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Interaction between environmental and genetic factors modulates schizophrenic endophenotypes in the Snap-25 mouse mutant *blind-drunk*

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To understand the pathophysiology of neuropsychiatric disorders such as schizophrenia requires consideration of multiple genetic and non-genetic factors. However, very little is known about the consequences of combining models of synaptic dysfunction with controlled environmental manipulations. Therefore, to generate new insights into gene–environment interactions and complex behaviour, we examined the influence of variable prenatal stress (PNS) on two mouse lines with mutations in synaptosomal-associated protein of 25 kDa (Snap-25): the *blind-drunk* (*Bdr*) point mutant and heterozygous *Snap-25* knockout mice. Neonatal development was analysed in addition to an assessment of adult behavioural phenotypes relevant to the psychotic, cognitive and negative aspects of schizophrenia. These data show that PNS influenced specific anxiety-related behaviour in all animals. In addition, sensorimotor gating deficits previously noted in *Bdr* mutants were markedly enhanced by PNS; significantly, these effects could be reversed with the application of anti-psychotic drugs. Moreover, social interaction abnormalities were observed only in *Bdr* animals from stressed dams but not in wild-type littermates or mutants from non-stressed mothers. These results show for the first time that combining a synaptic mouse point mutant with a controlled prenatal stressor paradigm produces both modified and previously unseen phenotypes, generating new insights into the interactions between genetics and the environment relevant to the study of psychiatric disease.

INTRODUCTION

In recent years, a considerable number of putative susceptibility genes for schizophrenia have been described, and the majority have central roles in synaptic function (1,2). However, according to the neurodevelopmental model of schizophrenia, polygenetic and environmental factors combine to influence early synaptic development and function, causing pathophysiological abnormalities long before the emergence of the symptoms required for diagnosis (3). For example, it is now well established that prenatal stress (PNS) has been directly associated with a number of significant long-term mental health problems, including schizophrenia (4–7). Consequently, a large number of studies in wild-type rodents have attempted to understand the important link between such early life 'programming' and neurological development (8). A range of approaches have been described to date, from the induction of viral infection to the application of physical or social stress during pregnancy (9,10). This work has shown that the timing and duration in addition to the type of environmental stressor can generate a wide variety of post-natal behavioural outcomes, including anxiety and memory deficits (11–20). In addition, PNS can elicit phenotypes in the adult offspring more specifically relevant to psychiatric disorders, such as abnormal sensorimotor gating or pre-pulse inhibition (PPI) responses (21). However, detailed interpretation or comparison of these datasets is problematic due to the inconsistency of the PNS paradigms used and the limited range of post-natal effects measured in a single study. Considering all these issues, a more heuristic approach to modelling

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the pathophysiology of schizophrenia would be to manipulate the prenatal environment of a mouse with a known genetic lesion and synaptic phenotype and investigate the behavioural consequences of the combined insults (22).

As alterations in synaptic connectivity and function are central to most of the currently favoured hypotheses regarding schizophrenia pathobiology, then genes that control these process warrant further attention (1). The neuronal soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) and SNARE-associated proteins play a central role in the neurotransmitter release, and the expression several, including SNAP-25 and syntaxin, is selectively affected in brains from schizophrenic patients (23–26). More compelling evidence of a role in disease causation comes from a meta-analysis of genome-wide association data in which the SNAP-25 genomic region emerges as strongly linked to schizophrenia (27). Mouse mutants of the SNARE-related proteins have shown a variety of complex behavioural phenotypes (28–32), although studies have been limited by the embryonic lethality of the *Snap-25*, *synaptobrevin-2* and *Munc-18* knockouts (33–35). To augment this resource, we have recently identified and characterized the *Snap-25* dominant mutant *blind-drunk (Bdr)*, named after its distinctive ataxic gait. A single amino acid substitution (I67T) results in an increased binding affinity within the core SNARE complex, preventing the normal recycling of synaptic vesicles (36). This in turn alters the amplitude of cortical neurotransmission, although no overt neuropathological abnormalities have been detected. Interestingly, *Bdr* mice display a range of behavioural defects including some aspects of anxiety and apathy but also a robust reduction in PPI response. Thus the *Bdr* mutant provides an important link between sensory behavioural abnormalities and a specific synaptic deficit caused by a single amino acid change. Taken together, these data suggest the SNARE complex and SNAP-25 may play a significant role in synaptic dysfunction in psychiatric disease. Moreover, the relatively mild behavioural phenotype of the *Bdr* mutant, in combination with the well-characterized synaptic deficit, renders it eminently suitable to examine the role of PNS in the modulation of behaviour.

The aim of this study was to investigate the combination of environmental and genetic factors on the development and behaviour of the *Bdr* mutant. The *Snap-25* knockout heterozygous (HET) mouse was added to the study to determine for the first time whether a 50% reduction in Snap-25 expression results in any specific behavioural abnormalities (33). In addition, we wanted to ascertain whether a reduction in expression of a synaptic protein would modulate sensitivity to PNS. Using a random, variable PNS paradigm, we carried out initially a detailed developmental screen on both strains. We then went on to assess the mutants in a battery of tests to model the psychotic, cognitive and negative aspects of schizophrenia and show that PNS influenced specific anxiety-related behaviour in all animals. Furthermore, the PPI and social interaction deficits noted in the *Bdr* mutants were markedly modulated by PNS. Thus for the first time we have demonstrated that PNS interacts with a genetic lesion in a SNARE protein to enhance schizophrenic-like behaviour.

Table 1. Pre- and post-partum measures of maternal behavior (mean ± SEM)

Male	*Bdr*		HET	
Stress group	NS	PNS	NS	PNS
Nesting	4.0 ± 0.7	4.1 ± 1.0	4.3 ± 0.9	4.0 ± 0.7
Latency to retrieve first pup (s)	29 ± 6.0	34 ± 4.5	10 ± 1.2	12 ± 2.1
Latency to retrieve last pup (s)	276 ± 20	302 ± 25	101 ± 14	96 ± 19
Number of dams retrieving all pups	8/8	8/8	8/8	8/8

RESULTS

Effect of PNS on breeding and development

PNS has been shown to cause subtle behavioural changes in the pregnant dams themselves that can influence development of the offspring (39,43), therefore this phenomenon was assessed by taking pre- and post-partum measurements of maternal activity. Data from all litters show that there was no significant effect of stress or genotype on average weight gain of the dams during pregnancy, litter size or sex ratio (Supplementary Material, Fig. S1 and Table S1). In addition, there was no difference in the nesting behaviour in either mutant group irrespective of PNS (Table 1). Pup retrieval was also measured, and there was also no significant effect of PNS on this parameter, although dams from the HET cross were quicker to instigate and complete the task than those from the *Bdr* group (Table 1).

Effect of PNS on corticosterone

To confirm that the chosen PNS protocol was causing measurable changes in stress hormones, the levels of corticosterone were measured 30 min after the second forced swim stressor at E14.5 and the third round of restraint stress at E15.5. The results show an approximate 4–5-fold increase in corticosterone levels after forced swimming and restraint in both *Bdr* and HET crosses compared with non-stress (NS) controls ($P < 0.01$; Fig. 1).

Effect of PNS on early development

The influence of PNS on neonatal *Snap-25* mutants was examined in detail to determine whether adult behavioural phenotypes might reflect the delay of specific developmental milestones or maturation of the nervous system. These data show that there were only very minor defects in motor function due to the PNS protocol, although those that were observed did not persist until weaning (Supplementary Material, Fig. S2).

