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Phenotypic characterisation of spatial cognition and social behaviour in mice with 'knockout' of the schizophrenia risk gene neuregulin 1

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Abbreviations: ANOVA, analysis of variance; CNS, central nervous system; DA, dopamine; EGF, epidermal growth factor; GABA, gamma-aminobutyric acid; Ig, immunoglobulin; KO, knockout; mRNA, messenger ribonucleic acid; nAChR, nicotinic acetylcholine receptor; NMDA, N-methyl-D-aspartic acid; NRG1, neuregulin 1; PCR, polymerase chain reaction; PFC, prefrontal cortex; TM, transmembrane; TMc, C-terminal transmembrane; TMn, N-terminal transmembrane; WT, wildtype.

Abstract- Neuregulin-1 (NRG1) has been identified as a candidate susceptibility gene for schizophrenia. In the present study the functional role of the NRG1 gene, as it relates to cognitive and social processes known to be disrupted in schizophrenia, was assessed in mice with heterozygous deletion of transmembrane (TM)-domain NRG1 in comparison with wildtypes (WT). Social affiliative behaviour was assessed using the sociability and preference for social novelty paradigm, in terms of time spent in: (i) a chamber containing an unfamiliar conspecific vs an empty chamber (sociability), or (ii) a chamber containing an unfamiliar conspecific vs a chamber containing a familiar conspecific (preference for social novelty). Social dominance and aggressive behaviour was examined in the resident-intruder paradigm. Spatial learning and memory was assessed using the Barnes maze paradigm, while spatial working memory was measured using the continuous variant of the spontaneous alternation task. Barnes maze data revealed intact spatial learning in NRG1 mutants, with elevated baseline latency to enter the escape hole in male NRG1 mutants reflecting an increase in activity level. Similarly, although a greater number of overall arm entries was found, spontaneous alternation was unaffected in NRG1 mice. Social affiliation data revealed NRG1 mutants to evidence a specific loss of WT preference for spending time with an unfamiliar as opposed to a familiar conspecific. This suggests that NRG1 mutants show a selective impairment in response to social novelty. While spatial learning and working memory processes appear intact, heterozygous deletion of TM-domain NRG1 was associated with disruption to social novelty behaviour. These data inform at a novel phenotypic level on the functional role of this gene in the context of its association with risk for schizophrenia.

Keywords: targeted gene deletion, mutant model, behavioural phenotype, social interaction, spatial working memory, psychosis

INTRODUCTION

Though schizophrenia is usually recognized and diagnosed in terms of positive symptoms such as hallucinations and delusions, evidence now indicates that cognitive deficits such as impairment in working memory, together with negative symptoms such as social incapacity, are primary determinants of holistic dysfunction (Thaker and Carpenter, 2001; Freedman, 2003; Bowie and Harvey, 2005; Addington et al., 2005; Malla and Payne, 2005; Waddington et al., 2006). Several genes have now been associated with risk for schizophrenia, in a manner suggesting the involvement of a number of genes of small effect (Harrison and Weinberger, 2005; Owen et al., 2005; Karayiorgou and Gogos, 2006). Among these, there is now strong evidence that neuregulin 1 [NRG1] is a risk gene for schizophrenia, although both the specific risk alleles and possible pathogenetic mechanism(s) are poorly understood (Stefansson et al., 2002; Corvin et al., 2004; Harrison and Law, 2006; Munafo et al., 2006).

The neuregulins are a family of growth factors whose effects are mediated via four neuregulin genes [NRG1-4] that bind to the ErbB family of tyrosine kinase transmembrane receptors [ErbB1-4]. Fifteen distinct isoforms of the NRG1 gene have been identified to date, and these isoforms have, until recently, been classified as Type I-III, depending upon N-terminal sequence, whether the isoforms express the α or ß epidermal growth factor [EGF]-like domain and whether they contain a transmembrane [TM] region (Falls, 2003; Harrison and Law, 2006). NRG1 types I and II contain one TM region, referred to as the C-terminal TM domain [TMc], while NRG type III contains an N-terminal TM domain [TMn] in addition to TMc. Types I and II NRG1 also contain

an immunoglobulin [Ig] domain and are designated Ig-NRG1. Two novel and as yet uncharacterised isoform types, designated Type IV-VI, have recently been identified (Steinthorsdottir et al., 2004; Law et al., 2006). NRG1 expression in the central nervous system [CNS] has been detected in many regions including the prefrontal cortex [PFC], hippocampus, cerebellum and substantia nigra, in both rodents (Kerber et al., 2003) and humans (Law et al., 2004). Numerous roles for NRG1 in CNS development and function have been identified, including synapse formation, neuronal migration, synaptic plasticity and the regulation of neurotransmitter expression and function (Falls, 2003; Harrison and Law, 2006).

It has been suggested that NRG1 polymorphisms associated with schizophrenia may do so via modulation of gene expression levels. One such polymorphism, SNP8NRG243177, originally identified as part of the so-called deCODE haplotype, has been found to be specifically related to disruption of normal frontal and temporal lobe function, premorbid intelligence levels and the emergence of psychotic symptoms (Hall et al., 2006). This NRG1 polymorphism has also be found to be associated with increased expression of the type IV transcript in postmortem brains of patients with schizophrenia (Law et al., 2006). A number of post-mortem studies have evidenced altered expression ratios of Type I / Type II and Type II / Type III mRNA in the prefrontal cortex of patients with schizophrenia (Law et al., 2004; Hashimoto et al., 2004). Also, an association between a missense mutation in the TM domain of the NRG1 gene and the development of psychosis has recently been reported (Walss-Bass et al., 2006). However, any relationship between such findings and the pathophysiology and symptoms of schizophrenia is, as yet, unclear.

One important approach to clarifying the functional roles of genes such as NRG1 is through the phenotype of mice with gene deletion [‘knockout’] (Arguello and Gogos, 2006; Chen et al., 2006; O'Tuathaigh et al., 2007). Thus, phenotypic characterisation of mice containing deletion of the TM domain of the NRG1 gene may inform on the involvement of this gene in the expression of a schizophrenia-like phenotype. Targeted deletion of NRG1 or its ErbB receptor results in midembryonic lethality, with homozygotes dying due to heart defects at embryonic day 10.5–11.5; however, heterozygous mice are viable and fertile (Gerlai et al., 2000; Stefansson et al., 2002). We have recently shown that heterozygous TM-domain NRG1 ‘knockouts’ display sex-specific abnormalities in the process by which individual elements of behaviour in the mouse repertoire change and interchange over an extended time-frame of interaction with the environment, from initial exploration, through habituation to quiescence (O’Tuathaigh et al., 2006). Other studies have reported heterozygous TM-domain ‘knockout’ to disrupt a number of behaviours of putative relevance to schizophrenia: impaired prepulse inhibition, spontaneous hyperactivity and reversal by clozapine of such hyperactivity (Stefansson et al., 2002). In contrast, no evidence for a hyperactive phenotype was observed in mutants heterozygous for an immunoglobulin domain-specific mutation of the NRG1 [Ig-NRG1] gene; however, these mutants displayed a putative selective attentional deficit (Rimer et al., 2005), suggesting a selective role for the TM-domain of the NRG1 gene in mediating features of a putative schizophrenia-like phenotype.

The purpose of the present experiments was to investigate whether heterozygous TM-domain NRG1 mutants demonstrate phenotypic differences across a variety of cognitive

and social interaction paradigms which access processes similar to those known to be disrupted in schizophrenia: spatial learning and working memory processes were assessed in the Barnes maze and spontaneous alternation memory tasks; social affiliative and aggressive behaviour was measured in the sociability and preference for social novelty and resident-intruder tasks.

