Corticosterone treatment during adolescence induces down-regulation of reelin and NMDA receptor subunit GLUN2C expression only in male mice: implications for schizophrenia

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Abstract

Stress exposure during adolescence/early adulthood has been shown to increase the risk for psychiatric disorders such as schizophrenia. Reelin plays an essential role in brain development and its levels are decreased in schizophrenia. However, the relationship between stress exposure and reelin expression remains unclear. We therefore treated adolescent reelin heterozygous mice (HRM) and wild-type (WT) littermates with the stress hormone, corticosterone (CORT) in their drinking water (25 mg/l) for 3 wk. In adulthood, we measured levels of full-length (FL) reelin and the N-R6 and N-R2 cleavage fragments in the frontal cortex (FC) and dorsal (DH) and ventral (VH) hippocampus. As expected, levels of all reelin forms were approximately 50% lower in HRMs compared to WT. In male mice, CORT treatment significantly decreased FL and N-R2 expression in the FC and N-R2 and N-R6 levels in the DH. This reelin down-regulation was accompanied by significant reductions in downstream N-methyl-D-aspartate (NMDA) GluN2C subunit levels. There were no effects of CORT treatment in the VH of either of the sexes and only subtle changes in female DH. CORT-induced reelin and GluN2C down-regulation in males was not associated with changes in two GABAergic neuron markers, GAD67 and parvalbumin, or glucocorticoids receptors (GR). These results show that CORT treatment causes long-lasting and selective reductions of reelin form levels in male FC and DH accompanied by changes in NMDAR subunit composition. This sex-specific reelin down-regulation in regions implicated in schizophrenia could be involved in the effects of stress in this disease.

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Introduction

Schizophrenia is a complex psychotic disorder that affects approximately 1% of the population worldwide (Tamminga and Holcomb, 2005). Its aetiology remains unclear but it is now well accepted that this neurodevelopmental disorder arises from the combination of genetic and environmental factors (for review see Brown, 2011). Thus, the ‘two-hit’ hypothesis of schizophrenia postulates that a first hit, such as a genetic defect, stress or maternal infection, produces subtle changes in brain development, rendering it more vulnerable to a second hit later in life, such as stress or drug abuse, together increasing the risk to develop schizophrenia (Bayer et al., 1999; Maynard et al., 2001).

Deficits in the expression of the extracellular matrix glycoprotein, reelin, may be a first neurodevelopmental ‘hit’. Reelin plays a fundamental role during brain development. Secreted by the Cajal–Retzius (CR) cells, it controls proper migration and positioning of cortical neurons. Although the mechanism by which reelin regulates neuronal migration is still not completely understood, it involves the reelin-disabled-1 (Dab1) signalling pathway. Reelin binds two transmembrane lipoprotein receptors, apolipoprotein E receptor 2 (ApoER2) and very-low-density lipoprotein receptor (VLDLR) leading to the subsequent tyrosine phosphorylation of the intracellular adaptor protein Dab1 (Herz and Chen, 2006).

In the adult brain, reelin expression shifts from CR cells to a subset of GABAergic neurons throughout the neocortex and hippocampus (Stranahan et al., 2013). Reelin signalling regulates synaptic plasticity notably by acting on N-methyl-D-aspartate receptors (NMDAR). NMDARs are ionotropic glutamate receptors whose activation...
leads to long-term potentiation (LTP), a mechanism thought to underlie learning and memory, and whose hypofunction is postulated to be involved in schizophrenia, particularly in parvalbumin (PV)-positive GABAergic neurons (Nakazawa et al., 2012). For example, reelin modulates NMDAR activity by increasing phosphorylation of GluN2A and GluN2B subunits (Chen et al., 2005) and by regulating NMDAR subunits composition as well as NMDAR surface trafficking (Groc et al., 2007). Both reelin-induced increased LTP and altered dendritic spine formation caused by reelin deficiency are accompanied by NMDARs changes (Ventrutti et al., 2011).

