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Altered motor activity, exploration and anxiety in heterozygous neuregulin 1 mutant mice: implications for understanding schizophrenia

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Human genetic studies have shown that neuregulin 1 (*NRG1*) is a potential susceptibility gene for schizophrenia. Nrg1 influences various neurodevelopmental processes, which are potentially related to schizophrenia. The neurodevelopmental theory of schizophrenia suggests that interactions between genetic and environmental factors are responsible for biochemical alterations leading to schizophrenia. To investigate these interactions and to match experimental design with the pathophysiology of schizophrenia, we applied a comprehensive behavioural phenotyping strategy for motor activity, exploration and anxiety in a heterozygous *Nrg1* transmembrane domain mutant mouse model (*Nrg1* HET) using different housing conditions and age groups. We observed a locomotion- and exploration-related hyperactive phenotype in *Nrg1* HETs. Increased age had a locomotion- and exploration-inhibiting effect, which was significantly attenuated in mutant mice. Environmental enrichment (EE) had a stimulating influence on locomotion and exploration. The impact of EE was more pronounced in Nrg1 hypomorphs. Our study also showed a moderate task-specific anxiolytic-like phenotype for *Nrg1* HETs, which was influenced by external factors. The behavioural phenotype detected in heterozygous *Nrg1* mutant mice is not specific to schizophrenia *per se*, but the increased sensitivity of mutant mice to exogenous factors is consistent with the pathophysiology of schizophrenia and the neurodevelopmental theory. Our findings reinforce the importance of carefully controlling experimental designs for external factors and of comprehensive, integrative phenotyping strategies. Thus, *Nrg1* HETs may, in combination with other genetic and drug models, help to clarify pathophysiological mechanisms behind schizophrenia.

Keywords: age, anxiety, behaviour, environmental enrichment, exploration, habituation, hyperactivity, neuregulin 1, schizophrenia

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Schizophrenia is characterized by positive, negative and cognitive symptoms, which are at least partially based on dopamine (DA) hyperstimulation (inducing mainly positive symptoms) and a dysfunction of either glutamate or its *N*-methyl-D-aspartate (NMDA) receptor subtype (mediating mainly negative and cognitive symptoms) (Coyle 1996; Wong & Van Tol 2003). The neurodevelopmental theory of schizophrenia suggests that genetic and/or environmental factors, which negatively affect the brain development during critical early periods of neuronal development, are responsible for these biochemical alterations in people diagnosed with schizophrenia (Weinberger & Marenco 2003).

Linkage and association studies have highlighted several candidate genes (e.g. dysbindin, *DISC1* and neuregulin 1), which increase the risk of developing schizophrenia, and have confirmed the polygenetic character of the disease (Owen *et al.* 2005). However, the schizophrenia concordance rate of 30–50% for monozygotic twins points to the importance of environmental factors in the development of schizophrenia (Kendler & Diehl 1993) such as obstetric complications (Boksa 2004) or cannabis abuse (Arseneault *et al.* 2004). Importantly, neither environment nor genetics are sufficient to cause the disorder in isolation (Mackay-Sim *et al.* 2004), but in combinations are likely to increase the risk of developing schizophrenia.

To investigate the effects of an interaction between genes and environment in more detail, we used a genetic animal model (Stefansson *et al.* 2002) for one of the most promising susceptibility genes for schizophrenia, neuregulin 1 (*NRG1*). The *NRG1* gene – also known as heregulin or neu differentiation factor (Falls 2003) – is located on chromosome 8p12-p21. Originally identified from a genomewide scan of schizophrenia families in Iceland (Stefansson *et al.* 2002), the significant role of *NRG1* in schizophrenia has been confirmed in many independent replication studies (Williams *et al.* 2003; Bakker *et al.* 2004; Corfas *et al.* 2004), including a recent meta-analysis (Munafo *et al.* 2006). The causative allele of *NRG1* has not been identified (Stefansson *et al.* 2004; Petryshen *et al.* 2005), although the transmembrane *NRG1* isoform (Falls 2003) has recently been associated with psychosis and schizophrenia (Walss-Bass *et al.* 2006). Using animal models that attempt to recapitulate some of the

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features of schizophrenia, a range of environmental factors may be related to the pathophysiology of this mental disorder including vitamin D deficiency (Burne *et al.* 2004) or maternal deprivation (Ellenbroek *et al.* 2004). Importantly, environmental enrichment (EE) has a significant impact on animal models of neurodegenerative diseases (van Dellen *et al.* 2000; Spires & Hannan 2005), as has age on the aetiology of schizophrenia (Thompson *et al.* 2004).

Heterozygous (hypomorphic) *Nrg1* transmembrane domain mutant mice were originally described as being hyperactive and having moderate deficits in prepulse inhibition (Stefansson *et al.* 2002). Another study identified an explorative-like phenotype and disruptions to the habituation of exploration (O'Tuathaigh *et al.* 2006b). Several other genetic animal models are available for the different isoforms of Nrg1 and its receptors $ErbB_{1-4}$ as well (Gerlai *et al.* 2000; Golub *et al.* 2004; Rimer *et al.* 2005). Unfortunately, behavioural phenotyping of animal models for schizophrenia has often been limited and unspecific (Ellenbroek & Cools 2000; van den Buuse *et al.* 2005), as a lot of studies focus exclusively on baseline prepulse inhibition deficits and general motor activity (GMA) screening (e.g. Hiroi *et al.* 2005; Stefansson *et al.* 2002). In contrast, the behavioural investigation of animal models for complex mental disorders such as schizophrenia demands a highly specific, multi-tiered and comprehensive phenotyping strategy (Crawley & Paylor 1997; Crawley 1999; Karl *et al.* 2003). In addition, environmental factors must be considered to match the experimental design with the pathophysiology of schizophrenia because impoverished standardized laboratory housing conditions may result in deprivation-like processes during development.

We have applied a comprehensive behavioural phenotyping strategy, focusing on the behavioural domains of motor activity, exploration and anxiety to evaluate the heterozygous transmembrane domain *Nrg1* mutant model of the original study that implicated Nrg1 as a model of schizophrenia (Stefansson *et al.* 2002). Furthermore, we investigated the effect of age and housing conditions on the behavioural profile. By examining the influence of age and environment on this schizophrenia-related animal model, we tried to mimic at least some of the complex interactions that may contribute to schizophrenia.

