Noldus Observer 5.0 keypad and event analysis software (Noldus, Leesburg, VA).

Investigators analyzed the videotapes while blind to the genotype of the pups. The number of events of the following mouse play behaviors was recorded: investigation (following, social sniffing), affiliation (social grooming), play soliciting (push under, crawl over, push past between cage wall and cage mate). In addition, the time spent in non-social activities (exploration and time at rest) was recorded.

Statistical analysis

The data were analyzed using the t test for simple comparisons, or Analysis of Variance (ANOVA), with repeated measures where appropriate (RMANOVA), followed by *post hoc* comparisons between the wild type (VIP +/+) offspring of WT (VIP +/+) mothers and all other groups with Fisher's PLSD for ANOVA, or Bonferroni/Dunn *post hoc* tests for RMANOVA, (Statview - Abacus Concepts, Berkeley, CA). Significance was set at $p < 0.05$ for ANOVA and Fisher's PLSD *post hoc* analyses, and was specified for each Bonferroni/Dunn *post hoc* analysis. In all analyses, the sample size was the number of litters in which the genotype occurred. The mean score obtained by the members of each genotype was used as the statistic for that genotype in the litter. All data are presented as mean $\pm$ one standard error of the mean.

Results

Developmental milestones

During testing in these, and all following behavioral measures, investigators were unaware of the identity of the genotype of both the mother and the offspring. There were no statistically significant sex differences in the rates of development of any group so the sexes were combined for analyses. Among the offspring of WT mothers there were no differences in the attainment of developmental milestones between the two genotypes (+/+ and +/-), and both genotypes attained developmental milestones at the same rate (Table 1). However, compared with the wild type (+/+) offspring of WT mothers, all offspring of VIP deficient mothers, exhibited significant delays of up to 2.5 days in the attainment of developmental milestones, regardless of their own genotype (+/+, +/-, or -/-) (Table 1). An exception was the performance of activity (first day the pups move outside of a circle 13 cm in diameter), which all groups executed on P12 ($F_{4,83} = 0.65$, $p = 0.63$). At P10, air righting had the greatest delays among the milestones related to strength and coordination with offspring of VIP deficient mothers delayed 2.5 days compared with wild type offspring of WT mothers ($F_{4,79} = 11.24$, $p < 0.0001$; Fisher's PLSD *post hoc* analysis: $p = 0.0002$ for +/+; $p < 0.0001$ for +/-; $p < 0.0001$ for -/-). Grasp was also delayed an average of 2 days ($F_{4,83} = 15.3$, $p < 0.0001$; Fisher's PLSD *post hoc* analysis: $p < 0.0001$ for +/+; $p < 0.0001$ for +/-; $p < 0.0001$ for -/-), whereas rooting ($F_{4,83} = 5.5$, $p = 0.0005$; Fisher's PLSD *post hoc* analysis: $p = 0.01$ for +/+; $p < 0.0001$ for +/-; $p = 0.0005$ for -/-) and surface righting ($F_{4,83} = 2.5$, $p = 0.04$; Fisher's PLSD *post hoc* analysis: $p = 0.01$ for +/+; $p = 0.007$ for +/-; $p = 0.02$ for -/-) were delayed the least, perhaps indicating a lesser degree of difficulty of these tasks. Cliff aversion ($F_{4,83} = 5.3$, $p = 0.0007$; Fisher's PLSD *post hoc* analysis: $p = 0.001$ for +/+; $p = 0.002$ for +/-; $p = 0.01$ for -/-) and negative geotaxis ($F_{4,83} = 3.8$, $p = 0.006$; Fisher's PLSD *post hoc* analysis: $p = 0.006$ for +/+; $p = 0.002$ for +/-; $p = 0.0007$ for -/-) were both delayed about one and half days in the offspring of VIP deficient mothers, and were the first behaviors to develop. Therefore, the severity of the delay was not related to the age at which the developmental milestones first appeared. Although auditory startle was delayed approximately 3 days in the offspring of VIP deficient mothers ($F_{4,83} = 22.6$, $p < 0.0001$; Fisher's PLSD *post hoc* analysis: $p < 0.0001$ for +/+; $p < 0.0001$ for +/-; $p < 0.0001$ for -/-) the other developmental markers of ear twitch ($F_{4,83} = 2.6$, $p = 0.04$; Fisher's PLSD *post hoc* analysis: $p = 0.03$ for +/+; $p = 0.02$ for +/-; $p = 0.04$ for -/-) and eye opening ($F_{4,83} = 4.2$, $p = 0.004$; Fisher's PLSD *post hoc* analysis: $p = 0.001$ for +/+; $p = 0.02$ for +/-; $p = 0.03$ for -/-) were delayed a day or less in the offspring of VIP deficient mothers (Table 1). The offspring of VIP deficient mothers did not differ significantly among themselves

in their rate of development (Table 1) indicating that the developmental delays were not due to the genotype of the offspring.

