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ARTICLE

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The schizophrenia susceptibility gene *neuregulin 1* modulates tolerance to the effects of cannabinoids

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Abstract

Cannabis increases the risk of schizophrenia in genetically vulnerable individuals. In this study we aim to show that the schizophrenia susceptibility gene *neuregulin 1* (*Nrg1*) modulates the development of tolerance to cannabinoids in mice. *Nrg1* heterozygous (HET) and wild-type (WT) mice were treated daily for 15 d with the synthetic analogue of Δ^9-tetrahydrocannabinol, CP55,940 (0.4 mg/kg). We measured the impact of this exposure on locomotor activity, anxiety, prepulse inhibition (PPI), body temperature and FosB/ΔFosB immunohistochemistry. Tolerance to CP55,940-induced hypothermia and locomotor suppression developed more rapidly in *Nrg1* HET mice than WT mice. Conversely in the light-dark test, while tolerance to the anxiogenic effect of CP55,940 developed in WT mice over days of testing, *Nrg1* hypomorphs maintained marked anxiety even after 15 d of treatment. Repeated cannabinoid exposure selectively increased FosB/ΔFosB expression in the lateral septum, ventral part (LSV) of *Nrg1* HET but not WT mice. On day 1 of exposure opposite effects of CP55,940 treatment were observed on PPI, i.e. it was facilitated in *Nrg1* hypomorphs and impaired in WT mice, despite the drug significantly impairing the acoustic startle reflex equally in both genotypes. These effects of CP55,940 on PPI were not maintained as both genotypes became tolerant to cannabinoid action with repeated exposure. Our results highlight that *Nrg1* modulates the development of cannabinoid tolerance dependent on the parameter being measured. Furthermore, these data reinforce the notion that the VLS is an important brain region involved in *Nrg1*–cannabinoid interactions.

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Introduction

Cannabis exposure increases an individual's risk of developing schizophrenia by 2-fold (Henquet *et al*. 2005). In healthy individuals cannabis use transiently recreates some of the psychological symptoms of the disorder and variation in the cannabinoid CB_1 receptor gene has been linked to schizophrenia (D'Souza *et al*. 2004; Ujike *et al*. 2002). Further, studies show that the endocannabinoid system is dysregulated in schizophrenia and in animal models of the disorder (Dean *et al*. 2001; Galve-Roperh *et al*. 2009; Leweke *et al*. 2007; Malone *et al*. 2008).

The observation that only a small proportion of cannabis users develop schizophrenia suggests that genetic vulnerability to the neuropharmacological actions of cannabis might explain the link between

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cannabis and psychosis (Henquet *et al*. 2008; Leweke & Koethe, 2008). Human studies support such a viewpoint demonstrating a role of the catechol-*O*-methyltransferase gene (Caspi *et al*. 2005; Henquet *et al*. 2009). However, research is required to isolate additional genes that might contribute to the polygenetic nature of cannabis-induced schizophrenia. Furthermore, the current literature fails to adequately describe the neurobiological mechanisms subserving this phenomenon. One strategy that might help address such shortcomings is the creation of better animal models of gene–cannabinoid interactions.

One of the most promising susceptibility genes for schizophrenia is *neuregulin 1* (*NRG1*) (Stefansson *et al*. 2002) and animal studies have shown that mutant mice heterozygous for the transmembrane domain of *Nrg1* (*Nrg1* HET mice) exhibit a schizophrenia-related behavioural phenotype, as well as abnormalities in underlying neurobiology (Boucher *et al*. 2007*a*; Dean *et al*. 2008; Stefansson *et al*. 2002). We have previously reported that *Nrg1* HET mice have an increased sensitivity to the acute neurobehavioural effects of cannabinoids (Boucher *et al*. 2007*a*, *b*). For example, the administration of the main psychoactive constituent of cannabis, Δ^9-tetrahydrocannabinol (THC), selectively facilitated prepulse inhibition (PPI) in *Nrg1* HET mice but not in wild-type (WT) littermates. We also showed that *Nrg1* HET mice display enhanced THC-induced c-Fos expression in stress and anxiety-related regions of the brain, with the most prominent effect being observed in the lateral septum, ventral part (LSV).

Frequent, long-term use of cannabis is more strongly linked to the development of schizophrenia (Linszen *et al*. 1994). Interestingly, a recent study reported that substance-dependent patients with psychotic disorders display more rapid development of tolerance to cannabis than substance-dependent individuals with no psychiatric comorbidity (Saddichha *et al*. 2010). Here we aim to assess whether *Nrg1* HET mice adapt differently to repeated cannabinoid administration as evidenced by tolerance. To achieve this we utilized a high-dose of the synthetic analogue of THC, CP55,940, and measured the physiological, behavioural and neurobiological responses of the mice over days of repeated administration.

Method

Animals

Male adult (age 22–25 wk) *Nrg1* HET mice (Karl *et al*. 2007) and WT littermates were pair-housed under standard housing conditions on a 12/12 h normal white/red light schedule with food and water available *ad libitum*. Male adult (age-matched $\pm$7 d) group-housed A/J mice (Animal Resources Centre, Australia) were used as standard opponents in the social interaction test (Boucher *et al*. 2007*a*). All research and animal care procedures were approved by the Garvan Institute/St Vincent's Hospital Animal Experimentation Ethics Committee.

Drug treatment

CP55,940 (Tocris, USA) was dissolved in 4% ethanol, 1% Tween 80, and 95% saline (Boucher *et al*. 2007*b*). CP55,940 or vehicle (Veh) were administered intraperitoneally at 0.4 mg/kg in a volume of 10 ml/kg body weight, 30 min prior to behavioural testing. CP55,940 promotes a similar constellation of neurobehavioural effects to THC (Arnold *et al*. 1998, 2001*a*, *b*; Gold *et al*. 1992) and has been used by numerous previous studies to demonstrate cannabinoid tolerance (Fan *et al*. 1996; Oliva *et al*. 2004; Rubino *et al*. 1997). The peak pharmacological effects of the drug occur 30 min post-injection and last for up to 4 h (Costa *et al*. 1996; McGregor *et al*. 1996).

Experimental procedure

Animals were treated once daily for 15 d. Every other day, mice were tested for body temperature (BT) and locomotor activity in the open field (OF). Mice were additionally tested for social interaction (SI), light-dark (LD) and PPI on days 1, 7 and 15. The order of testing in these paradigms (BT, OF, SI, LD then PPI) was always the same.