Materials and methods

Animals

The generation of Nrg1 transmembrane domain mutant mice was described previously (Stefansson *et al.* 2002). Test animals were heterozygous $Nrg1^{+/-}$ (*Nrg1* HET) and wild type (WT)-like control $Nrg1^{+/+}$ littermates (backcrossed in the 15th generation on C57BL/6 background). Genotypes were determined after weaning (postnatal day 21) using tail tip biopsy and polymerase chain reaction amplification of selective amplicons for the knockout allele (primers for mutant *Nrg1* mice: Neo173F: 5′-ATGAACTGCAGGACGAGGCA-3′ and Neo6301R: 5′-GCCACAGTCGATGAATCCAG-3′; primers for WT-like control mice: 5′-AACAGCCTGACTGTTAACACC-3′ and 5′-TGCTGTCCATCTGCACGAGACTA-3′). Age-matched (±12 days), male, adult test animals of similar genotype were pair-housed (thereby avoiding cohort removal effects: Kask *et al.* 2001) in Macrolon cages under a 12:12 h light:dark schedule [light phase: white light (illumination: 80 lx); dark phase: red light (illumination: <2 lx)]. Microbiological monitoring showed no infection of the specific pathogen free (SPF) facility holding room, with the exception of the pathogens commonly found in commercial and research facilities, *Pasteurella pneumotropica* and *Helicobacter* spp. All the research and animal care procedures were approved by the 'Garvan Institute/St Vincent's Hospital Animal Experimentation Ethics Committee' and were in accord with the 'Australian Code of Practice for the Care and Use of Animals for Scientific Purpose'.

Environmental/external factors

Different sets of WT and Nrg1 hypomorphic (*Nrg1* HET) mice were tested to investigate the influence of the environmental factor housing condition and of the factor age on behavioural performance. (1) Factor 'housing': different sets of mice were kept under standard laboratory housing conditions (experimental group: 'SH') or minimally enriched housing conditions (experimental group: 'EH') from birth onwards. Environmentally enriched home cages contained a red, transparent, polycarbonate igloo (certified polycarbonate mouse igloo: Bioserv, Frenchtown, NJ, USA) and a metal ring in the middle of the cage lid (3 cm in diameter). (2) Factor 'age': different sets of mice were tested at the age of 91–120 days ('3–4 months') or at the age of 130–151 days ('4–6 months'). This choice of time points was determined by the animal model as preliminary behavioural phenotyping showed that the hyperactive phenotype (described by Stefansson *et al.* 2002) was evident only from the age of 4 months onwards in several different cohorts of Nrg1 hypomorphic mice (kept in SH).

We tested eight separate sets of test mice (8–16 animals each) combining the different factors genotype (WT vs. *Nrg1* HET), age (3–4 months vs. 4–6 months) and housing (SH vs. EH) (Table 1). First, 3- to 4-month-old SH mice of both genotypes (set 1–2) were phenotyped behaviourally. Following this, 4- to 6-month-old SH test animals (WT and *Nrg1* HET; set 5-6) were analysed in regard to their behavioural performance. A few months later, WT and *Nrg1* HET mice of both age groups (3–4 months vs. 4–6 months) kept in EH were screened in identical test designs (set 3-4 and 7-8).

Behavioural phenotyping

For habituation purposes, all test animals were transported to the testing room 1 h prior to behavioural testing. Experiments were performed within a time limit of 3 h per day to avoid severe influences of the circadian rhythm on the animals' behavioural performance (Kopp 2001). For this reason, animals were tested on consecutive days at similar times of the circadian rhythm for some paradigms. Environmental odours were removed from the different test apparatus by cleaning the test arena after each trial with 30% ethanol solution.

Physical exam

General health, sensory abilities, neurological motor reflexes and motor function/co-ordination have a huge impact on the animals' behavioural performance (Crawley & Paylor 1997; Crawley 1999). Therefore, we used a wide range of basic tasks to check test mice of different genotypes (HET vs. WT) and housing conditions (SH vs. EH) (for details of test paradigms see Karl *et al.* 2003). Tests were performed after completion of all other handling-sensitive paradigms (Table 1) during the light phase (test age: 154–161 days).

General motor activity

General motor activity was evaluated by placing the mouse into an infrared photobeam-controlled open-field activity test chamber (MED Associates Inc., St Albans, VT, USA). This paradigm also mimics the natural conflict in mice between the tendency to explore a novel environment and to avoid an exposed open area (Crawley 1985; DeFries *et al.* 1966) and can be used as an anxiety-related open-field paradigm. The arena (43.2 × 43.2 cm) was divided into a central and a peripheral zone (MED Associates Inc. software coordinates for central zone: 3/3, 3/13, 13/3, 13/13). Animals were tested 1 h after onset of the dark phase for 30 min (illumination at floor level: <2 lx). The animal's horizontal activity (i.e. distance travelled), ambulatory

Table 1: Overview about the different experimental groups

Genotype	Set 1 WT	Set 2 *Nrg1* HET	Set 3 WT	Set 4 *Nrg1* HET	Set 5 WT	Set 6 *Nrg1* HET	Set 7 WT	Set 8 *Nrg1* HET
Housing	SH		EH		SH		EH	
Age	3–4 months				4–6 months			
	Test age (±4 days)	Behavioural paradigm			Test age (±12 days)	Behavioural paradigm		
	91	General motor activity						
	97	Hole board						
	103	Elevated plus maze						
	110	Light–dark						
	117	Cross maze						
	120	Marble burying						
		No testing (except sets 1, 2:see *Supplementary Material*: Fig. S1)			130	Light–dark		
					134	Cross maze		
					138	General motor activity		
					141	Marble burying		
					145	Elevated plus maze		
					151	Hole board		

Genotype (WT, wild type-like vs. *Nrg1* HET, heterozygous mutant), age (3–4 months = 91–120 days vs. 4–6 months = 130–151 days) and housing conditions (SH, standard laboratory housing vs. EH, enriched housing) are presented for the different sets of test mice. Test biography is shown including test age (days) and behavioural paradigm used (the different sets were tested chronologically in the following order: set 1-2, set 5-6, set 3-4 and set 7-8).

frequency, vertical activity, time spent in ambulation, or *resting* behaviour (no infrared photobeam-detectable movements) in the different zones as well as the overall velocity were recorded automatically (software settings: box size: 3; ambulatory trigger: 2; resting delay: 1000 ms; resolution: 100 ms). The ratio of central to total distance travelled, time spent in the central zone and the defecation score were taken as measures of anxiety (Denenberg 1969).