Evidence for a role of reelin in the physiopathology of schizophrenia also comes from post-mortem studies. Reelin mRNA and protein levels are decreased by approximately 50% in hippocampus, prefrontal cortex (PFC) and cerebellum of patients with schizophrenia (Impagnatiello et al., 1998; Fatemi et al., 2000; Guidotti et al., 2000). Reeler heterozygous mice (HRM), in which reelin expression is decreased by 50% similar to the deficit seen in schizophrenia, show neurochemical and neuroanatomical abnormalities and behavioural deficits relevant to this illness (Tueting et al., 1999, 2006).

Among ‘second hit’ environmental factors, stress exposure during pregnancy (Khashan et al., 2008) and childhood/adolescence (Schlosser et al., 2012) have been associated with higher onset of schizophrenia. Stress triggers the activation of the hypothalamic–pituitary–adrenal (HPA) axis which leads to the increased secretion in the plasma of glucocorticoid hormones; cortisol in humans and corticosterone (CORT) in rodents. HPA activity abnormalities have been detected in schizophrenia patients including the non-suppression of cortisol following deoxycorticosterone, increased basal cortisol levels (Walder et al., 2000; Bradley and Dinan, 2010) and a decreased expression of glucocorticoid receptor (GR) mRNA in post-mortem brains (Webster et al., 2002). In rodents, gestational and post-natal CORT exposure lead to brain abnormalities relevant to schizophrenia (van den Buuse et al., 2004; Meyer and Feldon, 2010).

The aim of the present study was to investigate the possible interaction of reelin levels and stress in adolescence, a critical period for brain development and schizophrenia onset. As previously described, we simulated a physiological stress response by chronic treatment with CORT (Klug et al., 2012), the main glucocorticoid component of the stress response, and assessed its long-term effect both in wildtype (WT) mice with normal baseline levels of reelin and in HRM with an already reduced level of reelin expression. Reelin undergoes proteolytic processing in vivo which leads to the generation of several reelin fragments (Lambert de Rouvroit et al., 1999). Therefore, we assessed levels of full-length (FL) reelin as well as its N-terminal fragments, N-R6 and N-R2. Although the physiological function of reelin cleavage is still not completely understood, it has been shown that the central domain and the N-terminal of reelin play differential roles in reelin signalling (Jossin et al., 2004; Kohno et al., 2009). We also assessed downstream consequences of potential changes in reelin levels in terms of NMDAR subunit composition and two GABAergic neuron markers, GAD67 and PV. Finally, we also assess levels of GR which mediate stress-responses in the brain.

Methods

Animals

Male and female reelin heterozygous mice on a C57Bl/J genetic background (D’Arcangelo et al., 1995; de Bergeyck et al., 1997) and wildtype littermate control mice were derived from a breeding colony at the Florey Neuroscience Institutes, Australia (van den Buuse et al., 2012). At 2 wk of age, a tail sample was taken from the offspring and used to genotype each animal using polymerase chain reaction (PCR) protocols (Hammond et al., 2006). After weaning at 3 wk of age, the mice were transferred to the Mental Health Research Institute, Melbourne, Australia, where they were housed in groups of 2–5 males or females per standard open-top mouse cage. The animals had ad libitum access to food and tap water available ad libitum and were kept on a regular light/dark period with lights on at 07:00 hours. All procedures were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes set out by the National Health and Medical Research Council of Australia and were approved by the Florey Neurosciences Institute Animal Experimentation Ethics Committee.

Corticosterone treatment

CORT treatment was dispersed in the drinking water. This non-invasive method has been shown to mimic the circadian rhythm of plasma CORT concentration being highest during the nocturnal active phase in mice and lower during the diurnal inactive phase (Karatsoreos et al., 2010). CORT powder (Sigma-Aldrich, USA) was dissolved in 100% ethanol and diluted with tap water to a final concentration of 25 mg/l (approximately 0.5% ethanol). Control animals received vehicle solution. In order to prevent CORT degradation, the water bottles were wrapped in aluminium foil to protect the solution from light. The CORT solution was freshly prepared and replaced once a week. Liquid intake was monitored and the CORT dose was calculated as 6–8 mg/kg/d.