Homing

ANOVA of homing data showed no main genotype effect ($F_{4,59} = 0.75$, $p = 0.56$) (Table 1), and no genotype of offspring of VIP deficient mothers differed significantly from the wild type (+/+) offspring of WT mothers (Fisher's PLSD *post hoc* analysis: $p = 0.84$ for +/+; $p = 0.67$ for +/-; $p = 0.39$ for -/-). These results indicate that, compared with the offspring of WT mothers, the offspring of VIP deficient mothers exhibited no locomotor or olfactory deficiencies at P11.

Weight gain

There were no sex differences in weight gain among the treatment groups throughout the testing period so the sexes were combined for analysis; however, there was a significant main effect of genotype (RMANOVA $F_{4,23} = 3.8$, $p = 0.01$) and a significant weight $\times$ genotype interaction ($F_{23,230} = 2.1$, $p = 0.0002$). Among offspring of WT mothers, there were no significant differences in weight gain between the two genotypes (+/+ or +/-), demonstrating that VIP deficiency did not influence weight gain in pups of WT mothers (Table 2). *Post hoc* analyses showed that the difference in weight between the offspring of WT mothers and the offspring of VIP deficient mothers was primarily contributed by the -/- offspring. The VIP null (-/-) offspring of VIP deficient mothers weighed significantly less than the wild type offspring of WT mothers until day 8 (Bonferroni/Dunn *post hoc* analysis, with significance level of $p = 0.005$: $p = 0.0002$ for P2; $p = 0.0002$ for P3; $p = 0.0002$ for P4; $p < 0.0001$ for P5; $p = 0.0023$ for P6; $p = 0.0017$ for P7; $p = 0.0014$ for P8) (Table 2). The heterozygote (+/-) offspring of VIP deficient mothers also weighed significantly less than the WT offspring of WT mothers on day 2 (Bonferroni/Dunn *post hoc* analysis, with significance level of $p = 0.005$: $p = 0.002$ for P2) (Table 2). Since the weight gain of +/- pups was not affected when born to WT mothers, but was delayed in the +/- offspring of VIP deficient mothers, maternal VIP deficiency may have contributed to the reduced weight gain of her +/- offspring.

Cross-fostered mice

To evaluate the potential that postnatal mothering by VIP deficient mothers might have contributed to the delays in the appearance of the developmental milestones of their offspring, litters of WT mothers and VIP deficient mothers were cross-fostered on P2. The offspring of VIP deficient mothers were raised until weaning by WT foster mothers and VIP deficient mothers raised the offspring of WT mothers. The cross-fostered offspring of VIP deficient mothers exhibited delays in the achievement of developmental milestones, despite being raised until P21 by WT foster mothers (Table 3). Also, the developmental milestones of the cross-fostered offspring of WT mothers appeared significantly earlier than the milestones of the offspring of VIP deficient mothers, despite being raised by VIP deficient foster mothers (Table 3). Therefore, postnatal mothering was not the cause of the delay in the appearance of developmental milestones in the offspring of VIP deficient mothers. In the cross-fostered litters, the greatest delays were seen in cliff aversion and negative geotaxis, two of the first behaviors to appear (Table 3). Cliff aversion was 2 to 3 days delayed in the offspring of VIP deficient mothers raised by WT foster mothers ($F_{3,20} = 3.9$, $p = 0.02$; Fisher's PLSD *post hoc* comparison with the offspring of WT mothers ($p = 0.03$ for +/+; $p = 0.02$ for +/-; $p = 0.002$ for -/-), and negative geotaxis was also delayed 2 to 4 days ($F_{3,20} = 5.1$, $p = 0.008$; Fisher's PLSD *post hoc* comparison with the offspring of WT mothers ($p = 0.04$ for +/+; $p = 0.003$ for +/-; $p = 0.002$ for -/-). The other milestones of grasp ($F_{3,20} = 7.3$, $p = 0.0017$), surface righting ($F_{3,20} = 5.7$, $p = 0.005$), air righting ($F_{3,20} = 4.3$, $p = 0.017$), activity ($F_{3,20} = 5.0$, $p = 0.009$) and eye opening ($F_{3,20} = 5.6$, $p = 0.0058$) were also delayed 1 or 2 days in the offspring of

VIP deficient mothers raised by WT foster mothers (Table 3). Although the ANOVA for rooting did not reach statistical significance ($F_{3,20} = 1.9$, $p = 0.15$), *post hoc* comparison with the offspring of WT mothers revealed that the $-/-$ offspring of VIP deficient mothers were significantly delayed compared with the WT offspring of WT mothers ($p = 0.04$). There was no significant delay in the appearance of ear twitch in the cross fostered offspring of VIP deficient mothers (Table 3). There were some delays in the appearance of developmental milestones of all cross-fostered offspring (Table 3) compared with non cross-fostered offspring (Table 1), especially those milestones that appear early in development such as cliff aversion and negative geotaxis suggesting that cross-fostering itself might have a temporary delaying effect on development. However, the later developing milestones, such as surface righting, air righting, activity, and eye opening, appeared on the same days for cross-fostered offspring (Table 3) as they had in the non cross-fostered offspring (Table 1) indicating that cross-fostering did not influence the rate of development of these milestones.