Hole board

The hole board (HB) test provides independent measures of locomotor activity and directed exploration (van Gaalen & Steckler 2000). Furthermore, it can be used as a task for anxiety (Pellow *et al.* 1985). Mice were placed into the open-field activity test chamber, which was equipped with a HB floor insert for mice (MED Associates Inc.: 16 holes; diameter: 1.6 cm). Animals were tested 1 h after onset of the dark phase (illumination at floor level: <2 lx). The infrared photobeams provided automated measures of the distance travelled, ambulatory frequency, *head dipping* frequency and working memory ratio (number of *head dippings* into novel holes divided by total number of *head dippings*) in a 7-min test session (Lister 1987).

Cross maze

The cross maze consisted of an eight-arm radial arm maze (Pathfinder maze system model 89000B: Lafayette Instrument, Sagamore Parkway North, IN, USA), with four of the arms blocked (walls of arms were red stained; illumination on arms: 70 lx). The maze was located in a room with different visual cues at the wall. In addition, unblocked arms were equipped with contrasting geometrical cues at the end of each arm. Animals were placed individually 3.5 h before onset of the dark phase into the centre arena of the apparatus. During the following 5 min, the order of arm visits (defined as all paws being placed inside the arm) was recorded. Parameters investigated were number of repeated arm entries during the first four arm entries, number of arm entries until all arms were visited once, overall number of arm entries, frequency of *rearing* and the defecation score. Furthermore, the percentages of triple and quadruple alternations – a triple/quadruple alternation was defined as the visit of three/four different arms consecutively without entering an already visited arm – were calculated (Stefansson *et al.* 2002).

Elevated plus maze

The elevated plus maze (EPM) represents the natural conflict between the tendency of mice to explore a novel environment and the tendency to avoid a brightly lit, elevated, open area (Montgomery 1958). The grey plus maze was '+' shaped, with a central platform (6 × 6 cm), two alternate enclosed arms (35 × 6 cm; height of enclosing walls 28 cm; dimly illuminated: 10 lx) and two alternate open arms (35 × 6 cm; without side walls; highly illuminated: 70 lx) with ledges (4 × 6 mm). The surface of the arms was raised 70 cm above the floor. Animals were tested 3 h before onset of the dark phase. The mouse was placed onto the centre field of the + (faced to an enclosed arm) and was allowed to explore the maze for 5 min. Behaviour was measured online. Frequency of *stretch-attend postures*, time spent on open arms and the percentage of open arm entries (Hogg 1996; Pellow *et al.* 1985) were recorded as measures of anxiety. An individual entry was recorded when the animal entered the arm with at least half of its body length. The number of total arm entries was recorded as a measure of locomotion. Frequencies of *head dipping* over the ledges and *rearing* in the junction area between arms were analysed as parameters for exploration.

Light-dark

In the light–dark (LD) test, the distance travelled and time spent in a brightly illuminated zone compared with a dark zone, and the occurrence of associated exploratory behaviours (e.g. *rearing*) can

be used to assess anxiety in rodents (Costall *et al.* 1989; Crawley 1999). During the light phase of the light cycle, animals were placed into the open-field activity test chamber, which was equipped with a dark box insert for mice (MED Associates Inc.). This insert (covering half the area of the chamber) was opaque to visible light, while allowing the infrared photobeams to pass through. Light (illumination: 20 lx) and dark (illumination: <2 lx) compartments were connected by an opening located in the centre of the partition. At the start of the experiment, mice were placed into the light compartment. The time spent in, entries into and distance travelled in the differentially illuminated compartments and vertical activity, time spent in ambulatory activity, ratio of distance travelled in the light compartment to total distance travelled (distance ratio) and the number of ambulatory episodes were recorded for the following 10 min.

Marble burying

The marble burying (MB) test was performed in the open-field activity test chambers (MED Associates Inc.). Glass marbles ($n = 12$, diameter: 1.5 cm) were spaced evenly on a 2-cm deep layer of sawdust in each chamber. Before the experiment, adult male A/J mice (Western Australia Animal Resources Center, Perth, Australia) were allowed to explore the chambers for 60 min to equalize the impact of olfaction on test animals' burying behaviour in subsequent test trials. After this period, test animals were placed individually into the test chambers and could explore the area without any disturbance for the following 30 min. Afterwards, the number of marbles completely buried or partially buried (marble covered with sawdust more than half but not completely) was recorded for each animal (Gyertyan 1995).

Statistical analysis

Analysis of the various behavioural parameters was assessed by applying three-way analysis of variance (ANOVA) to investigate the effects of 'genotype', 'age' and 'housing' on this schizophrenia-related animal model. This was followed by two-way ANOVAs (two factors separated by the third factor), if appropriate and necessary. In the GMA, habituation over time (overall distance travelled for 5-min blocks during a 30-min session – summed for the time intervals 0–5, 5–10, 10–15, 15–20, 20–25 and 25–30 min) was investigated using three-way ANOVA with repeated measures. Differences were regarded as statistically significant if $P < 0.05$. The number of animals (n) was 8–16. The *Results* section presents degrees of freedom, F values, P values, means and standard error of the mean (SEM) of the three- (and two-) way ANOVAs, while in figures and tables, means + SEM for the various experimental groups are shown.

Results

The parameters investigated in the 'physical exam' showed that all the animals were in a state of good health and physical condition and exhibited normal neurological reflexes and sensory abilities. Furthermore, motor function and co-ordination and MB behaviour were similar for the different experimental groups.

