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ARTICLE

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Cysteamine treatment ameliorates alterations in GAD67 expression and spatial memory in heterozygous reeler mice

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Abstract

Brain-derived neurotrophic factor (BDNF) signalling through its receptor, TrkB is known to regulate GABAergic function and glutamic acid decarboxylase (GAD) 67 expression in neurons. Alterations in BDNF signalling have been implicated in the pathophysiology of schizophrenia and as a result, they are a potential therapeutic target. Interestingly, heterozygous reeler mice (HRM) have decreased GAD67 expression in the frontal cortex and hippocampus and they exhibit many behavioural and neurochemical abnormalities similar to schizophrenia. In this study, we evaluated the potential of cysteamine, a neuroprotective compound to improve the deficits in GAD67 expression and cognitive function in HRM. We found that cysteamine administration (150 mg/kg.d, through drinking water) for 30 d significantly ameliorated the decreases in GAD67, mature BDNF and full-length TrkB protein levels found in frontal cortex and hippocampus of HRM. A significant attenuation of the increased levels of truncated BDNF in frontal cortex and hippocampus, as well as truncated TrkB in frontal cortex of HRM was also observed following cysteamine treatment. In behavioural studies, HRM were impaired in a Y-maze spatial recognition memory task, but not in a spontaneous alternation task or a sensorimotor, prepulse inhibition (PPI) procedure. Cysteamine improved Y-maze spatial recognition in HRM to the level of wide-type controls and it improved PPI in both wild-type and HRM. Finally, mice deficient in TrkB, showed a reduced response to cysteamine in GAD67 expression suggesting that TrkB signalling plays an important role in GAD67 regulation by cysteamine.

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Introduction

There is growing interest in the potential role of the gamma amino butyric acid (GABA) neurotransmitter system in the pathophysiology and behavioural abnormalities of schizophrenia. GABA has also been implicated in multiple processes of neural development (Owens & Kriegstein, 2002) such as cell proliferation (LoTurco *et al.* 1995), neurite growth (Spoerri, 1988) and adult neurogenesis (Ge *et al.* 2006). A number of studies have reported decrease in mRNA and protein levels of glutamic acid decarboxylase (GAD) 67, a rate-limiting enzyme in GABA synthesis in prefrontal cortex as well as other brain areas of schizophrenia subjects (Akbarian & Huang, 2006). In animal studies, GABA levels were reduced in adult heterozygotes of GAD67 knockout mice (Asada *et al.* 1997). Studies also suggest that the alterations in GABAergic system appear to have an important role in the cognitive abnormalities found in schizophrenia (Lewis & Moghaddam, 2006).

Brain-derived neurotrophic factor (BDNF) signalling through TrkB has been shown to be a key regulator in GAD67 expression and GABA function (Arenas *et al.* 1996; Mizuno *et al.* 1994). TrkB levels were found significantly lower in prefrontal cortex and hippocampus of schizophrenia subjects (Takahashi *et al.*

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2000; Thompson Ray *et al.* 2011; Weickert *et al.* 2005). In addition, a significant correlation between GAD67 and TrkB mRNA levels were found in the prefrontal cortex of schizophrenia subjects (Hashimoto *et al.* 2005). The relationship between GAD67 and TrkB was further supported by the evidence that TrkB hypomorphic mice, but not BDNF knockout mice have reduced GAD67 expression in prefrontal cortex (Hashimoto *et al.* 2005). These studies suggest that TrkB signalling is a possible molecular target to regulate GAD67 expression in cortex.

Heterozygous reeler mice (HRM) have many neuropathological and behavioural abnormalities homologous to schizophrenia, such as increased neuronal packing density and decreased spine density with GABAergic defect as reflected in down-regulation of GAD67 expression in frontal cortex (Liu *et al.* 2001) and hippocampus (Nullmeier *et al.* 2011), as well as deficits in prepulse inhibition (PPI) of startle (Tueting *et al.* 1999). Reelin is a large extracellular matrix protein that plays a critical role during brain development, but also continues to be expressed in adult cortex and hippocampus (D'Arcangelo *et al.* 1995). In the adult brain, reelin is secreted by GABAergic interneurons into the extracellular space surrounding dendrites, dendritic spines and axon boutons (Alcántara *et al.* 1998; Pappas *et al.* 2001). It has been shown that reelin expression is down-regulated in GABAergic neurons of the prefrontal (Brodmann areas 10 and 46) temporal and parietal cortices, hippocampus, caudate nucleus, and glutamatergic cerebellar neurons of schizophrenia patients (Guidotti *et al.* 2000). Our recent experiments have shown that HRM have decreased TrkB signalling in frontal cortex (Pillai & Mahadik, 2008).

Cysteamine, the FDA-approved drug currently prescribed for cystinosis, has antioxidant and anti-apoptotic properties (Lesort *et al.* 2003; Oliverio *et al.* 1999), and it increases brain as well as serum BDNF levels in rodents (Borrell-Pagès *et al.* 2006; Pillai *et al.* 2008). In the present study, we evaluated the potential of cysteamine to improve the deficits in GAD67 expression and cognitive function in HRM. Since TrkB plays an important role in the regulation of GAD67 expression, we investigated whether TrkB is necessary for cysteamine-induced effects on GAD67 expression using TrkB knockout mice.

Materials and methods

Animals and housing

Male HRM (B6C3Fe reln) and background strain wild-type (WT) mice, aged 2–3 months, were obtained from Jackson Laboratories (USA). TrkB knockout ($TrkB^{-/-}$) mice were provided by Dr Barbara Rohrer, Medical University of South Carolina, Charleston, SC, and the colony was maintained in our animal housing facility at the Georgia Health Sciences University. The generation of mice lacking TrkB has been described previously (Rohrer *et al.* 2004). Male WT and $TrkB^{-/-}$ mice used in a given experiment, originated from the same breeding series and were matched for age and weight (age 2–3 months, weight 25–30 g). Animals were housed ($n=4$ per cage) with food and water available *ad libitum*. Mice were maintained on a 12-h light/dark cycle (lights on 07:00 hours). All experimental procedures were performed during the light cycle. Animal use procedures were performed after being reviewed and approved by Medical College of Georgia, Committee on Animal Use for Research and Veterans Affairs Medical Center Subcommittee on Animal Use. Procedures were consistent with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines as per Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Drug treatment

Figure 1 describes the experimental design used for the drug treatment. Cysteamine (Sigma, USA) was dissolved in water and was delivered *ad libitum* in the drinking water (150 mg/kg body wt/d). Cysteamine dose was selected based on earlier studies where this concentration was found non-toxic, but showed neuroprotective effects (Borrell-Pagès *et al.* 2006; Pillai *et al.* 2008). The amount of drug intake was measured daily, and adjustments were made depending upon the fluid consumed and weight of the animals. Tap water was used for the control group to ensure that unanticipated effect of the vehicle was not present. All animals were monitored for change in body weight and food intake as possible adverse effects of the treatment.

