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## Behavioural characterization of neuregulin 1 (NRG1) type I over-expressing transgenic mice

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### Abstract

Neuregulin 1 (NRG1) is a pleiotropic growth factor involved in diverse aspects of brain development and function. In schizophrenia, expression of the NRG1 type I isoform is selectively increased. However, virtually nothing is known about the roles of this isoform in brain. We have studied transgenic mice over-expressing type I NRG1 (NRG1<sup>type I-tg</sup>) using a series of behavioural tests. NRG1<sup>type I-tg</sup> mice have a tremor, are impaired on the accelerating rotarod, and have reduced prepulse inhibition in the context of an increased baseline startle response. There is no overall anxiety or activity phenotype, although female NRG1<sup>type I-tg</sup> mice show mild increases in anxiety on some measures. The pattern of results shows both similarities and differences to those reported in hypomorphic NRG1 mice, and may be relevant for interpreting the increased NRG1 type I expression seen in schizophrenia.

### Keywords

neuregulin; schizophrenia; transgenic; behavioural phenotype

### Introduction

Neuregulin 1 (NRG1) is a pleiotropic growth factor of the epidermal growth factor family. It has been investigated extensively with regard to its roles in neuronal and glial development [1]. Most of these studies have focused on the peripheral nervous system, and on early developmental events, but more recently its roles in the brain, and in ongoing aspects of plasticity and function, have come under scrutiny. One reason for this is the evidence implicating NRG1 as a schizophrenia susceptibility gene [2-4], and its involvement in the pathobiology of the disorder [1,5,6].

NRG1 is expressed as multiple isoforms due to different promoters and alternative splicing. A key categorisation is into types I-VI NRG1, defined by their 5' exon usage [1]. These

isoforms differ in their expression and function, for example, with regard to the roles of types I and III NRG1 in myelination [7,8] and in cell migration [9]. In schizophrenia, types I and IV NRG1 appear of most relevance. Type IV NRG1 expression is linked to genetic risk for the disorder [10] and type I NRG1, but not other isoforms, is elevated in the hippocampus [10] and prefrontal cortex [11] of subjects with schizophrenia.

Identification of NRG1 as a possible schizophrenia susceptibility gene has encouraged behavioural and ethological testing of several NRG1 genetic mouse models. The results of these studies show a number of alterations which, broadly speaking, support a role for NRG1 in the schizophrenia phenotype (see Discussion). However, all existing data concern hypomorphs, in which one or more isoform of NRG1 is decreased. Given findings in schizophrenia, a mouse which over-expresses NRG1, especially the type I isoform, is of interest. Such a mouse exists, and exhibits hypermyelination of small diameter axons in the central but not peripheral nervous system [7,8]. Here we report their initial behavioural characterisation.

## Materials and Methods

The generation of NRG1<sup>type 1-tg</sup> mice has been described [7]. The mice used were heterozygous transgenic mice and their wildtype (wt) littermates obtained from the 6<sup>th</sup> and 7<sup>th</sup> generation of backcrossing of heterozygous male mice with C57/BL6 females. Animals were genotyped by PCR with primers specific for the transgene insert [7]. Same-sex littermates were group-housed in standard housing in a temperature-controlled room (21±1°C) on a 12hr light:dark cycle (off 19:00, on 07:00). Young adult mice were tested between 09:00-17:00 unless otherwise stated, with order of testing for genotypes and sexes counterbalanced where possible to avoid time of day effects. Animals were tested between 2 and 5 months of age. All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act, 1986. See Supplemental digital content 1 for detailed methods of the behavioural tests.

### In situ hybridization

Over-expression in the NRG1<sup>type 1-tg</sup> mice was measured and confirmed using *in situ* hybridization with a <sup>35</sup>S-labelled riboprobe (Riboprobe system, SP6/T7, Promega), specific to the NRG1 type I transgene on brain sections of mice aged 3 months, with standard procedures (probe sequence available on request).

### Motor Tests

**Locomotor activity**—Locomotor activity was monitored in transparent plastic cages (26 × 16 × 17cm), containing a thin layer of bedding with two photocell beams crossing the bottom of the cage. The total number of beam breaks was recorded in a 2 hour testing period (09:00-11:00). The following animals were tested: wt male, n=9, wt female, n=17, NRG1<sup>type 1-tg</sup> male, n=12, NRG1<sup>type 1-tg</sup> female, n=17.