EXPERIMENTAL PROCEDURES

Animals

TM-domain NRG1 heterozygous mutant mice were generated at the Victor Chang Cardiac Institute, University of New South Wales, Darlinghurst, Australia, as described previously (Stefansson et al., 2002), and maintained on a C57BL6 background [14 backcrosses]. Heterozygous [HET; $NRG1^{+/-}$] and wildtype [WT; $NRG1^{+/+}$] mutants were generated from heterozygous breeding pairs and genotyped using PCR analysis (O'Tuathaigh et al., 2006). They were housed in groups of 3-5 per cage and maintained on a standard 12:12 h light:dark cycle [08:00 on; 20:00 off] with ad libitum access to food and water. Mice used in these experiments were from litters of the same generational age. At time of testing, the mean body weight and age of NRG1 HETs [males: 29 ± 4 g, mean age 180 ± 32 days; females: 25 ± 3 g, mean age 167 ± 25 days] did not differ relative to WT [males: 31 ± 2 g, mean age 183 ± 28 days; females: 26 ± 3 g, mean age 158 ± 21 days]. These studies were approved by the Research Ethics Committee of the Royal College of Surgeons in Ireland and were conducted under licence from the Department of Health and Children in accordance with Irish legislation

and the European Communities Council Directive 86/609/EEC for the care and use of experimental animals.

Spontaneous alternation memory

The continuous variant of the Y-maze spontaneous alternation procedure was assessed during one 10 min session. The Y-maze apparatus consisted of three identical arms [40 × 12.5 × 40 cm]. Without prior habituation, each test mouse was placed at the centre of the Y-maze and allowed to move freely throughout the maze for a single 10 min period. Rodents possess a natural preference to explore areas previously un-explored; if a mouse has explored one arm of the Y-maze, it is not expected to enter the same arm during its next phase of exploration, but to enter one of the two alternate arms; this test has been suggested to measure several aspects of spatial working memory (Wall and Messier, 2002). A video camera, mounted centrally above the Y-maze, recorded each session and allowed alternation to be analysed using video tracking software [Ethovision®, Noldus Inc., the Netherlands]. Spontaneous alternation was defined as successive entries into the three arms, in overlapping triplet sets. It is expressed as a percentage and refers to the ratio of arm choices differing from the previous two choices to the total number of arm entries: percent alternation = [(number of alternations/total number of arm entries) – 2] x 100 (Wall and Messier, 2002). The total number of subjects was 42: 21 WT [10 male, 11 female] and 21 HET [10 male, 11 female].

Barnes maze

The Barnes maze paradigm exploits the natural inclination of small rodents to seek escape to a darkly lit, sheltered environment when placed in an open arena under bright, aversive illumination (Holmes et al., 2002). The maze comprised a white, wooden circular platform [1.3m in diameter], with a black cardboard perimeter wall [height 27cm], raised 45 cm above the floor. On the inner surface of this perimeter wall, but not directly over any one maze hole, were affixed four visuospatial cues made of rigid yellow paper [rectangle, circle, cross, triangle]; this increases the spatial component of the Barnes maze during training (Bach et al., 1995). Twenty circular holes [diameter 4.5 cm] were located equidistant around the perimeter of the platform. Two of these holes, located 180° apart, and therefore directly opposite to each other, led via ramps to escape boxes [9.5 × 9.5 × 12 cm]. Either one of these two escape boxes could be blocked by a barrier to leave a single functional escape box, into which was placed litter from the home cage of each test mouse and attractive food [Honey Loops ©, Kelloggs Inc., Battle Creek, MI, USA] as positive stimuli for entry into the escape box. The remaining 18 holes and the blocked escape box each led only to a false 'box' which, from the platform, appeared indistinguishable from an escape box but was too small to be entered; false boxes removed visual cues that might be observed through an open hole. Above the platform were two halogen lamps [height 47 cm, 180º apart] which gave bright illumination of the maze without causing shadows. A video camera, mounted above the platform, recorded each session.

(a) *Pre-training*: 24 hours prior to training, with room lights on and halogen lamps turned off, each test mouse was placed in a small glass start box in the centre of the platform for 1 min and then released. During the following 5 min the mouse was allowed to explore

the platform with 20 false boxes, i.e. with each of the two escape boxes blocked. After removing the mouse from the apparatus, one of the two escape boxes was then unblocked; the location of the unblocked escape box remained constant throughout the training sessions for a given mouse but was alternated *between* mice in a counterbalanced manner. The mouse was then placed for a further 5 min in a Plexiglas enclosure directed towards the escape box assigned to it and allowed to climb down into the escape box. If the mouse did not find the escape box within 5 min, the experimenter guided the mouse to the escape box, where it was allowed to remain for a further minute. The subject was then removed from the escape box and returned to its home cage. The arena was wiped clean using detergent [5% Virkon © diluted in water] both between each training session for a given mouse and between each mouse.

(b) *Training and testing*: On each day of training, with room lights off and halogen lamps on, the test mouse was placed in the start box for 1 min and then released. The mouse was then given 5 min to enter the escape box. A trial was terminated when the mouse had either entered the escape box or 5 min had passed; if the mouse did not find the escape box within 5 min, the experimenter removed the mouse and placed it into the escape box. Each mouse was given 3 trials per day, with an inter-trial interval of 2 h, over 5 consecutive days. Escape latency [time (sec) taken to find and enter escape box with front paws and trunk], total distance travelled prior to escape [distance (cm) moved before entering escape box] and total number of errors [number of complete insertions of front paws and trunk into an incorrect escape box] were analysed using video tracking software [Ethovision®, Noldus Inc., the Netherlands]. The total number of subjects was 52: 25 WT [13 male, 12 female] and 27 HET [13 male, 14 female].

Resident-intruder test

Following one week of single housing, mice were tested for social dominant and aggressive behaviour in the resident intruder paradigm. An intruder mouse [unfamiliar age-, weight-, and sex-matched C57BL6; four of each sex were used during testing] was placed in the resident's home cage. A transparent Plexiglas cover was placed on top of the home cage and each test session [10 min duration] was recorded on videotape using a camera placed 50 cm above the home cage. Order of testing was counterbalanced across the entire session such that WT and HET mice were exposed equally to each of the intruder mice, thereby minimising the possibility that phenotypic changes in behaviour might be related to intruder-related factors. After testing, behaviours were coded and quantified from videotapes using commercially-available behavioural analysis software [Observer Video Pro ©, Noldus inc., the Netherlands] by an observer blind to both sex and genotype. The social investigative and aggressive behaviors scored were: number of episodes and total time mice spent engaged in anogenital sniffing [time spent actively sniffing the partner's anogenital area] and non-agonistic social behaviors, including non-anogenital sniffing, grooming, following, or standing, sitting and lying down next to each other. The aggressive behaviours scored were: biting, pinning, aggressive following, tail-rattling. The total number of subjects was 40: 20 WT [10 male, 10 female] and 20 HET [10 male, 10 female].

Sociability and preference for social novelty

Social approach/avoidance behaviour was assessed using a recently developed procedure which provides a simple and easily quantifiable measure of affiliative behaviour in mice (Brodkin et al., 2004; Moy et al., 2004; Sankoorikal et al., 2006).

The apparatus was a rectangular, three-chambered box [left and right chambers 13.5 × 20 × 20 cm; centre chamber 9 × 20 × 20 cm; total size 36 × 20 × 20 cm]. Dividing walls were made from clear Plexiglas, 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 [see Fig. 1]:

(a) *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 10 minutes with access to the left and right chambers denied by Plexiglas doors.