Motor activity

Three-way ANOVA showed a significant effect of the factors genotype and age and a strong trend toward significance for the factor housing on the locomotion-related parameter overall distance travelled [genotype: $F(1,83) = 11.6$, $P = .001$; age: $F(1,83) = 25.5$, $P < 0.0001$; housing: $F(1,83) = 4.0$, $P = .05$; Fig. 1a]. Nrg1 hypomorphic mice exhibited a hyperlocomotive phenotype as shown by an increase in overall distance travelled. Motor activity was reduced in older animals.

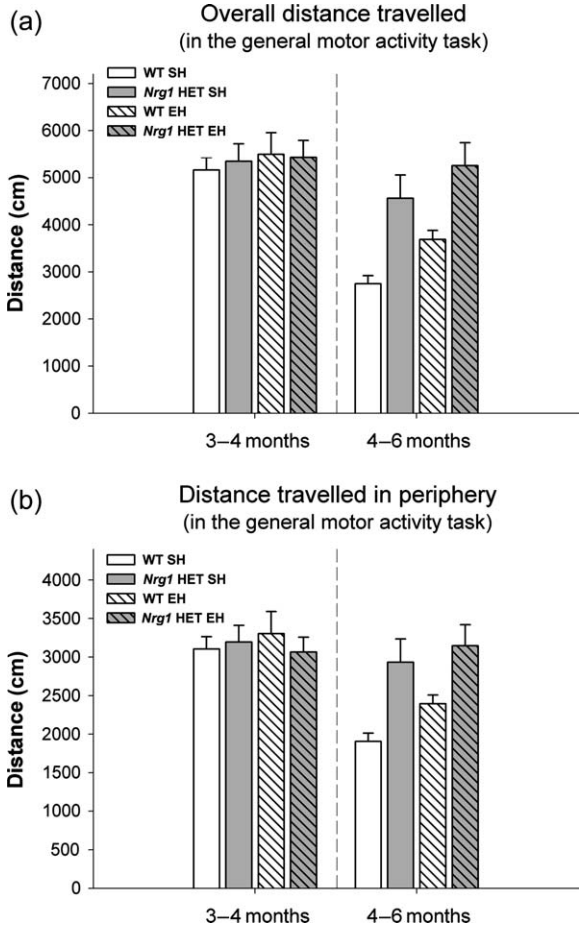

Figure 1: Motor activity in GMA: (a) The overall distance travelled (ODT) (cm) and (b) distance travelled (cm) in the periphery of the GMA paradigm are recorded. Three-way ANOVA showed a significant effect of the factors genotype and age and a strong trend for the factor housing ($P = .05$) and a significant genotype × age interaction in ODT. Furthermore, we detected an identical, even more pronounced phenotype in *Nrg1* HET compared with WT in regard to locomotion in the periphery. All data are presented as means + SEM.

Importantly, this effect was significantly more pronounced in WT animals as confirmed by a significant 'genotype × age' interaction [$F(1,83) = 10.1$, $P = .002$]. Mice in enriched housing conditions exhibited increased motor activity (Fig. 1a). This effect was most evident in WT-like animals at the age of 4–6 months (Fig. 1a).

To minimize the influence of anxiety or stress on GMA, we separately analysed distance travelled in the peripheral and therefore non-aversive zone of the GMA device. We detected an identical phenotype for this parameter in the mutant animals compared with WT. All the three factors investigated had a significant effect as shown by three-way ANOVA [genotype: $F(1,83) = 14.0$, $P = .0003$; age: $F(1,83) = 36.4$, $P < 0.0001$; housing: $F(1,83) = 7.5$, $P = 0.007$; Fig. 1b]. Reduction in Nrg1 expression led to a significantly attenuated age-dependent decrease in motor activity in mutant compared

with WT mice as shown by a significant genotype × age interaction [F (1,83) = 9.2, P = 0.003; Fig. 1b]. Enriched housing conditions enhanced motor activity in the periphery. This was most evident in 4- to 6-month-old WT animals and *Nrg1* HET animals, where EH actually increased peripheral motor activity to an extent, which depleted the locomotion-suppressant effects of age on these animals (Fig. 1b). Three-way ANOVA confirmed a similar hyperactive age-dependent phenotype of hypomorphic Nrg1 mice for the number of ambulatory episodes in the GMA (data not shown).

Habituation of motor activity over time

We also investigated the habituation of motor activity (overall distance travelled for consecutive 5-min blocks during a 30-min session) in the GMA. Repeated measures ANOVA showed significant effects of the three factors genotype, age and housing (data not shown, see *Motor activity* section) and, more importantly, significant time interval × genotype, time interval × age and time interval × housing interactions over the time course of 30 min [time interval × genotype: F (5,410) = 3.7, P = 0.003; time interval × age: F (5,410) = 13.4, P < 0.0001; time interval × housing: F (5,410) = 9.1, P < 0.0001; Fig. 2a,b]. Overall, distance travelled within 5-min blocks decreased over time for all animals. Focusing on genotype differences, relative to the first 0- to 5-min block, the reduction in locomotion over time was more prominent in *Nrg1* HET animals than their WT counterparts (Fig. 2a,b).

Exploration

Our analyses showed an effect of all three investigated factors on the exploration-related parameter vertical activity in the GMA [three-way ANOVA: genotype: F (1,83) = 4.8, P = 0.03; age: F (1,83) = 93.3, P < 0.0001; housing: F (1,83) = 21.4, P < 0.0001; Fig. 3a]. *Nrg1* HET animals showed an increased drive to explore their environment compared with control littermates. Mice of younger age or of enriched housing conditions exhibited enhanced exploration (Fig. 3a). Similar to the findings for motor activity, the increased explorative-like phenotype of Nrg1 hypomorphs was age dependent as confirmed by a significant genotype × age interaction [three-way ANOVA: F (1,83) = 5.7, P = .02].