**Rotarod**—The accelerating rotarod test was performed as described previously [12]. The speed at which the mouse fell from the rod was recorded. Data were analysed with ANCOVA, covarying for testing day body mass.

### Anxiety Tests

**Light-dark box (start in dark), anxiogenic open field, elevated plus maze, successive alleys**—Several ethological, unconditioned tests of anxiety were performed as described [12-14].

## Species-typical tests

Nesting and burrowing were assessed as described previously [15,16]. Both procedures began around 2 hours before the dark cycle commenced. Nest construction was scored with established criteria the following morning (~16 hours later; see Supplemental digital content 1). The mass of food pellets burrowed out of a tube was measured after 2 hours.

## Prepulse inhibition

Mice were tested for prepulse inhibition of the acoustic startle response. First, there were 10 presentations of a startle tone (105dB, 12kHz). Next there were 10 repetitions of four types of stimulus: (i) startle pulse alone, and a startle pulse preceded by 100ms by a prepulse of either (ii) 64dB, (iii) 68dB or (iv) 76dB (12kHz). The different types of trial were interleaved in a pseudorandom order with a variable inter-trial interval of 20-30s. Baseline acoustic startle response was calculated as the mean response of the 10 trials with no prepulse during the interleaved test trials, and the percentage prepulse inhibition calculated for each prepulse volume.

## Statistics

All data were inspected for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Levene's test). Data meeting these criteria were analysed with ANOVA, with between-subjects factors of genotype and sex.  $\alpha$  was set at 0.05. Significant sex\*genotype interactions were investigated by simple main effects, with "least significant difference" (LSD) correction. Non-spherical (Mauchly's test) repeated-measures within-subjects effects are reported with Huyn-Feldt correction. The nesting scores for each genotype were compared by a Mann Whitney U-test.

## Results

The  $NRG1^{type\ 1-tg}$  mice were viable and bred normally producing offspring at Mendelian ratios, and had comparable body mass to wt littermates between 1-5 months of age (data not shown). The only overt abnormality was a movement-related tremor (as reported previously [7,8]), which was visible from weaning, was similar in both sexes, and did not change in prominence or characteristics with age. The  $NRG1^{type\ 1-tg}$  mice showed robust over-expression of  $NRG1$  type I in the brain, notably in the hippocampus and cerebral cortex, particularly the deeper laminae (Fig. 1A,B). There was also over-expression in some brainstem nuclei, but not in the cerebellum (data not shown). The distribution of signal (i.e. the enhancement over grey versus white matter, and over the principal cell layer of the hippocampus) is indicative of a predominantly neuronal expression of the transgene, as would be predicted given the Thy-1 promoter construct [17].

## Motor tests

**Locomotor activity**— $NRG1^{type\ 1-tg}$  mice were not hyperactive ( $F < 1$ ; Fig. 1C, D). Mouse activity decreased across the session as the animals habituated to the test environment (block,  $F(23,1173)=11.49$ ,  $p < 0.001$ ). The change in activity during the test was different between genotypes (genotype\*block,  $F(23,1173)=2.45$ ,  $p=0.016$ ).  $NRG1^{type\ 1-tg}$  mice were less active than wt mice in the first 25 minutes of the test (blocks 1-3 and 5; all,  $F(1,51) > 3.5$ ,  $p < 0.05$ ), an effect that was more obvious in the female mice, although this did not reach significance (Fig. 1C and 1D).

**Rotarod**— $NRG1^{type\ 1-tg}$  mice showed a marked impairment on the rotarod, falling from the rod at a lower speed (Table 1).

## Anxiety tests

See Supplemental digital content 2 for detailed results of anxiety and motor tests.

**Light-dark box (start in dark)**—There was no effect of genotype, nor genotype\*sex interaction for any measure. Female mice had a greater latency to leave the dark section, spent more time in the dark section, and made fewer crossings between the dark and light sections than male mice, irrespective of genotype (Table 1).

**Open field**—The latency to enter the central, most anxiogenic region was not different according to genotype or sex, but there was a sex\*genotype interaction (Table 1), due to the transgene having opposite effects in males and females. However, investigation with simple main effects showed no significant difference between male wt and male  $NRG1^{type\ 1-tg}$  mice ( $F(1,59)=1.56$ ,  $p=0.216$ ) and only a trend towards increased latency in female  $NRG1^{type\ 1-tg}$  compared to female wt mice ( $F(1,59)=3.02$ ,  $p=0.087$ ). There were no significant differences between genotypes or sexes in the time spent in the central region. Total activity was lower in female than male mice, and there was a sex\*genotype interaction (Table 1) which arose because female  $NRG1^{type\ 1-tg}$  mice were less active than both female wt ( $F(1,59)=13.62$ ,  $p<0.001$ ) and male  $NRG1^{type\ 1-tg}$  mice ( $F(1,59)=16.34$ ,  $p<0.001$ ).