(b) *Stage 2: Sociability*

Following Stage 1, an unfamiliar C57BL6 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 × 10 × 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 twenty four hours 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 minutes. 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.

(c) *Stage 3: Preference for social novelty*

Following Stage 2, each test mouse was immediately returned to the centre chamber and the doors to the side chambers were closed. There followed a second 10 min session to quantify *social novelty* preference towards 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 allowed to leave the centre chamber and explore all three chambers of the apparatus for a second period of 10 minutes; it could therefore distribute its behaviour between the centre chamber, the chamber containing the previously investigated and now familiar mouse [*Stranger 1*] or the opposite chamber containing the novel, unfamiliar mouse [*Stranger 2*]. All other parameters and measures were as described above for stage 2. The total number of subjects was 40: 20 WT [10 male, 10 female] and 20 HET [10 male, 10 female].

Test of anosmia

Olfaction is essential for social recognition and social cognition in mice, such that the assessment of behaviour in such social paradigms is reliant upon intact olfactory

function. To determine whether anosmia might be a feature of the NRG1 mutant phenotype, olfactory function was assessed in the buried food localisation paradigm (Alberts and Galef, 1971; Stowers et al., 2002). For four days prior to testing, mice were introduced to a 23 h food restriction regimen and habituated to eat carbohydrate-rich snacks [Honey Loops©, Kelloggs Inc., Battle Creek, MI, USA] by placing 3 g in the home cage overnight. The test was conducted between 09.00 and 16.00 in clear glass cages [36 x 20 x 20 cm]. One of the corners of the cage was randomly selected as the area in which a sample of snack food would be buried. Location of the sample was counterbalanced across test sessions. The sample was buried by placing it on the floor of the cage 6 cm from the corner point, towards the centre, and then covering the entire floor of the cage with bedding [wood shavings] to an even depth of 2.0-2.5 cm. One mouse was then placed in the centre of the cage and latency to find the snack and commence eating was timed. A maximum time limit of 10 min was used; if the mouse had not located the food by this time, it was allocated the highest time score [i.e. 10 min]. The total number of subjects was 40: 20 WT [10 male, 10 female] and 20 HET [10 male, 10 female].

Statistical analysis

As described previously (O'Sullivan et al., 2006; O'Tuathaigh et al., 2006), data were subjected to square root transformation and analysed using two-way analysis of variance [ANOVA] with main factors of genotype [WT, HET] and sex [male, female]. For the sociability and preference for social novelty paradigms, a between-subjects ANOVA was carried out separately for each stage of the test. In the case of the Barnes maze, a repeated

measures ANOVA was used with genotype and sex as the between subjects factors and time [i.e. days of training] as the within subject factor. Spontaneous alternation data were analysed as (a) cumulative alternation percentage quantified over the 10 min session and (b) percentage alternation quantified over 5 x 2 min. time bins. Repeated measures ANOVA was used in the case of the latter measure in order to examine the interaction between time and main factors of genotype and sex; between-subjects ANOVA was used in the case of the former measure. Where appropriate, *post-hoc* comparisons were carried out using independent or paired t-tests, corrected for multiple comparisons. All statistical analysis was carried out using the SPSS software package [Version 14, SPSS Inc., Chicago, IL, USA].

RESULTS

Spontaneous alternation performance

NRG1 mutant mice alternated between the arms of the maze to the same extent as WT when examined over 5 x 2 min time bins or as a single, cumulative measure over 10 min [Fig. 2a & b: no effects of time, genotype or sex; no genotype × sex, genotype x time or genotype x sex x time interactions]. As expected on the basis of previous reports of hyperactivity in TM-domain NRG1 HETs (Stefansson et al., 2002; O'Tuathaigh et al., 2006), NRG1 mutants exhibited an increased number of arm entries relative to WT [Fig. 2c; effect of genotype, $F(1, 38) = 4.98$; $P < 0.05$; no effect of sex or genotype × sex interaction].

Barnes maze

Latency to enter the escape hole decreased across the five blocks of daily training, indicating that learning had occurred; this effect did not differ between the genotypes or between the sexes [Fig. 3a; effect of trial blocks, $F(4, 192) = 37.80$, $P<0.01$; no trial block × genotype or trial block × sex interactions, no trial block × genotype × sex interaction]. Analysis of between-group differences indicated overall escape latency to be higher in males than in females, with male NRG1 mutants evidencing higher escape latencies relative to female mutants and WT of both sexes [effect of sex, $F(1, 48) = 8.76$, $P<0.05$; genotype × sex interaction, $F(1, 48) = 6.33$, $P<0.05$; no effect of genotype].

In a complementary manner, number of errors decreased across the five days of training and this effect did not differ between the genotypes or between the sexes [Fig. 3b; effect of trial blocks, $F(4, 192) = 49.07$, $P<0.01$; no trial block × genotype or trial block × sex interactions, no trial block × genotype × sex interaction]. Analysis of between-group differences indicated number of errors to be higher in males than in females, with male NRG1 mutants evidencing more errors relative to female mutants and WT of both sexes [effect of sex, $F(1, 48) = 9.41$, $P<0.01$; genotype × sex interaction, $F(1, 48) = 5.06$, $P<0.05$; no effect of genotype]. In accordance with their greater escape latency and number of errors, male NRG1 mutants also evidenced more exploratory activity as indexed by greater overall distance traveled across the five days of training relative to female mutants and WT of both sexes [Fig. 3c; genotype × sex interaction, $F(1, 48) = 9.72$, $P<0.01$].

Resident-intruder test

More bouts of aggressive behaviours were recorded in NRG1 mutants than in WT; this effect did not differ between the sexes [Fig. 4a; effect of genotype, $F(1, 36) = 3.64$, $P < 0.05$; no effect of sex or sex × genotype interaction]. In a complementary, inverse manner, investigative sniffing was somewhat reduced in NRG1 mutants of both sexes [Fig. 4b; $P = 0.1$].

Sociability and preference for social novelty

During the *sociability* phase, mice spent more time in the chamber containing *Stranger 1* than in the opposite, empty chamber and spent least time in the centre chamber; this effect did not differ between the genotypes or between the sexes [Fig. 5a; effect of chamber, $F(2, 72) = 32.60$, $P < 0.01$; no chamber × genotype, chamber × sex or chamber × genotype × sex interactions]. However, consistent with their greater level of general activity, NRG1 mutants made more overall chamber entries relative to WT [effect of genotype, $F(1, 36) = 7.78$, $P<0.05$; no effect of sex or genotype × sex interaction]. No difference was observed between the genotypes in initial latency to enter either chamber [No effect of sex or genotype].

During the *social novelty* phase, WT spent more time in the chamber containing the new *Stranger 2* than in the opposite chamber containing the now familiar *Stranger 1* and least time in the centre chamber; this preference was lost in NRG1 mutants of both sexes, who spent similar times with *Stranger 1* and *Stranger 2* [Fig. 5b; chamber × genotype interaction, $F(2, 72) = 5.75$, $P<0.01$; no chamber × sex interaction, no chamber × genotype × sex interaction]. NRG1 mutants again made more overall chamber entries

relative to WT but this effect did not differ between the chambers [effect of genotype, $F(1, 36) = 5.56$, $P<0.05$; no chamber × genotype interaction].

Test of anosmia

Both NRG1 mutants and WT successfully located the carbohydrate-rich snack buried beneath the cage bedding with no difference in latency between the genotypes [Fig. 6]. No difference was observed between the genotypes in mean latency to locate the foodstuff [WT: 151 ± 24 s ; NRG1 HET: 155 ± 29 s] ; the snack was immediately consumed upon retrieval, indicating no effect on consummatory behavior.