Body temperature

BT was measured in each mouse using a mouse rectal temperature probe (AD Instruments, Australia) attached to a thermocouple. BT was recorded immediately prior to, and 30 min after, the injection. Data were expressed as difference between baseline BT and BT +30 min.

Open field, light-dark, and social interaction tests

For detailed information on these models, see Boucher *et al*. (2007*a*). Locomotor activity and anxiety-related behaviours were evaluated for 10 min by placing the mouse into an infrared photobeam-controlled OF test chamber (43.2 cm $\times$ 43.2 cm; Med Associates Inc., USA). The OF and LD paradigms mimic the natural conflict between the tendency of mice to explore a novel environment and to avoid an exposed, illuminated, open area (Crawley, 1985; DeFries *et al*. 1966)

and SI is widely used to measure social anxiety (File, 1988; Kask *et al*. 2001). For LD testing, the OF chamber was equipped with a dark box insert. Animals that did not explore the dark chamber for more than 10% of the total time on day 1 were excluded from the analysis (one *Nrg1* HET Veh, one WT Veh and one WT CP55,940). For SI, test animals were placed together with an unfamiliar A/J standard opponent into the OF. Locomotor activity was recorded in the OF as the total distance travelled. Anxiety was evaluated as the time spent in the centre area of the OF and in the light compartment in the LD test (Costall *et al*. 1989; Crawley, 1999; Denenberg, 1969) and as the total duration of active behaviours such as general and anogenital sniffing, allogrooming, following, and crawling.

Prepulse inhibition

PPI is the phenomenon where pre-exposure to a weak acoustic stimulus reduces the startle response to a loud tone (Wang *et al*. 2003). Animals were habituated for 3 d to the startle chambers (SR-Lab; San Diego Instruments, USA). After a 5-min acclimation period with a 70-dB background noise, each session consisted of 115 trials in a pseudorandomized order. Sessions started and ended with five consecutive startle presentations (120 dB), and the rest of the session consisted of five trials of startle alone of different stimuli (70, 80, 100, 120 dB) and five trials of startle (120 dB) preceded by various prepulse intensities (PIs: 4, 12 or 16 dB above background noise). Different PI-startle interstimulus intervals (32, 64, 128, 256 or 512 ms) were also used that varied randomly from 10–20 s, the prepulse duration was 20 ms and startle duration was 40 ms. Startle response was measured as the average mean amplitude and %PPI was calculated as average for the different delays and PI [(startle response 120 dB – PPI response) × 100/ startle response 120 dB].

Immunohistochemistry

We used c-Fos and FosB/ΔFosB immunohistochemistry in diverse brain regions to assess changes in neuronal activity following repeated CP55,940 exposure. While c-Fos is rapidly and transiently expressed to acute drug exposure, ΔFosB accumulates in the brain therefore reflecting long-term molecular changes (Nestler *et al*. 1999; Staples *et al*. 2009). However, the anti-FosB antibody used here is not specific to ΔFosB, a product of the *FosB* gene, but recognizes the full-length FosB isoform that is not necessarily specific to long-term neuronal changes.

On the last day, mice used for the behavioural assessments were perfused ~2 h after the injection. A more detailed overview of our Fos immunohistochemical protocol can be found in Boucher *et al*. (2007*b*). We used a primary antibody for c-Fos or FosB/ΔFosB (which does not cross react with c-Fos) (Santa Cruz Biotechnology, USA; rabbit polyclonal, sc-52 and sc-48, respectively).

Statistical analysis

For each behavioural dataset we first analysed the overall effects using three-way analysis of variance (ANOVA) with genotype (WT and *Nrg1* HET mice) and treatment (Veh and CP55,940) as between-subject factors and test day as the within-subject factor. Individual group comparisons on the different days were implemented using two-way ANOVA with genotype (WT and *Nrg1* HET mice) and treatment (Veh and CP55,940) as between-subject factors followed by Tukey's *post-hoc* test. For Fig. 1*c*, data were analysed using two-way ANOVA with genotype as the between-subject factor and test day as the within-subject factor. Genotype comparisons on the different days were implemented using Student's *t* test. For Fig. 3*b*, data were analysed using the non-parametric Kruskal–Wallis test followed by the Mann–Whitney *U* test for between-group comparisons. Fos counts in each brain region were analysed using two-way ANOVA followed by Tukey's *post-hoc* test. A significance level of $p < 0.05$ was chosen for all comparisons.

Results

Body temperature and locomotor activity

Three-way ANOVA indicated that CP55,940 administration decreased BT [$F(1,35) = 79.22$, $p < 0.001$] (Fig. 1*a*). An effect of test day [$F(7,245) = 16.89$, $p < 0.001$] showed that BT changed over days. This was due to the mice becoming progressively less sensitive to the hypothermic effects of CP55,940 with repeated administration [treatment × test day interaction [$F(7,245) = 18.47$, $p < 0.001$]. Importantly, a genotype × treatment × test day interaction [$F(7,245) = 2.46$, $p < 0.05$] supported the observation that *Nrg1* HET mice developed tolerance to cannabinoid-induced hypothermia more rapidly than WT mice. Further reinforcing this view, two-way ANOVA on the different days revealed genotype × treatment interactions on day 3 [$F(1,35) = 9.56$, $p < 0.01$], day 5 [$F(1,35) = 5.09$, $p < 0.05$] and day 9 [$F(1,35) = 6.47$, $p < 0.05$]. Tukey's *post-hoc* test showed that while no difference between CP55,940-treated *Nrg1* HET and WT mice was

