



The response of neuregulin 1 mutant mice to acute restraint stress

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ABSTRACT

Stress plays a role in the development and severity of psychotic symptoms and there may be a genetic component to stress vulnerability in schizophrenia. Using an established mouse model for schizophrenia, we investigated the behavioural and endocrine response of *Nrg1* transmembrane domain mutant mice (*Nrg1* HET) and wild type-like (WT) littermates to acute restraint stress. Animals were screened at 3–4 months and 6–7 months of age (before and after onset of hyperlocomotion) for open field behaviour and serum corticosterone levels. In younger mice, stress reduced locomotive and explorative measures and increased anxiety-like behaviour regardless of genotype. Older *Nrg1* mutants were less susceptible to the effects of stress on anxiety-related behaviours. All mice responded to restraint stress with robust increases in serum corticosterone. Importantly, the stress-induced increase in corticosterone was more pronounced in *Nrg1* mutant than WT mice at the younger but not the older age. Our results suggest that transmembrane domain *Nrg1* has only a moderate effect on the acute stress response of mice. The behavioural differences detected between WT and *Nrg1* HET mice at the older age were evident without parallel modifications to the glucocorticoid system.

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1. Introduction

Neuregulin 1 (*NRG1*) has been associated with an increased risk to develop schizophrenia ([27,35]; meta-analyses: [23,26,30]). The protein is involved in axon guidance, myelination, and synapse formation. Alternative promoter usage results in numerous splice variant types and more than 30 isoforms [11,21]. The isoform variants most commonly expressed in the brain contain a transmembrane domain [10,11,35].

Studies suggest stress plays a role in the development and severity of psychotic symptoms. Indeed, stress can precipitate symptom onset [7] and trigger relapse in schizophrenia patients [13]. Importantly, there may be a genetic component to stress vulnerability in schizophrenia, because (1) schizophrenia patients handle negative life events more poorly than healthy subjects [12] and have an underlying vulnerability to stress [34], (2) first degree relatives of schizophrenia patients demonstrate increased stress sensitivity [24], and (3) a *NRG1* polymorphism interacts

with psychosocial stress to affect reactivity to expressed emotions [18].

The heterozygous *Nrg1* transmembrane domain mutant mouse (*Nrg1* HET) is an animal model of schizophrenia providing face, construct and predictive validity. *Nrg1* HETs display an age-dependent hyperactivity (reversible by clozapine [15,27]). Additionally, mutant mice exhibit an anxiolytic-like and cognitive phenotype [6,9,15]. Interestingly, *Nrg1* modulates the effects of the psychoactive cannabis constituent Δ^9 -tetrahydrocannabinol on stress-related brain circuitry in mice [3,4] and is expressed in brain regions controlling stress reactivity [5]. Furthermore, observations from animal caretakers suggest that *Nrg1* mutant mice are more susceptible to transport stress.

The current study aimed to evaluate the behavioural and endocrine (i.e. serum corticosterone) response of *Nrg1* mutants and WT littermates to acute restraint stress. Test animals were screened before and after onset of the schizophrenia-relevant hyperlocomotive phenotype for baseline and stress-induced open field behaviour. This paradigm has produced the most consistent schizophrenia-relevant *Nrg1* phenotype in the past and enables the analysis of anxiety-related behaviours [15].

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2. Material and methods

2.1. Animals

Test animals were male *Nrg1* HET and WT littermates (C57BL6/JArc background) [27], as they have shown more pronounced phenotypes than females [4,6,9,20]. Age-matched test mice (± 7 days) were used at the age of 3–4 months (8 *Nrg1* HETs, 11 WT) and the age of 6–7 months (10 *Nrg1* HETs, 17 WT). Test mice were pair-housed in cages equipped with an igloo (Bioserv, Frenchtown, USA) and a metal ring (3 cm diameter) in the cage lid. Mice were kept under a 12:12 h light:dark schedule (red light permanently present: illumination < 5 lx). Research and animal care procedures were approved by the University of New South Wales Animal Care and Ethics Committee and were in accordance with the EC Directive 86/609/EEC for animal experiments.