Behavioural studies

All behavioural experiments were conducted in rooms equipped with white-noise generators (San Diego Instruments, USA) set to provide a constant background level of 70 dB, and ambient lighting of approximately 25–30 lx (lumen/m^2). Animals were transferred (in their home cages) to the behavioural testing rooms each morning approximately 30 min before the beginning of experiments. Significant efforts were also made to minimize the total number of

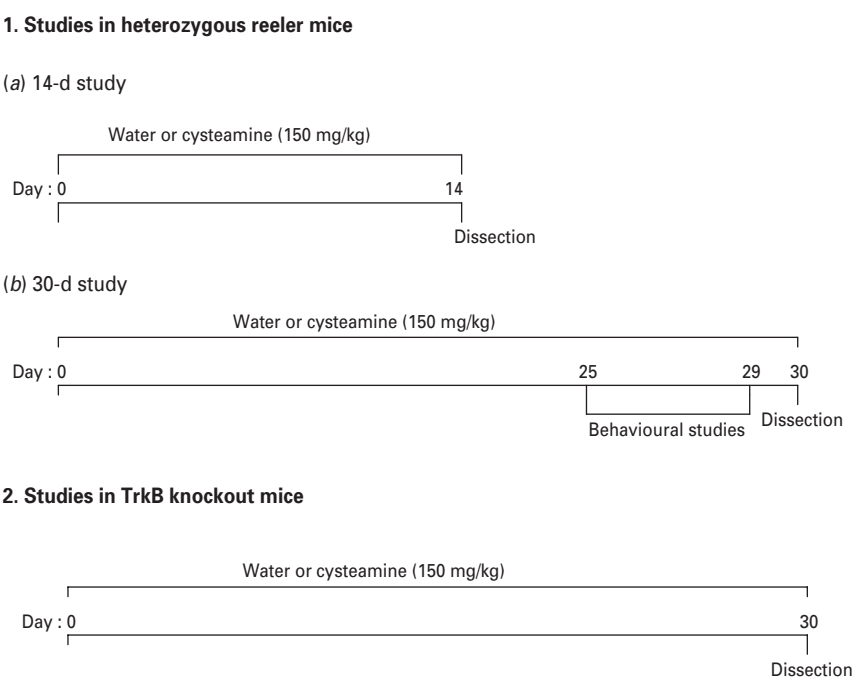

Fig. 1. Experimental designs used for testing the behavioural and biochemical responses to cysteamine treatment in mice. (*a*) Cysteamine (150 mg/kg) or water (vehicle) was administered through drinking water to heterozygous reeler mice (HRM) and wild-type (WT) mice for 14 d. On day 14, mice were killed and brains removed for biochemical analyses. (*b*) Cysteamine (150 mg/kg) or water (vehicle) was administered through drinking water to HRM and WT mice for 30 d. In order to study the behavioural responses to cysteamine or vehicle treatment, animals were tested for behavioural studies from day 25 to day 29. On day 30, mice were killed and brains removed for biochemical analyses. (*b*) Cysteamine (150 mg/kg) or water (vehicle) was administered through drinking water to TrkB knockout and WT mice for 30 d. On day 30, mice were killed and brains removed for biochemical analyses.

animals used while maintaining statistically valid group numbers.

Three behavioural tests were performed during the last week of drug treatment, conducted in the following order: Y-maze spontaneous alternation, Y-maze two-trial recognition memory test, and PPI. All behavioural tests were conducted between 09:00 and 17:00 hours.

Y-maze tests

To assess the effects of genotype and cysteamine on memory-related behaviours, two Y-maze tasks were used, spontaneous alternation and the two-trial recognition test.

Apparatus. The Y-maze test apparatus consisted of three arms made of acrylic glass (Plexiglas) (painted black) and joined in the middle to form a symmetrical 'Y' shape. The arms of the apparatus were 40 cm long $\times$ 12 cm wide. The walls of the arms were 35 cm high, allowing the mouse to see distal spatial landmarks while the inside of the arms were identical, providing no intramaze cues.

Spontaneous alternation test. In this task, test animals were placed in the Y maze (with all arms open) for 10 min and all arm entries were sequentially scored. The dependent variables were defined as the number of arms entered, and percent alternation, calculated as the number of alternations (entries into three different arms consecutively) divided by the total possible alternations, i.e.: (no. of arms entered $-2) \times 100$.

Two-trial recognition memory test. With one arm of the Y-maze blocked, animals were placed in one of the arms (start arm) and allowed to explore the two open arms for 10 min. The animal was then returned to the maze 2 h later with all arms open and scored for 5 min. The amount of time spent in each arm, and the number of entries into each arm was recorded. The time spent and entries into the previously unexplored arm were assessed as measures of recognition memory.

PPI

To assess the effects of genotype and cysteamine on sensorimotor gating a PPI procedure (modified for mice) was conducted as described previously (Terry *et al.* 2005). Four startle chambers (San Diego Instruments, USA) were used consisting of a Plexiglas tube (diameter 2.8 cm, length 8.9 cm) placed in a sound-attenuating chamber, in which the mice were individually placed. The tube was mounted on a plastic frame, under which a piezoelectric accelerometer was mounted, which records and transduces the motion of the tube. Two days before PPI testing the experimental animals were each placed in one of the startle test chambers for a period of 10 min (without any startle stimuli) as an initial period of acclimation to the apparatus. One day before PPI testing, the animals were again placed in the test chamber and then exposed to 12 startle stimuli and to each prepulse level three times. This procedure is performed to reduce the highly variable responses to the initial exposures to the startle stimuli as well as to ensure that the prepulse stimuli (alone) have no significant effect on the startle response. On the day of PPI testing, experimental animals were transported to the startle chamber room and left undisturbed for at least 30 min. Next, the mice were placed in the chamber, and allowed to habituate for a period of 5 min, during which time a 70-dB background white noise was present. After this period, the mice received 12 startle trials, 12 no-stimulus trials, and 12 trials of each of the prepulse/startle trials for a total of 60 trials. The inter-trial interval ranged from 10 to 30 s, and the total session lasted about 25–30 min. The startle trials consisted of single 120-dB white-noise bursts lasting 20 ms.

The PPI trials consisted of a prepulse (20-ms burst of white noise with intensities of 75, 80, or 85 dB) followed, 100 ms later, by a startle stimulus (120 dB, 20-ms white noise). During the no-stimulus trial, no startle noise was presented, but the movement of the mouse was recorded. This represents a control trial for detecting differences in overall activity. The 60 different trials were presented pseudorandomly, ensuring that each trial was presented 12 times and that no two consecutive trials were identical. The resulting movement of the mouse in the startle chamber was measured during 100 ms after startle stimulus onset (sampling frequency 1 kHz), rectified, amplified, and fed into a computer that calculates the maximal response over the 100-ms period. Basal startle amplitude was determined as the mean amplitude of the 12 startle trials. Prepulse inhibition was calculated according to the formula: $100 - 100\% \times (\mathrm{PPx}/\mathrm{P120})$, in which PPx is the mean of the 12 prepulse inhibition trials (i.e. for each individual prepulse intensity), and P120 is the basal startle amplitude.