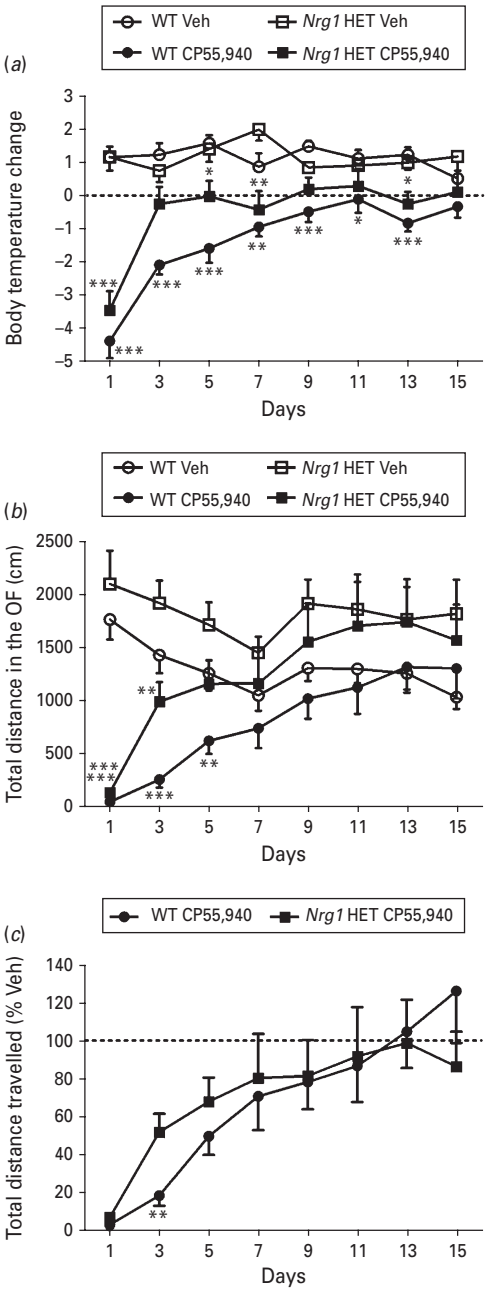

Fig. 1. Tolerance to the effects of CP55,940 (0.4 mg/kg) compared to vehicle (Veh) of wild-type (WT) and *Nrg1* HET mice on (*a*) body temperature and (*b*) locomotor activity in the open field (OF) (WT Veh, $n=12$; WT CP55,940, $n=11$; *Nrg1* HET Veh, $n=8$; *Nrg1* HET CP55,940, $n=8$). Panel (*c*) represents the locomotor activity in the OF as a % of the Veh group for WT and *Nrg1* HET mice. Data are presented as means ± S.E.M. Significant effects of CP55,940 compared to Veh within the same genotype group are indicated by * $p<0.05$, ** $p<0.01$ and *** $p<0.001$. For clarity of exposition, *post-hoc* genotype effects are not demarcated in this figure, but they are all reported in the Results section.

observed on day 1, *Nrg1* HET mice displayed significantly less CP55,940-induced hypothermia than WT animals on day 3 ($p<0.01$) and day 5 ($p<0.05$). On day 9, Tukey's *post-hoc* test revealed a decreased BT in Veh-treated *Nrg1* HET mice compared to Veh-treated WT animals ($p<0.05$). Tukey's *post-hoc* tests detecting treatment effects within each genotype on the different days are demarcated in Fig. 1*a*.

In the OF (Fig. 1*b*), *Nrg1* HET mice displayed higher locomotor activity than WT mice over days as revealed by three-way ANOVA [genotype: $F(1,35)=6.77$, $p<0.05$]. This analysis also showed that CP55,940 reduced locomotor activity [treatment: $F(1,35)=8.15$, $p<0.01$]. Effect of test day [$F(7,245)=9.13$, $p<0.001$] and a treatment × test day interaction [$F(7,245)=22.64$, $p<0.001$] highlighted that the mice developed tolerance to the locomotor suppressant action of CP55,940. However unlike for BT, no significant genotype × treatment × test day interaction was observed, making it less clear whether *Nrg1* HET mice developed tolerance more rapidly than WT mice to cannabinoid-induced locomotor activity suppression. We then assessed the differential development of tolerance between CP55,940-treated *Nrg1* HET mice and WT mice on the different days of treatment using two-way ANOVA. Overall genotype effects were observed on day 3 [$F(1,35)=13.98$, $p<0.001$], day 5 [$F(1,35)=7.92$, $p<0.01$], day 9 [$F(1,35)=6.64$, $p<0.05$] and day 11 [$F(1,35)=4.17$, $p<0.05$]. Tukey's *post-hoc* test showed that locomotor suppression was less in CP55,940-treated *Nrg1* HET mice than in CP55,940-treated WT mice on day 1 ($p<0.05$), day 3 ($p<0.001$) and day 5 ($p<0.05$). In the Veh group, hyperactivity of *Nrg1* HET mice compared to WT mice was seen on day 9 ($p<0.05$), day 11 ($p<0.05$) and day 15 ($p<0.05$). For *post-hoc* treatment effects within each genotype on days 1, 3 and 5, see Fig. 1*b*.

As genotype differences in the effects of CP55,940 are complicated by Veh-treated *Nrg1* HET mice showing hyperactivity compared to WT mice, we examined the rate of induction of tolerance to the effects of CP55,940 on the total distance travelled as a % of the Veh group (Fig. 1*c*). Two-way ANOVA showed an effect of test day [$F(7,119)=16.3$, $p<0.001$]. Further analysis of the different days using Student's t test revealed no difference between CP55,940-treated *Nrg1* HET mice and WT mice on day 1, indicating that the animals were equally affected by the drug. However on day 3, CP55,940-treated *Nrg1* HET mice travelled significantly more than WT mice [$t(17)=3.27$, $p<0.01$]. This indicates that on day 3, *Nrg1* HET mice displayed a more rapid rate of decline in the suppressant effect of CP55,940 on locomotor activity than WT mice.