Chronic CORT treatment started at 6 wk of age and lasted for 21 d until 9 wk of age based on previous results (Klug et al., 2012) and observations of seminal vesicles and uterus weight changes reflecting adolescent hormonal fluctuations (Hill et al., 2012).

Antibodies and reagents

Primary antibodies used were: anti-reelin G10 clone (MAB5364, Merck Millipore, Germany), anti-NMDAR2C
which also detects GluN2A, GluN2B and GluN1 (ab110, Abcam, UK), anti-GAD67 (G5419, Sigma, Australia), anti-Parvalbumin (MAB1572, Millipore), anti-Glutocorticoid receptor (ab2768, Abcam) and anti-β-actin (A5316, Sigma). To verify results for the small GluN2A band detected by the anti-NMDAR2C antibody, we also used a specific anti-NMDAR2A antibody (ab14596, Abcam). Anti-mouse or anti-rabbit HRP-linked secondary antibodies were from Cell Signalling Technology Inc. The BCA (bicinchoninic acid) protein assay kit was from Thermo Scientific (USA). Detection systems used were LumiGLO® Reagent (Cell Signalling, USA) and Ultra Western Lightning Ultra (Perkin Elmer).

Western blot analysis

In order to assess the long-term effect of CORT and compare our results to previous data produced from similarly treated behavioural cohorts, mice were killed by cervical dislocation at 16 wk of age, 7 wk after the end of CORT treatment (Klug et al., 2012; Klug and van den Buuse, 2013). The oestrous cycle stage of female mice at the time of tissue collection was not determined. The brain was removed, frozen on dry ice, and stored at −80°C. For protein extraction, the brains were thawed on a cold plate and rapidly dissected into frontal cortex (FC), dorsal hippocampus (DH) and ventral (VH) hippocampus. Tissue samples were weighed and lysed with 100 μl/0.01 g RIPA buffer (50 mM Tris pH 8.0, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 150 mM sodium chloride, dH2O, phosphatase inhibitor (1:50) and protease inhibitor (1:200)) and maintained on ice. Samples were then sonicated, left on a rotator for 45 min and centrifuged for 18 min at 13 000 g at 4°C to remove debris. Protein concentrations were determined by the BCA method. Volumes containing 50 μg of protein were mixed with an equal volume of loading buffer (0.4 M Tris pH 6.8, 37.5% glycerol, 10% SDS, 1% 2-mercaptoethanol, 0.5% Bromphenol Blue, dH2O, phosphatase inhibitor (1:50) and protease inhibitor (1:200)) and then denatured at 95°C (3 min for reelin, 10 min for other proteins). Samples were resolved by SDS-polyacrylamide gel,(Reelin: 4–15% Mini-PROTEAN® TGX™ precast polyacrylamide gels (BIORAD, Regents Park, Australia), NMDAR: 8% acrylamide gels, GAD67 and PV: 15% acrylamide gels) 2 h at 120 V. The proteins were transferred onto nitrocellulose membranes: overnight at 30 V and +4°C then 1 h at 30 V the next morning for reelin, NMDAR and glucocorticoid receptor; 1.5 h at 120 V and +4°C for GAD67 and PV. Membranes were blocked for 1 h at room temperature for reelin, and NMDAR, or overnight at 4°C for GAD67, PV and glucocorticoid receptor in tris-buffered-saline/TWEEN 20 (TBST) (20 mM Tris, 150 mM sodium chloride, 0.1% TWEEN 20, dH2O) with 5% non-fat milk. Primary antibodies, anti-reelin (1:1000), anti-NMDAR2C (1:500), anti-NMDAR2A (1:1000), anti-Parvalbumin (1:1000), anti-GAD67 (1:1000), anti-Glutocorticoid receptor (5 μg/ml) and anti-β-actin (1:10000), were incubated in TBST with 5% BSA overnight at 4°C. After 1 h incubation at room temperature with either anti-mouse or anti-rabbit HRP-linked secondary antibodies, images were captured using a Luminescence Image Analyzer (LAS-4000; FujiFilm Life Science, USA) and analysed using Image Quant software (GE Healthcare, Australia).