**Elevated plus maze**—The time in the open arms and percentage of entries made into the open arms was not different according to genotype or sex (Table 1). Total activity (measured by the total number of entries into any section) showed no main effect of genotype or sex, but a sex\*genotype interaction (Table 1). Investigation with simple main effects showed that female  $NRG1^{type\ 1-tg}$  mice made fewer arm entries than female wt mice ( $F(1,59)=8.25$ ,  $p=0.006$ ), and compared to male  $NRG1^{type\ 1-tg}$  mice ( $F(1,59)=8.09$ ,  $p=0.006$ ).

**Successive Alleys**—The latency to enter the most aversive alley (Alley 4) was greater in  $NRG1^{type\ 1-tg}$  mice than wt mice (Table 1). Female mice had greater latencies to enter the more aversive alleys than male mice (Table 1). All mice spent more time in the less aversive alleys (Table 1). There was an alley\*sex interaction, which was because female mice spent more time in the first alley ( $F(1,59)=21.43$ ,  $p<0.001$ ) and less time in the third and fourth alleys (both,  $F(1,59)>26$ ,  $p<0.001$ ).

The total number of entries into each alley was greater in wt mice than  $NRG1^{type\ 1-tg}$  mice, and in male mice than female mice (Table 1). There were fewer entries into the more anxiogenic alleys and a significant alley\*genotype interaction (Table 1). Wt mice made more entries into all of the arms than  $NRG1^{type\ 1-tg}$  mice (all alleys,  $F(1,59)>10$ ,  $p<0.002$ ). Both wt and  $NRG1^{type\ 1-tg}$  mice made more entries into the less aversive alleys than the more aversive alleys (effect of alley, both genotypes,  $F(3,57)>55$ ,  $p<0.001$ ).

## Species-typical tests

$NRG1^{type\ 1-tg}$  mice built less structured nests than wt mice and burrowed out a lower mass of pellets from a tunnel (Table 1).

## Prepulse inhibition

Prepulse inhibition was impaired in  $NRG1^{type\ 1-tg}$  mice (Fig. 1E; main effect of genotype,  $F(1,38)=4.98$ ,  $p=0.032$ ). The impairment was present with prepulse volumes of 64dB and 68dB, but not at 76dB (genotype  $\times$  prepulse volume,  $F(2,76)=5.09$ ,  $p=0.011$ ; investigated by simple main effects: 64dB,  $F(1,38)=9.09$ ,  $p=0.005$ ; 68dB,  $F(1,38)=7.20$ ,  $p=0.011$ ; 76dB,  $F(1,38)=0.06$ ,  $p=0.810$ ). However, the baseline acoustic startle response was greater in

NRG1<sup>type 1-tg</sup> mice (Fig. 1F;  $F(1,38)=28.58$ ,  $p<0.001$ ), evident from the first exposure to the startle stimulus (Fig. 1G).

## Discussion

We studied the behaviour of mice over-expressing the type I isoform of NRG1 and found several differences from their wt littermates. We confirmed the presence of a tremor [7], which we assume may be a manifestation of their altered central myelination [8], and which in turn underlies the poor performance of the NRG1<sup>type 1-tg</sup> mice on the rotarod (Table 1). NRG1<sup>type 1-tg</sup> mice have decreased prepulse inhibition, in the context of an increased acoustic startle response (Fig. 1E-G). No consistent anxiety phenotype is seen across tests, but there is some indication of increased anxiety in female NRG1<sup>type 1-tg</sup> mice (Table 1). Table 2 summarises the present results and compares them with those found in the four NRG1 genetically modified mice studied previously.