Adult behavioural test battery

The open-field locomotor activity test showed that both PNS and NS mice from the HET group were generally more active than the *Bdr* group, although there was no effect of stress on this particular parameter (Fig. 2A). However, animals from the PNS litters spent significantly less time in the central area of the open field than NS animals (Fig. 2B).

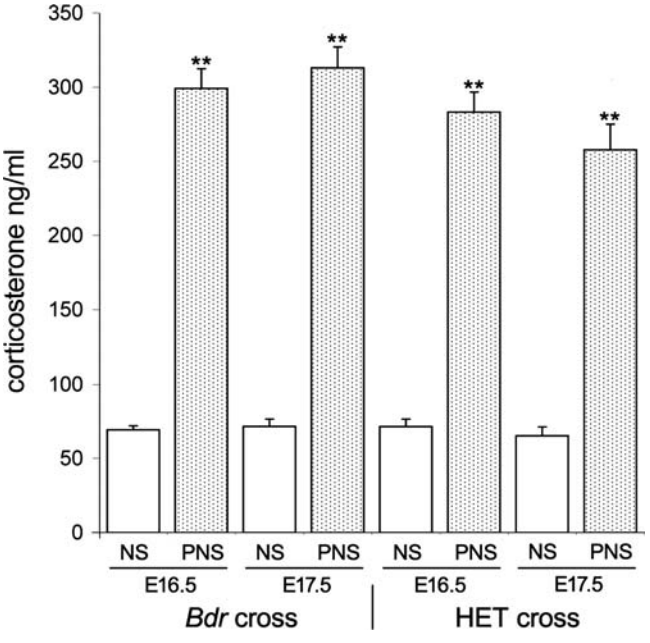

Figure 1. Corticosterone levels are increased in PNS over NS dams. Blood corticosterone levels were significantly increased in pregnant dams at E14.5 and 15.5, 30 min after the administration of PNS, compared with NS control wild-type (WT) dams. Data are shown as mean ± SEM; NS versus PNS animals, $^{**}P < 0.01$.

This anxiety-related behaviour was tested further in the elevated plus maze (EPM). As in the open field, mice from the HET cross were generally more active than those in the *Bdr* group on the apparatus, although there was no effect of PNS on total distance travelled (data not shown). Measures of unconditioned anxiety, however, were influenced by PNS in mice from both genotypic groups; including a reduction in the time spent in the open arms (Fig. 2C) and number of entries in the open arms (Fig. 2D). However, there was no significant interaction between stress and genotype on either of these parameters.

The Y-maze continuous alternation test was chosen as a test of short-term memory acquisition and exploratory behaviour. All mice alternated throughout the apparatus during the testing period, and there was no effect of stress or genotype on performance (Fig. 3A). There was also no influence of stress on the activity of mice in the forced swim test, a common method used to analyse behavioural despair in mice (Fig. 3B), and no alteration in fear-conditioned memory (Fig. 3C).

Olfaction is essential for normal rodent social recognition and importantly, none of the mice tested showed any impairment in their ability to find buried food in the test for anosmia, other than background strain differences as expected (Fig. 4A) (44). In the social testing apparatus, both *Snap-25* lines showed a strong preference for the first stranger (Stranger 1) versus the empty cage during sociability testing (Fig. 4B) and spent more time with the novel stranger versus the familiar mouse in the social novelty stages of the protocol. Interestingly, however, *Bdr* mutants from PNS dams spent significantly more time than PNS controls and NS *Bdr* mice in proximity to the empty cage in the first part of the test revealing a significant interaction between stress and genotype in the *Bdr* mice (stress × genotype $P = 0.040$; Fig. 4B). Furthermore, PNS *Bdr* mice spent approximately equivalent time with the second intruder mouse (Stranger 2) and Stranger 1 in the social novelty analysis compared with wild-types (stress × genotype $P = 0.035$; Fig. 4B). To determine whether that this effect related to contact between the animals or empty cages as opposed to a measure of activity in each chamber of the apparatus, sniffing time was also recorded for all experimental animals from the *Bdr* strain. These data confirmed the activity data, showing an interaction between stress and genotype on measures in both the sociability ($P = 0.038$; Fig. 4C) and social novelty ($P = 0.042$; Fig. 4C) parts of the test. Control parameters such as number of chamber entries and time in the central chamber were not significantly altered between genotypes or stress conditions (data not shown). In addition, apathy in the form of novel object recognition was assessed. *Bdr* mutants and controls spent similar lengths of time interacting with two identical objects in the first open-field trial and both groups spent significantly longer interacting with a novel object introduced in the second trial (Fig. 5). There was, however, no effect of PNS in either parameter.

Pre-pulse inhibition

To assess sensorimotor gating response, mice were tested using a combined acoustic startle and pre-pulse protocol. As previously described, *Bdr* mice show a significant reduction in PPI of ~15% compared with controls at 8 and 16 dB above background, although no such effect was observed in HET mice (Fig. 6A). In all wild-type and HET animals, PNS resulted in a small, but non-significant decrease in PPI. Surprisingly, however, PNS caused a further, significant 20–25% decrease in PPI of *Bdr* mice compared with non-stressed mutants at both higher pre-pulse levels, revealing a significant interaction between stress and genotype (78 dB, $P = 0.025$; 86 dB, $P = 0.012$; Fig. 6A). There was no effect of genotype or stress on the weight-correct startle response amplitude (data not shown). In order to observe the response of *Bdr* mutants to psychoactive drugs during PPI analysis, acute doses of the commonly used anti-psychotics clozapine and haloperidol were administered along with a saline control on an independent cohort of NS and PNS mice. The doses were selected to be in line with similar studies in mice that cause measurable enhancement in PPI to assess validity of the model, without a significant attenuation of startle response (45–47). Saline-injected animals generated highly similar results to untreated mice, confirming that the PPI deficits in *Bdr* mutants were reproducible (Fig. 6B). The results after clozapine treatment show a non-significant increase in PPI of ~10% for wild-type mice irrespective of stress, but a striking and significant 35–40% increase for *Bdr* mutants from PNS dams at 78 and 86 dB, almost restoring the response to wild-type levels (genotype × drug: 76 dB, $P = 0.030$; 86 dB, $P = 0.019$; Fig. 6B). Although haloperidol had a more moderate effect than clozapine on PPI in *Bdr* mice, a significant increase was observed at the 86 dB pre-pulse (Fig. 6B). These data show that an enhanced PPI deficit occurs when combining