Tissue sample preparation

Animals were sacrificed by cervical dislocation, and frontal cortex and hippocampal samples from vehicle as well as drug-treated mice were collected according to a mouse brain atlas (Paxinos & Franklin, 2001). Tissue homogenates were made from frozen tissue sonicated in radioimmune precipitation assay (RIPA) buffer [10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, 1% sodium deoxycholate] for Western blotting. This buffer was supplemented with a protease inhibitor cocktail (Sigma). After 15 min incubation on ice, the extracts were clarified by centrifugation at 16 000 *g* for 15 min at 4 °C and stored at −70 °C. Protein concentration was determined by the bicinchoninic acid method (BCA Protein Assay kit, Sigma).

Western blot analysis

Equal amounts of protein were resolved in SDS–polyacrylamide gels and transferred electrophoretically onto a nitrocellulose membrane. The membrane was blocked for 1 h in phosphate buffered saline (PBS) solution with the detergent Tween-20 [PBST; 3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl, 0.05% Tween-20, (pH 7.4.)] and 5% non-fat milk or 5% BSA. The membranes were incubated overnight with the indicated primary antibodies. The primary antibodies used were anti-BDNF (1:500; no. sc-546; Santa Cruz Biotechnology, USA), anti-TrkB (1:300; no. 4603; Cell Signaling, USA), anti-TrkB.TK- (1:500; no. sc-119; Santa Cruz Biotechnology), anti-GAD67 (1:500; no. sc-5602; Santa Cruz Biotechnology), and anti-β-actin (1:1000; no. A5316; Sigma). The membranes were washed with PBST then incubated with secondary antibody for 1 h. Proteins were visualized by enhanced chemiluminescence. The films were subsequently scanned, and band intensity was quantified by densitometry software (Image J, NIH, USA).

Statistical analysis

Results are expressed as the mean ± S.E. The significance of differences was determined by one-way analysis of variance (ANOVA) in experiments analysing protein levels and Y-maze analysis. Repeated-measures ANOVAs were used in PPI response measurement followed by *post-hoc* Bonferroni's test. p values <0.05 were regarded as statistically significant.

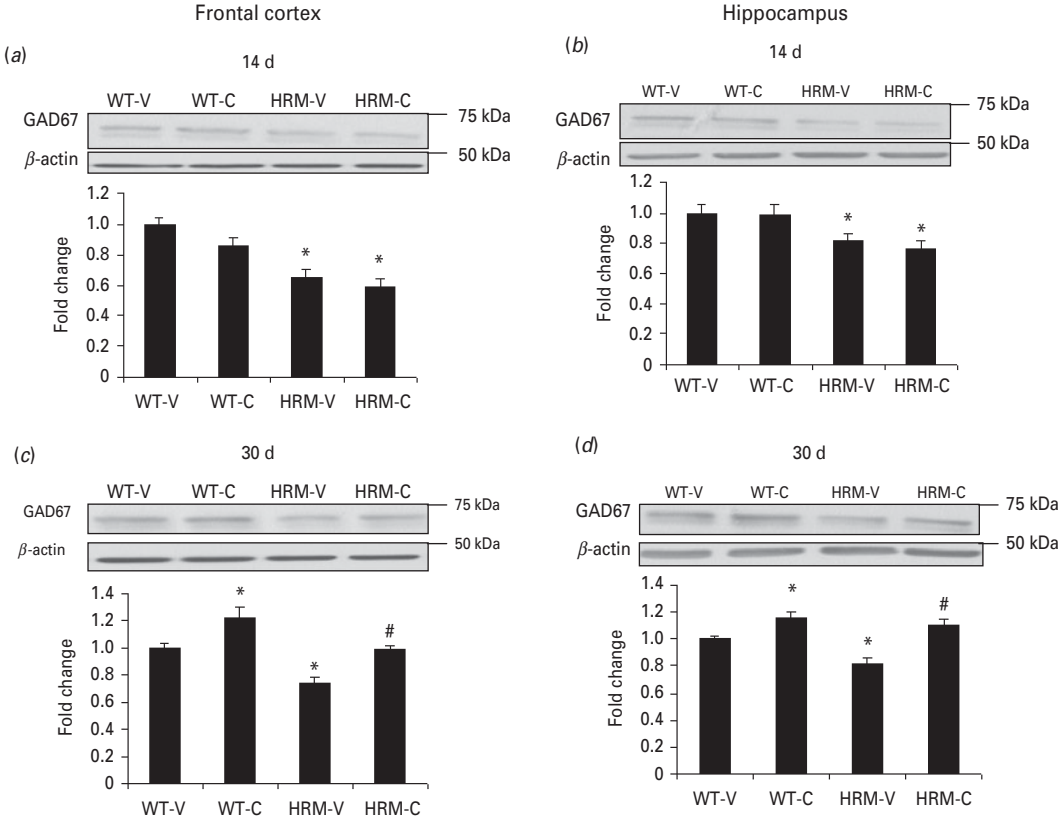

Fig. 2. Cysteamine treatment ameliorates the reduction in GAD67 expression in frontal cortex and hippocampus of heterozygous reeler mice (HRM). Cysteamine (150 mg/kg) or water (vehicle) was administered through drinking water to HRM and wild-type (WT) mice for 14 d (*a*, *b*) or 30 d (*c*, *d*). GAD67 expression was determined by Western blot analysis in frontal cortex (*a*, *c*) and hippocampus (*b*, *d*). WT-V, Wild-type treated with vehicle (water); WT-C, wild-type treated with cysteamine; HRM-V, heterozygous reeler mice treated with vehicle; HRM-C, heterozygous reeler mice treated with cysteamine. Data represent mean ± S.E. ($n = 5$ per group) expressed as fold change in GAD67 protein levels compared to WT-V (* $p < 0.05$ *vs.* WT-V; # $p < 0.05$ *vs.* HRM-V; Bonferroni's test). β-actin is the loading control.

Results

Behavioural studies were performed only in mice treated with vehicle or cysteamine for 30 d whereas biochemical analyses were performed in both 14-d and 30-d treatment groups. There were no differences in relative body weight gain or water intake in mice during the treatment.