In the four anxiety tests used, there were no main effects of genotype in any of the anxiety-related measures (latency to enter, and time spent in, anxiogenic areas). However, there were genotype\*sex interactions, in the anxiogenic open field and successive alleys, for latency to enter the more anxiogenic sections, because female NRG1<sup>type 1-tg</sup> mice were slower than the other groups. Hence there was some suggestion of increased anxiety in female NRG1<sup>type 1-tg</sup> mice, but this was not consistently found in all tests, and may be secondary to changes in locomotor activity or initiation of movement. That is, the NRG1<sup>type 1-tg</sup> mice, especially females, were less active during the initial period of testing in the photocell activity cages, and in the successive alleys test (in terms of total crossings). There were also genotype\*sex interactions in activity measures in the anxiogenic open field and elevated plus maze, largely driven by decreased activity in female NRG1<sup>type 1-tg</sup> mice. Notably, sex differences were reported in NRG1-TM<sup>+/-</sup> mice (Table 2) in terms of their habituation of exploratory behaviour [18], but not in the elevated plus maze or light-dark box [19].

The prepulse inhibition deficit in the NRG1<sup>type 1-tg</sup> mice is potentially of interest (Fig. 1E), as decreased prepulse inhibition is a frequent finding in rodent models of schizophrenia – including some NRG1 hypomorphs (Table 2) – and in schizophrenia itself. As such, the present finding is in keeping with the proposed involvement of type I NRG1 in the disorder. However, the marked baseline difference in acoustic startle response (Fig. 1F, G) makes the difference difficult to interpret [20].

Finally, comparing under- with over-expressing mice might be expected to show either a gene dosage effect, such that reciprocal changes from wt mice are seen, or an ‘inverted U’, such that similar changes are seen in both types of mutant compared to wt. An inverted U has been hypothesised for NRG1 effects on synaptic plasticity [21]. Table 2 suggests that neither simple model fits the behavioural data. For example, the hypoactive phenotype in female NRG1<sup>type 1-tg</sup> mice contrasts with the hyperactive female NRG1-TM<sup>+/-</sup> mice, whereas both lines, as well as NRG1-CRD<sup>+/-</sup> mice, show impaired prepulse inhibition. Clearly, such comparisons are limited since the different isoforms affected in the hypomorphs are confounded with the direction of the genetic manipulation. Nevertheless, it is notable that there is a similarly complex pattern of results for multiple types of mutant DISC-1 mice [22], DISC-1 being another schizophrenia susceptibility gene. These data emphasise the unpredictability of phenotypes observed in mice wherein different modifications of a gene have been made, and hinder extrapolation back to complex human phenotypes such as schizophrenia, especially when the mouse models are only crude approximations to the genetic alteration being modelled in the first place [23,24].

## Conclusions

NRG1<sup>type 1-tg</sup> mice are impaired on the rotarod, and show decreased prepulse inhibition against a background of an increased startle response. There is no consistent anxiety or activity phenotype, though there is some evidence that female NRG1<sup>type 1-tg</sup> mice exhibit increased anxiety, accompanied by reduced activity. The results indicate that there are behavioural consequences of over-expressing type I NRG1, which may be relevant to the role that NRG1 plays in brain disorders such as schizophrenia.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

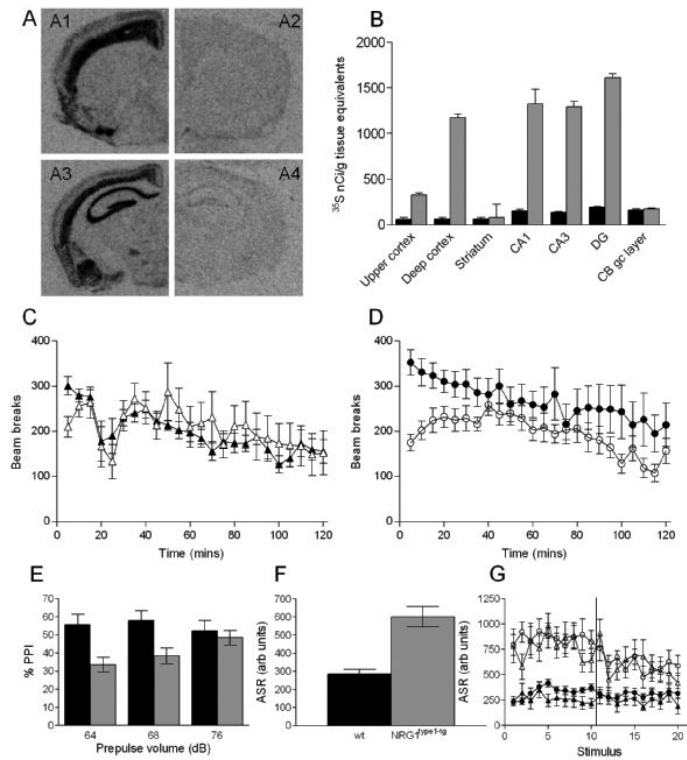
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**Figure 1.**