DISCUSSION

The primary aim of the present studies was to characterise the functional role of the NRG1 gene as it relates to the social interaction and spatial cognition abnormalities observed in schizophrenia. It was found that heterozygous TM-domain NRG1 mutants exhibit a constellation of social interaction deficits suggestive of disruption to social recognition memory and/or discrimination of socially novel stimuli, with an increase in aggressive behaviour. Both spatial learning and memory, assessed in the Barnes maze, and spatial working memory, as measured by non-delay Y-maze alternation, were unaffected in NRG1 mutants relative to WT. Consistent with the previously reported hyperactive phenotype following disruption of NRG1 gene function (TM-domain: Stefansson et al., 2002; O'Tuathaigh et al., 2006; EGF-like domain: Gerlai et al., 2000), changes across certain performance measures in tests of spatial cognition, some of which

were sex-specific, were found to be attributable to an increase in basal activity levels in NRG1 mutants.

NRG1, spatial learning and working memory

It has been suggested (Koopmans et al., 2003) that different aspects of working memory are accessed in the two tasks applied: the short-term storage and retrieval of previous trial choices in Y-maze alternation and the retrieval, short-term storage and manipulation of goal-directed information (e.g. route planning) in the Barnes maze; specifically, Barnes maze performance is dependent upon the temporary storage of the remembered escape hole location while the mouse traverses the maze.

Analysis of Barnes maze data in NRG1 mutants revealed that rate of learning, as indexed by latency to reach the escape hole and number of errors committed across the five daily blocks of sessions, was unaltered. However, male NRG1 mutants showed increased latency to enter the escape hole across days 1-4 of training compared to all other experimental conditions. It has been noted that training performance of mice in the Barnes maze may be subject to considerable variability; in contrast with the water-based Morris maze, which examines similar memory processes, mice do not go directly toward the escape position but, rather, often inspect other holes briefly prior to entering the correct hole (Pompl et al., 1999). Barnes maze error data revealed that male NRG1 mutants investigated a greater number of 'false' escape holes relative to other groups across days 1-4 of training. This may reflect disruption to one or more of several processes, including impaired attention, increased exploration of a novel environment and/or decreased anxiety.

A study of the effect of prenatal cocaine exposure on Barnes maze performance suggested that group differences in attentional function might account for increased latency to enter the escape hole across initial learning sessions (Inman-Wood et al., 2000). Furthermore, Ig-domain NRG1 mutants exhibit a selective deficit in latent inhibition, a task which measures ability to ignore irrelevant stimuli (Rimer et al., 2005). Thus, elevated escape latencies in male NRG1 mutants might reflect sex-specific disruption to such attentional processes. Alternatively, using ethologically based assessment of all topographies of behaviour in the murine repertoire (Waddington et al., 2005), we observed in male TM-domain NRG1 mutants an increase in specific elements of exploration in an open-field environment (O'Tuathaigh et al., 2006). Thus, increases in escape latency, number of holes investigated and non-escape-directed activity, as indexed by distance travelled, might indicate a similar context- and sex-specific increase in exploration. While distinguishing between these possibilities and/or decreased anxiety is a topic for future studies, the present findings do not indicate a major role for NRG1 in spatial learning and working memory.

Similarly, no effect of genotype on alternation rate was observed in the Y-maze alternation task. As for the Barnes maze, a higher level of activity in NRG1 mutants was found, in terms of an increase in overall Y-maze arm entries. However, in contrast with the Barnes maze, this hyperactive phenotype was not found to be sex-specific. These data are in general agreement with a previous report in EGF-like-domain NRG1 mutants (Gerlai et al., 2000) of indistinguishable T-maze alternation, with faster time to complete fifteen alternation trials indicating a greater level of activity. However, the basis for increases in activity in NRG1 mutants being specific for males both at an ethological

level (O'Tuathaigh et al., 2006) and in the Barnes maze but similar for males and females in the Y-maze remains to be determined. Sex-specific phenotypic effects in mutants are increasingly recognised and likely reflect novel neuronal mechanisms that remain to be elucidated (Waddington et al., 2005; O'Tuathaigh et al., 2007; Yang et al., 2006). Additionally, the present data elaborate the argument that phenotypic effects in a given sex cannot be assumed to apply to the other sex unless demonstrated in that sex (Waddington et al., 2005; O'Tuathaigh et al., 2007), by indicating that sex specificity in phenotype can depend on unknown differences between paradigms that are presumed to access the same or similar processes.

NRG1 and sociability vs preference for social novelty

In the *sociability* phase, NRG1 mutants distributed their time between the chamber containing *Stranger 1*, the opposite, empty chamber and the centre chamber in a manner indistinguishable from WT; that is, NRG1 mutants shared the preference of WT for the chamber containing the novel conspecific rather than the empty chamber. Social approach behaviour in this *sociability* phase is likely attributable to a variety of occasionally conflicting, approach- and avoidance-related motivations (Brodkin et al., 2004), including social investigation, aggressive behaviour and defensive avoidance. In the resident-intruder test, we found NRG1 mutants to commit an increased number of aggressive incidents, with minimal disruption to affiliative behaviours. Thus, although NRG1 mutants and WT evidenced indistinguishable sociability behaviour in the present paradigm, differences in resident-intruder behaviours suggest that in NRG1 mutants

distinct phenotypic effects on conflicting motivating factors might oppose each other to sustain such sociability.

However, in the subsequent *social novelty* phase, NRG1 mutants distributed their time between the chamber containing *Stranger 1*, the opposite chamber containing *Stranger 2* and the centre chamber in a manner that distinguished them from WT; that is, NRG1 mutants lost the preference of WT to spend more time in the chamber containing the novel conspecific [*Stranger 2*] rather than in the chamber containing the now familiar conspecific [*Stranger 1*] and spent similar time in both chambers. An activity-based explanation of these findings is unlikely, as although NRG1 mutants made more chamber entries than WT, these excess entries were not distributed differentially across the chambers. Rather, NRG1 mutants may evidence disruption to recognition of *Stranger 1* as familiar; more specifically, detection of *Stranger 2* as socially novel in comparison with *Stranger 1* is dependent on retrieval of the socially based memory of the initial encounter with *Stranger 1* (Petrulis and Eichenbaum, 2003). Therefore, disruption to social novelty preference in NRG1 mutants might involve deficits in aspects of social recognition memory.

Alternatively, NRG1 mutants may be unable to detect and/or respond to the novelty status of *Stranger 2* in comparison with *Stranger 1*; more specifically, NRG1 mutants might be impaired in olfactory cues that are important determinant of social behaviour in rodents. Indeed, in the developing rodent brain the expression profiles of ErbB receptors and their ligands in the olfactory bulb suggest a putative role for neuregulin in olfactory bulb maturation (Anton et al., 2004; Bovetti et al., 2006), while in the adult rodent brain NRG1 is expressed in distinct layers of the olfactory bulb (Corfas et al., 1995; Longart et

al., 2004). However, using the buried pellet localization test, we found no difference between NRG1 mutants and WT in latency to locate a buried food pellet or in subsequent consumption of that pellet. This indicates no prominent disruption to olfactory function in NRG1 mutants. Thus, though these findings cannot exclude more subtle effects of NRG1 deletion on olfaction, they implicate other mechanisms.