Cross-foster weight gain

Although repeated measure ANOVA of the weight gain of the cross-fostered offspring through the entire 21 days of post natal examination did not reveal a significant genotype effect, during the first 7 postnatal days, there was a significant main genotype effect ($F_{3,20} = 4.2$, $p = 0.019$). As a group, the offspring of VIP deficient mothers weighed less than the offspring of WT mothers during this period (Table 4) ($F_{1,22} = 8.4$, $p = 0.008$), despite having been raised by foster WT mothers. As in non-cross-fostered mice, *post hoc* analysis showed that the difference in weight was primarily contributed by the VIP null $-/-$ offspring of the VIP deficient mothers. The VIP null $-/-$ offspring of VIP deficient mothers weighed significantly less than the wild type offspring of WT mothers until day 6 (Bonferroni/Dunn *post hoc* analysis, with significance level of $p = 0.008$; $p = 0.0029$ for P3; $p = 0.0011$ for P4; $p = 0.0007$ for P5; $p = 0.005$ for P6) (Table 4). The heterozygote ($+/-$) offspring of VIP deficient mothers also weighed significantly less than the WT offspring of WT mothers on day 1 (Bonferroni/Dunn *post hoc* analysis, with significance level of $p = 0.008$; $p = 0.0065$ for P3 (Table 4).

Maternal Behavior

Maternal behaviors of WT and VIP deficient mothers are shown in Table 5. There were no differences between WT mothers and VIP deficient mothers in total percent time exhibiting maternal behaviors ($F_{1,27} = 0.85$, $p = 0.36$) or in any of the measures of maternal behavior including nest score ($F_{1,27} = 0.9$, $p = 0.35$), pup location ($F_{1,27} = 0.6$, $p = 0.44$) and huddling ($F_{1,27} = 0.6$, $p = 0.45$), nursing ($F_{1,27} = 0.25$, $p = 0.62$), forced nursing ($F_{1,27} = 0.24$, $p = 0.62$), crouching-over ($F_{1,27} = 0.65$, $p = 0.42$), licking and grooming ($F_{1,27} = 0.76$, $p = 0.39$), and nest-building ($F_{1,27} = 1.0$, $p = 0.31$). Both groups of mothers also spent the same amount of time performing non-maternal behaviors ($F_{1,27} = 0.09$, $p = 0.76$) including self grooming ($F_{1,27} = 0.0001$, $p = 0.99$), feeding ($F_{1,27} = 0.11$, $p = 0.74$), and out of nest activity ($F_{1,27} = 0.09$, $p = 0.33$). In addition, sonic pup vocalization did not vary between the two groups ($F_{1,27} = 0.73$, $p = 0.39$); however, it was the only observation that changed in frequency over the observation period, as it increased with age of the pups. These data, combined with the demonstration that cross-fostered offspring exhibited the behaviors typical of the offspring of their birth mother, indicate that the differences between offspring of WT mothers and the offspring of VIP deficient mothers are not due to postnatal maternal care.

Maternal preference

In the analysis of maternal preference data ANOVA demonstrated that there were significant genotype effects in both the time spent on the mother's shavings (Figure 1A) ($F_{4,71} = 7.4$, $p < 0.0001$), and in the time spent on the stranger's shavings (Figure 1B) ($F_{4,71} = 5.8$, $p = 0.0004$). Fisher's *post hoc* comparisons with WT offspring of WT mothers (VIP $+/+$) showed that $+/-$

offspring of WT mothers did not differ significantly from their WT littermates in time spent on mother's shavings (Figure 1A, $p = 0.38$), nor on the time spent on the stranger's shavings (Figure 1B, $p = 0.25$). However, *post hoc* comparisons with the WT offspring of WT mothers, demonstrated that all genotypes of offspring of VIP deficient mothers (VIP +/-) spent significantly less time on the mother's shavings ($p = 0.0007$ for +/+ offspring; $p < 0.0001$ for +/- offspring; $p = 0.0007$ for -/- offspring) (Figure 1A) and significantly more time on the strangers' shavings ($p = 0.001$ for +/+ offspring; $p = 0.001$ for +/- offspring; $p = 0.0005$ for -/- offspring) (Figure 1B). Since the homing test indicated no deficiency in locomotion or olfactory capability in the offspring of VIP deficient mothers, the reduced preference for the odor of the mother's shavings suggests that the offspring of VIP deficient mothers express a reduction in maternal affiliation regardless of their own genotype.