A: In situ hybridization showing expression of type I NRG1 mRNA in NRG1<sup>type 1-tg</sup> mice (A1, A3) and wildtype mice (A2, A4).  
 B: Quantitation of the autoradiograms showing relative expression of type I NRG1 mRNA in NRG1<sup>type 1-tg</sup> mice (grey bars) and wild type mice (black bars). DG: dentate gyrus. CB gc layer: granule cell layer of the cerebellum.  
 C and D: Locomotor activity monitoring, mean number of beam breaks  $\pm$  S.E.M. (C) Male wildtype, filled triangles (n=9) and NRG1<sup>type 1-tg</sup> mice, open triangles (n=12). (D) Female wildtype, filled circles (n=17) and NRG1<sup>type 1-tg</sup> mice, open circles (n=17).  
 E - G: Acoustic startle response and prepulse inhibition. E: Mean ( $\pm$ s.e.m.) percent prepulse inhibition (PPI) of the acoustic startle response (total wt mice, black bars, n=24; total NRG1<sup>type 1-tg</sup> mice, grey bars n=18). F: Mean ( $\pm$  s.e.m.) acoustic startle response (ASR) from 10 “no prepulse” trials interleaved during the prepulse inhibition testing. G: Baseline startle response shown trial-by-trial, including the first ten trials, which preceded the prepulse inhibition trials. Symbols as in Fig. 1C and D.



Table 1

**Results of the basic behavioural screen**

Data are mean  $\pm$  s.e.m unless otherwise stated. Genotype and sex main effects or interactions only shown if significant ( $p < 0.05$ ). Time in seconds.

Test and measure	Male wt	Female wt	Male NRG1 <sup>tg</sup> 1-4g	Female NRG1 <sup>tg</sup> 1-4g	Statistics
<b>Rotarod</b>					
Speed at fall (rpm)	11.2 $\pm$ 1.4	13.0 $\pm$ 1.0	6.4 $\pm$ 0.4	8.3 $\pm$ 0.5	genotype, $F(1,56)=30.19$ , $p < 0.001$
<b>Open field</b>					
Latency to centre	68.2 $\pm$ 7.3	39.0 $\pm$ 7.9	45.9 $\pm$ 7.9	63.1 $\pm$ 14.5	sex*genotype, $F(1,59)=4.23$ , $p=0.044$
Time in central area	21.5 $\pm$ 3.2	27.0 $\pm$ 5.5	21.6 $\pm$ 2.2	16.6 $\pm$ 1.9	
Total activity (m)	3.97 $\pm$ 0.22	4.33 $\pm$ 0.35	4.50 $\pm$ 0.26	3.04 $\pm$ 0.17	sex, $F(1,59)=6.18$ , $p=0.016$ sex*genotype, $F(1,59)=9.67$ , $p=0.003$
<b>Light-dark box (start in dark)</b>					
Latency to cross	7.9 $\pm$ 1.7	35.9 $\pm$ 6.9	14.0 $\pm$ 2.1	53.2 $\pm$ 17.5	sex, $F(1,59)=26.55$ , $p < 0.001$
Time in dark	169.8 $\pm$ 9.1	203.6 $\pm$ 8.1	162.7 $\pm$ 7.0	215.9 $\pm$ 10.9	sex, $F(1,59)=20.07$ , $p < 0.001$
Total crossings	21.9 $\pm$ 1.5	17.5 $\pm$ 1.6	21.3 $\pm$ 1.8	13.1 $\pm$ 1.7	sex, $F(1,59)=13.35$ , $p < 0.001$
<b>Elevated plus maze</b>					
Time in open	58 $\pm$ 7.6	49 $\pm$ 4.3	41 $\pm$ 5.3	34 $\pm$ 4.4	
% open entries	32.5 $\pm$ 3.0	31.7 $\pm$ 2.4	27.5 $\pm$ 3.1	28.7 $\pm$ 2.6	
Total entries	59.8 $\pm$ 4.6	65.3 $\pm$ 4.9	67.2 $\pm$ 3.3	47.6 $\pm$ 5.0	sex*genotype, $F(1,59)=6.32$ , $p=0.015$
<b>Successive Alleys</b>					
Alley 2 latency	15 $\pm$ 2.6	31 $\pm$ 4.3	15 $\pm$ 1.6	36 $\pm$ 5.6	sex, $F(1,59)=26.95$ , $p < 0.001$
Alley 3 latency	44 $\pm$ 9.4	102 $\pm$ 20	53 $\pm$ 21	169 $\pm$ 26	sex, $F(1,59)=20.43$ , $p < 0.001$
Alley 4 latency	69 $\pm$ 20	154 $\pm$ 21	97 $\pm$ 28	243 $\pm$ 5	genotype, $F(1,59)=6.07$ , $p=0.017$ sex, $F(1,59)=23.60$ , $p < 0.001$