Data analysis

All data were expressed as mean±S.E.M. Differences within and between groups were analysed by analysis of variance (two-way analysis of variance (ANOVA), Systat, version 9, SPSS Inc., USA) followed by Tukey’s test for pairwise comparisons. Genotype, CORT treatment and sex of the animals were between-group factors.

Results

Sex- and form-specific decreases in reelin levels after corticosterone treatment in adolescence

Using an antibody against an N-terminal epitope, we assessed by Western blot the levels of full-length reelin and two of its proteolytic fragments, N-R6 and N-R2 (approximately 350, 300 and 165 kDa, respectively). As expected, levels of all reelin forms were approximately 50% lower in HRMs compared to WT (Figs. 1–3).

In the FC of male mice, CORT treatment significantly decreased full-length and N-R2 expression (main effect of treatment: F(1,21)=10.1, p=0.004 and F(1,20)=7.0, p=0.015, respectively) irrespective of the genotype (Fig. 1a, c). A trend for a similar decrease was seen for N-R6 expression but this did not reach significance (p=0.064, Fig. 1c). CORT treatment did not have any effect on levels of full-length reelin or its fragments in the FC of female mice (Fig. 1b, d, f).

CORT treatment significantly decreased N-R2 and N-R6 levels in male DH (F(1,23)=5.8, p=0.024 and F(1,23)=4.6, p=0.043, respectively) compared to control mice (Fig. 2c, e). CORT treatment also reduced N-R2 levels in female DH (F(1,20)=4.7, p=0.043) (Fig. 2f). There were no significant changes in reelin levels or its fragments in the VH of either male or female mice (Fig. 3).

The ratios of N-R6/FL and N-R2/FL did not differ among the groups, indicating that although CORT treatment did not affect the cleavage of reelin, it did most likely influence the production/secretion/availability of full-length reelin (Table 1).

Down-regulation of reelin levels by corticosterone treatment is accompanied by selective changes in NMDAR subunit levels

In the FC of male mice, CORT treatment decreased levels of the GluN2C subunit (main effect of treatment, F(1,22)=9.9, p=0.005) irrespective of the genotype (Fig. 4e). ANOVA revealed a significant genotype×treatment interaction for levels of the GluN1 subunit.
heterozygous mice (HRM). Data are mean±S.E.M of 4–7 animals per group. Levels of full-length reelin (FL, ≈350 kDa, panels a and b) and its N-terminal fragments, N-R6 (≈300 kDa, panels c and d) and N-R2 (≈165 kDa, panels e and f), were decreased by approximately 50% in HRM compared to WT in both sexes (not indicated, p<0.001). Full-length (FL) and N-R2 levels were significantly decreased in males treated with corticosterone (CORT) in comparison with vehicle (Veh) controls (F(1,21)=10.1, *p=0.004 and F(1,20)=7.0, *p=0.015, respectively) irrespective of the genotype (a and c). A trend for a similar decrease was seen for N-R6 expression but this did not reach significance (fp=0.064) (panel i). CORT treatment did not have any effect on levels of full-length reelin or its fragments in the FC of female mice (panels b, d, and f). Representative images of the Western blots of full-length reelin and its N-terminal fragments are shown for FC of males (panel g) and females (panel h). The lower intensity of these three bands in HRM in comparison to WT reflects the approximate 50% expected decrease of reelin in HRM.