Play behavior

There was a significant genotype effect in total number of play events (Figure 2) ($F_{4,50} = 5.1$, $p = 0.0016$) and Fisher's *post hoc* comparisons with WT offspring of WT mothers showed that the total number of play interactions did not differ between the two genotypes of the offspring of WT mothers ($p = 0.14$ for +/- offspring of WT mothers). However, all three genotypes of offspring of VIP deficient mothers had significantly fewer total play events compared with WT offspring of WT mothers ($p = 0.023$ for +/+ offspring; $p = 0.037$ for +/- offspring; $p = 0.04$ for -/- offspring). Play interactions included a number of specific types of play including follow, social sniff, social groom, push under, crawl over, and push past, and in most of these all three genotypes of offspring of VIP deficient mothers exhibited significantly fewer play events than the offspring of WT mothers. Follow: ANOVA, $F_{4,46} = 4.7$, $p = 0.0028$, Fisher's *post hoc* comparisons with WT offspring of WT mothers: $p = 0.03$ for +/+ offspring; $p = 0.04$ for +/- offspring; $p = 0.19$ for -/- offspring (Table 6). Social sniff: $F_{4,46} = 3.5$, $p = 0.01$, Fisher's *post hoc* comparisons with WT offspring of WT mothers: $p = 0.01$ for +/+ offspring; $p = 0.04$ for +/- offspring; $p = 0.001$ for -/- offspring (Table 6). Social groom: $F_{4,46} = 3.8$, $p = 0.008$, Fisher's *post hoc* comparisons with WT offspring of WT mothers: $p = 0.002$ for +/+ offspring; $p = 0.02$ for +/- offspring; $p = 0.003$ for -/- offspring (Table 6). Push under: $F_{4,46} = 4.0$, $p = 0.007$, Fisher's *post hoc* comparisons with WT offspring of WT mothers: $p = 0.01$ for +/+ offspring; $p = 0.15$ for +/- offspring; $p = 0.07$ for -/- offspring (Table 6). Crawl over: $F_{4,46} = 3.2$, $p = 0.02$, Fisher's *post hoc* comparisons with WT offspring of WT mothers: $p = 0.03$ for +/+ offspring; $p = 0.26$ for +/- offspring; $p = 0.04$ for -/- offspring (Table 6). Push past: $F_{4,46} = 4.1$, $p = 0.006$, Fisher's *post hoc* comparisons with WT offspring of WT mothers: $p = 0.008$ for +/+ offspring; $p = 0.007$ for +/- offspring; $p = 0.003$ for -/- offspring (Table 6). However, time spent in non-social activities such as exploring the cage and at rest did not differ among groups ($F_{4,46} = 0.74$, $p = 0.57$ for explore time (Table 6), and $F_{4,46} = 0.78$, $p = 0.54$ for time at rest (Table 6).

Discussion

The major findings of this study are: 1) the rate of achievement of developmental milestones was determined by the VIP genotype of the mother, not the genotype of the offspring; 2) both maternal and offspring VIP genotype contributed to rate of weight gain; 3) deficits in maternal preference were determined by maternal genotype; 4) deficits in play behavior were determined by maternal genotype; 5) examination of maternal behavior and the effects of cross-fostering litters indicated that the developmental deficits found in the offspring of VIP deficient mothers were not due to post-natal mothering.

VIP deficient mothers have approximately 50% of the VIP of WT mothers (Girard et al., 2006), and in the current study, these mothers produced +/+, +/- and -/- offspring, all of which exhibited developmental delays, regardless of their own VIP genotype. The corollary was also

true; both $+/+$ and $+/-$ offspring of WT mothers developed normally, regardless of their own VIP genotype. An important comparison is between the VIP $+/-$ offspring of VIP deficient mothers and WT mothers, as these mice inherited the same genetic capability to produce VIP, but experienced different prenatal exposure to VIP. Since the abnormal gene in these mice could have been inherited from either the mother or the father, there may have been a potential issue of imprinting. However, since the variances in the data from VIP $+/-$ offspring were of the same magnitude as those of its WT and VIP null siblings it did not appear that the VIP $+/-$ offspring of VIP deficient mothers represented two populations. The comparison of WT offspring of WT mothers with WT offspring of VIP deficient mothers is important since these mice are the most similar genetically but they experienced different prenatal conditions. The above comparisons show that: 1) the developmental performance of VIP $+/-$ offspring was due to maternal factors, not their own level of VIP; VIP $+/-$ offspring of VIP deficient mothers had deficits; VIP $+/-$ offspring of WT mothers did not; and 2) the developmental performance of WT offspring differed depending on their maternal environment, WT offspring of VIP deficient mothers had deficiencies, WT offspring of WT mothers did not. Furthermore, 1) no differences were detected in the maternal behaviors of WT mothers and VIP deficient mothers; and 2) the offspring of VIP deficient mothers exhibited developmental delays regardless of whether they were raised by their own mother or cross-fostered to a WT mother. These results indicated that postnatal mothering was not a factor in this study, and further support the conclusion that the level of maternal VIP during pregnancy was the significant determinant in the rate of development of the offspring. Among the offspring of VIP deficient mothers, genotype differences were seen in growth rate where VIP $-/-$ mice weighed significantly less than their $+/+$ littermates for the first few postnatal days. These data indicate that the VIP generated by the pup contributed to growth stimulation. However, since the VIP $+/-$ offspring of WT mothers showed no delay in growth rate, and the $+/-$ offspring of VIP deficient mothers did show a slight delay, indications are that both offspring and maternal VIP influenced the rate of growth during the first few postnatal days. No differences were seen among the offspring of WT and VIP deficient mothers in the latency to home in the homing test, demonstrating that all mice in the study had equivalent olfactory and locomotor capabilities in this task.