Test and measure	Male wt	Female wt	Male NRG1 type 1-1-fg	Female NRG1 type 1-fg	Statistics
Time					
Alley 1	194±7.3	214±8.5	190±8.9	243±4.6	alley, F(2,177)=920.71, p<0.001
Alley 2	69±5.0	67±7.7	69±5.3	46±3.0	alley*sex, F(2,177)=16.13, p<0.001
Alley 3	27±2.4	16±1.9	27±3.6	12±2.1	
Alley 4	17±1.9	9.4±1.8	21±3.6	4.2±1.9	
Total entries					
Alley 1	9.7±1.0	9.0±0.8	8.6±0.7	5.1±0.6	genotype, F(1,59)=17.86, p<0.001
Alley 2	14.9±1.5	12.4±1.0	12.4±1.2	6.4±0.8	sex, F(1,59)=17.87, p<0.001
Alley 3	8.0±0.9	4.9±0.7	5.5±0.8	1.7±0.4	alley, F(3,177)=274.69, p<0.001
Alley 4	3.3±0.4	1.9±0.4	1.7±0.3	0.5±0.2	alley*genotype, F(3,177)=5.31, p=0.008 alley*sex, F(3,177)=6.55, p=0.003
<b>Species typical</b>					
Nesting (median score)	4	4	2	1	genotype, U=9.5, p<0.001
Burrowing (mass burrowed)	124±24	58±29	15±14	13±7	genotype, F(1,24)=14.30, p=0.001

Table 2

**Behavioural phenotypes of NRG1 mouse mutants**

Heterozygous mutants with deletion of the epidermal growth factor (EGF) domain (NRG-EGF<sup>+/-</sup>), the trans-membrane domain (NRG1-TM<sup>+/-</sup>), the immunoglobulin domain of types I and II NRG1 (NRG1-Ig<sup>+/-</sup>), or with disruption of the cysteine-rich domain of type III NRG1 (NRG1-CRD<sup>+/-</sup>). OFT, open field test; EPM, elevated plus maze; LD, light-dark box; HB, holeboard; TM, RW, running wheel; CM, cross maze; SA, successive alleys; PPI, prepulse inhibition; LI, latent inhibition; ASR, acoustic startle response. See *Supplemental digital content 3* for supplementary references S1-S7. Table adapted from [25] and ref. S7.

Behaviour	NRG1-EGF <sup>+/-</sup>	NRG1-TM <sup>+/-</sup>	NRG1-Ig <sup>+/-</sup>	NRG1-CRD <sup>+/-</sup>	NRG1type I-tg
<b>Locomotor activity</b>	Hyperactive (OFT, EPM, LD, HB).	Hyperactive (OFT, HB, LD).	Normal (RW).	Normal (OFT).	Generally normal but hypoactive initially.
<b>Motor tasks</b>	Normal horizontal bar. Normal rotarod, but increased learning with training. Normal marble burying.	Normal fine motor skills (chewing, rearing to wall).	Normal grip strength. Normal rotarod.	-	Impaired on rotarod. Species-specific skills impaired.
<b>Anxiety</b>	Normal OFT thigmotaxis. Normal LD and EPM.	Increased entries in all arms of EPM. Decreased anxiety in OFT. Normal LD, EPM.	-	-	Slight increase, in females only (OFT, EPM, SA).
<b>PPI</b>	Normal PPI.	Impaired PPI [2]. Normal PPI and ASR [27,31].	Normal PPI. Impaired LI.	Impaired PPI. Normal ASR.	Impaired PPI. Increased ASR.
<b>References</b>	[2,7,25, S1]	[2, 18,19,S2-S4]	[S5,S6]	[S7]	