2.2. Open field (OF)

Baseline OF testing was conducted to confirm the age-dependent hyperlocomotive phenotype of *Nrg1* mutants [15–17]. The response to restraint stress was investigated after an inter-test interval of at least seven days. Mice were placed in OF activity chambers (Med Associates Inc., St Albans, USA) for 30 min. Distance travelled and vertical activity (as an indirect measure of rearing) as well as resting in central and peripheral zones was recorded [15,16]. The ratio of the distance travelled in the center relative to the total distance travelled ('distance ratio') and the time spent in the center ('center time') were taken as measures of anxiety [8].

2.3. Corticosterone analysis

Mice were taken from the home cage 60 min after onset of the light phase and placed in restraint tubes for 15 min (Broome Rodent Restraint: Harvard Apparatus, Holliston, USA). Following a 5 min recovery period, mice were tested in the OF. Blood samples were taken prior to and following restraint stress within 3 min from the tail vein. Samples were stored on ice until centrifugation (13,000 rpm for 5 min). Serum corticosterone was analysed using a radioimmunoassay kit from ICN Biomedicals (Costa Mesa, USA) [25].

2.4. Statistical analysis

Results were analysed as published previously [15,16] by three-way repeated measures (RM) analysis of variance (ANOVA) using the within factor 'restraint stress' and 'age' and the between factor 'genotype'. This was followed by lower-degree ANOVAs (i.e. two-way and one-way ANOVAs) split by corresponding factors where appropriate. One-way ANOVAs were also chosen for Δ (Restraint–Baseline). Analyses were conducted using Statview Version 5.0. Differences were regarded as statistically significant if $p < .05$. All data are presented as means \pm standard error of the mean (SEM) or Δ mean (Restraint–Baseline).

3. Results

3.1. Open field behaviour

Nrg1 mutant mice exhibited hyperlocomotion compared to WT mice ($p = .001$). However, looking at age effects separately, OF locomotion was only significantly different between *Nrg1* HET mice and WT littermates at the age of 6–7 months ($p = .003$; Fig. 1B), whereas total distance travelled of young *Nrg1* HET mice was not significantly increased ($p > .05$; Fig. 1A). Exposure to acute stress had no

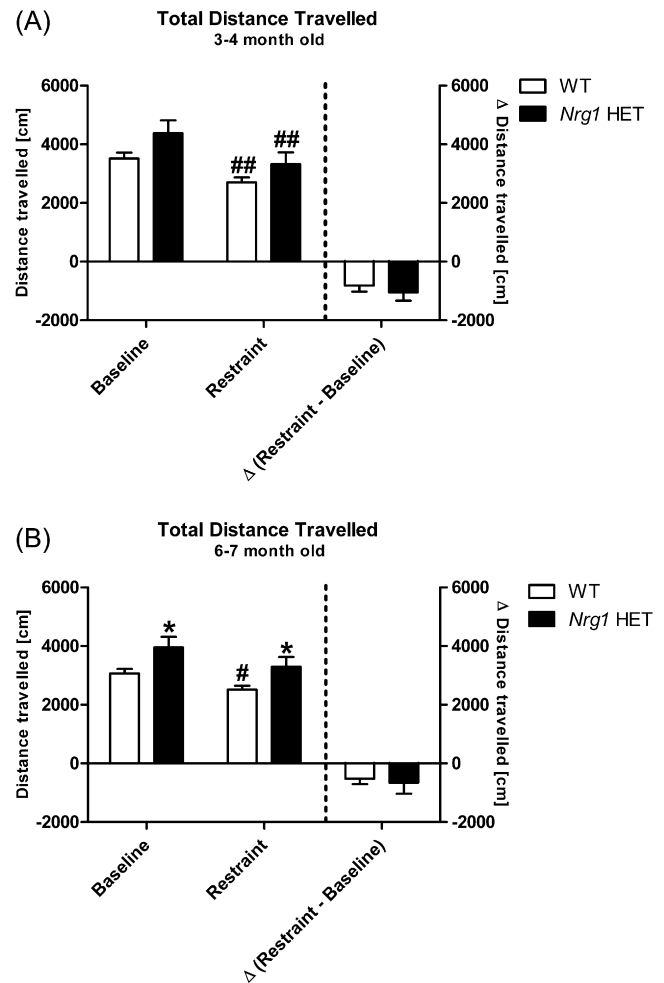


Fig. 1. Open field locomotion. Total distance travelled [cm] is shown in (A) 3–4-month old mice and (B) 6–7-month old mice at baseline and following acute restraint stress. Significant one-way ANOVA effects of stress versus baseline of the corresponding genotype are indicated by * ($p < .05$, ** $p < .01$) whereas significant effects of *Nrg1* versus WT mice of the corresponding stress group are indicated by asterisks (* $p < .05$).