Effect of cysteamine on GAD67 protein levels in HRM

Figure 2 shows the GAD67 protein levels in prefrontal cortex and hippocampus determined after 14 d or 30 d cysteamine administration. One-way ANOVA showed a significant group effect in GAD67 protein levels in frontal cortex [$F(3, 16) = 16.06$, $p < 0.0001$] and hippocampus [$F(3, 16) = 6.5$, $p = 0.0045$]. Subsequent comparisons by Bonferroni's multiple comparison test indicated significant reduction in GAD67 protein levels in both frontal cortex (Fig. 2*a*) and hippocampus (Fig. 2*b*) of HRM compared to WT mice ($p < 0.05$). A comparison between vehicle- and cysteamine-treated animals after 14 d treatment revealed no significant difference in the levels of GAD67 protein in the frontal cortex (Fig. 2*a*) and hippocampus (Fig. 2*b*). Mice treated with cysteamine or vehicle for 30 d showed a statistically significant group effect in GAD67 protein levels in frontal cortex [$F(3, 16) = 13.88$, $p = 0.0001$] and hippocampus [$F(3, 16) = 12.24$, $p = 0.0002$]. Subsequent analysis showed that WT mice treated with cysteamine for 30 d showed significant increases in GAD67 protein levels in frontal cortex (Fig. 2*c*) and hippocampus (Fig. 2*d*). The increases in GAD67 levels were also found in the frontal cortex (Fig. 2*c*) and hippocampus (Fig. 2*d*) of HRM ($p < 0.05$).

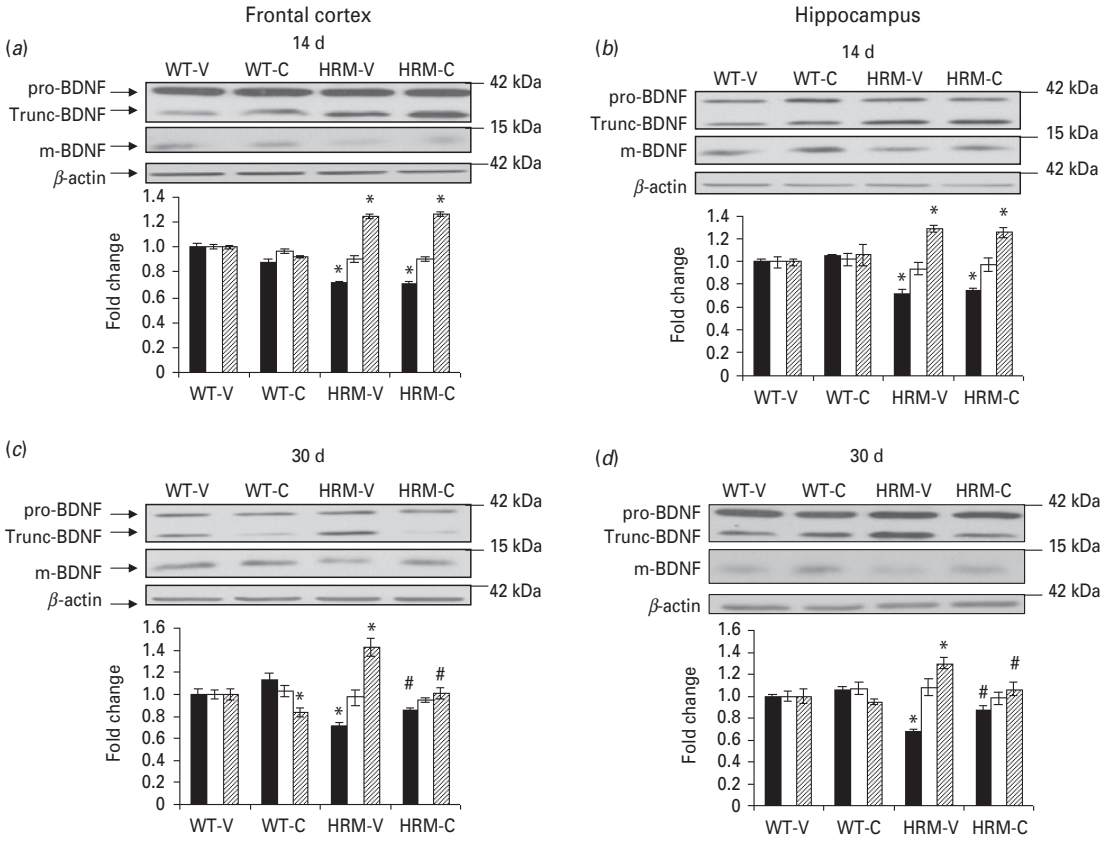

Fig. 3. Cysteamine treatment ameliorates the changes in brain-derived neurotrophic factor (BDNF) expression in frontal cortex and hippocampus of heterozygous reeler mice (HRM). Cysteamine (150 mg/kg) or water (vehicle) was administered through drinking water to HRM and wild-type (WT) mice for 14 d (*a*, *b*) or 30 d (*c*, *d*). Mature BDNF (m-BDNF), truncated BDNF (trunc-BDNF) and pro-BDNF were determined by Western blot analysis in frontal cortex (*a*, *c*) and hippocampus (*b*, *d*). WT-V, Wild-type treated with vehicle (water); WT-C, wild-type treated with cysteamine; HRM-V, Heterozygous reeler mice treated with vehicle; HRM-C, Heterozygous reeler mice treated with cysteamine. Solid bars represent m-BDNF, open bars represent pro-BDNF and hatched bars represent trunc-BDNF. Data represent mean ± S.E. ($n = 5$ per group) expressed as fold change in BDNF protein levels compared to WT-V (* $p < 0.05$ *vs.* WT-V; # $p < 0.05$ *vs.* HRM-V; Bonferroni's test). β-actin is the loading control.

Effect of cysteamine on BDNF protein levels in HRM

Our immunoblot analysis with BDNF antibody showed pro-BDNF (~32 kDa), truncated BDNF (~27 kDa) and mature BDNF (~14 kDa). One-way ANOVA showed a significant group effect in BDNF protein levels in frontal cortex [mature BDNF: $F(3,16) = 34.55$, $p < 0.0001$; truncated BDNF: $F(3,16) = 108.44$, $p < 0.0001$] and hippocampus [mature BDNF: $F(3,16) = 53.81$, $p < 0.0001$; truncated BDNF: $F(3,16) = 7.115$, $p < 0.003$] of mice treated with vehicle or cysteamine for 14 d. *Post-hoc* analysis revealed a significant decrease in mature BDNF protein and increase in truncated BDNF levels in frontal cortex and hippocampus of HRM compared to WT mice ($p < 0.05$). Cysteamine treatment for 14 d did not result in any significant change in BDNF protein levels in any of the brain regions examined (Fig. 3*a*, *b*). Next, we evaluated the effects of 30 d cysteamine treatment on BDNF proteins in frontal cortex and hippocampus of HRM and WT mice. One-way ANOVA showed a significant group effect in BDNF protein levels in frontal cortex [mature BDNF: $F(3,16) = 15.84$, $p < 0.0001$; truncated BDNF: $F(3,16) = 17.59$, $p < 0.0001$] and hippocampus [mature BDNF: $F(3,16) = 45.54$, $p < 0.0001$; truncated BDNF: $F(3,16) = 8.895$, $p = 0.0011$] of mice treated with vehicle or cysteamine for 30 d. *Post-hoc* analysis showed that cysteamine treatment could significantly attenuate the changes in mature BDNF and truncated BDNF protein levels in frontal cortex of HRM ($p < 0.05$; Fig. 3*c*). In addition, we found a significant decrease in