There were no differences in the latency of performance of developmental milestones among the three genotypes of the offspring of VIP deficient mothers, demonstrating that the reduced maternal VIP impacted all offspring equally. However, the delay in appearance of milestones was not uniform. Some milestones were delayed only 1.5 days and others up to 2.5 days. Also, no pattern was apparent in the delay in the appearance of the milestone relative to the age at first appearance of that milestone in WT mice, which suggested that the offspring of VIP deficient mothers were neither catching up to the offspring of WT mothers, nor were they falling further behind. Perhaps the delay in performance of each task was related to its complexity and the amount of strength or degree of coordination required. The behaviors of cliff aversion, negative geotaxis, surface righting and air righting require maturation of labyrinthine and body righting mechanisms in addition to strength and coordination, and all of these factors may contribute to delayed performance of these tasks by offspring of VIP deficient mothers. Significant delays in the appearance of auditory startle, ear twitch and eye opening, due to a slower maturation of the neural circuits underlying these milestones, further indicated that retardation in neural development was probably a factor in several of the tests administered. Locomotor ability, as measured by open field and homing, did not differ among groups, indicating that although reduced strength and coordination contributed to the developmental delays seen in offspring of VIP deficient mothers, the deficiency was not of a nature to result in reduced activity. These data are consistent with the results from studies of adult VIP null mice that were seen to be weaker in grip strength, but more active in the open field than wild type mice (Girard et al., 2006).

Although the development of cross-fostered litters was not influenced by being raised by the mother of a different genotype, compared with non cross-fostered litters, all cross-fostered litters exhibited delays in the appearance of those developmental milestones that appeared within the first few days of cross-fostering. These results indicate that the event of cross-fostering alone had a short-term effect on post-natal development.

Maternal preference and play behavior, the tests used here to assess social behaviors in pre-weaned mice, revealed deficits in all offspring of VIP deficient mothers, and no deficits were seen in the offspring of WT mothers, regardless of genotype suggesting that, like developmental milestones, the VIP genotype of the mother was the main contributor to the deficits in social behavior of offspring. Compared with the offspring of WT mothers, the offspring of VIP deficient mothers spent less time on maternal shavings and more time on stranger shavings in the maternal preference test. Since the homing test showed that the offspring of VIP deficient mothers had no olfactory or motor deficits at P11, the results of the maternal preference test indicate that the offspring of VIP deficient mothers exhibited reduced maternal affiliation (Moles et al, 2004). All genotypes of offspring of VIP deficient mothers had deficits in play behavior at P17. Since time exploring and time at rest did not differ between the offspring of WT mothers and the offspring of VIP deficient mothers, the deficit in play behavior by offspring of VIP deficient mothers was not due to reduced activity or due to increased exploration at the expense of social interactions. The offspring of VIP deficient mothers apparently expressed significantly less motivation to play.

The developmental delays and growth restriction seen in the offspring of VIP deficient mothers in the current experiment are also similar to the results obtained when mice experienced blockage of VIP actions through VIP hybrid antagonist treatment of pregnant females during E9–E11 (Wu et al., 1997) or E8–E10 (Hill, 2007), the period when VIP is apparently supplied by maternal sources. These VIP antagonist treated offspring exhibited retardation in the achievement of several developmental milestones (Wu et al., 1997; Hill, 2007) and weighed less than WT pups throughout the first 21 days of life (Hill, 2007). These results also revealed that the earlier the blockage of VIP, the greater the postnatal effects (Hill, 2007). While blockage from E9–E11 influenced fetal weight (Gressens et al., 1994), postnatal growth restriction was not apparent (Wu et al., 1997). However, treatment from E8–E10 resulted in not only retardation of a greater number of milestones, it caused growth restriction that was still apparent at P21 (Hill, 2007). In the current study, the offspring of VIP deficient mothers were exposed to reduced levels of VIP throughout pregnancy and exhibited growth restriction and retardation in all developmental milestones and indicators examined. The more severe deficits exhibited by the offspring of VIP deficient mothers compared with VIP antagonist treated offspring could be because the VIP deficiency of the VIP deficient mothers was greater than that produced by blockage of the VIP receptors with the VIP antagonist, that it occurred throughout the entire embryonic period, or that it occurred throughout pregnancy. The fact that VIP mRNA was no longer detectable in the decidual, trophoblast or placental tissues by E11 in the mouse (Spong et al., 1999), and that VIP antagonism treatment after E11 did not inhibit fetal growth (Gressens et al., 1994), indicates that by E11 maternal uterine VIP was no longer a regulatory factor in embryonic growth. In addition, since all offspring of VIP deficient mothers were equally retarded in development, the expression of VIP initiated by E12 in those offspring with one or more VIP genes did not ameliorate the developmental deficiencies reported here. These data support the conclusion that the deficiency of maternal VIP during embryogenesis contributed greatly to the retarded development, growth restriction and deficits of social behaviors in the offspring of VIP deficient mothers. Importantly, the current study illustrates the importance of comparing the behavior and neurochemistry of knockout mice to wild type mice whose prenatal environment has not been compromised by potential deficits in the gene under study.