significant impact on the hyperlocomotive phenotype of older *Nrg1* mutant mice (no interaction of 'restraint stress' with 'genotype'). Furthermore, 'genotype' had an impact on resting time ($p = .03$) and distance travelled in the periphery ($p = .003$). Two-way ANOVAs for the older cohort revealed that mutants exhibited a decrease in resting time ($p = .02$; significant at baseline) and increase in locomotion in the periphery ($p = .02$; significant post restraint stress) compared to WT (Table 1).

Acute restraint stress had a significant impact on the behavioural performance of mice. Stress inhibited locomotive and explorative behaviours and increased resting time. Three-way ANOVAs detected an overall effect of stress on total distance travelled ($p < .001$), distance travelled in the periphery ($p < .001$), rearing frequency ($p < .001$), and resting time ($p = .001$) (Fig. 1 and Table 1). This impact of stress on OF behaviour was evident in both test cohorts: mice at the age of 3–4 months showed a significant stress response in total and peripheral distance travelled (both $p < .001$), frequency of rearing ($p = .002$) and resting time ($p < .001$) (Fig. 1A and Table 1). Similarly, mice in the 6–7 month old test group exhibited stress-induced changes to distance travelled ($p = .003$), distance travelled in the periphery ($p = .003$), frequency of rearing ($p < .001$), and resting time ($p = .004$) (Fig. 1B and Table 1).

Table 1

Effect of restraint stress on open field behaviours in *Nrg1* HET and WT mice. Data for distance travelled in periphery (PerDistance), rearing frequency, time spent resting (i.e. RestTime), and time in the center of the OF (CenterTime) are presented as means \pm SEM.

	Baseline		Restraining stress		Δ (Restraining – Baseline)	
	WT	<i>Nrg1</i> HET	WT	<i>Nrg1</i> HET	WT	<i>Nrg1</i> HET
3–4-month old mice						
PerDistance [cm]	2264.4 \pm 133.7	2808.7 \pm 279.4	1825.2 \pm 114.9 ^{##}	2320.1 \pm 292.1 [#]	–439.2 \pm 130.6	–488.6 \pm 175.0
Rearing [n]	218.8 \pm 23.1	303.6 \pm 52.9	174.9 \pm 19.2	215.0 \pm 25.2 [#]	–43.9 \pm 21.0	–88.6 \pm 32.6
RestTime [s]	1147.2 \pm 2.0	1077.0 \pm 52.4	1258.5 \pm 20.8 ^{##}	1207.2 \pm 41.5 [#]	111.3 \pm 27.8	130.2 \pm 43.4
CenterTime [s]	462.7 \pm 52.5	475.6 \pm 67.3	348.2 \pm 49.9	341.9 \pm 49.9	–114.6 \pm 67.9	–133.7 \pm 67.8
6–7-month old mice						
PerDistance [cm]	2029.8 \pm 108.2	2432.5 \pm 227.1	1593.5 \pm 93.2 ^{##}	2083.1 \pm 205.5 [*]	–436.3 \pm 132.6	–349.4 \pm 214.5
Rearing [n]	237.0 \pm 16.0	259.8 \pm 24.9	128.6 \pm 9.6 ^{###}	126.4 \pm 16.6 ^{###}	–108.4 \pm 19.1	–133.4 \pm 21.8
RestTime [s]	1182.5 \pm 19.3	1096.6 \pm 35.9 [*]	1254.6 \pm 19.8 ^{##}	1188.2 \pm 40.1	72.1 \pm 24.2	91.6 \pm 43.2
CenterTime [s]	480.6 \pm 45.6	576.7 \pm 67.1	525.2 \pm 51.0	481.3 \pm 69.6	44.6 \pm 36.8	–95.4 \pm 72.1

Significant one-way ANOVA effects of 'restraint stress' are indicated by ^{##} (^{*} $p < .05$, ^{###} $p < .01$) whereas significant effects of the *Nrg1* mutation versus control mice of the corresponding stress group (baseline or restraint stress) are indicated by asterisks (^{*} $p < .05$).