NRG1 and schizophrenia

There is now a substantial body of evidence indicating NRG1 to be a risk gene for schizophrenia (Harrison and Law, 2006; Munafo et al., 2006). However, it far from clear how the genetic risk variants identified impact on neuronal function and behaviour. Disturbance in the documented roles of neuregulin in neural migration, synaptic development, synaptic plasticity and the regulation of neurotransmitter expression and function (Harrison and Law, 2006; Esper et al., 2006) would be compatible with contemporary theories of schizophrenia that involve early perturbation in brain development and disruption to synaptic connectivity in critical neuronal networks (Waddington et al., 1999; Andreasen, 2000; Harrison and Weinberger, 2005). Furthermore, there is initial evidence for dysregulation of neuregulin-ErbB4 function in schizophrenia (Hahn et al., 2006; Law et al., 2006).

However, schizophrenia appears to be an oligogenic rather than a single gene disorder, with several risk genes of small effect having been identified (Harrison and Weinberger 2005; Owen et al., 2005; Karayiorgou and Gogos, 2006). This raises a fundamental question: does each gene, including NRG1, contribute to overall risk for diagnosis of

schizophrenia, which usually follows the emergence of positive, psychotic symptoms, or do specific genes such as NRG1 influence risk for distinct aspects [endophenotypes] of the overall schizophrenia syndrome, for example cognitive deficits such as impairment in working memory or negative symptoms such as social incapacity? In the present study, a primary finding is that while NRG1 is not involved in choosing between social vs non-social investigation, it is critically involved in choosing between social investigation of a familiar vs a non-familiar conspecific.

Regarding mechanisms that might underlie this social deficit, N-methyl-D-aspartate [NMDA] hypofunction is a contemporary hypothesis for schizophrenia (Coyle and Tsai, 2004; Millan, 2005). Reduction in forebrain NMDA receptors has been reported in TM-NRG1 mutants (Stefansson et al., 2002) and abnormalities in social behaviour have been reported in hypomorphic NMDA mutants (Mohn et al., 1999; Duncan et al., 2004); however, these studies did not resolve sociability vs social novelty. There is also evidence for α7 nicotinic acetylcholine receptor [α7nAChR] dysregulation in schizophrenia (Leonard and Freeman, 2006; Olincy et al., 2006), for NRG1 modulation of α7nAChR currents (Chang and Fischbach, 2006) and for the involvement of the α7nAChR in social recognition (van Kampen et al., 2004). Regarding the long-standing dopamine [DA] hyperfunction hypothesis of schizophrenia (Kapur et al., 2005; Seeman et al., 2006), NRG1 can regulate aspects of DAergic neurotransmission (Yurek et al., 2004) and DAergic dysfunction, particularly in the medial prefrontal cortex, has been implicated in the detection of salient social and non-social stimuli (O'Tuathaigh et al., 2003; Bassareo et al., 2002; De Leonibus et al., 2006). However, while there is some evidence to suggest that DA plays a more significant role in the detection of novel,

unfamiliar stimuli rather than in the discrimination of conspecifics based on prior exposure (De Leonibus et al., 2006), the present findings indicate NRG1 to be involved in the latter process.

CONCLUSION

Mutants with heterozygous TM-domain deletion of the schizophrenia risk gene NRG1 showed largely intact spatial learning and memory but were characterised by an increase in aggressive behaviours and a specific social interaction deficit: when faced with a choice between investigating an unfamiliar mouse or an empty cage, the normal murine preference for sociability with the unfamiliar mouse is unaltered; however, when faced with a choice between investigating a unfamiliar mouse or a familiar mouse, the normal murine preference for sociability with the unfamiliar mouse is lost. These findings suggest a role for the NRG1 gene in mediating selective aspects of a schizophrenia-like phenotype.

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Figure Legends

Fig. 1. The sociability and social novelty test apparatus.

Fig. 2. Y-maze spontaneous alternation performance in NRG1 HET [10 male, 11 female] and WT [10 male, 11 female] mice. (a) Mean alternation ratios ± SEM across 5 x 2 min time bins [Time 1-5]; no significant difference was observed between the genotypes. (b) Mean cumulative alternation ratios ± SEM for the overall 10 min session. (c) Average number of arm entries ± SEM. NRG1 HET mutants made significantly more entries relative to WT; * $P < 0.05$ vs. WT.

Fig. 3. Barnes maze performance in NRG1 HET [13 male, 14 female] and WT [13 male, 12 female] mice across five blocks of training on successive days [3 trials per block]. (a) Mean latencies to enter the escape hole ± SEM. No significant difference in rate of learning was found between the genotypes; significantly higher overall latency in male

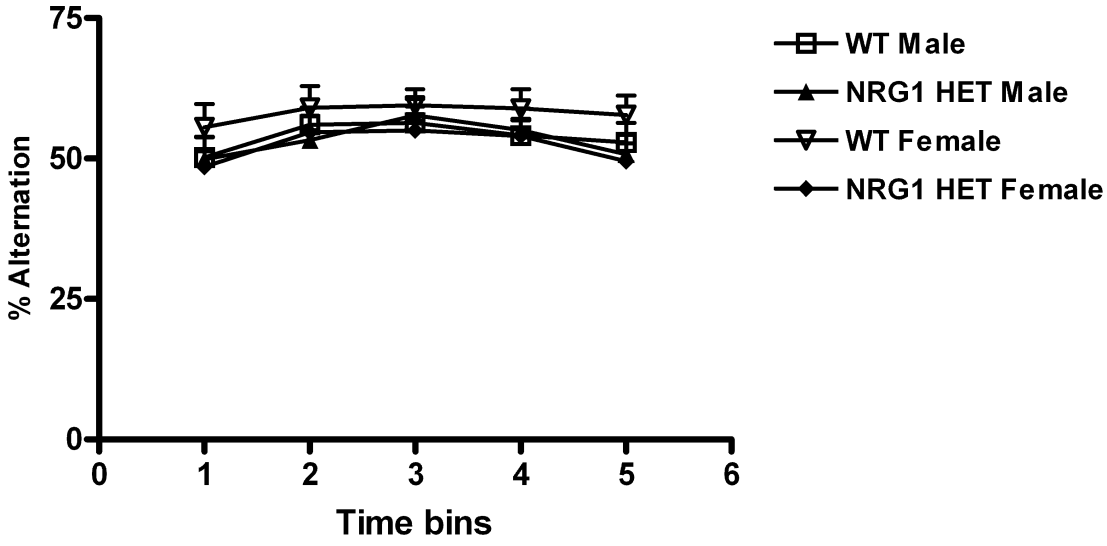
NRG1 mutants relative to the other three groups. (b) Mean number of errors ± SEM. No significant difference in decline in errors was found between the genotypes; significantly higher overall errors in male NRG1 mutants relative to the other three groups. (c) Mean distance travelled ± SEM. No significant difference in decline in distance travelled was found between the genotypes; significantly greater distance travelled in male NRG1 mutants relative to the other three groups.

Fig. 4. Resident-intruder test in NRG1 HET [10 male, 10 female] and WT [10 male, 10 female] mice. (a) Mean number of aggressive behaviours ± SEM by resident towards intruder mouse during a 10 min session. NRG1 mutants evidenced significantly more aggressive behaviours than WT; * $P < 0.05$ vs. WT. (b) Mean time ± SEM engaged in social investigative sniffing by resident towards intruder mouse during the same 10 min session.