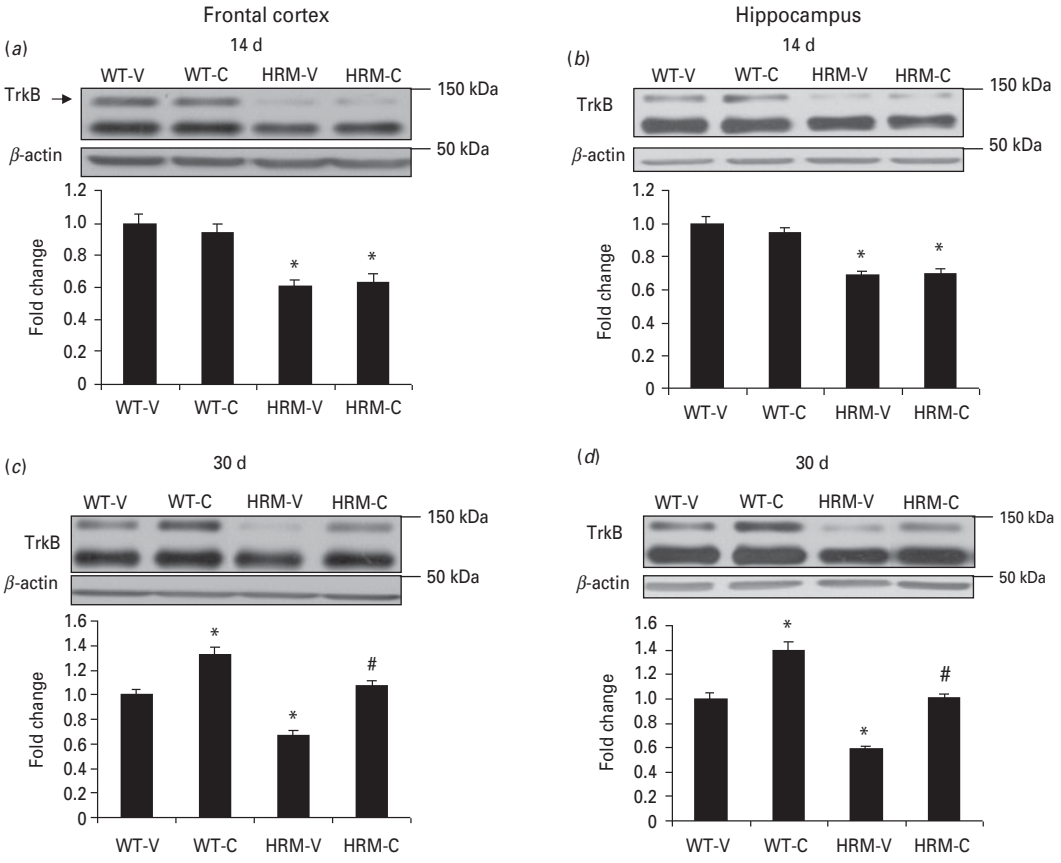

Fig. 4. Cysteamine treatment ameliorates the reduction in TrkB expression in frontal cortex and hippocampus of heterozygous reeler mice (HRM). Cysteamine (150 mg/kg) or water (vehicle) was administered through drinking water to HRM and wild-type (WT) mice for 14 d (*a*, *b*) or 30 d (*c*, *d*). TrkB expression was determined by Western blot analysis in frontal cortex (*a*, *c*) and hippocampus (*b*, *d*). WT-V, Wild-type treated with vehicle (water); WT-C, wild-type treated with cysteamine; HRM-V, heterozygous reeler mice treated with vehicle; HRM-C, heterozygous reeler mice treated with cysteamine. Data represent mean ± S.E. ($n = 5$ per group) expressed as fold change in TrkB protein levels compared to WT-V (* $p < 0.05$ *vs.* WT-V; # $p < 0.05$ *vs.* HRM-V; Bonferroni's test). β-actin is the loading control.

truncated BDNF levels in hippocampus following cysteamine treatment whereas mature BDNF levels were significantly increased following cysteamine treatment in this brain region of HRM ($p < 0.05$; Fig. 3*d*). We did not find any change in proBDNF levels in frontal cortex and hippocampus between HRM and WT mice (Fig. 3*a*, *b*). Moreover, cysteamine treatment did not have any effect on proBDNF levels in HRM and WT mice in the above brain regions.

Effect of cysteamine on TrkB protein levels in HRM

TrkB proteins exist as full-length (~148 kDa) and truncated (~98 kDa) forms. We used two separate antibodies to detect these two different TrkB forms in our samples. One-way ANOVA showed a significant group effect in full-length TrkB protein levels in frontal cortex [$F(3, 16) = 17.05$, $p < 0.0001$] and hippocampus [$F(3, 16) = 24.77$, $p < 0.0001$] of mice treated with vehicle or cysteamine for 14 d. *Post-hoc* analysis showed a significant decrease in full-length TrkB protein levels in frontal cortex and hippocampus of HRM compared to WT mice ($p < 0.05$; Fig. 4). No significant change in full-length TrkB protein levels was observed following 14 d cysteamine treatment (Fig. *a*, *b*). Mice treated with cysteamine or vehicle for 30 d showed a statistically significant group effect in TrkB protein levels in frontal cortex [$F(3, 16) = 39.77$, $p < 0.0001$] and hippocampus [$F(3, 16) = 40.11$, $p < 0.0001$]. Full-length TrkB levels were significantly increased in frontal cortex and hippocampus of WT mice following 30 d cysteamine treatment ($p < 0.05$). Full-length TrkB levels were increased also in the frontal cortex and hippocampus of HRM ($p < 0.05$).