Although development was delayed in all offspring of VIP deficient mothers, these mice appeared to recover sufficiently well to have no apparent olfactory or motor deficits in the behavioral tasks under study, perhaps indicating that reduced maternal VIP brought about a generalized retardation of growth and development. However, the deficits in social behavior may be representative of a more long lasting, and perhaps more specific, effect of exposure to reduced VIP during pregnancy that has impacted on the development of the neural foundations of social behavior. The postnatal deficits caused by the VIP deficiency of the mother during pregnancy may be reflecting the broad range of actions of VIP; including its indirect actions on neurotrophic factors during embryogenesis. As outlined above, VIP is known to regulate numerous factors during embryogenesis, including IGF-1, ADNF, NGF, and ADNP, a requirement for neural tube closure (Pinhasov et al., 2003). Recent work with the VIP knockout mouse has indicated that the maternal VIP genotype can influence the ADNP expression in the brains of her offspring (Giladi et al., 2007). Also, the presence of abundant VIP binding sites on the floor plate of the neural tube (Hill et al., 1999; Spong et al., 1999) suggests that VIP plays a significant role in embryonic neural organization. The above-mentioned factors indicate potential mechanisms through which the dysfunction of VIP regulation during critical periods of development could have widespread and long lasting deleterious effects. Embryos are especially vulnerable during early pregnancy, including implantation and the early postimplantation period of embryogenesis. It has been estimated that more than 20% of pregnancies are lost in humans (Wilcox et al., 1988) and in domesticated animals (Heap et al., 1992) during these vulnerable periods of early pregnancy. It is not surprising that interference with developmental processes during this period could result in embryonic loss, or in the persistent and diverse deficiencies evident offspring experiencing VIP deficiencies or blockage of VIP during embryogenesis. VIP has been implicated in the neurodevelopmental disorders including Down syndrome. The cord blood of newborns, later shown to have Down syndrome and autism, have been reported to have higher than normal concentrations of VIP (Nelson et al., 2001). Astrocytes from Down syndrome cortex exhibit abnormalities in VIP chemistry, as do the cortical astrocytes of the segmental trisomy mouse model of Down syndrome (Hill et al., 2003; Sahir et al., 2006). VIP was further dysregulated in the brain of the trisomy mouse model which showed increased VIP binding, VIP mRNA and mRNA for the VPAC-1 receptor (Sahir et al., 2006). The deficits in the social behaviors of maternal affiliation and play in the offspring of VIP deficient mothers indicate that the VIP knockout mouse may be useful in further understanding the development of the neural foundations of social behavior. The results of this study further suggest that the VIP knockout mouse may have use as a model for the social behavior deficits in those neurodevelopmental disorders that are characterized by deficits in social behavior, such as autism.

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Figure 1.

Maternal preference of offspring of WT (VIP +/+) mothers and VIP deficient (VIP +/-) mothers. On P14, the amount of time pups spend on maternal shavings, and on the shavings of an unfamiliar lactating female mouse, were measured during three 1 min trials. The time spent on mother's shavings, and on stranger shavings, was averaged across the three trials. The statistic used was the mean score obtained by each genotype in each litter. Offspring of WT mothers: 76 +/+ offspring in 21 litters and 51 +/- offspring in 16 litters. Offspring of VIP deficient mothers: 21 +/+ offspring in 13 litters, 41 +/- offspring in 14 litters and 18 -/- offspring in 12 litters. * = significantly different from wild type (+/+) offspring of WT mothers, $p < 0.05$.

Figure 2.

Play behavior events on P17. Total number of play events during a 15 min observation period, of +/+ (n = 18 pairs) and +/- (n = 12 pairs) offspring of WT (VIP +/+) mothers, and +/+ (n = 6 pairs), +/- (n = 10 pairs) and -/- (n = 10 pairs) offspring of VIP deficient (VIP +/-) mothers. * = significantly different from wild type (+/+) offspring of WT mothers, $p < 0.05$.

Table 1

Developmental milestones of neonatal mice from WT (VIP $+/+$) mothers (mated with either VIP $+/+$ males or VIP $+/-$ males) compared with neonatal mice from VIP deficient (VIP $+/-$) mothers (mated with VIP $+/-$ males).