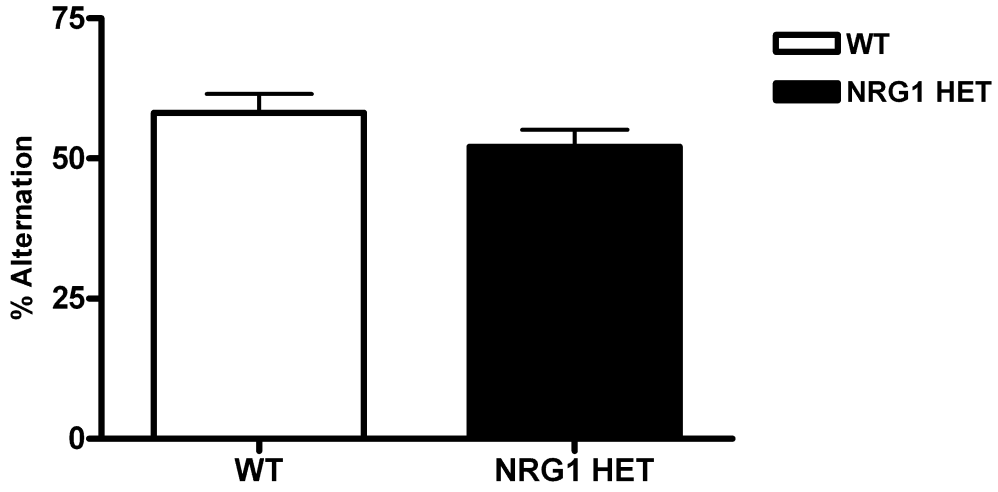
Fig. 5. Sociability and preference for social novelty in NRG1 HET [10 male, 10 female] and WT [10 male, 10 female] mice. (a) Mean time ± SEM spent in chamber containing *Stranger 1*, centre chamber and empty chamber. All groups spent more time in chamber containing *Stranger 1* relative to the other chambers. * * Preference for *Stranger 1* vs. empty chamber, $P < 0.01$ (b) Mean time ± S.E.M. spent in chamber containing [now familiar] *Stranger 1*, centre chamber and chamber containing [novel] *Stranger 2*. NRG1 mutants lost the preference of WT to spend more time in chamber containing *Stranger 2*. * * Preference for *Stranger 2* vs. *Stranger 1*, $P < 0.01$.

Fig. 6. Test of anosmia in NRG1 HET [10 male, 10 female] and WT [10 male, 10 female] mice. Mean latency to locate buried snack ± SEM did not differ between the genotypes.

(a)


75
50
25
0
% Alternation
0
1
2
3
4
5
6
Time bins
WT Male
NRG1 HET Male
WT Female
NRG1 HET Female

(b)


75
50
25
0
% Alternation
WT
NRG1 HET
WT
NRG1 HET

(c)

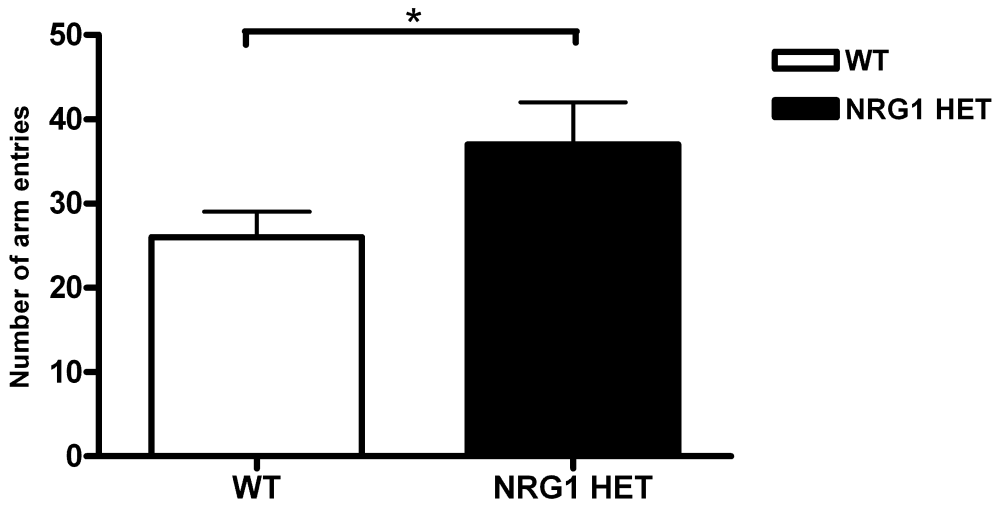
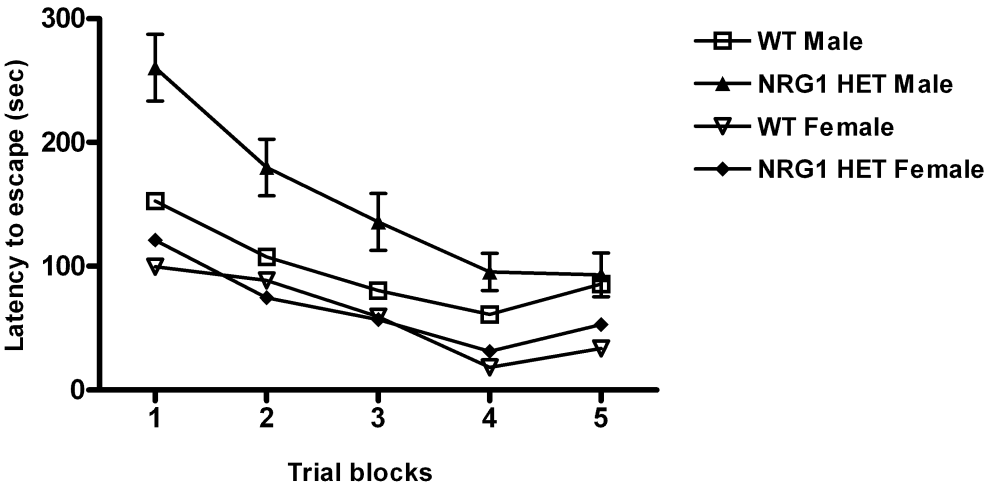
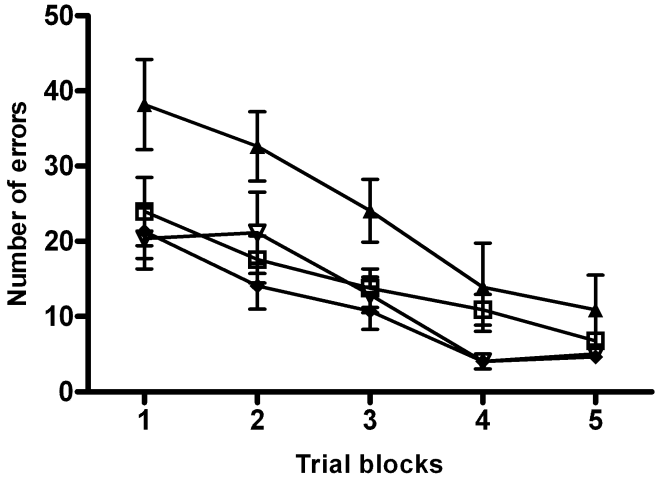

*
50
40
30
20
10
0
Number of arm entries
WT
NRG1 HET
WT
NRG1 HET

Fig. 2(a) & (b) & (c)

(a)


300
200
100
0
Latency to escape (sec)
1
2
3
4
5
Trial blocks
WT Male
NRG1 HET Male
WT Female
NRG1 HET Female

(b)


50
40
30
20
10
0
Number of errors
1
2
3
4
5
Trial blocks

(c)