The anxiety-related LD test showed similar effects on exploration. Mutation in the *Nrg1* gene, younger age (trend) and EE significantly enhanced the exploratory drive in test mice [three-way ANOVA: genotype: F (1,83) = 23.3, P < 0.0001; age: F (1,83) = 3.4, P = 0.07; housing: F (1,83) = 52.8, P < 0.0001; Fig. 3b]. Importantly, using three-way ANOVA, we demonstrated that the factor housing had a differential impact on exploration of *Nrg1* HET and WT mice [genotype × housing interaction: F (1,83) = 8.4, P = 0.005]. Enrichment stimulated the exploratory drive of *Nrg1* HET but not WT mice (Fig. 3b). Possibly, this exploration-enhancing effect of enriched housing interferes with the suppressive influence of age on the explorative phenotype as we only found a significant genotype × age interaction for SH [two-way ANOVA interaction split by housing: F (1,44) = 6.1, P = 0.02].

The frequency of *head dipping* within the HB task, another strong exploration-related paradigm, confirmed an overall hyperexplorative-like phenotype of *Nrg1* mutant mice and an attenuating influence of age on exploration [three-way ANOVA: genotype: F (1,83) = 8.7, P = .004; age: F (1,83) = 35.2, P < 0.0001; Fig. 3c].

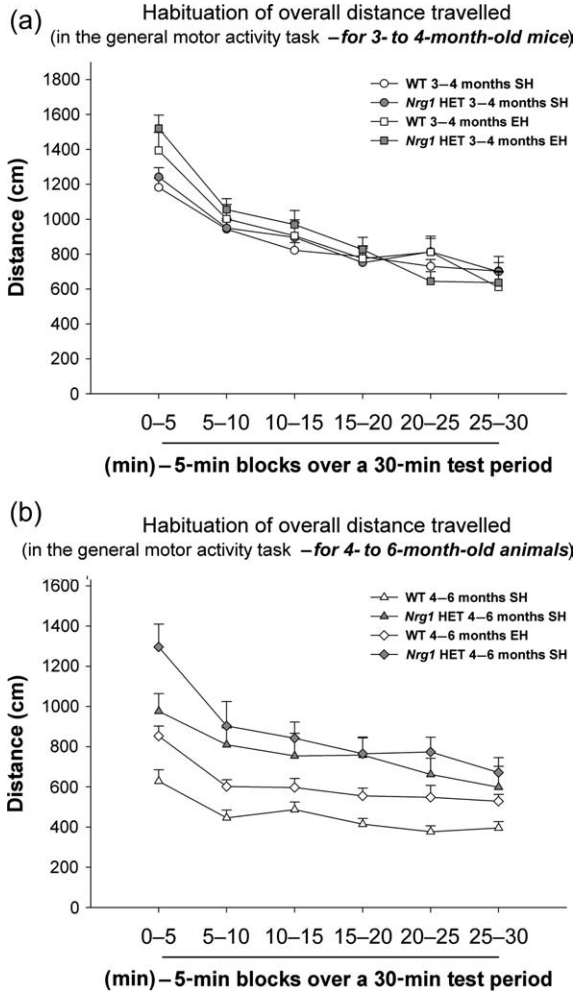

Figure 2: Habituation processes in the GMA: Overall distance travelled (ODT) during a 30-min trial in the GMA – separated for 5-min blocks each (summed ODT for 0–5, 5–10, 10–15, 15–20, 20–25 and 25–30 min). Repeated measures ANOVA showed a significant effect of genotype, age and housing and significant time interval × genotype, time interval × age and time interval × housing interactions over the time course of 30 min. All data are presented as means + SEM.

Anxiety

Using a multi-tiered phenotyping strategy, we observed a task-specific involvement of Nrg1 in anxiety-related domains (Fig. 4a, b and Fig. 5a, b). In the GMA, we found a significant effect of genotype, age and housing on the anxiety-related parameters time spent in the central zone (zone time) and ratio of central to total distance travelled (ratio) [three-way ANOVA: for zone time: genotype: F (1,83) = 22.8, P < 0.0001;

age: $F(1,83) = 60.8$, $P < 0.0001$; housing: $F(1,83) = 50.6$, $P < 0.0001$ – for ratio: genotype: $F(1,83) = 9.9$, $P = 0.002$; age: $F(1,83) = 27.4$, $P < 0.0001$; housing: $F(1,83) = 8.0$, $P = 0.006$; Fig. 4a, b]. Heterozygous *Nrg1* mutant animals were less anxious compared with WT. Increasing age and standard housing conditions had an anxiety-elevating effect in test animals (Fig. 4a, b). Interestingly, housing conditions had a more pronounced anxiolytic-like impact on *Nrg1* HETs compared with WT as shown by a significant genotype × housing interaction for zone time [$F(1,83) = 9.2$, $P = 0.003$]. The parameter defecation score confirmed the findings in regard to zone time (data not shown).

Although anxiety-related differences between *Nrg1* HET and WT mice were not confirmed in the LD and EPM test