Distance ratio and center time were chosen as measures of anxiety-related behaviours. Acute stress had a significant impact on distance ratio ($p = .01$; Fig. 2) and center time ($p = .02$; Table 1). Importantly, our analysis revealed that the effect of acute restraint stress on distance ratio was dependent on the genotype of the test animals (interaction between 'restraint stress' and 'genotype': $p = .03$) (Fig. 2). Looking at age effects separately, stress decreased distance ratio in 3–4-month old mice ($p = .002$) but not in older mice ($p > .05$). A significant interaction between 'restraint stress'

and 'genotype' was evident in the 6–7-month old cohort ($p = .01$): *Nrg1* mutant mice did not exhibit a pronounced anxiety response to acute restraint stress whereas WT mice actually showed a decrease in anxiety behaviour following stress (Fig. 2B). Analysing Δ distance ratio revealed a significant 'genotype' effect ($p = .02$; Fig. 2B). Center time was significantly reduced by restraint stress in younger ($p = .02$) but not older mice ($p > .05$), although their stress response was moderately affected by the genotype (trend for interaction between 'restraint stress' and 'genotype': $p = .07$; Table 1).

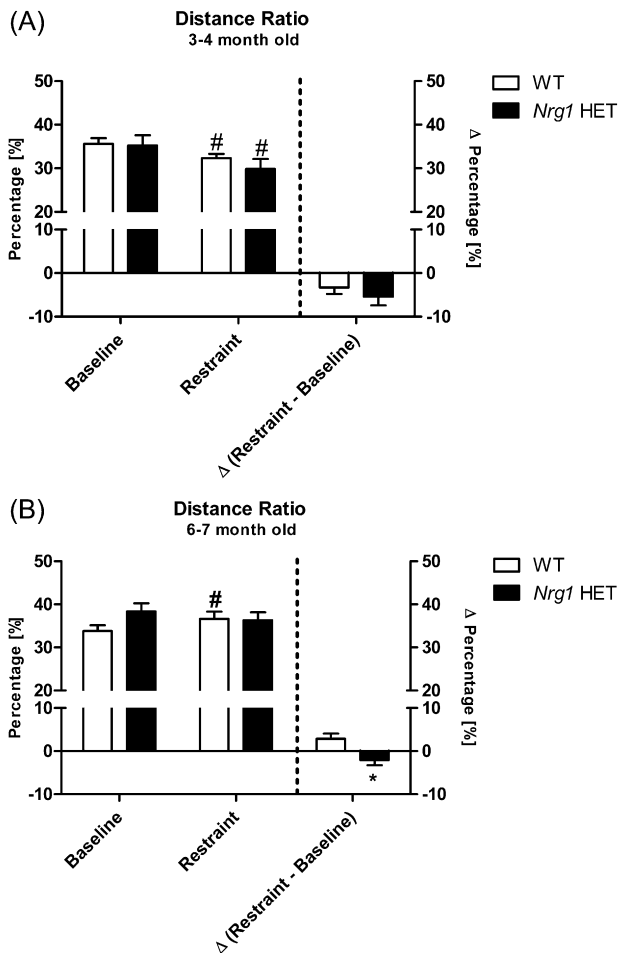


Fig. 2. Anxiety-like open field behaviour. Distance ratio [%] is shown in (A) 3–4-month old mice and (B) 6–7-month old mice at baseline and following acute restraint stress. Significant one-way ANOVA effects of stress versus baseline of the corresponding genotype are indicated by ^{##} (^{*} $p < .05$) whereas significant genotype effects versus WT mice are indicated by asterisks (^{*} $p < .05$).