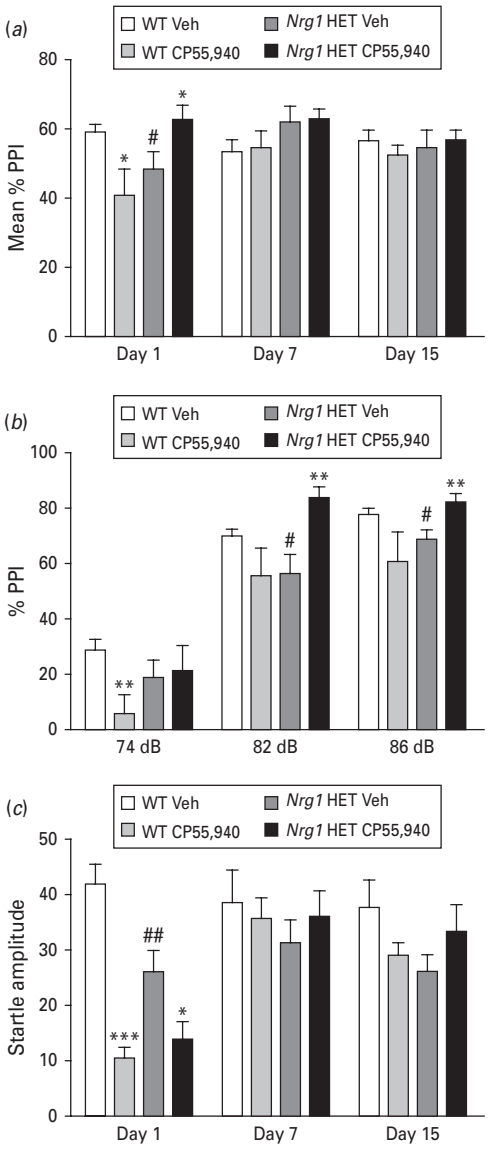

Fig. 2. Tolerance to the effects of CP55,940 (0.4 mg/kg) compared to vehicle (Veh) of wild-type (WT) and *Nrg1* HET mice on (*a*) %PPI (mean of the different prepulse intensities), (*b*) %PPI for the different prepulse intensities on day 1 and (*c*) startle response (WT Veh, $n=12$; WT CP55,940, $n=11$; *Nrg1* HET VEH, $n=8$; *Nrg1* HET CP55,940, $n=8$). Data are presented as means ± S.E.M. Significant effects of CP55,940 compared to Veh within the same genotype group are indicated by * $p<0.05$, ** $p<0.01$ and *** $p<0.001$. For clarity of exposition, we have only demarcated significant effects of *Nrg1* HET mice compared to WT mice in Veh-treated animals using $^{\#}p<0.05$ and $^{\#\#}p<0.01$.

Complete tolerance developed to CP55,940-induced locomotor suppression in both genotypes by days 5–15.

Prepulse inhibition

In the PPI paradigm (Fig. 2*a*) three-way ANOVA revealed no overall effect of genotype, treatment or test day on %PPI. However, genotype × treatment [$F(1,35)=4.49$, $p<0.05$] and genotype × treatment × test day [$F(2,70)=5.42$, $p<0.01$] interactions were observed. On day 1, two-way ANOVA revealed a genotype × treatment interaction [$F(1,35)=9.57$, $p<0.01$] and *post-hoc* analysis using Tukey's test showed that acute CP55,940 exposure decreased %PPI in WT mice ($p<0.05$) while it increased %PPI in *Nrg1* HET mice ($p<0.05$). Tukey's *post-hoc* test on day 1 also revealed that Veh-treated *Nrg1* HET mice displayed significantly less %PPI compared to Veh-treated WT mice ($p<0.05$), while CP55,940-treated *Nrg1* HET mice have an increased %PPI compared to CP55,940-treated WT mice ($p<0.05$). An overall genotype effect was observed on day 7 with two-way ANOVA [$F(1,35)=4.26$, $p<0.05$]. However, no *post-hoc* genotype or treatment effects were observed on days 7 and 15.

As individual group effects were only observed on day 1, we also analysed %PPI on this day for each PI (Fig. 2*b*). Three-way ANOVA revealed a genotype × treatment interaction on the different PIs [$F(1,35)=9.57$, $p<0.01$] and an effect of the different PIs [$F(2,70)=145.75$, $p<0.001$]. Analysis of the different PIs using two-way ANOVA showed that the genotype × treatment interaction was only found for the highest intensities, i.e. 82 dB [$F(1,35)=9.69$, $p<0.01$] and 86 dB [$F(1,35)=5.45$, $p<0.05$]. Tukey's *post-hoc* test revealed that at 74 dB, CP55,940 decreased %PPI only in WT animals ($p<0.01$). This effect on WT mice was not observed for the other intensities of prepulse. Increased %PPI was revealed only in *Nrg1* HET mice for PIs of 82 dB ($p<0.01$) and 86 dB ($p<0.01$). Tukey's *post-hoc* test also revealed genotype effects where a decreased %PPI was observed in Veh-treated *Nrg1* HET mice compared to WT at the 82 dB ($p<0.05$) and 86 dB ($p<0.05$) PIs. In CP55,940-treated mice, an increased %PPI was seen in *Nrg1* HET mice compared to WT for a PI of 82 dB ($p<0.01$).

During the PPI test, we also measured the startle response of the animals to a 120 dB acoustic stimulus (Fig. 2*c*). Three-way ANOVA showed CP55,940 administration decreased the startle response [$F(1,35)=4.2$, $p<0.05$]. No effect of genotype was observed but a significant genotype × treatment interaction [$F(1,35)=4.14$, $p<0.05$], an effect of test day [$F(2,70)=20.67$, $p<0.001$] and a treatment × test day interaction [$F(2,70)=21.03$, $p<0.001$] were observed. Analysis of the different days using two-way ANOVA revealed both a treatment effect [$F(1,35)=45.12$,