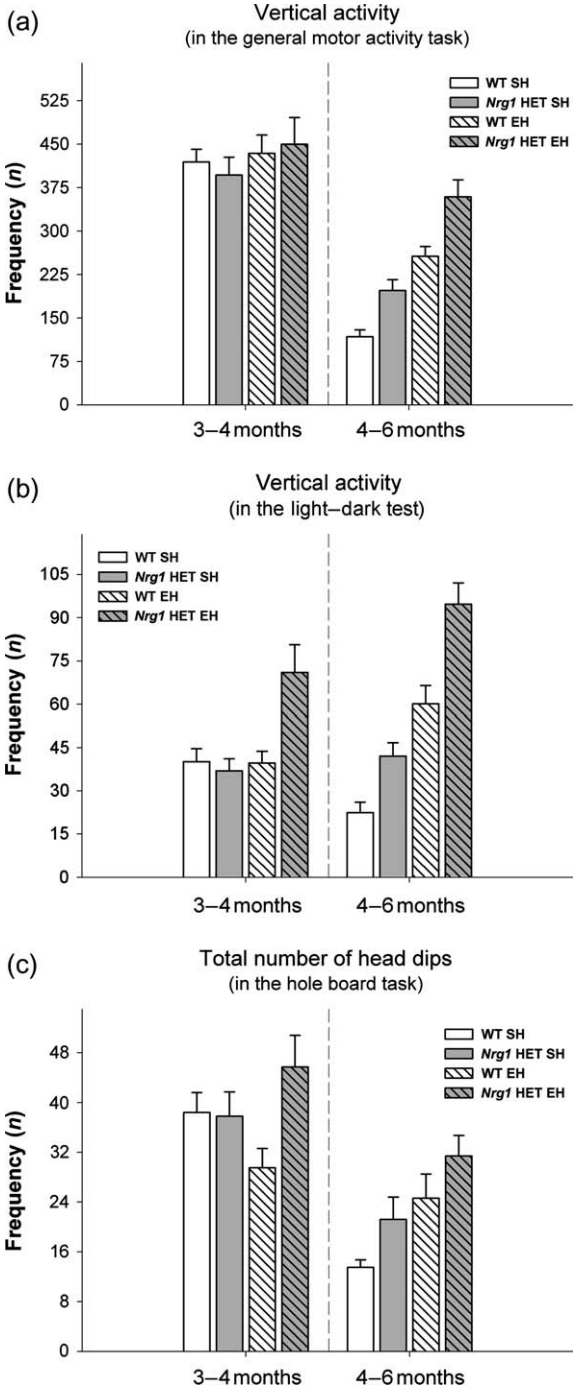

Figure 3: Exploration: Vertical activity (*n*) in (a) the GMA paradigm and (b) the LD test and (c) total number of head dips in the HB task are shown. Three-way ANOVA showed a significant effect of genotype, age (strong trend: $P = 0.07$ for LD) and housing and a significant genotype × age interaction (for GMA) and genotype × housing interaction (for LD) on vertical activity in GMA and LD. In the HB task, statistical analyses detected a significant effect of genotype and age and a significant genotype × housing for 3- to 4-month-old mice. All data are presented as means + SEM.

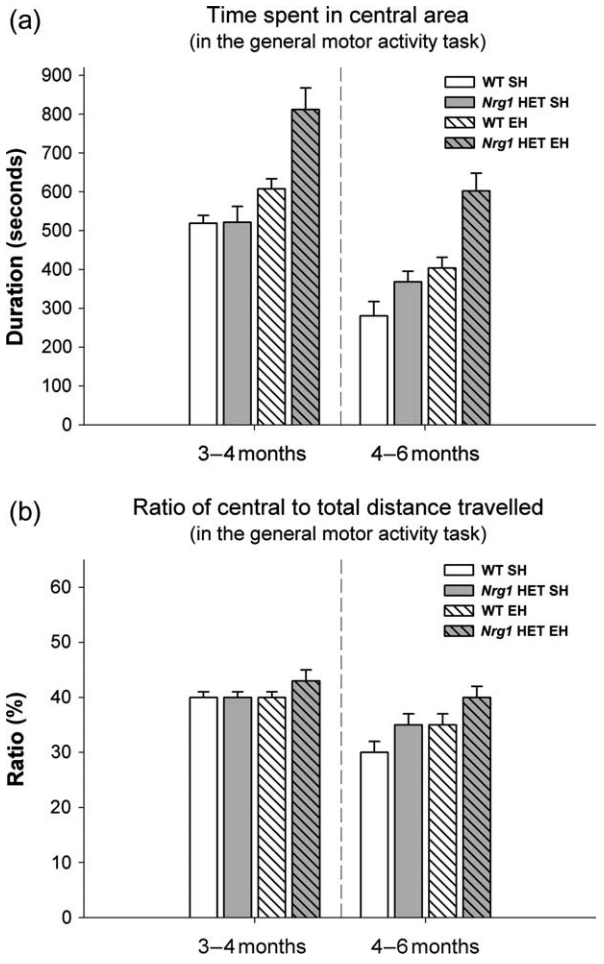

Figure 4: Anxiety-related parameters in GMA: (a) Time spent in the central zone of the arena (seconds) (zone time) and (b) ratio of central to total distance travelled (%) (ratio) are presented. Three-way ANOVA showed a significant effect of the factors genotype, age and housing for both parameters and a significant genotype × housing interaction for zone time. All data are presented as means + SEM.

(Fig. 5a, b), we found an interesting differential effect of genotype × housing on the time spent on open arms of the EPM in our animal model [three-way ANOVA: $F(1,83) = 4.4$, $P = 0.04$; Fig. 5a], showing that 4- to 6-month-old *Nrg1* HET mice were more susceptible to the anxiety-suppressing actions of enriched housing than control mice. In addition, three-way ANOVA showed a trend for genotype on the distance ratio in the LD test [$F(1,83) = 3.8$, $P = 0.06$; Fig. 5b].

Discussion

We observed a locomotion- and exploration-related hyperactive phenotype in heterozygous *Nrg1* transmembrane domain mutant mice. Increased age had a locomotion- and exploration-inhibiting effect on mice. Importantly, this suppression

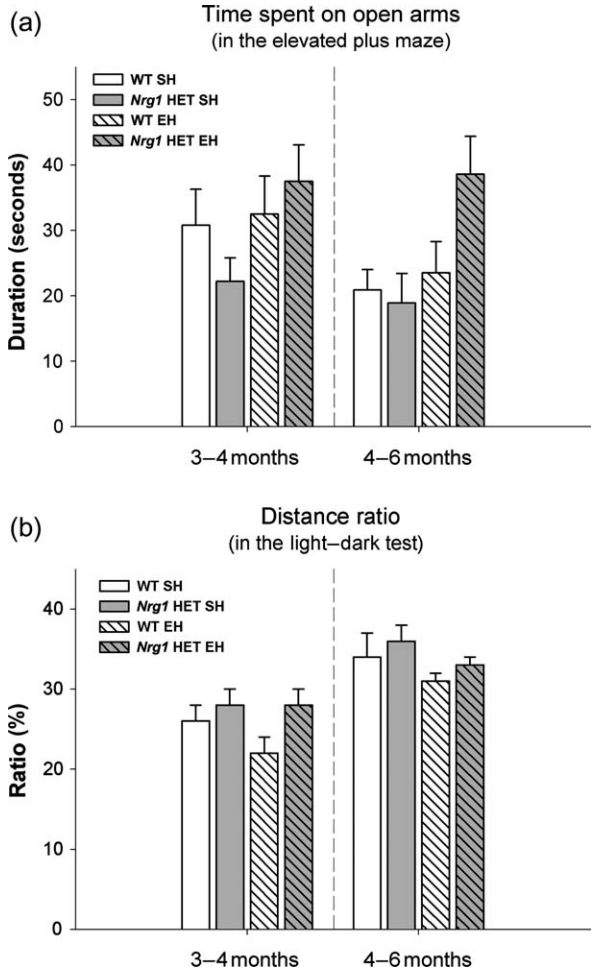

Figure 5: Anxiety-related parameters in EPM and LD: (a) The time spent on the open arms (seconds) of the EPM and (b) ratio of distance travelled in the light compartment to total distance travelled (cm) (distance ratio) in the LD are shown. Three-way ANOVA showed a significant genotype × housing interaction for time spent on open arms of the EPM and a strong trend for genotype on the distance ratio in the LD. All data are presented as means + SEM.