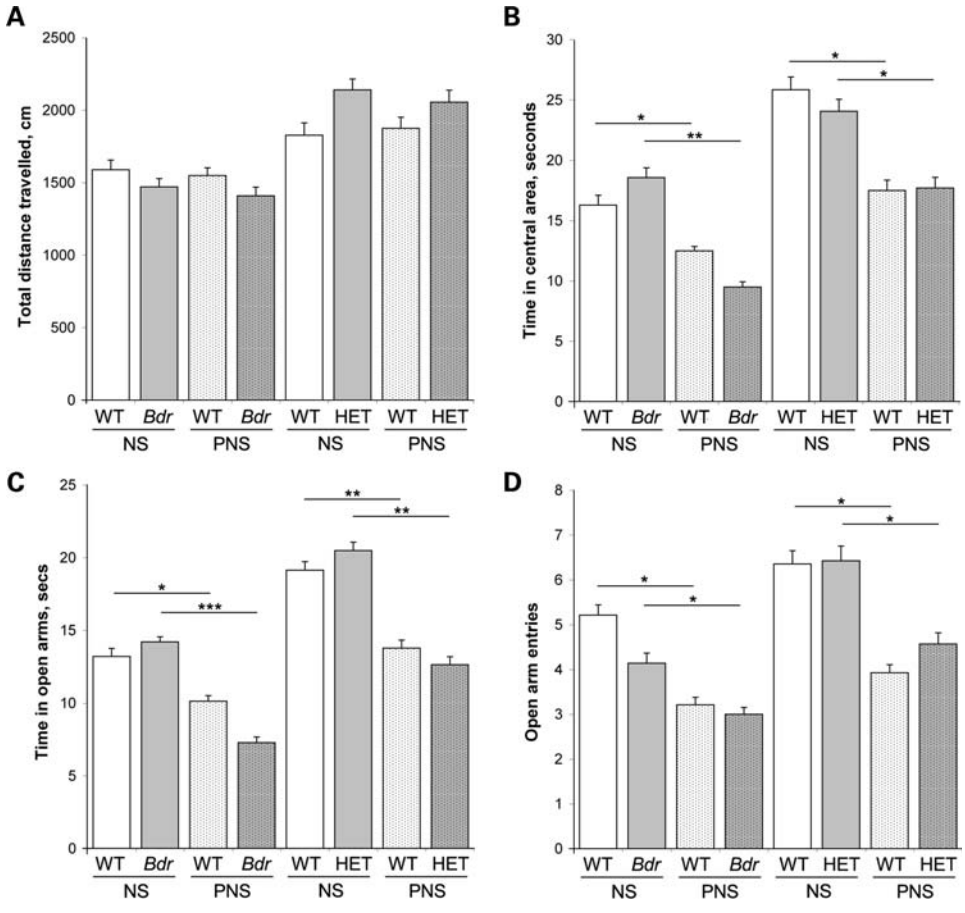

Figure 2. PNS influences anxiety irrespective of genotype. (**A** and **B**) Open field. (A) Offspring from the HET cross were generally more active than those from *Bdr* cross, however this difference was not significant or influenced by PNS. (B) PNS caused a significant reduction in time spent in the central area of the open field in mice from both crosses, although there was no interaction between stress and genotype. (**C** and **D**) Elevated plus maze. PNS caused a significant reduction in the time spent in the open arms (C) and on the number of arm entries (D) in mice from both crosses, although there was no interaction between stress and genotype. Data are shown as mean ± SEM; NS versus PNS animals, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

the *Bdr* phenotype with PNS and that this behaviour is hypersensitive to psychoactive drugs.

DISCUSSION

To generate new insights into interactions between genes and the environment using synaptic protein mutations and PNS, we analysed neonatal and adult offspring from two independent *Snap-25* mutant lines. The application of PNS during gestation in rodents is a well-established method for modelling the influence of environmental factors on behaviour; however, using repeated or prolonged PNS paradigms has been shown to cause habituation to the stressful conditions and limit the effectiveness of the experiment due to desensitization of the hypothalamic–pituitary–adrenal (HPA) response (48). Consequently, we chose a random, variable PNS regime similar to one used in rats that generated a range of behavioural abnormalities in the offspring (21,37,49). It was, however, necessary to adapt the stress paradigm to mice. For example, due to the size difference between the species, stress induced by prolonged exposure to a cold environment was not carried out here. Furthermore, overnight fasting was not used to avoid developmental defects in the offspring due to the potentially reduced body weight of the stressed dams (50).

Depending on the source of the environmental stressor concerned, clinical data suggests that the foetal brain is highly sensitive to insults at time points throughout pregnancy (4,6,51,52). With specific reference to neuropsychiatric disorders, maternal stress during the first and second trimester appears to have the greatest influence on an increased risk of schizophrenia, as exemplified by retrospective studies of prenatal exposure to influenza (53), war (54) and familial adverse life events (55). Indeed, in mice it has been established that the post-natal behavioural outcomes of PNS are sensitive to the timing of the stressors employed. For example, variable stress both early [E0.5–6.5 (56)] and late [E15–21 (57)] in gestation can elicit differing responses in mice. To better interpret such data, it is useful to extrapolate the timing of neurodevelopment from humans to rodents. Combining morphological (58,59), evolutionary and computer modelling methods (60) places the end of gestation in mice (typically E19.5) at ∼90–120 days post-conception (DPC) in humans. Here we applied the variable PNS protocol for 7 days from mid- to late gestation (E11.5 –17.5) to correspond from the end of the first (50–60 DPC) to beginning of the

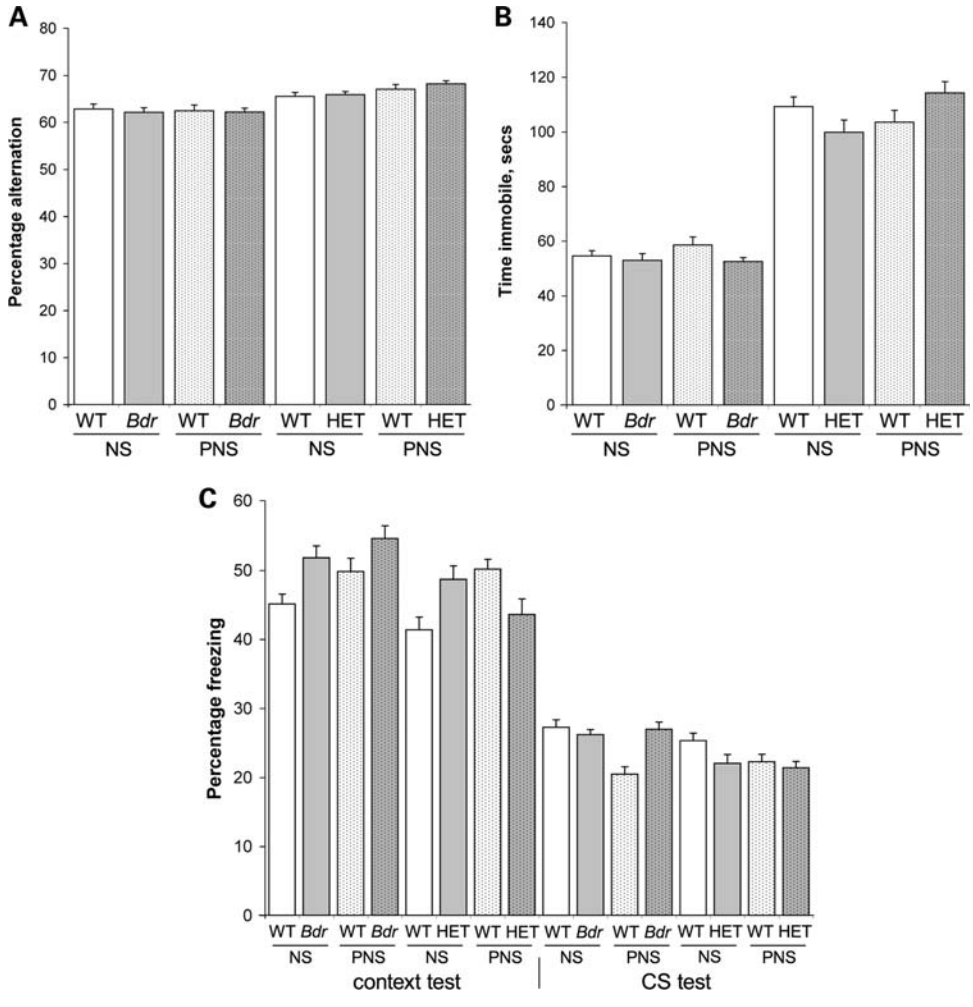

Figure 3. Behavioral tests unaffected by PNS. Alternation in the Y-maze (**A**), immobility in the forced swim test (**B**) and fear conditioning (**C**) testing showed that none of these behaviors were significantly influenced by PNS. Data are shown as mean ± SEM.