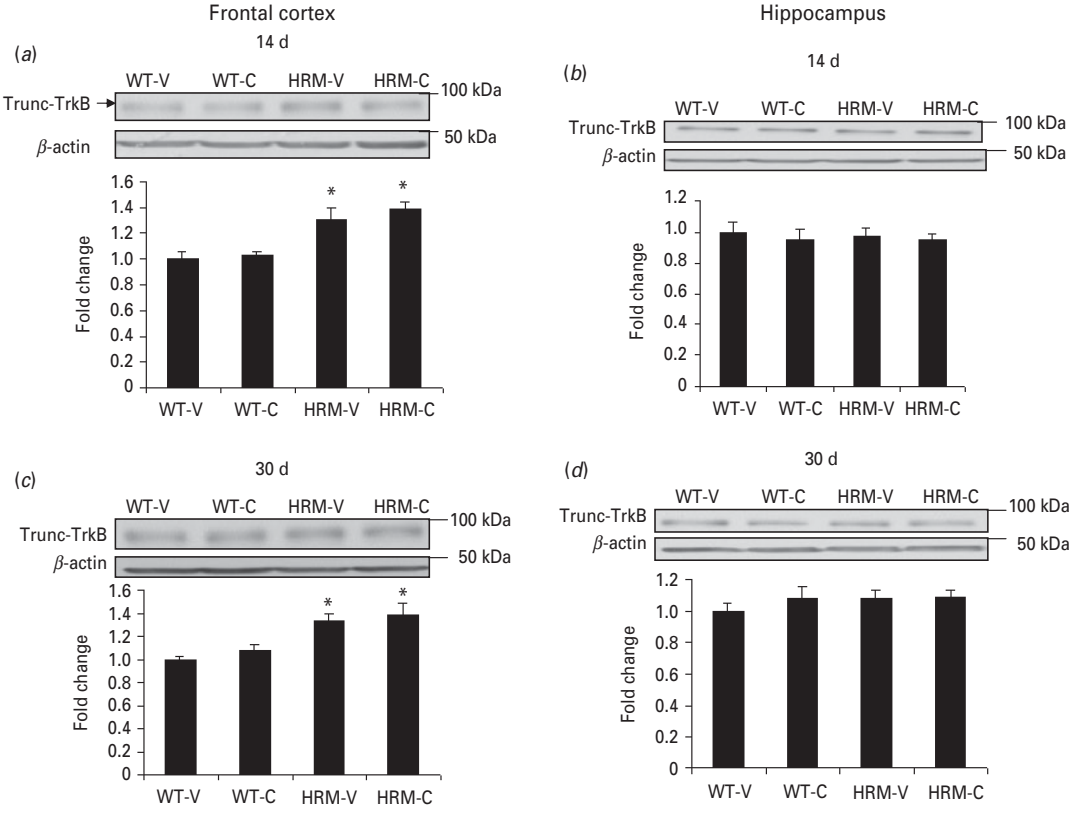

Fig. 5. Cysteamine treatment has no effect on truncated TrkB expression in frontal cortex of heterozygous reeler mice (HRM). Cysteamine (150 mg/kg) or water (vehicle) was administered through drinking water to HRM and wild-type (WT) mice for 14 d (*a*, *b*) or 30 d (*c*, *d*). Truncated TrkB (trunc-TrkB) expression was determined by Western blot analysis in frontal cortex (*a*, *c*) and hippocampus (*b*, *d*). WT-V, Wild-type treated with vehicle (water); WT-C, wild-type treated with cysteamine; HRM-V, heterozygous reeler mice treated with vehicle; HRM-C, heterozygous reeler mice treated with cysteamine. Data represent mean ± S.E. ($n = 5$ per group) expressed as fold change in trunc-TrkB protein levels compared to WT-V (* $p < 0.05$ *vs.* WT-V; Bonferroni's test). β-actin is the loading control.

Next, we examined the truncated TrkB levels in HRM and WT mice. A statistically significant group effect was revealed in one-way ANOVA in truncated TrkB levels in frontal cortex [$F(3,16) = 10.06$, $p = 0.0006$], but not hippocampus [$F(3,16) = 0.1987$, $p = 0.8958$] of mice treated with vehicle or cysteamine for 14 d. *Post-hoc* analysis showed a significant increase in truncated TrkB protein levels in frontal cortex of HRM compared to WT mice ($p < 0.05$; Fig. 5*a*). We did not find any significant change in truncated TrkB protein levels in hippocampus of HRM (Fig. 5*b*). Mice treated with cysteamine or vehicle for 30 d showed a statistically significant group effect in truncated TrkB protein levels in frontal cortex [$F(3,16) = 8.12$, $p = 0.0016$], but not hippocampus [$F(3,16) = 0.557$, $p = 0.651$]. No significant effect on truncated TrkB levels was found following cysteamine treatment for 14 d or 30 d (Fig. 5*a*, *d*). Taken together, these results indicate that cysteamine treatment could significantly attenuate the change in full-length TrkB levels, but not in truncated TrkB levels in HRM.

Effect of cysteamine on GAD67 protein levels in TrkB knockout mice

Since we found a significant increase in GAD67 and TrkB protein levels following cysteamine treatment and TrkB is a known regulator of GAD67 expression we next examined whether TrkB is necessary for cysteamine-induced effects on GAD67 expression. A statistically significant group effect was revealed in one-way ANOVA in GAD67 protein levels in frontal cortex [$F(3,15) = 22.84$, $p < 0.0001$] and hippocampus [$F(3,15) = 28.18$, $p < 0.0001$] of mice treated with vehicle or cysteamine. We found a significant reduction in GAD67 protein levels in frontal cortex and

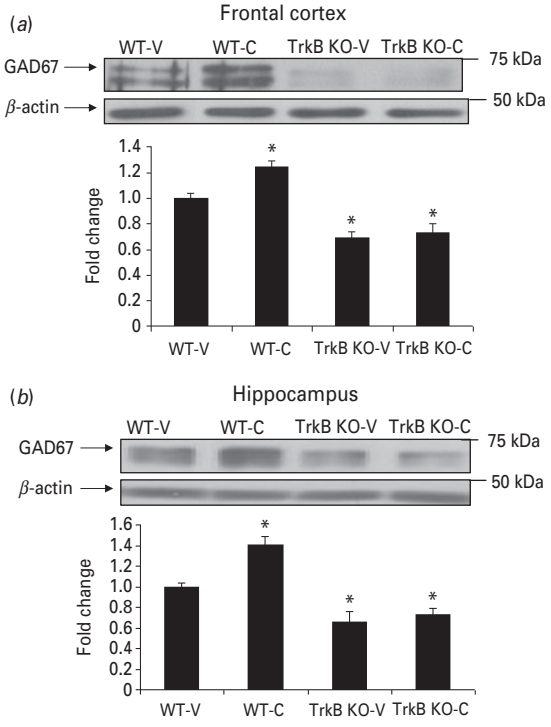

Fig. 6. Cysteamine treatment has no effect on GAD67 expression in frontal cortex and hippocampus of TrkB knockout ($TrkB^{-/-}$) mice. Cysteamine (150 mg/kg.d) or water (vehicle) was administered through drinking water to $TrkB^{-/-}$ and wild-type (WT) mice for 30 d. GAD67 expression was determined by Western blot analysis in (*a*) frontal cortex and (*b*) hippocampus. WT-V, Wild-type treated with vehicle (water); WT-C, wild-type treated with cysteamine; $TrkB^{-/-}$-V, TrkB knockout mice treated with vehicle; $TrkB^{-/-}$-C, TrkB knockout mice treated with cysteamine. Data represent mean ± S.E. ($n=4$ for WT-V and $n=5$ each for WT-C, $TrkB^{-/-}$-V and $TrkB^{-/-}$-C) expressed as fold change in GAD67 protein levels compared to WT-V (* $p<0.05$ *vs.* WT-V; Bonferroni's test). β-actin is the loading control.