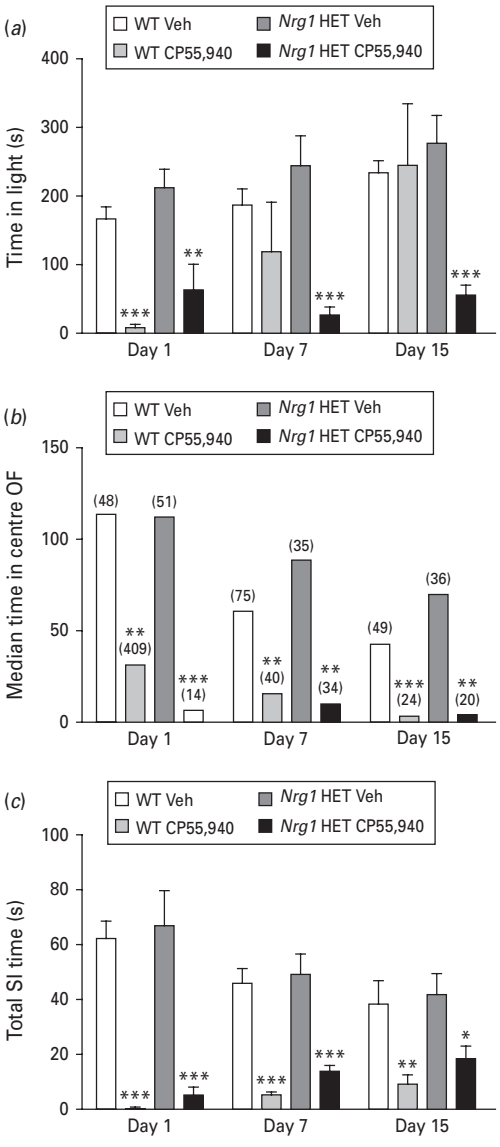

Fig. 3. Tolerance to the effects of CP55,940 (0.4 mg/kg) compared to vehicle (Veh) of wild-type (WT) and *Nrg1* HET mice on (*a*) the light-dark test (WT Veh, $n=11$; WT CP55,940, $n=10$; *Nrg1* HET Veh, $n=7$; *Nrg1* HET CP55,940, $n=8$), (*b*) the open field (OF) test and (*c*) social interaction (SI) test (WT Veh, $n=12$; WT CP55,940, $n=11$; *Nrg1* HET Veh, $n=8$; *Nrg1* HET CP55,940, $n=8$). Data are presented as means ± S.E.M. for panels (*a*) and (*c*), and as medians (interquartile range) for panel (*b*). Significant effects of CP55,940 compared to Veh within the same genotype group are indicated by * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

$p<0.001$] and a genotype × treatment interaction [$F(1,35)=8.91$, $p<0.01$] on day 1 only. Tukey's *post-hoc* test showed that CP55,940 treatment decreased startle response on day 1 in both *Nrg1* HET and WT mice ($p<0.05$ and $p<0.001$, respectively), with no effect seen on days 7 and 15. Veh-treated *Nrg1* HET mice exhibited less startle than Veh-treated WT mice to the 120 dB stimulus on day 1 ($p<0.01$), but not on days 7 or 15.

Anxiety in light-dark, open field and social interaction tests

Anxiety-related behaviours as measured in the LD, OF and SI tests are summarized in Fig. 3. In the LD test (Fig. 3*a*), three-way ANOVA showed that CP55,940 decreased the time spent in the light compartment over days [treatment: $F(1,32)=17.02$, $p<0.001$]. An effect of test day was also observed [$F(2,64)=6.55$, $p<0.01$] as the animals became less averse to exploring the light compartment. A differential effect of CP55,940 on WT and *Nrg1* HET mice was supported by a trend for a genotype × treatment interaction [$F(1,32)=3.78$, $p=0.06$]. This appeared to be due to altered development of tolerance to cannabinoid-induced anxiety over days with trends towards a significant genotype × test day [$F(2,64)=2.97$, $p=0.06$] and genotype × treatment × test day [$F(2,64)=2.95$, $p=0.06$] interactions. Two-way ANOVA on the different days revealed a genotype effect on day 1 [$F(1,32)=4.65$, $p<0.05$] and treatment effects on day 1 [$F(1,32)=44.52$, $p<0.001$] and day 7 [$F(1,32)=9.43$, $p<0.01$]. Interestingly, a genotype × treatment interaction was observed on day 15 [$F(1,32)=4.56$, $p<0.05$]. Tukey's *post-hoc* test reinforced the notion that *Nrg1* HET mice displayed a lack of tolerance to cannabinoid-induced anxiety, as CP55,940 decreased the time spent in the light of *Nrg1* HET mice compared to Veh on day 1 ($p<0.01$), day 7 ($p<0.001$) and day 15 ($p<0.001$), while it only decreased time in the light on day 1 in WT mice ($p<0.001$). *Post-hoc* comparisons between Veh-treated *Nrg1* HET and WT mice showed no difference in time spent in the light compartment in the LD test on any test day.

For time spent in the centre of the OF, an overall group effect was observed using the non-parametric Kruskal–Wallis test ($p<0.001$, Fig. 3*b*). Between-group comparisons using the Mann–Whitney *U* test on the different days showed that CP55,940 decreased the time spent in the centre of the OF of both WT and *Nrg1* HET mice on day 1 ($p<0.01$ and $p<0.001$, respectively), day 7 ($p<0.01$ and $p<0.01$, respectively) and day 15 ($p<0.001$ and $p<0.01$, respectively). Comparisons between *Nrg1* HET and WT mice for each treatment group showed that CP55,940-treated *Nrg1* HET mice spent less time in the centre of the OF on day 1 than CP55,940-treated WT mice ($p<0.01$).

Table 1. FosB/ΔFosB and c-Fos counts following 15 d repeated treatment with CP55,940 (0.4 mg/kg) or vehicle (Veh) in wild-type (WT) and *Nrg1* HET mice (WT Veh, $n=12$; WT CP55,940, $n=11$; *Nrg1* HET Veh, $n=8$; *Nrg1* HET CP55,940, $n=8$)

			WT		*Nrg1* HET	
Region	Bregma	Marker	Veh	CP55,940	Veh	CP55,940
mPFC	+1.98	FosB	22.3±3.2	24.4±3.8	22.1±3.4	21.7±4.1
		c-Fos	29.5±6.3	22.6±4.8	24.1±6.7	12.0±2.7
NAc shell	+0.98	FosB	47.5±9.4	41.5±10.9	50.4±6.2	55.3±10.8
		c-Fos	13.8±3.7	9.7±2.3	12.9±4.0	11.6±5.3
NAc core	+0.98	FosB	56.4±13.4	50.6±9.3	53.4±6.1	59.6±14.9
		c-Fos	5.0±1.7	2.3±0.8	3.6±1.5	3.1±1.8
LSV	+0.98	FosB	56.8±5.5	53.7±7.3	41.6±4.7	69.1±7.0**
		c-Fos	67.5±9.5	86.1±10.0	69.8±8.5	67.1±9.1
dlBNST	+0.26	FosB	6.2±0.7	7.1±1.2	5.7±2.0	6.9±1.5
		c-Fos	5.8±1.2	3.5±0.9	5.8±0.7	2.9±1.1*
PVN	−0.94	FosB	6.9±1.8	16.9±5.6	4.6±0.8	7.4±1.6
		c-Fos	19.5±3.8	38.4±9.9	11.9±4.4	22.1±7.2
PVT	−0.94	FosB	0.8±0.5	6.2±1.4**	1.9±0.6	4.5±1.1
		c-Fos	39.1±6.9	84.1±17.6*	41.7±9.5	53.9±14.4
CEA	−1.34	FosB	14.9±2.7	29.3±5.5*	14.3±3.2	25.5±3.3*
		c-Fos	6.8±1.4	7.7±1.4	3.4±1.3	6.6±2.1
VTA	−3.28−3.40	FosB	0.1±0.1	0.7±0.3	0.7±0.4	0.8±0.3
		c-Fos	3.1±0.8	5.5±1.4	3.0±1.5	2.5±1.3

mPFC, Medial prefrontal cortex; NAc, nucleus accumbens; LSV, lateral septum, ventral part; dlBNST, dorsolateral bed nucleus of the stria terminalis; PVN, paraventricular nucleus of the hypothalamus; PVT, paraventricular nucleus of the thalamus; CEA, central nucleus of the amygdala; VTA, ventral tegmental area.
Data are presented as means ± S.E.M. Significant effects of CP55,940 compared to Veh within the same genotype group are indicated by * $p<0.05$ and ** $p<0.01$.