second (80–110 DPC) trimester in humans, consistent with the foetal developmental time points most vulnerable to PNS. During optimization of the PNS experiments, we attempted to stress the dams closer to parturition similar to related studies (37). However, this lead to spontaneous early abortions and/or the birth of underdeveloped offspring in a considerable number of litters (data not shown); therefore PNS was not applied beyond E17.5 here.

Importantly, the chosen PNS regime successfully generated measurable stress hormone enhancement, but did not elicit significant confounding pre- or post-partum behavioural defects in the treated dams that may have influenced subsequent phenotypic testing of the offspring. Other studies have also showed that gestational stress does not influence maternal behaviour (61,62), although the subtle effects of PNS on parameters not observed in the current study have been noted elsewhere, such as licking, sniffing and nursing of the pups (43,63,64). The development of affiliative behaviour between a rodent mother and her offspring is undoubtedly highly complex and other studies have attempted to circumvent such stress-induced maternal effects by cross-fostering pups from stressed dams to unstressed females. For example, a study in rats using cross-fostering in combination with variable PNS showed no effect of adoption on the social behavioural deficits observed in the offspring from stressed dams (49). However, studies in mice have demonstrated that cross-fostering itself can have significant effects on both the maternal activity (39) and the long-term emotional behaviour of the offspring, including social interaction (65). For these reasons, we avoided this particular breeding method, in addition to the potential problems of infanticide that we have observed ourselves during pilot experiments with this particular PNS protocol (data not shown).

Our study of neonates showed that PNS only had a minor effect on neurodevelopment and none of the measurements were significantly altered by weaning age. This part of the study also provided, for the first time, a description of the neurodevelopmental profile of both *Snap-25* mutant lines. For example, *Bdr* mice are typically 70–80% of the size of wild-type littermates at adulthood (36), but we show here that this is not due to a failure to thrive or feed during the early post-natal weeks. However, some reduction in general locomotor activity was seen in mutant mice, which is likely to be the first signs of the ataxia that is more clearly observed at 4–5 weeks of age.

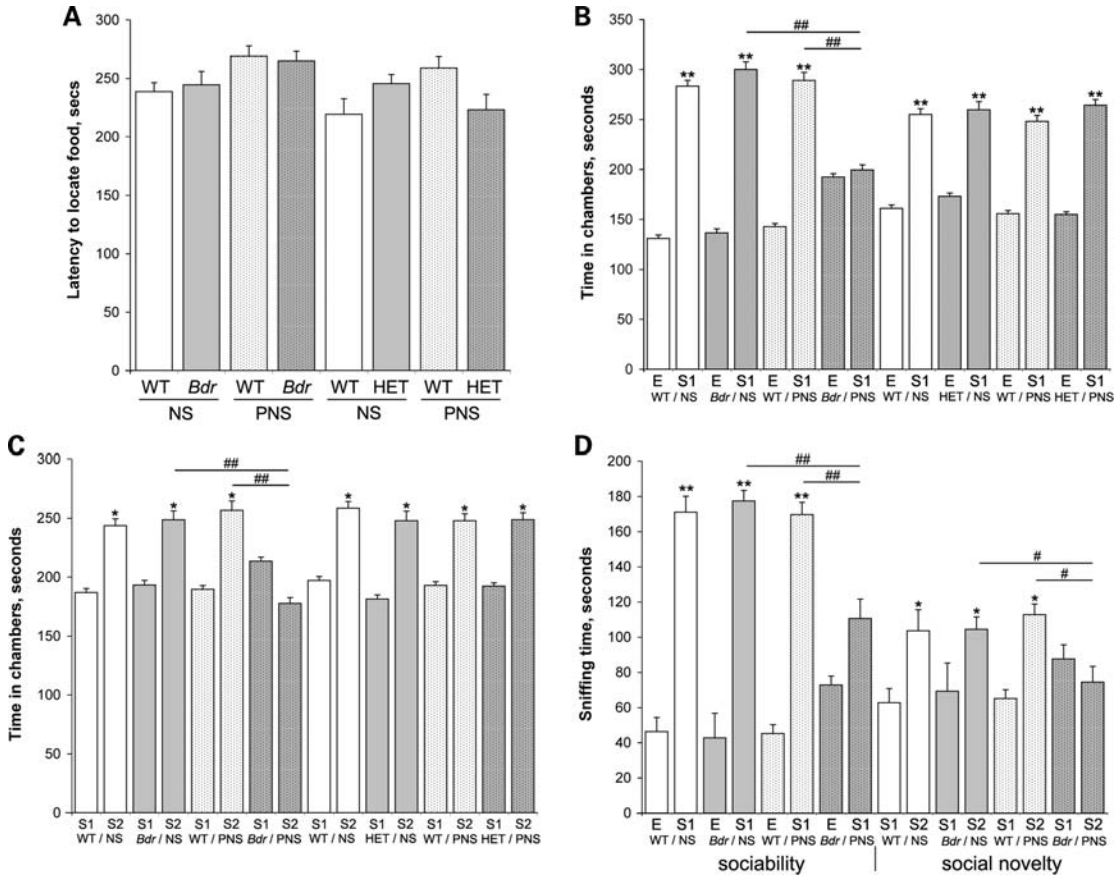

Figure 4. *Bdr* mutants subjected to PNS show social interaction abnormalities. (**A**) Test for anosmia. All mice tested were able to locate a hidden food treat, suggesting no olfactory deficits. (**B**–**D**) Social testing. (B) Sociability: mice typically spent more time in the chamber containing the first stranger mouse (labelled S1) compared with the chamber containing the empty cage (labelled E) ($^{**}P < 0.01$). In contrast, *Bdr* mutant mice subjected to PNS spent approximately equivalent time in both side chambers compared to *Bdr* mutants and PNS controls ($^{\#\#}P < 0.01$). (C) Social novelty: mice typically spent more time in chamber 2 containing a novel second stranger mouse (labelled S2) compared with the first stranger (S1) ($^{*}P < 0.05$). As in (B), *Bdr* mutant mice subjected to PNS spent approximately equivalent time in both chambers compared to NS *Bdr* mutants and PNS controls ($^{\#\#}P < 0.01$). (D) The performance of the animals from the *Bdr* cross was re-scored from the same experiments (B and C) according to sniffing time. Mice spent significantly more time interacting with Stranger 1 compared with the empty cage ($^{**}P < 0.01$) and more time with Stranger 2 than stranger 1 ($^{*}P < 0.05$). In contrast, *Bdr* mutants subjected to PNS showed defects in social interaction behavior compared with NS *Bdr* mutants and PNS control littermates in both sections of the test (sociability, $^{\#\#}P < 0.01$; social novelty, $^{\#\#}P < 0.01$). In addition, there was a significant stress × genotype interaction on reduction in normal social interactions in *Bdr* mutants from stressed dams across all parameters tested (see text). Data are shown as mean ± SEM.