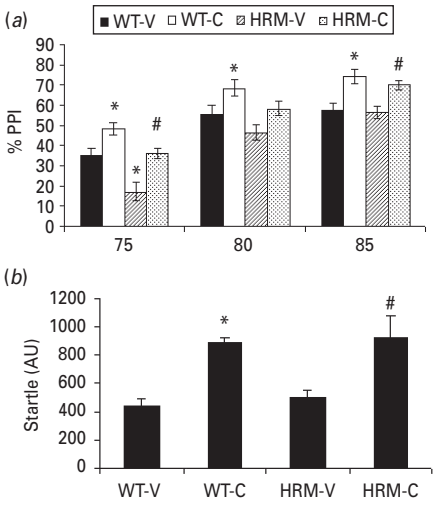

Fig. 7. Effects of cysteamine on prepulse inhibition (PPI) of the auditory startle response in heterozygous reeler mice (HRM). Cysteamine (150 mg/kg.d) or water (vehicle) was administered through drinking water to HRM and wild-type (WT) mice for 30 d. WT-V, Wild-type treated with vehicle (water); WT-C, wild-type treated with cysteamine; HRM-V, heterozygous reeler mice treated with vehicle; HRM-C, heterozygous reeler mice treated with cysteamine. Data represent mean ± S.E. ($n=8$ per group) expressed as (*a*) %PPI and (*b*) startle response (* $p<0.05$ *vs.* WT-V; # $p<0.05$ *vs.* HRM-V; Bonferroni's test).

hippocampus of $TrkB^{-/-}$ mice compared to WT mice ($p<0.05$; Fig. 6). Next, we tested the effect of cysteamine on GAD67 protein levels in $TrkB^{-/-}$ and WT mice. Cysteamine treatment for 30 d increased GAD67 protein levels in WT mice ($p<0.05$). However, in knockout mice, cysteamine failed to increase GAD67 protein levels. The above data indicate that TrkB is necessary for cysteamine-mediated effects on GAD67 expression.

Effect of cysteamine on PPI and Y-maze in HRM

We used a combination of auditory-evoked startle (120 dB) and three levels of prepulses (75, 80, and 85 dB). A repeated-measures ANOVA revealed a significant effect of prepulse [$F(2,60)=111.9$, $p<0.0001$], but no significant prepulse × treatment interaction [$F(2,60)=0.643$, n.s.] or main effect of treatment [$F(1,30)=0.02$, n.s.]. We found a significant decrease in %PPI in HRM compared to WT mice at the 75-dB level ($p<0.05$; Fig. 7*a*). Next, we examined the effect on %PPI of cysteamine treatment for 30 d in HRM and WT mice compared to vehicle-treated mice. Individual planned comparisons at each level of prepulse intensity revealed significant differences in PPI between vehicle and cysteamine at all prepulse levels in WT mice whereas only at the 75-dB and 85-dB prepulse levels in HRM ($p<0.05$; Fig. 7*a*). We found a significant difference in startle response to 120-dB stimuli following cysteamine treatment [$F(3,28)=6.69$, $p=0.0015$]. *Post-hoc* analysis showed that cysteamine treatment for 30 d increased startle response in both HRM and WT mice ($p<0.05$; Fig. 7*b*).

The data from Y-maze test analysed by one-way ANOVA showed a significant group effect in spatial memory function [$F(3,29)=16.41$, $p<0.001$; Fig. 8]. *Post-hoc* analysis showed that HRM have impaired spatial memory, spending less time in the novel arm

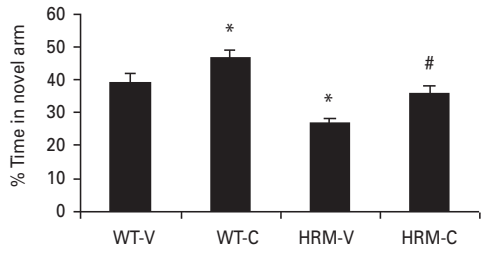

Fig. 8. Effects of cysteamine on spatial recognition memory in heterozygous reeler mice (HRM). Cysteamine (150 mg/kg) or water (vehicle) was administered through drinking water to HRM and wild-type (WT) mice for 30 d. WT-V, Wild-type treated with vehicle (water); WT-C, wild-type treated with cysteamine; HRM-V, heterozygous reeler mice treated with vehicle; HRM-C, heterozygous reeler mice treated with cysteamine. Data represent mean ± S.E. ($n=9$ for WT-V and $n=8$ each for WT-C, HRM-V and HRM-C) expressed as % time in novel arm (* $p<0.05$ *vs.* WT-V; # $p<0.05$ *vs.* HRM-V; Bonferroni's test).

than WT mice ($p<0.05$). Cysteamine-treated HRM spent significantly more time in novel arms than vehicle-treated HRM ($p<0.05$). Working memory was studied by measuring spontaneous alternation in the Y-maze. There was no significant effect of genotype or treatment on the percentage of alternation (data not shown). This suggests that although hippocampus-dependent spatial memory is changed following cysteamine treatment in HRM, non-hippocampal working memory is intact with cysteamine treatment in both WT and HRM.

Discussion

In this study, we evaluated the effects of cysteamine treatment in HRM on the levels of GAD67 and BDNF/TrkB proteins in two different brain regions (frontal cortex and hippocampus) that have been implicated in schizophrenia. We also evaluated the drug for its effects on domains of cognition known to be adversely affected in schizophrenia (information processing and spatial memory). In the neurochemical studies, we observed increases in GAD67 and TrkB protein levels upon exposure of cysteamine in frontal cortex and hippocampus of HRM. Other studies have shown alterations in TrkB signalling (Pillai & Mahadik, 2008) and GAD67 expression (Liu *et al.* 2001; Nullmeier *et al.* 2011) in HRM. The involvement of TrkB in cysteamine-induced GAD67 expression is strengthened by our data demonstrating that cysteamine failed to increase GAD67 expression in TrkB$^{-/-}$ mice.

TrkB signalling has been shown to induce GABA function and GAD67 expression in neurons (Arenas *et al.* 1996; Mizuno *et al.* 1994). TrkB exists in two forms, full-length TrkB and truncated TrkB (which lacks tyrosine kinase domain). We have previously reported a significant increase in truncated TrkB levels in the frontal cortex of HRM (Pillai & Mahadik, 2008). In the present study, we found a significant increase in truncated BDNF levels in both frontal cortex and hippocampus of HRM. The 28-kDa form of BDNF has been shown to be a cleaved form of pro-BDNF in both humans and animals (Carlino *et al.* 2011; Seidah *et al.* 1999). However, the biological role of truncated BDNF is not known. Further studies are needed to elucidate the role of truncated BDNF in the regulation of TrkB signalling as well as GAD67 expression in view of the fact that we did not observe significant changes in pro-BDNF levels in HRM.