The results from the total time in the SI test are shown in Fig. 3*c*. Three-way ANOVA showed that repeated administration of CP55,940 decreased overall SI time [$F(1,35)=107.18$, $p<0.001$]. A significant drug × test day interaction was observed [$F(2,70)=11.11$, $p<0.001$]. No overall genotype difference was observed over days. Analysis of the different days separately by two-way ANOVA revealed treatment effects on day 1 [$F(1,35)=89.87$, $p<0.001$], day 7 [$F(1,35)=64.79$, $p<0.001$] and day 15 [$F(1,35)=15.72$, $p<0.001$]. Little CP55,940-induced tolerance was observed, as significant *post-hoc* effects of treatment were observed on all days for both WT [day 1 ($p<0.001$), day 7 ($p<0.001$), day 15 ($p<0.01$)] and *Nrg1* HET mice [day 1 ($p<0.001$), day 7 ($p<0.001$), day 15 ($p<0.05$)]. A genotype effect in SI was seen on day 7 with CP55,940-treated *Nrg1* HET mice showing increased SI compared to CP55,940-treated WT mice ($p<0.001$).

Fos immunohistochemistry

Table 1 shows c-Fos and FosB/ΔFosB counts in different brain areas. Of all brain regions analysed, two-way ANOVA revealed only one significant genotype × treatment interaction [$F(1,34)=5.6$, $p<0.05$] which was for FosB/ΔFosB expression in the LSV. Tukey's *post-hoc* test showed that chronic CP55,940 treatment selectively increased FosB/ΔFosB expression in the LSV of *Nrg1* HET mice ($p<0.01$) but not WT mice (Fig. 4). No concomitant effect of repeated cannabinoid exposure was observed for c-Fos expression in this brain region. Repeated CP55,940 treatment increased FosB/ΔFosB expression in only two other regions, the paraventricular nucleus of the thalamus (PVT) [$F(1,35)=15.7$, $p<0.001$] and in the central nucleus of the amygdala (CEA, Fig. 5) [$F(1,34)=9.2$, $p<0.01$]. In the CEA, *post-hoc* test showed that CP55,940 increased FosB/ΔFosB in both WT and *Nrg1* HET mice equally (p values <0.05), without modulating c-Fos expression. In the PVT, two-way ANOVA showed a significant effect of treatment on both c-Fos and FosB/ΔFosB expression. Tukey's *post-hoc* test highlighted a selective increase in FosB/ΔFosB and c-Fos expression in WT animals ($p<0.01$ and $p<0.05$, respectively).

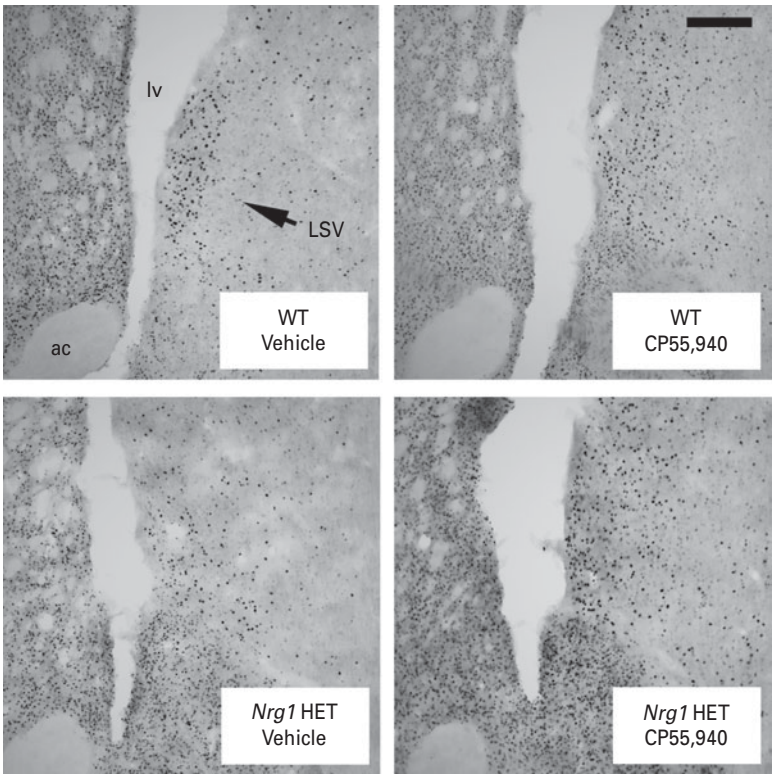

Fig. 4. FosB/ΔFosB-labelled neurons within the lateral septum, ventral part (LSV) in representative sections from wild-type (WT) *Nrg1* HET mice treated with vehicle or CP55,940 (0.4 mg/kg). The lateral ventricle (lv) and anterior commissure (ac) are also indicated. Scale bar, 200 μm.

Two-way ANOVA showed that repeated CP55,940 exposure decreased c-Fos expression in the dorsolateral bed nucleus of the stria terminalis [$F(1,34) = 6.16$, $p < 0.05$] which was only significant for *Nrg1* HET mice compared to WT mice as highlighted by Tukey's *post-hoc* test ($p < 0.05$). Two-way ANOVA indicated that CP55,940 treatment increased c-Fos expression in the paraventricular nucleus of the hypothalamus (PVN) [$F(1,32) = 4.2$, $p < 0.05$] albeit equally in both *Nrg1* HET and WT mice. The regions where two-way ANOVA found no significant genotype, treatment or genotype × treatment interaction on either c-Fos and FosB/ΔFosB expression included the medial prefrontal cortex (mPFC), the shell and core of the nucleus accumbens (NAC) and the ventral tegmental area (VTA).