Interestingly, a detailed developmental study of the *complexin-I* knockout mouse has been carried out (41). These mice display ataxia as early as P7, which understandably affected motor control and exploration although additional neurodevelopmental milestones were also altered, including those related to sensory behaviour. Importantly, we have confirmed here that the mild ataxia of adult *Bdr* mice does not result in significantly reduced locomotor activity in any of the apparatus used. In addition to a measure of activity, data from the open field and EPM tests show that PNS generated anxiety-related behaviour in all mice tested, regardless of genotype. This observation is consistent with several studies of both prenatal and perinatal stress in rodents (16,20,66). Moreover, one study showed that anxiolytic phenotypes in both the open field and EPM observed in rats from stressed dams were essentially reversed in pups that had been handled during neonatal development, demonstrating the sensitivity of rodents to environmental manipulation, and illustrating why neonatal mice tested here could not be used for subsequent adult behavioural studies (67).

The sociability paradigm used here was originally developed to model autistic-like behaviour, although social communication problems are common in many neuropsychiatric disorders including schizophrenia (68,69). Social defects are also common to many mutant mouse models of synaptic dysfunction or schizophrenia susceptibility genes (30,47,70). Surprisingly, during social novelty testing, *Bdr* mutants from stressed dams spent approximately the same time with the now familiar intruder mouse (Stranger 1) than the new intruder mouse (Stranger 2). This suggests that PNS induces deficits in social recognition memory in *Bdr* mutants; a phenotype not observed in PNS wild-type mice or NS *Bdr* animals. In addition, our data from the object novelty testing show that the activity of *Bdr* mutants from stressed dams was not indicative of general apathy in the social testing apparatus. In contrast to studies of anxiety and cognitive behaviour, relatively few investigations have examined the role of PNS on social

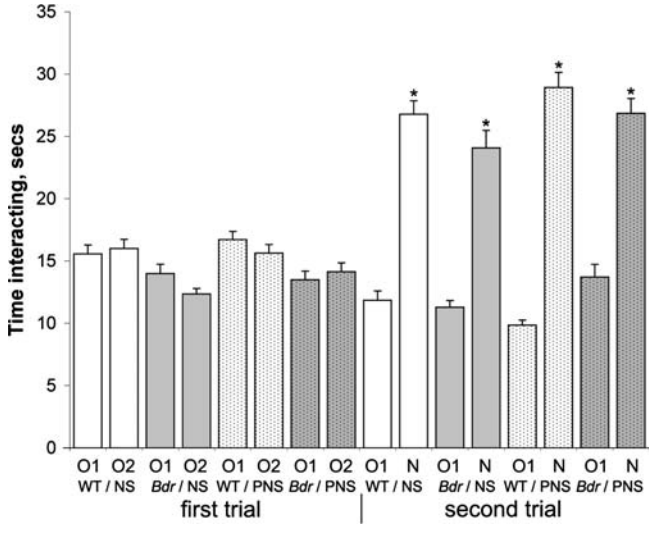

Figure 5. *Bdr* mutants show no deficit in novel object recognition. All animals spent equivalent amount of time interacting with both identical objects 1 and 2 (O1 and O2) during the first trial and significantly more time with the novel object (N) compared with the familiar object (O1) in the second trial ($^*P < 0.05$). Data are shown as mean ± SEM.

interaction. Recent work has shown that wild-type rats subjected to a variable PNS paradigm exhibit social impairments during a simple open-field observational test (49). Interestingly, the same study demonstrated both pre-pubertal and adult offspring from stressed dams displayed a decline in relative physical contact as well as overall time spent with the intruder rat, suggesting a reduction in social drive that may model the initial clinical signs of schizophrenia observed in adolescents (71). It has also been shown in rats that electric shock treatment throughout pregnancy lead to an increased latency to initiate social play in the offspring (72).

Initially from studies using pharmacological intervention it has been proposed that more anxious animals are less likely to engage in social activity (73,74). Furthermore, anxiolytic behaviour in rats caused by PNS has been shown to correlate with reduced social interaction and aggressiveness (75). The intricacies of social response in rodents are likely to be influenced by a considerable number of factors, including motivation and sensory cues as well as anxiety. Indeed, there are many examples of rodent models that are in fact less anxious than their respective controls, but still display social deficits (76,77). Therefore, as there was a non-significant difference in anxiety measures between *Bdr* mice and controls subjected to PNS and no social impairment in the same control animals, anxiety is unlikely to have influenced the social testing results described here.

Sensorimotor gating deficits are commonly reported in schizophrenic patients, and PPI is therefore considered as valuable endophenotype to study in mouse models of psychiatric disease (78). As previously described, we observed a reproducible reduction in PPI in *Bdr* mutants (36), although here we dissected this response further by using a range of prepulses. *Bdr*, but not HET mice, were also hypersensitive to PNS, with a significant interaction between stress and genotype on PPI at the two highest pre-pulse intensities. To assess predictive validity of these responses pharmacological studies were carried out, showing an almost complete rescue of the PPI deficits in both the NS and PNS *Bdr* mutants with the atypical anti-psychotic clozapine, although the typical anti-psychotic haloperidol had a more modest effect on PPI improvement. These data are consistent with a number of neurodevelopmental rodent models of schizophrenia generated by genetic mutations (79), hippocampal lesions (45) or drug administration (80). PPI defects are also associated with neuropsychiatric disease models in rodents that apply environmental stressors, including prenatal infection (81) and isolation rearing on wild-type (82) or *Nurr1* mutant mice (83). Uniquely, the response of *Bdr* mutants to PNS shows not only that a synaptic mutation in combination with prenatal factors can significantly modulate PPI, but this interaction has predictive validity as a psychiatric disease model.

Considering the control animals screened as part of this study, PNS induced no deficits in PPI, social interaction or cognitive and depressive behaviours. These data are in disagreement with previous work in wild-type rodents demonstrating that each of these behavioural domains can be influenced by gestational factors, including the application of either chronic repetitive stress (84) or a variable stress paradigm similar to that used here (21,49,57). The fact that we only identified anxiety-like behaviour from dams subjected to PNS might suggest that our chosen stress protocol was generally less severe than those highlighted above, despite the obvious increase in blood corticosterone levels measured in all mice tested. Nonetheless, the fact that no PPI or social deficits were observed in control mice facilitated the identification of interactions between *Bdr* mutants and PNS; the additive effects of several behavioural abnormities would have likely confused or even masked the combined influence of genetic and environmental manipulation.