was significantly attenuated in Nrg1 hypomorphs. Environmental enrichment had a stimulating influence on locomotion and exploration. The increased explorative-like phenotype of animals kept in enriched housing was more pronounced in *Nrg1* HETs. In addition, we found an increased habituation of the locomotive response to a novel environment in *Nrg1* HETs compared with control mice. Heterozygous *Nrg1* mutant mice also showed a moderate task-specific anxiolytic-like phenotype in the GMA paradigm. In this context, ageing and deprived housing conditions (i.e. standard laboratory housing) elevated anxiety levels. Interestingly, *Nrg1* mutant mice were more susceptible to the anxiolytic-like effects of enriched housing than WT-like mice. Thus, our detailed multi-tiered phenotyping strategy using heterozygous transmembrane domain *Nrg1* mutant animals and integrating the external factors age and housing condition significantly extends the findings of two recent studies on the same Nrg1 isoform mutant line (Stefansson *et al.* 2002; O'Tuathaigh C *et al.*2006b). Furthermore, it has shown a more complex hyperactive and anxiety-related phenotype, which is affected by external modifications.

Locomotion-related hyperactivity in animal models is a behavioural characteristic considered to be relevant to the psychotic features of schizophrenia. NMDA antagonists (e.g. MK-801 or ketamine) and DA agonists (e.g. amphetamine) induce a broad spectrum of schizophrenia-like symptoms in humans and animals (O'Tuathaigh C *et al.* 2006a), including an increase in locomotor activity (Wong & Van Tol 2003; Javitt & Coyle 2004). Phenotyping studies using mouse models for NMDA receptor subunits, DA receptor subtypes and the dopamine transporter (DAT) support the idea that the dopaminergic and especially glutamatergic system can be involved in rodent hyperactivity (Smith *et al.* 1998; Dulawa *et al.* 1999; Mohn *et al.* 1999; Zhuang *et al.* 2001). Importantly, Nrg1 up-regulates the expression of NMDA receptor subunits and may modulate the kinetic properties of NMDA channels (Ozaki *et al.* 1997; Stefansson *et al.* 2004). In line with this, Nrg1 hypomorphic mice exhibit a reduced expression of functional NMDA receptors (Stefansson *et al.* 2002). Accordingly, reduced NMDA receptor levels in heterozygous *Nrg1* mutant animals may be responsible for their observed hyperactive phenotype – either directly or indirectly by facilitating the sensitization/activation of the dopaminergic system (Duncan *et al.* 1999).

Although the glutamatergic system may not be directly involved in the moderately reduced task-specific anxiety state of Nrg1 hypomorphic mice (Ikeda *et al.* 1995; Mohn *et al.* 1999), findings from knockout mice for the dopamine D_{1A} and D_4 receptor indicate that the dopaminergic system is involved in the anxiety-related behavioural response of mice to novelty (Smith *et al.* 1998; Dulawa *et al.* 1999). Interestingly, hyperdopaminergic DAT knockdown mice exhibit increased exploration of a novel stimulus confirming that overstimulation of the dopaminergic system influences the anxiety-related response to novelty in rodents. Therefore, the NMDA receptor hypofunction in the *Nrg1* HETs could result in an increased activation of dopaminergic pathways (Duncan *et al.* 1999), leading at least partially to the described anxiolytic-like phenotype in these mice. Further research should concentrate on site-specific expression analyses of DA and its

different receptor subtypes in heterozygous *Nrg1* transmembrane domain mutant mice.

The finding of an age-dependent locomotion- and exploration-related hyperactivity in the *Nrg1* mutant mice was strengthened by a preliminary study, in which the standardized housed animals of set 1-2 were screened not only at the age of 3–4 months but also at 4–6 months of age. Similar to the naively tested 4- to 6-month-old mice of sets 5 and 6, the repeatedly tested 4- to 6-month-old *Nrg1* mutants (set 2) showed a hyperexplorative-like phenotype compared with their WT littermates (set 1). Furthermore, a separate cohort of WT and *Nrg1* mutant mice was tested at the age of 4–6 months and again at the age of 10 months. Exploration-related hyperactivity was evident in Nrg1 hypomorphic animals at both ages (Figure S1). Thus, the age-dependent hyperactive phenotype in *Nrg1* mutants seems to be long term and independent of test pre-experience. Importantly, both previous studies using this Nrg1 transmembrane domain mutant mouse model (Stefansson *et al.* 2002; O'Tuathaigh C *et al.* 2006b) provide indirect support for our findings as both tested animals at the age of 5 months or older. It is known that ageing can reduce rodent motor activity and exploration (Sprott & Eleftheriou 1974; Goodrick 1975; Lamberty & Gower 1992), which may be related to altered dopaminergic neurotransmission (Lalonde 2002) and reduced expression of the GABAergic synthetic enzyme glutamic acid decarboxylase, the rate-limiting step in GABA synthesis (Frick *et al.* 2002). Furthermore, older mice are reported to have elevated anxiety levels (Chen *et al.* 2004). However, these observations are based on aged mice (20–25 months), whereas our mice were 3–6 months old. One explanation may be that their age-dependent characteristics are related to a phenomenon in rats, which show a peak in susceptibility to NMDA receptor hypofunction at the age of four months (Farber *et al.* 1995). Assuming our 4-month-old Nrg1 hypomorphs show a similar peak, NMDA receptor hypofunction may abolish GABAergic inhibition and release excessive excitatory activity. This could result in damaged cortical neurons (neurotoxic effect), thereby influencing the onset of psychotic-like symptoms (Farber *et al.* 1995). A second hypothesis for our age-dependent characteristics (Feinberg 1982; Duncan *et al.* 1999) considers a two-stage model, in which genetic factors (such as a *Nrg1* reduction) could lead to a failure of normal neuronal development, migration and synaptogenesis. In early adulthood, redundant synaptic connections are eliminated through synaptic pruning affecting glutamatergic synapses. Because Nrg1 is a key player in synaptic activation and expression (Keshavan *et al.* 1994), excessive pruning could be evident in mice with reduced levels of this gene. Future research will explore whether differential developmental modification processes occur between WT-like and Nrg1 mutant mice at the critical age of 3–6 months. We will focus on the glutamatergic and dopaminergic system and the expression profile of the different Nrg1 isoforms during this period.