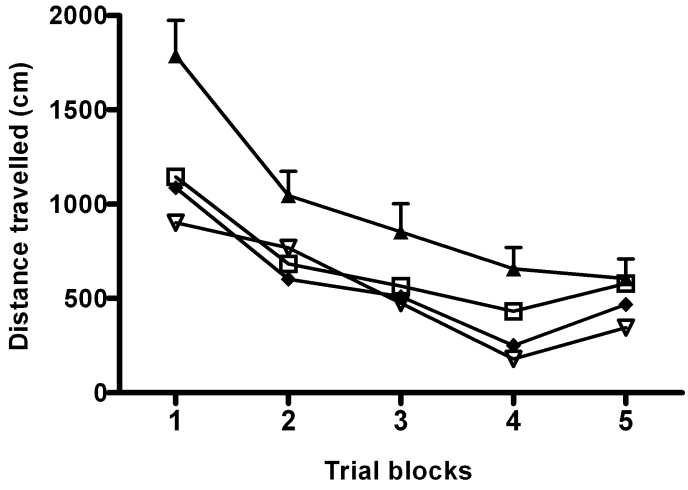
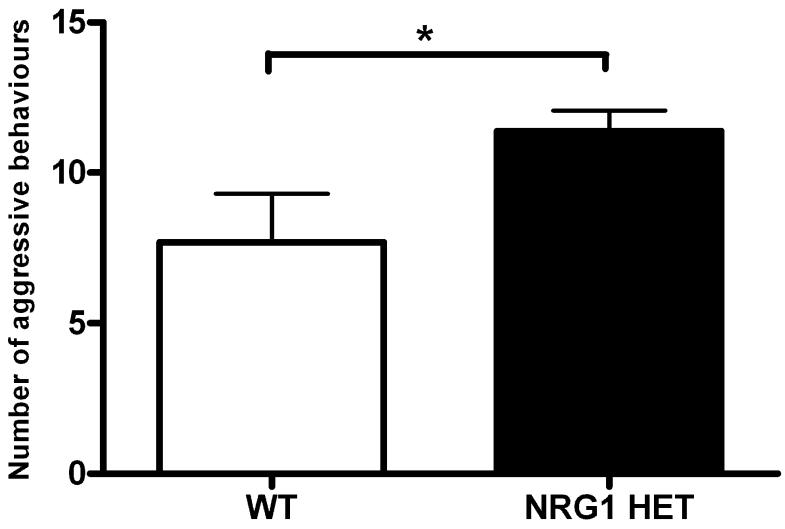

2000
1500
1000
500
0
Distance travelled (cm)
1
2
3
4
5
Trial blocks

Fig. 3(a) & (b)

(a)


Number of aggressive behaviours
15
10
5
0
*
WT
NRG1 HET

(b)

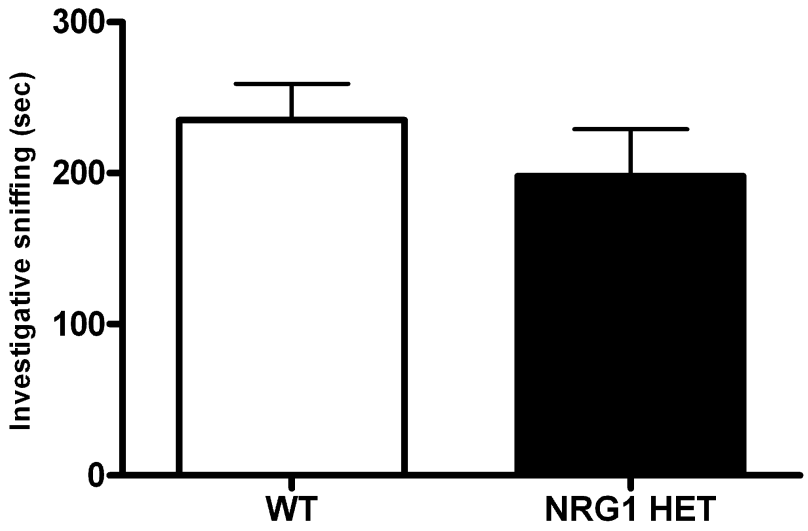

Investigative sniffing (sec)
300
200
100
0
WT
NRG1 HET

Fig. 4 (a) & (b)

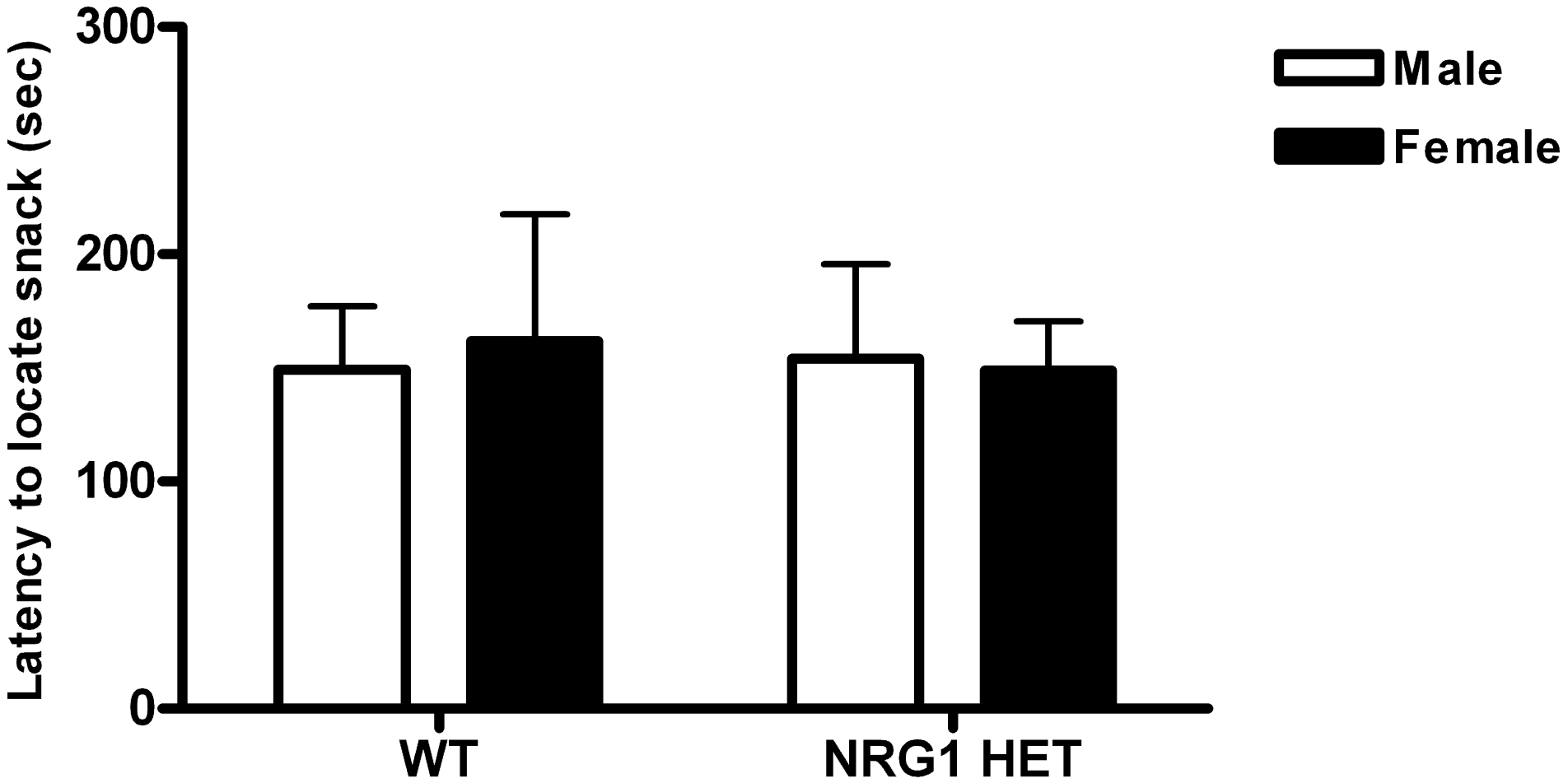
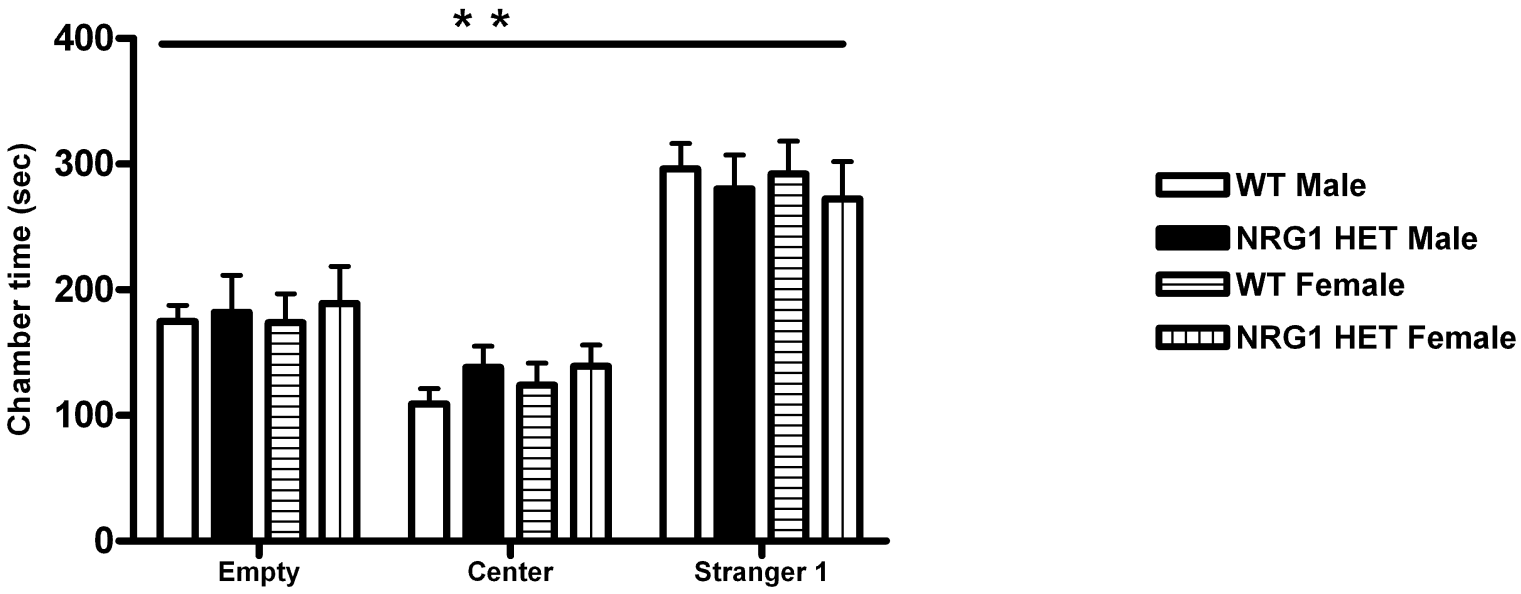