Offspring genotype Litters	WT mother		VIP deficient mother	
	$+/+$ n = 19	$+/-$ n = 8	$+/+$ n = 23	$+/-$ n = 17
Cliff aversion, first day	2.8 $\pm$ 0.2	2.4 $\pm$ 0.4	4.5 $\pm$ 0.4 *	4.3 $\pm$ 0.5 *
Negative geotaxis, first day	3.3 $\pm$ 0.4	4.7 $\pm$ 0.3	5.1 $\pm$ 0.4 *	5.5 $\pm$ 0.6 *
Rooting, first day	4.6 $\pm$ 0.2	5.1 $\pm$ 0.3	6.4 $\pm$ 0.3 *	6.3 $\pm$ 0.3 *
Grasp, first day >1 second	8.2 $\pm$ 0.2	8.0 $\pm$ 0.3	10.4 $\pm$ 0.2 *	10.3 $\pm$ 0.3 *
Surface righting, first day < 1 second	9.5 $\pm$ 0.2	9.9 $\pm$ 0.2	10.5 $\pm$ 0.2 *	10.4 $\pm$ 0.3 *
Auditory startle, first day	10.2 $\pm$ 0.5	9.0 $\pm$ 0.5	13.1 $\pm$ 0.3 *	13.1 $\pm$ 0.3 *
Ear Twitch, first day	10.4 $\pm$ 0.2	10.4 $\pm$ 0.1	10.9 $\pm$ 0.1 *	11.0 $\pm$ 0.2 *
Air right, first day	10.7 $\pm$ 0.5	10.1 $\pm$ 0.3	12.9 $\pm$ 0.3 *	13.3 $\pm$ 0.3 *
Activity, first day	12.1 $\pm$ 0.3	12.8 $\pm$ 0.4	12.3 $\pm$ 0.5 *	12.7 $\pm$ 0.3 *
Eye opening	13.4 $\pm$ 0.1	13.5 $\pm$ 0.1	14.2 $\pm$ 0.2 *	14.2 $\pm$ 0.2 *
Homing, seconds	54.0 $\pm$ 3.0	67.9 $\pm$ 8.1	58.6 $\pm$ 11.1	57.8 $\pm$ 12.4

* = significantly different from $+/+$ offspring of WT mothers, $p < 0.05$. The mean score obtained by the members of each genotype was used as the statistic for that genotype in the litter.

Table 2

Weight of neonatal mice from WT (VIP +/+) mothers (mated with either VIP +/+ males or VIP +/- males) compared with neonatal mice from VIP deficient (VIP +/-) mothers (mated with VIP +/- males).

Offspring genotype Litters	WT mother		VIP deficient mother	
	+/+ n = 15	+/- n = 9	+/+ n = 15	-/- n = 15
Day 2	1.64±0.03	1.63±0.03	1.45±0.03*	1.40±0.05*
Day 3	1.98±0.03	1.96±0.05	1.87±0.05	1.67±0.05*
Day 4	2.48±0.06	2.46±0.08	2.22±0.08	2.11±0.09*
Day 5	3.06±0.07	3.08±0.11	2.81±0.07	2.56±0.10*
Day 6	3.62±0.08	3.60±0.12	3.40±0.08	3.12±0.12*
Day 7	4.14±0.09	4.13±0.12	4.01±0.10	3.55±0.14*
Day 8	4.75±0.09	4.63±0.14	4.49±0.11	4.09±0.15*
Day 9	5.19±0.10	5.07±0.14	5.02±0.13	4.60±0.18
Day 10	5.78±0.12	5.24±0.34	5.73±0.12	5.21±0.19
Day 15	7.42±0.29	7.32±0.16	7.73±0.24	6.91±0.29
Day 20	8.54±0.38	8.82±0.28	8.96±0.26	7.56±0.37

Repeated measure ANOVA followed by Bonferroni/Dunn *post hoc* comparisons with +/+ offspring of VIP +/- mothers.

* = p < 0.005. The mean weight obtained by the members of each genotype was used as the statistic for that genotype in the litter.

Table 3

Developmental milestones of cross-fostered neonatal mice. Offspring of WT (VIP +/+) mothers (mated with VIP +/+) were raised from P2 by VIP deficient mothers and the offspring of VIP deficient mothers (VIP +/- females mated with VIP +/- males) were raised by WT mothers.