The major finding of the present study is the potential of cysteamine to normalize GAD67 expression in HRM. It has been suggested that reelin through DAB1 signalling selectively regulates GAD67, but not GAD65 expression in interneurons (Guidotti *et al.* 2000). Interestingly, we found a significant increase in TrkB protein levels following cysteamine treatment in both frontal cortex and hippocampus. Our data show that the cysteamine treatment induces GAD67 expression in the frontal cortex and hippocampus of WT mice, but no change in GAD67 protein levels was found in TrkB$^{-/-}$ mice following cysteamine exposure. Proper development and regulation of GABAergic interneurons are important for normal cognitive function of the adult brain (Woo & Lu, 2006). In addition, significant positive correlations between TrkB and GAD67 mRNAs were observed in the dorsolateral prefrontal cortex of schizophrenia subjects (Hashimoto *et al.* 2005). Together, the above data indicate that TrkB might be a molecular target for cysteamine action on the GABAergic system.

To assess the effects of genotype and cysteamine on behaviours that have relevance to schizophrenia we utilized a PPI procedure and two Y-maze tasks. The rationale for selecting the PPI procedure was based on several factors: (1) The test is quite useful for identifying sensory information-processing deficits, a common feature in several neuropsychiatric conditions including schizophrenia. Auditory (sensory) gating deficits in schizophrenia are in fact thought to contribute to the deficits in attention, cognitive impairment, and even hallucinations (Adler *et al.* 1998). (2) PPI of the acoustic startle reflex is a cross-species phenomenon (identical in all mammal studies to date) and thus it is easily studied in animals as well as humans (Braff *et al.* 1992). (3) The PPI model in mice has been found to be particularly useful for studying

the effects of gene alterations on sensorimotor gating (Geyer *et al.* 2002).

In the present study, we used a combination of auditory-evoked startle (120 dB) and three levels of prepulses (75, 80 and 85 dB). We found a significant decrease in %PPI in HRM compared to WT mice only at the 75-dB level. The literature show contradictory data on PPI deficits in HRM. The study by Tueting *et al.* (1999) found alterations in PPI in HRM, but the above data were not replicated in two other studies (Podhorna & Didriksen, 2004; Salinger *et al.* 2003). However, Qiu *et al.* (2006) found a significant diminution of the force of a 120-dB startle response for HRM with the application of an acoustic stimulation of 82-dB intensity, but not of other intensities (70, 76 or 88 dB). These inconsistencies between data from different laboratories may result from differences in the age, behavioural training, rearing conditions, genetic background and interpretation, all of which can influence the outcome of behavioural tests (Wahlsten *et al.* 2003). Regardless, cysteamine treatment could increase %PPI in both HRM and WT mice. In addition, a significant increase in startle response to 120-dB stimuli was found in cysteamine-treated mice compared to vehicle-treated mice. Our data on the potential of cysteamine to increase %PPI is in agreement with an earlier report that cysteamine blocks amphetamine-induced decreases in PPI (Feifel & Minor, 1997). We found increases in both %PPI and startle response following cysteamine treatment. It has been suggested that the effects of centrally active drugs on startle response and PPI in mice tend to be dissociated (Geyer *et al.* 2002), reflecting the difference in the nature of complexity in cellular mechanisms between the startle response and PPI. It has been reported that increased startle reactivity is not necessarily associated with decreased PPI (Paylor & Crawley, 1997). The data from the above study is supported by the findings that nicotine treatment increases both startle reactivity and PPI (Acri *et al.* 1994; Schreiber *et al.* 2002). Furthermore, haloperidol increased PPI, but did not alter the increased startle reactivity in NMDA receptor knockout mice (Duncan *et al.* 2006). The above data suggest that there does not appear to be a predictable relationship between startle amplitude and PPI.

For memory-related assessments we employed a Y-maze and two tasks, spontaneous alternation and the two-trial recognition test. Both tasks make use of the normal navigation and exploratory behaviours of rodents, do not require rule learning, and, other than the potential fear-related response associated with the exposure to a novel environment, they do not have significant aversive components (i.e. food restriction or water immersion). In addition, both tasks are thought to rely on short-term spatial memory processes (Hughes, 2004). In the continuous or free-running version of the task (i.e. spontaneous alternation) the tendency for rodents to alternate their non-reinforced choices of the maze arms on successive opportunities is employed and working memory is required. As the name implies, the two-trial recognition memory test is thought to employ spatial recognition memory. Each of the Y-maze tests described here has been shown to be sensitive to hippocampal damage, gene manipulation, and pharmacological interventions (Conrad *et al.* 1997; Dellu *et al.* 1992, 2000; Hughes, 2004; Martin *et al.* 2003).

In the spatial memory test in Y-maze, HRM spent less time in the novel arm than WT mice. However, we did not find any difference in spontaneous alternation behaviour, a working-memory test between HRM and WT mice. Our data on the absence of working-memory deficits in HRM are in agreement with previous studies (Krueger *et al.* 2006; Salinger *et al.* 2003). Cysteamine treatment could increase spatial recognition memory in both HRM as well as WT mice.

Our study has a few limitations. BDNF signalling through TrkB has been shown to promote inhibitory synaptogenesis, the development of GABAergic interneurons and induces the expression of GAD67 (Huang *et al.* 1999; Yamada *et al.* 2002). It is known that GAD67 abnormalities affect parvalbumin-containing GABA neurons involved in gamma oscillations (Gonzalez-Burgos & Lewis, 2008). The gamma-band oscillations are associated with information transmission and processing (Herrmann *et al.* 2010). Moreover, schizophrenia is associated with impaired performance and reduced frontal gamma activity in individuals during cognitive tasks (Cho *et al.* 2006) and pharmacologically enhanced $GABA_AR$ activity simultaneously improves cognitive performance and frontal gamma-band power in individuals with schizophrenia (Lewis *et al.* 2008). It is possible that increase in GAD67 expression following cysteamine treatment improves GABAergic neurotransmission and thereby cognitive function, but further studies are warranted. It is also possible that decreases in GAD67 in TrkB knockout mice are due to a loss of interneurons, as reported by reductions in the number of GABAergic synapses and GAD65 expression (Carmona *et al.* 2006). In that case, the interneurons involved in the cysteamine response in HRM may no longer be rescuable in TrkB knockout mice due to an entirely cysteamine-independent mechanism. It is also important to determine whether there is an overall loss of interneurons in HRM. Such an

experiment would be helpful in elucidating whether the changes in GAD67 expression observed with cysteamine results from a specific increase in the remaining 'resistant' cells, not just a change in all GAD67-positive cells.

BDNF/TrkB signalling is an important mediator of neuronal development and function, and numerous studies suggest that abnormal BDNF/TrkB signalling may be involved in the pathophysiology of neuropsychiatric disorders including schizophrenia. Identification of potent modulators of TrkB signalling provides tools for further evaluating the role of BDNF/TrkB signalling in these disease states. Furthermore, the finding that cysteamine can modulate GAD67 expression through TrkB suggests that TrkB signalling could be a possible therapeutic target in schizophrenia. Compounds like cysteamine, which can increase TrkB signalling could be possible adjunctive therapy for treating this disorder.

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Statement of Interest

None.

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