300
200
100
0
Latency to locate snack (sec)
Male
Female
WT
NRG1 HET

Fig. 6

(a) Sociability



(b) Preference for social novelty

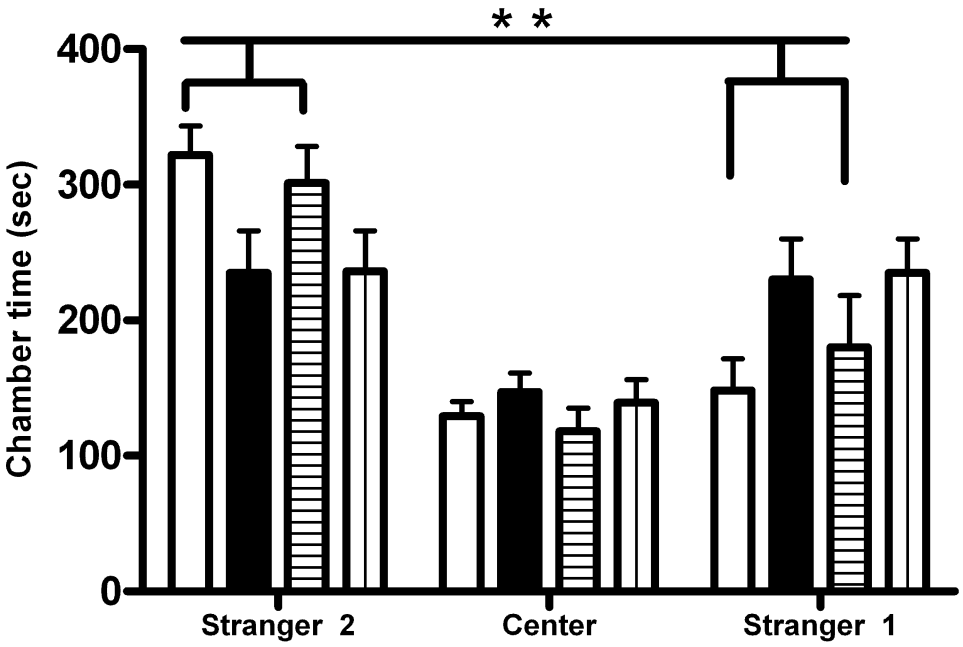


Fig. 5(a) & (b)

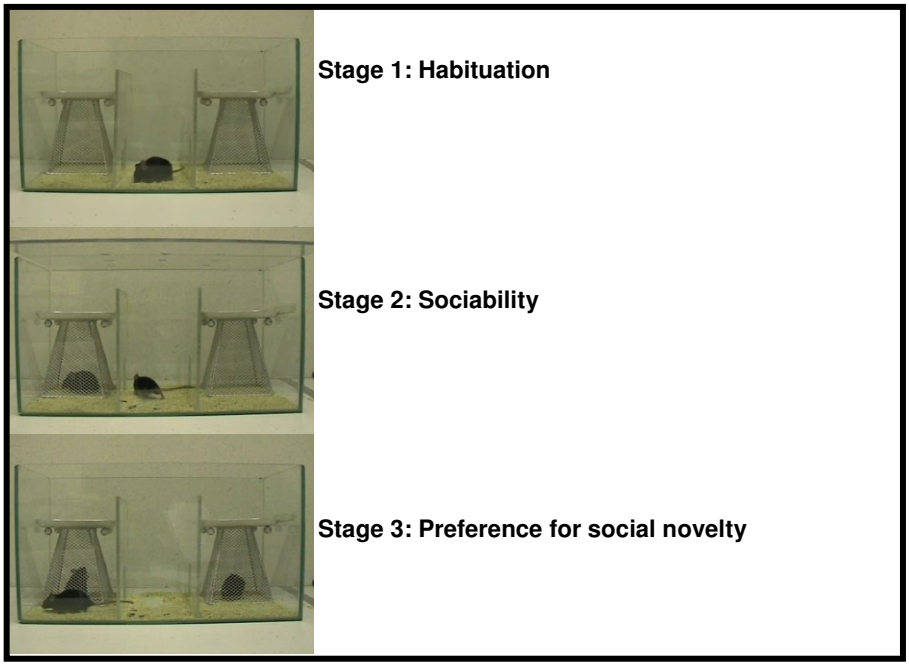

Stage 1: Habituation
Stage 2: Sociability
Stage 3: Preference for social novelty

Fig. 1