3.2. Serum corticosterone

All mice exhibited a significant increase in serum corticosterone levels in response to restraint stress. Three-way ANOVAs confirmed the effect of acute stress on corticosterone levels ($p < .001$), which was evident in both cohorts (3–4 months old: $p < .001$ – 6–7 months old: $p < .001$). *Nrg1* deficiency had no impact on stress hormone levels ($p > .05$). However, the level of corticosterone release in response to stress was genotype-dependent (interaction between 'restraint stress' and 'genotype': $p < .001$): in the younger cohort, stress induced a more pronounced increase in *Nrg1* HET mice (10-fold increase) than in WT mice (3.5-fold increase), which was confirmed by a significant interaction between 'restraint stress' and 'genotype' ($p = .02$; Fig. 3A) and by analysing Δ corticosterone, which revealed a significant 'genotype' effect in the younger cohort ($p = .03$; Fig. 3A). No such genotype-dependent effect was detected in the older cohort (Fig. 3B).

Interestingly, older mice exhibited generally lower corticosterone levels than 3–4-month old mice ($p < .001$). Importantly, age also influenced the stress-induced release of corticosterone as evidenced by an interaction between 'restraint stress' and 'age' ($p < .001$). Older mice responded with a less severe increase in corticosterone than younger mice, which was confirmed by an interaction between 'restraint stress' and 'age' (*Nrg1* HET: $p < .001$; WT: $p = .02$).

4. Discussion

This study investigated the behavioural and endocrine response of a mouse model for *Nrg1* to acute restraint stress before and after the onset of its characteristic hyperlocomotive phenotype. In the younger mice, acute restraint stress reduced locomotive and explorative measures and increased anxiety-related behaviour in the OF regardless of genotype. Older *Nrg1* mutants did not respond with increased anxiety to stress. Mice showed robust stress-induced increases in serum corticosterone regardless of genotype or age.

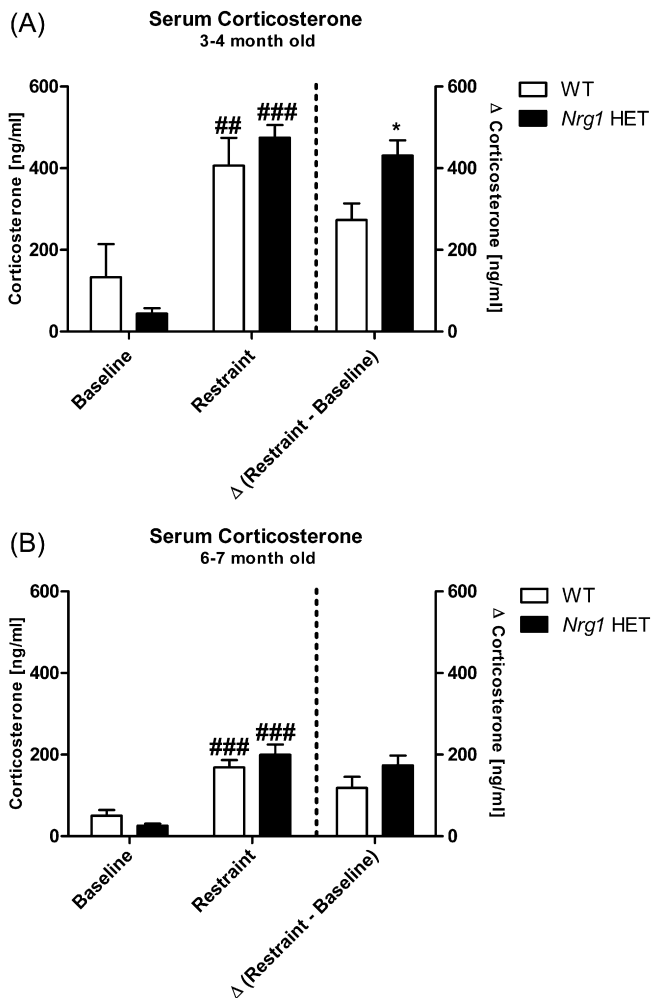


Fig. 3. Serum corticosterone. Serum corticosterone levels [ng/ml] measured at baseline and following restraint stress are shown in (A) 3–4-month old mice and (B) 6–7-month old mice. Significant one-way ANOVA effects of stress versus baseline of the corresponding genotype are indicated by ‘##’ ($p < .01$) and ‘###’ ($p < .001$) whereas significant genotype effects versus WT mice are indicated by asterisks (* $p < .05$).

