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Abstract

Clinical genetic studies have implicated neuregulin-1 [NRG1] as a leading susceptibility gene for schizophrenia. NRG1 is known to play a significant role in the developing brain, which is consistent with the prevailing neurodevelopmental model of schizophrenia. Thus, the emotional and social phenotype of adult mice with heterozygous 'knockout' of transmembrane [TM]-domain NRG1 was examined further in both sexes. Emotional/anxiety-related behaviour was assessed using the elevated plus-maze and the light-dark test. Social behaviour was examined in terms of dyadic interactions between NRG1 mutants and an unfamiliar C57BL6 conspecific in a novel environment. There was no effect of NRG1 genotype on performance in either test of emotionality/anxiety. However, previous reports of hyperactivity in NRG1 mutants were confirmed in both paradigms. In the test of social interaction, aggressive following was increased in NRG1 mutants of both sexes, together with an increase in walkovers in female mutants. These findings elaborate the specificity of the NRG1 phenotype for the social rather than the emotional/anxiety-related domain. They indicate that NRG1 is involved in the regulation of reciprocal social interaction behaviour and thus suggest a putative role for NRG1 in a schizophrenia-related endophenotype.

Keywords

Neuregulin-1; Targeted gene deletion; Emotionality/anxiety; Social interaction; Phenotype; Schizophrenia

Abbreviations

ANOVA – Analysis of variance; DA – Dopamine; HET – Heterozygous; NMDA – N-methyl-D-aspartic-acid; NRG1 - Neuregulin-1; TM- Transmembrane; WT- Wildtype

Introduction

Among several candidate genes of current interest (Harrison and Weinberger, 2005; Owen et al., 2005; Karayiorgou and Gogos, 2006; Waddington et al., 2007), two recent meta-analyses of case-control and family-based association studies have confirmed a role for neuregulin-1 [NRG1] as a susceptibility gene for schizophrenia (Li et al., 2006; Munafo et al., 2006). However, as no coding mutation has yet been identified, the functional implications of genetic variation at NRG1 for schizophrenia are far from clear. A recent study has reported an association between a mis-sense mutation in the transmembrane domain of the NRG1 gene and schizophrenia (Walss-Bass et al., 2006). Furthermore, the relative expression of three of several NRG1 isoforms is altered in the dorsolateral prefrontal cortex of patients with schizophrenia (Hashimoto et al., 2004; Law et al., 2006).

NRG1 plays a role in several aspects of brain development and plasticity, as well as in NMDA glutamatergic and dopaminergic receptor expression and function (Corfas et al., 2004; Harrison and Law, 2006). While these characteristics are consistent with a role for NRG1 in the pathobiology of schizophrenia, studies in mice with targeted gene deletion of schizophrenia risk genes are useful tools for identifying more directly any role for such genes in the regulation of behaviours relevant to psychosis (Arguello and Gogos, 2006; Chen et al., 2006; O'Tuathaigh et al., 2007a; Waddington et al., 2007). While homozygous deletion is lethal due to cardiac defects, mice with heterozygous deletion of NRG1 display locomotor hyperactivity that is sensitive to attenuation by the

antipsychotic clozapine, impairment in habituation processes and disruption of prepulse inhibition and latent inhibition (Gerlai et al., 2000; Stefannsson et al., 2002; O'Tuathaigh et al., 2006; Rimer et al., 2005).

We have reported recently that while social affiliative behaviour is intact in NRG1 heterozygous mutants, they evidence a selective disruption in behavioural response to social novelty (O'Tuathaigh et al., 2007b). Also, in the resident-intruder paradigm it was found that NRG1 mutants display a moderate increase in aggressivity towards a conspecific placed in the home cage. In contrast, NRG1 mutants did not display abnormalities in murine paradigms of spatial learning and working memory (O'Tuathaigh et al., 2007b). The importance of considering motivation and traits related to emotionality when interpreting performance in rodent models of social functioning and cognition, many of which rely on behaviours evoked in anxiogenic contexts, has been noted by several authors (e.g. Galsworthy et al., 2002; Jacobsen et al., 2007). Therefore, the present studies were undertaken for two complementary reasons: (i) given that emotionality/anxiety can influence rodent performance in tests of social functioning and cognition, to assess NRG1 mutants in paradigms sensitive to disruption in emotional/anxiety-related behaviour; (ii) to define the breadth of social deficit by extending phenotypic studies of NRG1 mutants to additional social paradigms. Such studies may also identify additional, distinctive phenotypic features that merit further investigation.

Methods

Animals

TM-domain NRG1 'knockout' mice were generated at the Victor Chang Cardiac Research 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; $NRG1^{+/-}$] mutants and wildtypes [WT; $NRG1^{+/+}$] were generated from heterozygous breeding pairs and genotyped using PCR analysis (O'Tuathaigh et al., 2006, 2007b). 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. All testing was conducted between 10:00-14:00. Mice used in these experiments were all from litters of the same generational age. At time of testing, the mean body weight and age of NRG1 HET mutants [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. They 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, and from the Environmental Protection Agency in relation to the contained use of genetically modified organisms.