Offspring genotype Litters	VIP deficient foster mother +/+ n = 6	+/+ n = 6	WT foster mother	
			+/- n = 6	-/- n = 6
Cliff aversion, first day	4.0±0.4	6.7±0.9*	6.8±0.6*	7.6±1.0*
Negative geotaxis, first day	4.1±0.6	6.7±0.7*	8.2±0.7*	8.3±0.7*
Rooting, first day	4.6±0.5	4.9±0.5	4.8±0.7	6.6±0.8
Grasp, first day >1 second	9.6±0.2	11.5±0.4*	11.0±0.3*	11.5±0.3*
Surface righting first day < 1 second	9.2±0.2	12.1±0.4*	11.4±0.5*	11.9±0.6*
Ear twitch, first day	10.4±0.4	10.3±0.6	11.1±0.4	10.5±0.5
Air right, first day	10.1±0.3	12.3±0.5*	12.2±0.4*	12.1±0.6*
Activity, first day	12.4±0.4	14.5±0.2*	14.2±0.5*	14.6±0.5*
Eye opening, first day	13.5±0.2	14.6±0.2*	14.4±0.3*	14.9±0.3*

* = significantly different from wild type (VIP +/+) offspring of WT mothers, $p < 0.05$. The mean score obtained by the members of each genotype was used as the statistic for that genotype in the litter.

Table 4

Weight of cross-fostered neonatal mice. Offspring of WT (VIP +/+) mothers (mated with VIP +/+) were raised from P2 by VIP deficient mothers and the offspring of VIP deficient mothers (VIP +/- females mated with VIP +/- males) were raised by WT mothers.

Offspring genotype Litters	VIP deficient foster mother +/+ n = 6	+/+ n = 6	WT foster mother	
			+/- n = 6	-/- n = 6
Day 3	1.90±0.10	1.62±0.06	1.57±0.07*	1.54±0.06*
Day 4	2.39±0.16	1.97±0.13	2.01±0.09	1.74±0.08*
Day 5	2.90±0.18	2.52±0.15	2.46±0.15	2.06±0.09*
Day 6	3.42±0.19	3.07±0.15	3.03±0.21	2.64±0.21*
Day 7	4.02±0.20	3.68±0.15	3.58±0.25	3.18±0.21
Day 8	4.54±0.22	4.21±0.16	4.10±0.29	3.88±0.37
Day 9	5.23±0.15	5.05±0.12	4.61±0.25	4.58±0.40
Day 10	5.57±0.23	5.42±0.18	5.16±0.37	4.99±0.45
Day 15	7.58±0.21	7.14±0.31	6.99±0.54	7.38±0.75
Day 20	8.48±0.31	8.36±0.39	8.07±0.52	8.73±0.78

Repeated measure ANOVA of daily weight gain followed by Bonferroni/Dunn *post hoc* comparisons with wild type (VIP +/+) offspring of WT mothers.

* = $p < 0.008$. The mean score obtained by the members of each genotype was used as the statistic for that genotype in the litter.

Table 5

Percent of time WT mothers (VIP +/+) and VIP deficient mothers (VIP +/-) spent performing maternal and non-maternal behaviors. Behaviors were observed daily for 10, 30 s sessions, separated by 1 min intervals on days P1 to P13. Data represents the mean percent of time the behavior was observed per day across 13 days.

	WT mother n = 16	VIP deficient mother n = 11
Nest Score (scale of 0–3)	2.1±0.17	2.0±0.17
Pups in Nest	99.6±0.33	100
Pups Huddled	99.1±0.83	100
Parental	79.9±2.9	83.9±3.9
Nursing	70.0±3.4	71.8±3.4
Forced Nursing	7.7±1.4	9.0±2.5
Crouching over	45.1±4.6	49.8±4.8
Licking and Grooming	8.5±1.2	7.0±1.3
Nest-building	5.0±0.8	3.7±1.1
Non-Parental	25.2±2.8	25.8±3.8
Grooming	4.9±0.8	5.5±0.9
Feeding	12.9±2.3	14.1±2.9
Active	10.6±1.5	9.5±1.3
Pup Vocalization	11.3±2.1	14.2±2.2

Table 6
Play behaviors (number of events) of neonatal mice from WT (VIP +/+) mothers (mated with either VIP +/+ males or VIP +/- males) compared with neonatal mice from VIP deficient (VIP +/-) mothers (mated with VIP +/- males).

Offspring genotype Pairs	WT mothers		VIP deficient mothers	
	+/+ n=12	+/- n=12	+/+ n=6	+/- n=11
Follow	3.7±0.6	5.6±1.5	0.3±0.2*	1.1±0.6*
Social sniff	16.6±1.7	12.8±1.9	8.5±1.6*	11.2±2.2*
Social groom	7.1±0.9	5.5±0.7	2.8±1.0*	4.4±0.7*
Push under	9.1±1.2	11.5±2.2	1.6±0.7*	5.5±2.1
Crawl over	8.8±0.7	10.3±1.7	3.0±0.8*	6.3±2.3
Push past	3.2±0.5	2.5±0.5	0.8±0.6*	0.9±0.5*
Time explore	9.7±0.3	10.3±0.3	9.8±0.3	10.3±0.7
Time rest	2.75±0.3	2.15±0.3	2.6±0.3	3.2±0.6

ANOVA followed by Fisher's *post hoc* comparisons with +/+ offspring of VIP +/+ mothers.

* = p < 0.05.