It is well established that enriched housing of laboratory animals such as mice and rats results in various molecular and cellular changes in the central nervous system (CNS) (especially cortex and hippocampus), including enhanced neuro-, glio-, angio- and synaptogenesis and altered gene expression (Wurbel 2001; Kempermann *et al.* 2002; Spires & Hannan 2005). Furthermore, EE plays a significant role in learning and memory (van Praag *et al.* 2000), can reduce anxiety levels (Chapillon *et al.* 1999), and increase exploratory and locomotor activity (Roy *et al.* 2001; Benaroya-Milshtein *et al.* 2004). It also modifies or even rescues knockout-specific impairments of genetic animal models (van Dellen *et al.* 2000; Rampon *et al.* 2000). One major issue with the use of EE in medical research is standardization. Most studies use different, unspecific environmental structures and toys, continuously changing the composition and collection of objects throughout the experimental period (e.g. Rampon *et al.* 2000; Zhu *et al.* 2006). Furthermore, researchers use different cage sizes for standardized and enriched housing conditions, which on its own could have an impact on the animals' performance (see Olsson & Dahlborn 2002). For this reason, we selected a highly standardized enrichment design using identical cage sizes and a consistent EE composition. In addition, we limited the enrichment components to a minimum to show that EE can be an affordable and standardized procedure even for large animal facilities.

Our results support the notion that even limited EE stimulates motor activity and exploration and produces an anxiolytic-like phenotype in mice, suggesting that novelty may not be an essential component of EE (Kempermann *et al.* 2002). This is important, given that the stress-protective impact of EE on mouse behaviour is still discussed controversially (Chapillon *et al.* 1999; Benaroya-Milshtein *et al.* 2004; Brenes Saenz *et al.* 2006; Zhu *et al.* 2006). In addition, our minimal enrichment design had a beneficial effect on the motor activity-suppressant effects of age, confirming findings from a previous study (van Praag *et al.* 2000). The biochemical mechanisms underlying the differential potency of EE on *Nrg1* HETs and WT mice have to be investigated in further detail, although we hypothesize that EE has an impact on dopamine and glutamate circuits as well as directly affecting Nrg1 expression. The phenotype-strengthening effects of EE in this animal model are inconsistent with previously described phenotype-rescuing characteristics (van Dellen *et al.* 2000; Olsson & Dahlborn 2002; Spires & Hannan 2005). Importantly, the Nrg1 hypomorphs may respond differentially to, or even be disadvantaged by, the exposure to a more complex environment as a potential animal model for schizophrenia would be expected to be more reactive to changes in the environment. Furthermore, because EE was in place from birth onwards, we cannot rule out a differential impact of maternal behaviour on the performance of mice kept in SH and EH. Ongoing investigations will help to clarify these issues.

The heterozygous *Nrg1* transmembrane domain mutant mouse has been proposed as an animal model for schizophrenia (Stefansson *et al.* 2002; O'Tuathaigh *et al.* 2006b). Consistent with its potential role in schizophrenia, Nrg1 plays a critical role in how the brain adapts and responds to the environment. It is involved in the expression and function of CNS neurotransmitter receptors for NMDA and GABA, has an impact on glial cell activation and regulates myelin gene expression (Stefansson *et al.* 2004). Importantly, impaired neuronal development and myelination has been described in schizophrenia patients (Hakak *et al.* 2001). Furthermore, the expression profiles of various isoforms of Nrg1 mRNA are

altered in the post-mortem dorsolateral prefrontal cortex and hippocampus of schizophrenia patients (Hashimoto *et al.* 2004; Law *et al.* 2006) and the *NRG1* genotype may be related to the response to antipsychotic treatment (Kampman *et al.* 2004). Further research should target more schizophrenia-specific behavioural domains and focus not only on positive but also on the negative symptoms of schizophrenia (Ellenbroek & Cools 2000; van den Buuse *et al.* 2005). Including the use of female mice would enable the exploration of the sexually dimorphic phenotype in more detail (O'Tuathaigh C *et al.* 2006b). Together, these varied approaches will help to address the question of whether the *Nrg1* HETs are an adequate and valid model for some features of schizophrenia.

In conclusion, we detected an effect of Nrg1 on locomotion, exploration and task-specific anxiety. These behavioural domains are not specific characteristics of schizophrenia *per se*, but the increased behavioural sensitivity of *Nrg1* HET animals to exogenous factors is consistent with the pathophysiology of schizophrenia and the neurodevelopmental theory. Interestingly, *Nrg1* mutants exhibited altered habituation mechanisms, which are probably related to disruptions to hippocampal development and function (Okada & Corfas 2004; Clark *et al.* 2005; Lee *et al.* 2005). The disruption of such processes and impaired processing of novel stimuli have long been considered relevant to the cognitive deficits observed in schizophrenia (Grossberg 2000). There are no animal models that capture all the features of schizophrenia. However, the heterozygous *Nrg1* mutant animal model may – in combination with other genetic and drug models and the use of multitiered phenotyping approaches – help us to clarify certain pathophysiological mechanisms behind this complex mental disorder.

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Supplementary material

The following supplementary material is available for this article:
Figure S1: Age-dependent exploration in the light–dark test
This material is available as part of the online article from:
http://www.blackwell-synergy.com/doi/abs/10.1111/j.1601-1848.2006.00298.x
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