We chose to maintain the two *Snap-25* mutant lines on their original genetic backgrounds to allow direct comparisons with previously published work without the potential of confounding modifier effects. Thus the general behavioural profiles of the C3H (background for the *Bdr* cross) and C57BL/6 (background for the HET cross) inbred strains were evident during the study. All mice from the HET cross were generally more active and less anxious than the entire *Bdr* group, as indicated by results from the open field and EPM; these data are consistent with former studies of the C57BL/6 and C3H strains (85,86). This is also likely to explain why that HET group required less time to retrieve their pups than mice from the *Bdr* cross. In addition, mice from the HET cross spent more time immobile than the *Bdr* group during the forced swim test, a result described previously for their respective genetic backgrounds (87). Strain comparisons in the social interaction paradigm used here have also been carried out, revealing that the C3H strain showed a more overt sociability response to the first part of the test than C57BL/6 mice (44). Conversely, in the same study, whereas mice from the C57BL/6 strain showed a significant preference for social novelty, C3H animals did not. Although we did detect a slight enhancement in sociability from the *Bdr* cross versus mice from the HET cross as expected, more importantly we observed a robust social novelty response from NS *Bdr* mutant and control animals. The effect of PNS on social activity parameters

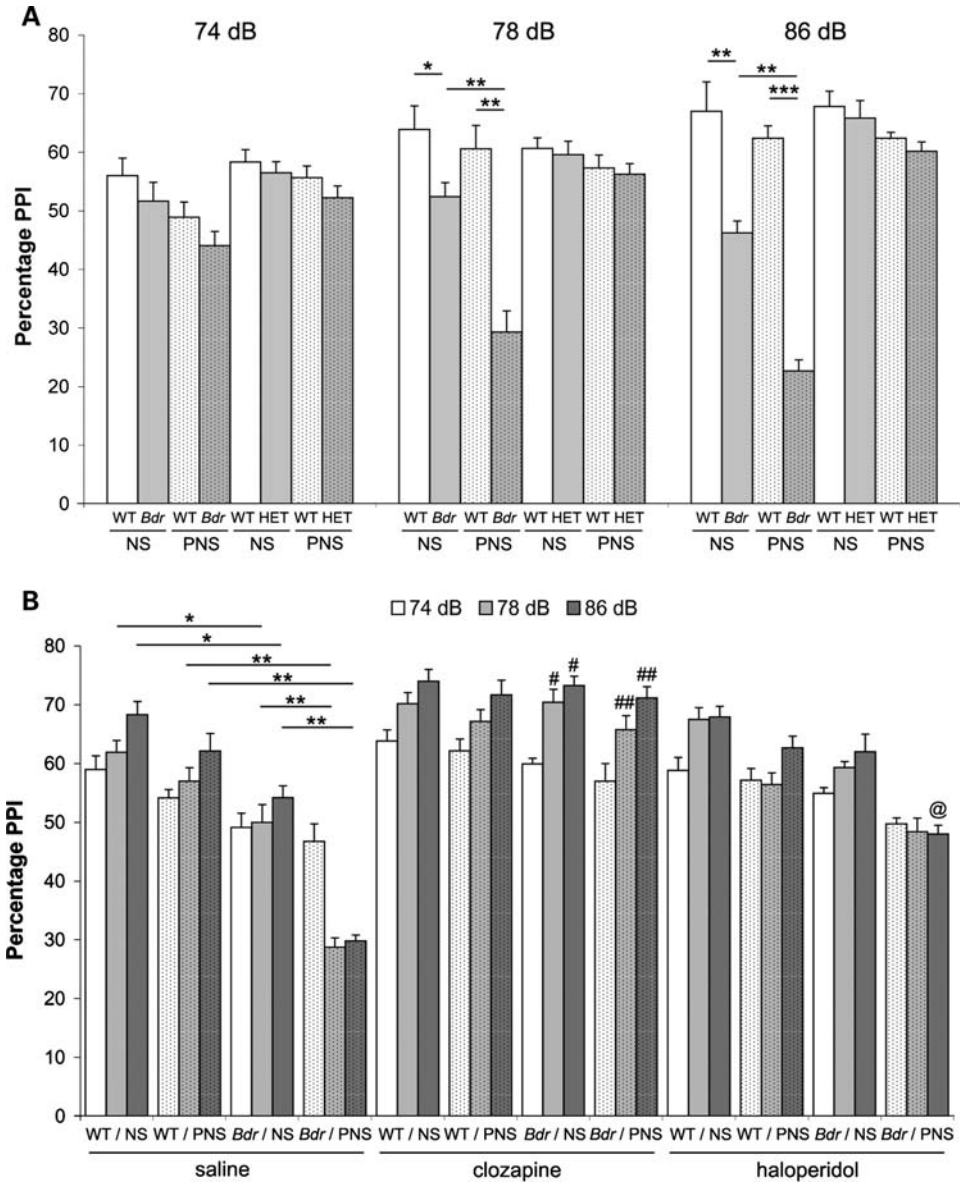

Figure 6. PPI deficits in *Bdr* mutants are enhanced by PNS. (**A**) PPI testing: *Bdr* mutants show a significant PPI deficit using a pre-pulse intensity at both 8 and 16 dB above background (70 dB) compared with WT controls ($^{*}P < 0.05$, $^{**}P < 0.01$). The application of PNS to *Bdr* mice further enhanced the PPI deficit compared with NS mutants ($^{**}P < 0.01$) and PNS controls ($^{**}P < 0.01$, $^{***}P < 0.001$). There was also significant genotype × stress interaction in *Bdr* mutants (see text). Mice from the HET cross did not show any PPI abnormalities. (**B**) Pharmacological responses: vehicle (saline) treated animals showed PPI responses equivalent to those in (A), with a reduction in *Bdr* mutants at 78 and 86 dB prepulse intensities ($^{*}P < 0.05$) and further enhancement of the deficit in NS mutants or PNS WT animals ($^{**}P < 0.01$). Clozapine administration reversed the PPI deficits in *Bdr* mutants from both NS ($^{\#}P < 0.05$) and PNS ($^{\#\#}P < 0.01$) dams versus vehicle-treated *Bdr* mice at 78 and 86 dB. Haloperidol administration caused a significant increase in PPI only at 86 dB in *Bdr* mutants compared with saline-treated mutants ($^{@}P < 0.05$). Data are shown as mean ± SEM.

could therefore be determined in the *Bdr* line, despite the C3H background. It is unclear why our results differ from the original analysis of inbred strains, although it may reflect subtle differences in the size and content of the apparatus used (44). Consequently, by controlling for strain differences by using littermates from each *Snap-25* cross throughout, our study demonstrates that a comparison of the behavioural data from independent mutant lines requires careful consideration of the genetic backgrounds involved (88).

The data from HET mutants did not deviate significantly from wild-type controls in any of the tests carried out here, suggesting that a 50% reduction in Snap-25 expression does not influence a range of behavioural domains. Synaptic function has been shown to be normal in HET animals, although possible functional compensation by related synaptic proteins has been considered (33,89). In mice, Snap-23, Snap-29 and Snap-47 are all expressed in neurons and are capable of supporting exocytosis, although when assayed *in vitro*, each displays functional idiosyncrasies distinct from Snap-25 (90–92). If complete functional compensation by related proteins does not occur *in vivo*, a more straightforward explanation may be that the absolute levels of Snap-25 in HET mice are not limiting to synaptic function.