Apparatus and Experimental procedures

Elevated plus-maze

Emotional/anxiety-related behaviour was assessed in the elevated plus-maze test (Pellow and File, 1986). The plus-maze consists of four perpendicular arms: two opposing arms are surrounded by cream-coloured, chipboard walls [closed arms; 12 cm high]; the other two arms are devoid of walls [open arms]. The plus-maze was elevated 25 cm above ground level, with testing conducted under dim lighting conditions. Mice were placed individually in the centre of the maze facing one of the open arms and the start of each trial defined by first arm entry. Cumulative time spent in open [aversive] and closed [non-aversive] arms was recorded during a 5 min session, together with the number of entries into each arm as defined by all four paws being placed in that arm; an increase or decrease in time spent in the open arm reflects, respectively, a decrease or increase in 'anxiety', with total number of arm entries reflecting level of 'activity'. The apparatus was cleaned between trials using 3% Virkon ™ [Antec International, USA]. All measures were made by an observer blind to genotype.

Light-dark emergence

Emotional/anxiety-related behaviour was further assessed in the light-dark test (Bourin and Hascoet, 2003). The chamber is divided two compartments: one [14 x 16 x 19 cm] is brightly lit; the other [28 x 16 x 19 cm] is covered and in darkness. A small open door [diameter 5.5 cm] allows access between the two compartments. To start the test, each mouse was placed in the dark compartment and then allowed to explore the apparatus for 10 min. Cumulative time spent in the bright [aversive] and dark [non-aversive] compartments was then recorded during a 5 min session, together with the number of entries into each compartment as defined by all four paws being placed in that

compartment; an increase or decrease in time spent in the bright compartment reflects, respectively, a decrease or increase in 'anxiety', with total number of entries reflecting level of 'activity'. The apparatus was cleaned between trials using 3% Virkon. All measures were made by an observer blind to genotype.

Social interaction in a novel environment

Social interaction in a novel environment was tested in a clear Perspex chamber [28 x 28 x 16 cm]. Each mouse was paired with an unfamiliar age-, weight- and sex-matched C57BL6 mouse. Both the test mouse and the unfamiliar C57BL6 conspecific were placed in the chamber simultaneously for a 10 min trial that was captured and recorded using a digital camcorder mounted above the chamber at ceiling level. Between each test, the chamber floor and walls were cleaned with 3% Virkon and clean bedding material placed on the floor. For each animal, the investigator was blind to genotype during both testing and subsequent coding of behaviours.

All social and nonsocial exploratory behaviours were later coded using video analysis software [Observer ®, Noldus Inc., The Netherlands]. Eight behaviours were coded and organised into the following behavioural domains: social investigation [anogenital sniffing: sniffing by test mouse of anogenital region of conspecific]; social dominance [walkover: test mouse places its front paws on head or back of conspecific; aggressive following: test mouse rapidly follows conspecific from behind, forcing it to retreat]; agonistic behaviours [pinning: test mouse pins conspecific to floor; clawing]; non-social exploration [rearing to wall; rearing free: test mouse is upright with front paws raised

away from wall; sifting: test mouse sifts through chamber bedding]. Total number of episodes, total duration and latency to display were determined for each behaviour initiated by the test mouse.

Statistical analysis

For each test paradigm, data were subjected to square root transformation (Babovic et al., 2007; O'Tuathaigh et al., 2006, 2007b) and then analysed using two-way analysis of variance [ANOVA], with main factors of genotype and sex, Where appropriate, *post-hoc* comparisons were carried out using independent or paired t-tests, corrected for multiple comparisons. Statistical significance was accepted at the 0.05 level of probability. All statistical analyses were carried out using the SPSS software package [Version 14, SPSS Inc., Chicago, IL, USA].

Results

Elevated plus-maze

Heterozygous deletion of NRG1 did not influence percentage time spent in the open arms [effect of genotype, $F(1, 52) = 0.07$, $P = 0.88$; no genotype × sex interaction; Fig. 1a]. NRG1 mutants exhibited more entries into both the open arms [marginal effect of genotype, $F(1, 52) = 3.23$, $P = 0.08$; no genotype × sex interaction; Fig. 1b] and the closed arms [effect of genotype, $F(1, 52) = 6.31$, $P < 0.02$; no genotype × sex interaction; Fig. 1c], indicating an increased level of 'activity'.

Light-dark emergence

Heterozygous deletion of NRG1 did not influence percentage time spent in the bright compartment of the light-dark chamber [effect of genotype, $F(1, 40) = 1.02$, $P = 0.32$; no genotype × sex interaction; Fig. 2a]. NRG1 mutants exhibited more entries into the bright compartment [effect of genotype, $F(1, 40) = 5.43$, $P = 0.02$; no genotype × sex interaction; Fig. 2b], indicating an increased level of 'activity'.