Discussion

Within 5 d of repeated cannabinoid exposure *Nrg1* hypomorphic mice more rapidly developed tolerance to cannabinoid-induced hypothermia and locomotor suppression than WT mice. However, *Nrg1* HET mice maintained consistent cannabinoid-induced anxiety in the LD test over days, unlike WT mice who developed complete tolerance to this effect by day 15 of exposure. By contrast, no tolerance was observed for both genotypes in social anxiety promoted by CP55,940 as measured in the SI test, even after 15 d of drug exposure. The most striking neurobiological correlate of repeated cannabinoid treatment was that FosB/ΔFosB was selectively increased in the LSV of *Nrg1* HET mice but not WT mice.

Here we replicated the finding of Stefansson *et al*. (2002) by showing that on the first day of testing, Veh-treated *Nrg1* HET mice display deficits in PPI. However, this PPI deficit may be confounded by *Nrg1* HET mice also showing an impaired startle response. In our previous work we observed no PPI or startle deficit in *Nrg1* HET mice which might be explained by the experimental design implemented (Boucher *et al*. 2007*a*). In our previous study a within-subjects, counterbalanced design was used, where most Veh-treated animals had prior test exposure to the PPI chambers. Therefore familiarity with PPI testing may determine whether deficits in acoustic startle and PPI are observed in *Nrg1* HET mice. Reinforcing this view is the fact that *Nrg1* HET mice only showed a deficit in

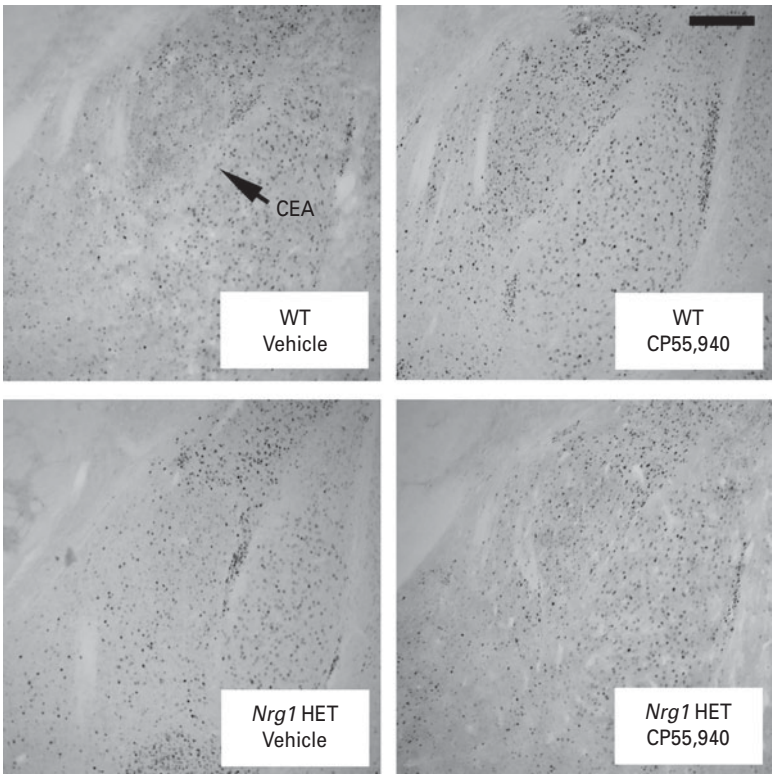

Fig. 5. FosB/ΔFosB-labelled neurons within the central nucleus of the amygdala (CEA) in representative sections from wild-type (WT) *Nrg1* HET mice treated with vehicle or CP55,940 (0.4 mg/kg). Scale bar, 200 μm.

startle response and PPI on day 1, which was not replicated on days 7 and 15 of testing.

At face value our results support research showing cannabinoids promote PPI deficits in WT mice (Martin *et al*. 2003; Nagai *et al*. 2006). However, our data and the work of others have frequently failed to dissociate PPI deficits from cannabinoid-induced acoustic startle reflex impairments. For example, Nagai *et al*. (2006) reported PPI deficits following THC exposure; however, this effect was confounded by a concomitant decrease in startle reactivity. Further, Martin *et al*. (2003) showed CP55,940 impaired PPI utilizing a high dose that also blunted the acoustic startle response. No deficits in PPI or the startle reflex were observed at lower doses, consistent with work showing cannabinoids have no effect on PPI (Boucher *et al*. 2007*a*; Malone & Taylor, 2006). Together this highlights that an unambiguous demonstration of cannabinoid-induced PPI deficits are rare in the literature. One exception to this is the report that a synthetic cannabinoid, structurally unrelated to THC, promoted PPI deficits without affecting the acoustic startle response (Schneider & Koch, 2002).

It is of note that acute cannabinoid exposure differentially affected PPI depending on the genotype of the mice. In contrast to WT mice where CP55,940 promoted PPI deficits, it facilitated PPI in *Nrg1* HET mice. This is consistent with our previous study showing PPI facilitation in THC-treated *Nrg1* HET mice, even in mice that showed no cannabinoid-induced reduction in acoustic startle (Boucher *et al*. 2007*a*). However, here the high dose of CP55,940 suppressed acoustic startle in both *Nrg1* HET and WT mice, confounding the differential PPI effect we observed on day 1. Although, it is of note that the opposite effects of CP55,940 on PPI in WT *vs. Nrg1* HET mice were observed despite CP55,940 suppressing acoustic startle in both genotypes. This underscores that suppression of the acoustic startle response can correlate with completely opposite effects on PPI. As heavy cannabis abuse is more reliably associated with worsening of symptoms in patients with schizophrenia (Linszen *et al*. 1994) we predicted that repeated high-dose cannabinoid exposure might alter the responsivity of *Nrg1* HET mice in the PPI paradigm. However, no such effects were observed, and by day 7 both genotypes were completely tolerant to the effects of CP55,940 on startle and PPI.