Our data are apparently at odds with the description of the hyperactive *coloboma* mutant containing a deletion spanning four genes, including *Snap-25* (93). These mice also have a 50% reduction in expression of Snap-25 protein, although genetic rescue of the hyperactivity was seen using a *Snap-25b* transgene (94). Considering our data from HET mice, this discrepancy may involve subtleties of the isoform-specific *Snap-25* mini-gene used for transgenic rescue (94). However, it would be interesting to determine whether the additional genes deleted in *coloboma* influenced cognitive behaviours common to psychiatric disorders and conditions, where hyperactivity is a feature, such as attention-deficit hyperactivity disorder. The picture regarding SNAP-25 expression levels in human post-mortem tissue from schizophrenic patients is somewhat complex (23). Although there is some consensus that reduced levels of SNAP-25 occur in certain regions of the brain such as the hippocampus (26), it is possible such observations may be a result of prior neuropatholoigcal insults altering synaptic morphology or density. Nevertheless, it is intriguing that changes in neonatal Snap-25 protein levels have been reported recently in a mouse expressing a human DISC-1 transgene (95) and prenatal infection models of schizophrenia (96).

If the apparently 'normal' phenotype of HET mice is a consequence of functional compensation, in contrast, the behavioural profile of *Bdr* mice is caused by changes in synaptic vesicle recycling without any reduction in the expression of Snap-25 itself (36). It is still unclear why the global synaptic deficit in *Bdr* mice would influence certain behavioural parameters and not others, although we hypothesise that events causing the continual, rapid firing of neurons would result in depletion of the reserve pool of vesicles, ultimately preventing normal synaptic function. Clearly sensorimotor gating and social interaction, those behavioural domains most sensitive to PNS in the *Bdr* mutant, are highly complex tasks that require the integration of many brain circuits (97,98), although studies are only just beginning to isolate which neurodevelopmental pathways are most susceptible to environmental factors (52,99). We can speculate, for example, that inhibitory feedback loops to the HPA axis from the hippocampus or frontal cortex are adversely affected in *Bdr* mice, thus perpetuating the stress response (100). Our strategy has therefore investigated the consequences of altering two-independent aspects of synaptic function relevant to the pathophysiology of psychiatric disease, namely changes in protein expression and protein interaction at the synapse.

A consensus is emerging that to understand the pathogenic mechanisms of complex neurodevelopmental disorders will require consideration of environmental as well as multiple genetic factors; and as a consequence, improved animal models are required (101–103). We have demonstrated here that combining a synaptic mouse mutant with a controlled prenatal stressor paradigm produces both modified and completely new phenotypes relevant to the study of psychiatric disease. Our work also exemplifies the importance of studying point mutations in addition to gene knockouts to accurately model human genetic lesions (47,104). With the limitations and complexities surrounding retrospective human studies (6), the analysis of mouse mutants such as *Bdr* subjected to environmental manipulation will become an increasingly important tool for the study of neuropsychiatric disorders.

MATERIALS AND METHODS

Breeding

Bdr males (from serial backcross to C3H/HeH >12 generations) and HET males (backcross to C57BL/6 >10 generations) were mated to virgin wild-type C3H/HeH or C567BL/6 females, respectively. The presence of a copulation plug denoted the first day of gestation (E0.5), when the females were then house individually. On the day of birth (P0), litters sizes were counted and culled to between six and eight pups, with a minimum of two males, on the second day post-partum (P1). Litters of less than six pups were not used. Pregnant females were randomly assigned to either the PNS or the NS groups in each experiment. All experiments were carried out using procedures approved by the UK Home Office and the Departmental Ethical Review Committee (University of Oxford).

Genotyping

Bdr mice were genotyped using polymorphic microsatellite markers and sequencing as previously described (36). *Snap-25* HET mice were identified in a mutiplex PCR reaction using primers for specific for the neomycin resistance marker 5′ ATCCATCATGGCTGATGCAATG and 5′ CATGATATT CGGCAAGCAGGCA with the wild-type Snap-25 locus 5′ GAAGAAGGCATGAACCATATCAAC, 5′ CCCGCAGAA TTTTCCTAGGTCC (33).

Random variable PNS paradigm

A repeated prenatal variable stress paradigm similar to that described by Kinnunen *et al*. (37) for rats was employed; however, the duration of each stressor was suitably adjusted for mice and was carried out during days E11.5–17.5 (Table 2). Restraint was carried out using a polypropylene plastic tube 4 cm in diameter for 30 min. A large (90 × 70 × 40 cm) brightly illuminated white arena was used for the open-field stressor and swim stress was conducted in a container (40 × 60 × 30 cm) containing water at 18°C for a period of 5 min. Social stress consisted of placing individual pregnant dams in a cage of four or more wild-type non-pregnant unfamiliar females overnight. Home cages were also changed at E12.5, 14.5 and 16.5. Non-stressed females were left in their own home cages throughout the stress procedures. To prevent bias in the subsequent experiments, pregnant dams were randomly assigned to the maternal, neonatal, adult behavioural battery or PPI testing groups (see below).

Corticosterone measurements

To measure the stress response of dams, corticosterone levels were compared 30 min after the third restraint stressor carried out at E15.5 and the second forced swim stressor at E14.5 ($n = 8$ each group). Blood was taken alternately from individual animals from both groups and assayed using the Corticosterone EIA kit (Immunodiagnostic Systems) according to the manufacturer's instructions.

Table 2. Timing of PNS paradigm

	AM	PM	Overnight
E11.5	Restraint	Forced swim	
E12.5		Open field	Cage change
E13.5	Restraint		Social stress
E14.5	Open field	Forced swim	Cage change
E15.5		Restraint	
E16.5		Open field	Cage change
E17.5	Forced swim		Social stress

Maternal measurements

For a quantitative measure of nesting behaviour, a single nestlet was placed in the home cage of E16.5 dams at 1600 h following normal cage cleaning, but without the addition of alternative nesting material. Nest quality was scored based on the amount of shredded material and the shape that was created overnight: (i) material not shredded; (ii) flat nest with less than 50% shredded; (iii) flat nest with more than 50% shredded; (iv) more than 90% shredded with shallow walls; (v) more than 90% shredded with walls more than 3 cm high (38). At P3, the same dams were tested for pup retrieval activity. The mothers were first sequestered on the nest using a cardboard barrier, whereas the pups were placed equally around the home cage in a 3×2 pattern. The barrier was then removed and each dam was scored for the total number of pups retrieved in 10 min and the latency to retrieve the first and all of the pups back to the nest (39).

Neonatal measurements

In addition to the pups being weighed every 2 days from P3–21, a range of commonly used developmental milestones were quantified (40,41).

(1) Eye opening: day when complete opening of both eyelids occurred.
(2) Ear twitch response: day when sudden ear movement was observed after stroking the pinna with a cotton bud.
(3) Surface righting: time taken for pups to return to an upright position after being placed supine.
(4) Negative geotaxis: time taken for pups placed head down on a ramp covered in rough paper inclined at 45° to turn around and begin walking up the slope.
(5) Forelimb strength: day when a pup suspended by the forelimbs on a wire of 1 mm in diameter could remain hanging for more than 1 s before losing grip.
(6) Walking: day when movement of the pup was first seen where the entire body was lifted from the floor supported by both forelimbs and hindlimbs.
(7) Linear movement/activity: time for a pup to demonstrate forward movement from a central area 5×5 cm in an empty plastic open-field chamber (30×30 cm). For the measure of locomotor activity, the total number of 6 cm^2 squares marked on the floor that were crossed in a 180 s period was recorded.

Adult behavioural test battery

A range of tests were carried out on male mice from 8 weeks of age over a 3 weeks period ($n = 14$ each genotype and stress group) in the following order.