However, the increase in corticosterone was more pronounced in *Nrg1* mutants than WT mice at 3–4 months of age.

Nrg1 mutant mice exhibit a hyperactive phenotype by the age of 6–7 months as has been described previously (e.g. [15,32]). In our study, hyperlocomotion was accompanied by decreased resting behaviour in baseline conditions and increased distance travelled in the periphery post restraint stress.

We hypothesize that mutated *Nrg1*, once it becomes “active”, interferes with how the organism responds to environmental manipulations, as was suggested by earlier research [15]. Indeed, hyperactive *Nrg1* HETs (6–7 months old) exhibited no stress-induced changes in open field anxiety. However, their endocrine susceptibility to restraint stress was similar to WT mice. Interestingly, younger mutants exhibited a more pronounced stress-induced increase in corticosterone than WT mice (2.8-fold) but showed a WT-like behavioural response to restraint stress. This might suggest the specificity of age-dependent effects of mutated *Nrg1* on behavioural domains. The fact that this endocrine phenotype of younger mutants is missing in older *Nrg1* hypomorphs demands further investigation. Our finding of an apparently disconnected endocrine and behavioural stress response in *Nrg1* mutant mice was unexpected but is consistent with other mouse models [19,31]. This phenomenon could be related to an involvement of *Nrg1* in stress reactivity downstream from the release of

glucocorticoids. *Nrg1* might impact on the expression of glucocorticoid receptors or the release of corticotrophin-releasing or adrenocorticotropin hormone, as has been shown in a rat model for Type II *Nrg1* [29]. Taken together, these findings suggest that transmembrane domain *Nrg1* impacts on the behavioural stress responses independently of its involvement in the activation of the glucocorticoid system.

The observation that older WT mice exhibited decreased anxiety-like behaviour as a response to stress was surprising, in particular as these mice showed a stress-related response in other behavioural and endocrine measures. A similar phenomenon has been described in another mouse study [2]. Elevated exploration of open spaces has been interpreted as a strain-specific more active stress coping mechanism of C57BL/6J mice [22].

There is a paucity of studies on the behavioural and endocrine effects of acute stress in mouse models for schizophrenia. A recent study suggested the involvement of *Nrg1* in the neuro-endocrine response of *Nrg1* transgenic rats to acute stress [29]. Rats with reduced levels of Type II *Nrg1* displayed sex-specific increased basal corticosterone levels but a similar peak endocrine response to restraint stress as non-transgenic animals [28]. The transgenic rats also exhibited changes in glucocorticoid receptor expression. The difference in the endocrine stress response between this and our study (apart from species differences [1]) is probably due to the particular stress designs used (i.e. rats were exposed to 30 min of restraint stress after an extended habituation period) and the characteristics of the *Nrg1* modification (i.e. the rat model targets the 5' end of the *Nrg1* gene, whereas our model targets the 3' end). Interestingly, in a mouse model for Type III *Nrg1*, a blunted increase in corticosterone release in response to mild acute stressors was found in mutant mice [5]. Conflicting findings between different *Nrg1* mouse models are in line with the observation that the behavioural impact of *Nrg1* mutations in mice is highly specific [14].

Although not specific to mutations in *NRG1*, it is interesting to note that non-medicated first episode psychosis patients show impaired cortisol and hypothalamic-pituitary-adrenal (HPA) axis activity in stressful situations [33], confirming that it is important to investigate the link between schizophrenia and stress in more detail.

Our results suggest that transmembrane domain *Nrg1* has only a minor effect on the behavioural response of mice to acute restraint stress and only once the *Nrg1* mutation has become ‘behaviourally active’. It is important to note that those behavioural differences were evident without accompanying modifications to the glucocorticoid system. The endocrine response to stress was increased in *Nrg1* mutant mice but only prior to the onset of hyperlocomotion. This suggests that the behavioural differences observed are not related to endocrine effects. Future research should address the impact of chronic stress on *Nrg1* mutant mice before and after the development of hyperlocomotion thereby also considering additional aspects of HPA functions.

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