It is well-established that cannabinoids have acute biphasic effects with low doses being anxiolytic and

high doses producing an anxiogenic profile in animal models (Berrendero & Maldonado, 2002; Boucher *et al*. 2007*a*; Hill & Gorzalka, 2004; Viveros *et al*. 2005). However, few studies have addressed tolerance to cannabinoid modulation of emotional behaviour in rodents. In the LD test, the first administration of CP55,940 decreased the time spent in the light compartment in both WT and *Nrg1* HET mice, although this effect is possibly confounded by the locomotor suppression promoted by such a high-dose of CP55,940. In WT mice, cannabinoid-induced anxiety progressively decreased over days 7 and 15 indicative of tolerance, consistent with one study that showed tolerance develops to cannabinoid-induced anxiety in the elevated plus-maze (Caberlotto *et al*. 2004). Surprisingly, no such tolerance to cannabis-induced anxiety was observed in *Nrg1* HET mice in the LD test, with these mice maintaining a pronounced aversion to the light compartment over days. Importantly, as the animals were tolerant to the locomotor-suppressant effects of CP55,940 by days 7 and 15, the persistent cannabinoid-induced anxiety observed in *Nrg1* HET mice cannot be explained by locomotor impairment.

While *Nrg1* HET but not WT mice displayed persistent cannabinoid-induced anxiety-related behaviour in the LD test, in the OF and SI models both genotypes showed consistent reductions in prosocial behaviours and exploration of the centre of the OF on days 1, 7 and 15. This supports that various animal models of anxiety assay distinct forms of anxiety (Rodgers, 1997) which are differentially susceptible to cannabinoid tolerance. Our study also confirms research showing that tolerance to the various pharmacological effects of cannabinoids develops at different rates. That is, tolerance to cannabinoid-induced hypothermia develops first, followed by locomotor suppression, anxiety-related behaviour with the memory-impairing effects of the drug being the most resistant to tolerance (Boucher *et al*. 2009; Frischknecht *et al*. 1982; Gonzalez *et al*. 2005; Whitlow *et al*. 2003).

Our results showing that *Nrg1* HET mice develop tolerance to cannabinoid-induced hypothermia and locomotion suppression may seem contradictory given that these animals show resistance to tolerance to anxiety promoted by cannabinoids in the LD test. However, this suggests that *Nrg1* deficiency may trigger different alterations in the discrete brain regions that subserve cannabinoid tolerance to anxiety, motor behaviour and BT. A recent human study has reported that substance-abusing individuals with a psychotic disorder displayed a more rapid development of tolerance to cannabis than people with substance dependence with no comorbid psychiatric disorder (Saddichha *et al*. 2010). Given our findings here it would be interesting to further investigate such psychotic, substance-abusing patients to observe whether their increased vulnerability to tolerance development is explained by an increased incidence of variation in *Nrg1* compared to the non-psychiatric, substance-dependent patients.

Here we report for the first time, that 15 d of repeated cannabinoid treatment selectively enhanced FosB/ΔFosB expression in the LSV in *Nrg1* HET mice but not WT mice. This finding is in accord with our previous data showing acute THC treatment increased c-Fos expression in the LSV of *Nrg1* HET mice but not WT mice and this selective induction of c-Fos was dependent on the stress of behavioural testing (Boucher *et al*. 2007*b*). Indeed, the three-way interaction between *Nrg1*, cannabinoids and stress appeared to activate a stress-related circuit in the brain with the LSV being of central importance. Our current result with FosB/ΔFosB further supports that the LSV is an important site of cannabinoid-promoted dysregulation in *Nrg1* HET mice. In addition, our results suggest that the LSV might be involved in the lack of tolerance to cannabis-induced anxiety observed in these animals.

The current study implies that the acute, selective induction of c-Fos in the LSV of *Nrg1* HET mice by THC (Boucher *et al*. 2007*b*) is susceptible to tolerance, with no such effect in the LSV being observed in *Nrg1* HET mice that were repeatedly exposed to CP55,940. This is consistent with c-Fos being more robustly expressed to acute drug exposures (Hughes & Dragunow, 1995). Exceptions to this view are reported here, with WT mice selectively exhibiting an increase in CP55,940-induced c-Fos expression in the PVT after repeated exposure. Furthermore, in the PVN, a site of acute cannabinoid-induced c-Fos expression, continued c-Fos expression was observed following 15 d of exposure as supported by a main effect of CP55,940 treatment. Unlike c-Fos, the selective induction of FosB/ΔFosB we observed here in *Nrg1* hypomorphs in response to repeated cannabinoid exposure might be resistant to tolerance. Alternatively it may be that ΔFosB is induced by repeated exposure to CP55,940 that might contribute to lasting behavioural modifications such as persistent cannabinoid-induced anxiety (Nestler *et al*. 1999). However, our antibody does not discriminate between FosB and ΔFosB, thus it cannot be ruled out that this effect is merely due to FosB. Unfortunately, current immunohistochemical methods are limited in their ability to directly stain ΔFosB. Our future studies will attempt to disentangle the contribution of FosB and ΔFosB by using other

techniques which allow direct assessment of these isoforms.

As cannabinoids activate the HPA axis and stress-related regions of the brain (Arnold *et al*. 2001*b*; Rodriguez de Fonseca *et al*. 1996), it is possible that the mere 'stress' of cannabinoid exposure is responsible for *Nrg1* HET mice showing up-regulation of FosB/ΔFosB in the LSV and persistent anxiety-related behaviour. Repeated stress exposure in rats strongly induces the expression of ΔFosB in the LSV and more weakly in the CEA (Perrotti *et al*. 2004; Sheehan *et al*. 2004). Here we showed cannabinoid-induced FosB/ΔFosB in the CEA of both WT and *Nrg1* HET mice, but only in the LSV of *Nrg1* hypomorphic mice. Thus, it is possible that neurodevelopmental defects accrued through a deficiency in *Nrg1* in hypomorphic mice that makes them vulnerable to gene transcription changes promoted by cannabinoids in the LSV.

Our results highlight that *Nrg1* modulates the repeated neurobehavioural effects of cannabinoids and provide an animal model to further explore the neurobiological basis of genetic vulnerability to the adverse effects of cannabis. These data support the view that partial deletion of *Nrg1* may predispose to, on one hand accelerated development of tolerance to cannabinoid sedation and hypothermia, while on the other facilitating persistent aversive emotional responses evoked by cannabinoids. Interestingly, a recent clinical study showed that schizophrenia patients with a polymorphism in *NRG1* were more likely to have delusional thoughts in response to psychosocial stress (Keri *et al*. 2009). Thus, it would be interesting to further examine whether individuals with polymorphisms in *NRG1* are more susceptible to negative emotional responses to the stress of cannabinoid exposure.

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Statement of Interest

None.

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