Social interaction in a novel environment

As the total number of observations categorised into the domain of agonistic behaviour was very few, these behaviours were summed; thus, agonistic behaviour was considered a unitary measure. Heterozygous deletion of NRG1 was without effect on total number of observations for the following behaviours: anogenital sniffing, walkover, agonistic behaviour, rearing free, rearing to wall and sifting [no effects of genotype or genotype × sex interactions, all $P > 0.05$). NRG1 mutants exhibited more aggressive followings [effect of genotype, $F(1, 40) = 4.86$, $P = 0.03$; no genotype × sex interaction; Fig. 3a] and spent more time engaging in aggressive following [effect of genotype, $F(1, 40) = 6.27$, $P = 0.02$; no genotype × sex interaction; Fig. 3b]; they also evidenced reduced latency to initiate aggressive following [effect of genotype, $F(1, 40) = 5.97$, $P = 0.02$; no genotype × sex interaction; data not shown]. In exploratory analyses, female but not male NRG1 mutants spent more time engaging in walkover behaviour [female NRG1 *vs*. WT: $t(19) = 2.02$, $P < 0.05$; Fig. 3b].

Discussion

The present studies extend the behavioural phenotype of the TM-domain heterozygous NRG1 mutant mouse. Absence of any substantive genotypic differences in indices of emotionality/anxiety in NRG1 mutants of both sexes elaborates a recent report, confined to male NRG1 mutants, that noted no pronounced differences using similar paradigms (Karl et al., 2007). It is essential to clarify NRG1 mutant phenotype in both sexes as we have recently reported sexually dimorphic phenotypic effects of NRG1 deletion during both exploration of and subsequent habituation to a novel environment (O'Tuathaigh et al., 2006) and in other test paradigms (O'Tuathaigh et al., 2007b). A subtle, task-specific anxiolytic phenotype has been noted for male NRG1 mutants in terms of anxiety-related parameters in the open-field test (Karl et al., 2007); this may be reflected in the present non-significant trend towards decreased anxiety in NRG1 mutants of both sexes using the light-dark test. However, the interpretation of such findings is made more complex by a consistently observed hyperactivity profile (O'Tuathaigh et al., 2006, 2007a). In the present report, both male and female NRG1 mutants demonstrated an increased number of arm entries in the elevated plus-maze and of chamber entries in the light-dark test, both being indices of general activity in these paradigms.

In contrast, increases in aggressive following and, in females, walkovers indicate a social phenotype for NRG1 mutants. Using the resident-intruder paradigm, we have reported a moderate increase in aggressivity towards a conspecific placed in the home cage, as well as disruption to social novelty but not social preference (O'Tuathaigh et al., 2007b). The present results elaborate this finding, as aggressive following and walkovers serve a

predominantly social dominance-related rather than investigative function in rodents (Panksepp, 1981; Almeida and De Araujo, 2001); indeed, investigative sniffing was unaltered in NRG1 mutants, in accordance with our recent finding of intact social approach behaviour (O'Tuathaigh et al., 2007b). This social dominance-related NRG1 mutant phenotype may be manifest differently depending on environmental factors: it may take a milder form [i.e. aggressive following, walkovers] in a socially neutral context such as a novel environment, but a more robust form [including biting, pinning, tail rattling] in the more socially confrontational context of the home cage. If confirmed, increase in walkovers in female but not in male NRG1 mutants would be consonant with our previous findings of certain phenotypic effects of NRG1 deletion being sex-specific (O'Tuathaigh et al., 2006, 2007b).

Conclusions

In summary, there appear to be two specific aspects to the social phenotype associated with heterozygous deletion of TM-domain NRG1: (i) there is a moderate increase in milder dominance-related behaviour in a non-territorial environment, while an increase of more robust aggressive behaviour is evident in a confrontational, territorial environment; (ii) though social affiliative behaviour is intact, behavioural response to social novelty is disrupted. Critically, they occur in the absence of any marked deficits in emotional/anxiety-related behaviour, spatial learning, working memory or olfactory-based behaviours. These findings indicate that the schizophrenia risk gene NRG1 may mediate endophenotypic aspects of the disorder that pertain to social functioning.

Acknowledgments

These studies were supported by Science Foundation Ireland and the Health Research Board.

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Fig. 1. (a) Time spent in open arm of elevated plus-maze, as percentage of total trial time. (b) Number of entries into open arm. (c) Number of entries into closed arm. Data are means ± SEM for male and female WT and NRG1 HET mice; * $P < 0.05$, HET *vs.* WT.

Fig. 2. (a) Time spent in bright chamber of light-dark apparatus, as percentage of total trial time. (b) Number of entries into bright chamber. Data are means ± SEM for male and female WT and NRG1 HET mice; * $P < 0.05$, HET *vs.* WT.

Fig. 3. (a) Counts in social interaction paradigm for each of the following behaviours: AS, anogenital sniffing; WKR, walkover behaviour; FW, aggressive following; AG, agonistic behaviour. (b) Time (s) engaged in each of the following behaviours: AS, WKR, FW, AG. Data are means ± SEM for male and female WT and NRG1 HET mice; * $P < 0.05$, HET *vs.* WT.