(1) Open field: mice were placed in the same corner of a brightly lit arena $50 \times 50 \times 30$ cm with markings every 10 cm on the floor to create a 5×5 grid. Movement of the mice over 10 min was recorded using the Ethovision XT software (Noldus) with a monochrome camera suspended directly above the arena. Distance travelled and time spent in the central 3×3 squares was recorded.

(2) EPM: the EPM consisted of four arms, two open ($36 \times 6 \times 0.5$ cm) and two closed ($36 \times 6 \times 25$ cm). The mouse was placed on an open arm facing a central area where all four arms meet. Mice were observed over a 5 min period and the number of entries and time spent in open and closed arms was recorded. Entries were defined as the passing of all four paws into an arm. Activity was measured using the Ethovision system as above.

(3) Y-maze: the maze consisted of three identical arms ($40 \times 10 \times 20$ cm) placed at 120° from each other. Each mouse was placed at the end of one arm and allowed to explore the maze freely for 6 min. The total number of arm entries was recorded in addition to the calculation of the spontaneous alternation score; this was calculated as the number of alternations (entries in three different arms consecutively) divided by the total possible number of alternations (total number of arm entries—two).

(4) Olfactory test: before testing commenced, all mice were habituated to an unfamiliar food source (Chocolate Weetos, Kellogs) for 3 days. For the test, after 8 h of food deprivation, mice were placed in a clean cage with 3 cm of normal bedding and allowed to explore for 5 min. The animal was removed from the cage while a single piece of cereal was buried 1 cm below the surface. The mouse was then returned to the cage and the latency to find the hidden food was recorded.

(5) Sociability and preference for social novelty behaviour was assessed based on a previously described method (42). The apparatus was a rectangular, three-chambered (left and right chambers $20 \times 30 \times 30$ cm; centre chamber $20 \times 20 \times 30$ cm; total size $60 \times 30 \times 30$ cm). Dividing walls were made from clear acrylic, with small square openings (4×4 cm) allowing access from the centre chamber into left and right chambers. Each chamber was cleaned and fresh bedding added between trials. A video camera, mounted in front of the apparatus, recorded each session. The paradigm consisted of a three-stage procedure:

Stage 1: habituation: in the initial stage, the test mouse was first placed in the centre chamber and allowed to explore all three chambers of the apparatus for 5 min. It was then replaced in the centre chamber for a further 5 min with access to the left and right chambers denied by Plexiglasss doors.

Stage 2: sociability: an unfamiliar BALB/c mouse (Stranger 1: age-, weight- and sex-matched) was placed in either the left or right chamber enclosed in a small, internal wire cage ($10 \times 10 \times 12$ cm) which allowed nose contact but prevented fighting; placement of Stranger 1 in the left or right chamber alternated between trials, with an empty but otherwise

identical wire cage in the opposite chamber. Each stranger mouse had been habituated to placement in the small wire cage 24 h before testing. Following placement of Stranger 1 into the left or right chamber, both doors to these side chambers were then opened and the test mouse was allowed to leave the centre chamber and explore all three chambers of the apparatus for 10 min. The test mouse could therefore distribute its behaviour between the centre chamber, the chamber containing Stranger 1 or the opposite, empty chamber. Time spent in each compartment was recorded, with entry into any chamber defined as all four paws in that chamber.

Stage 3: Preference for social novelty: each test mouse was immediately returned to the centre chamber and the doors to the side chambers were closed. Then followed a second 10 min session to quantify social novelty preference toward a novel stranger. With the initial stranger (Stranger 1; now familiar) retained in its original chamber, a second, unfamiliar mouse (Stranger 2) was placed in the previously empty but otherwise identical small wire cage in the opposite chamber. Following placement of Stranger 2 into the chamber opposite to that still containing Stranger 1, both doors to the side chambers were opened and the test mouse was allowed to leave the centre chamber and explore all three chambers of the apparatus for a second period of 10 min; it could therefore distribute its behaviour between the centre chamber, the chamber containing the previously investigated and now familiar mouse (Stranger 1) and the opposite chamber containing the novel, unfamiliar mouse (Stranger 2). All other parameters and measures were as described above for Stage 2.

(6) Novel object recognition: two identical objects (solid plastic cylinders 10 × 3 cm) were secured to the floor of the open-field apparatus (see above) equidistant from the walls at either end. Mice were placed in the arena and allowed to explore for 5 min, after which they were transferred to their home cage. One hour later one of the objects was replaced with a novel object (plastic doll 10 cm long) and the mouse was returned to the arena for 5 min. The time spent exploring each object was recorded from both trials. The arena and objects were cleaned between trials.

(7) Forced swim test: each mouse was placed into a transparent 4l glass beaker (45 cm high × 15 cm diameter) filled to a depth of 20 cm with water (maintained at 30°C) and remained there for 6 min. The duration of immobility, which was defined as floating in an upright position without additional activity other than that necessary for the animal to keep its head above water, was recorded during the last 4 min of the test period.

(8) Fear conditioning: Pavlovian fear conditioning was measured using apparatus composed of test chamber with the facilities for emitting white noise and electrical shock (MED Associates). The test chamber (56 × 38 × 36 cm) was made of clear Plexiglass, the bottom composed of a grid floor used to delver a mild electric shock. This was housed inside a sound-attenuated chamber with a viewing lens to allow the experimenter to see into the test chamber. A mouse was placed into the test chamber and allowed to explore freely for 2 min. A white noise (80 dB) serving as the conditioned stimulus (CS) was aired for 30 s, followed by a mild (2 s, 0.5 mA) foot shock (unconditioned stimulus, US). This CS–US pairing was repeated 2 min later and this comprised the training period. For the context text, 24 h later the mouse was placed back into the test chamber and the presence of freezing behaviour was recorded every 10 s for 5 min. Two hours later the mouse was evaluated for its response to the CS. Contextual cues for this test were altered; the shape of the test chamber was modified by insertion of a triangular piece of cardboard, the grid floor covered with Plexiglass and vanilla essence used to alter the smell. Mice were observed for freezing behaviour 3 min prior to the CS tone being turned on and for 5 min following the CS. Freezing interval was converted to percent freeze value.

PPI/antipsychotic treatment

For PPI testing ($n = 14$, 8-week-old male mice of each genotype and stress group), startle and pre-pulse response studies were carried out using the Med Associates Startle Reflex system. Startle response amplitude was calculated from an initial block of 10 105 dB 20 ms pulses. For the PPI testing, a second block of 32 additional identical startle trials in total were preceded 100 ms by a pure tone pre-pulse at 4, 8 and 16 dB above background. Trial types were presented in a pseudo-randomized order with eight presentations of each pre-pulse and startle combination and eight startle only trials. PPI for each frequency was calculated using the following formula: PPI = 100 − [(mean startle amplitude for pre-pulse trials/mean startle amplitude for startle trials) × 100]. For drug treatments, an additional independent group of mice from the *Bdr* cross was used ($n = 12$, 8-week-old male mice from each stress group). The typical anti-psychotic clozapine and the atypical anti-psychotic haloperidol (Tocris) were administered by i.p. injection at 5 and 0.3 mg/kg, respectively, 20 min prior to testing in the PPI apparatus. Control animals were injected with vehicle (0.9% NaCl). Mice from each group were tested once every 7 days with injection of saline or either drug in a randomized order, so that each animal received each treatment only once.

Statistics

Data were analysed using two-way analysis of variance with genotype and environment as main factors with *post hoc* comparisons carried out using independent or paired *t*-tests.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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