(F(1,16)=7.8, p=0.013, Fig. 4g); however, post-hoc analysis did not reveal any group differences. No effects of CORT treatment were observed on GluN2A and GluN2B expression in either male or female mice (Fig. 4).

Also in the DH of male mice, GluN2C expression was significantly decreased by CORT treatment (F(1,20)=5.3, p=0.032, Fig. 5e), whereas it tended to be increased in females (p=0.062) (Fig. 5f). No significant changes were observed in the expression of GluN2A, GluN2B or GluN1 in the DH of either males or females (Fig. 5).

Verification of the levels of GluN2A with a specific NMDAR2A antibody confirmed the lack of any differences between the groups in either the FC or DH (Supplementary Fig. S1).
there was a signifi
FC (Supplementary Fig. S2). However, in female FC
female DH (Supplementary Fig. S3) as well as in male
Fig. S2) or DH (Supplementary Fig. S3).
not affect GAD67 levels either in the FC (Supplementary
between WT and HRM. Moreover, CORT treatment did
No differences in GAD67 protein levels were observed
GAD67 levels
Down-regulation of reelin levels by corticosterone
expression of reelin in ventral hippocampus
heterozygous mice (HRM). Data are mean±S.E.M of 4
(VH) of male and female wildtype controls (WT) and reelin
Protein expression of reelin in ventral hippocampus
Fig. 3.
Reelin and GluN2C down-regulation by corticosterone
Sex- and form-specific decreases in reelin levels after
corticosterone treatment
Stress triggers the activation of the HPA axis which leads
to the increased secretion in the plasma of glucocorticoid
hormones; cortisol in humans and CORT in rodents.
Glucocorticoids can then reach the brain and bind to
two types of receptors, the GR and the mineralocorticoid
receptors (MR), which are localised in regions important
for memory such as the hippocampus and the PFC
(de Kloet et al., 2005; Joels et al., 2006). Our observations
of a down-regulation of reelin in dorsal hippocampus and
frontal cortex of mice treated with CORT may mimic ef-
effects normally evoked by activation of the HPA axis
after stress exposure and the involvement of stress feed-
back loops in these two brain regions. Interestingly,
we did not observe changes of reelin levels in the ventral
hippocampus. Evidence suggests that the dorsal and ven-
tral poles of the hippocampus are functionally different
(Fanselow and Dong, 2010). The dorsal hippocampus is
thought to be involved in learning and memory, whereas
the ventral hippocampus is associated with fear and
anxiety (Moser and Moser, 1998; Bannerman et al., 2004).
Therefore, the differential effect of CORT in these sub-
regions could have selective behavioural consequences,
although further studies are needed to confirm this.
Some previous studies have investigated the sub-
regional localisation of glucocorticoid receptors in the
hippocampus. For example, Robertson and co-workers
reported that GR expression is twice higher in dorsal
hippocampus than ventral hippocampus and that MR ex-
pression is twice higher in ventral hippocampus than dor-
sal hippocampus of rats (Robertson et al., 2005). Our
present study showed that the same ratio occurs for GR

**Fig. 3.** Protein expression of reelin in ventral hippocampus (VH) of male and female wildtype controls (WT) and reelin heterozygous mice (HRM). Data are mean±S.E.M of 4–7 animals per group. Levels of full-length reelin (FL, ≈350 kDa, panels a and b) and its N-terminal fragments, N-R6 (≈300 kDa, panels c and d) and N-R2 (≈165 kDa, panels e and f), were decreased by approximately 50% in HRM compared to WT in both sexes (not indicated, p<0.012). No significant changes were observed between the corticosterone (CORT)-treated and vehicle (Veh) controls groups for either males or females. Representative images of the Western blots of full-length reelin and its N-terminal fragments are shown for FC of males (panel g) and females (panel h). The lower intensity of these three bands in HRM in comparison to WT reflects the approximate 50% expected decrease of reelin in HRM.

**Down-regulation of reelin levels by corticosterone treatment is not accompanied by changes in PV or GAD67 levels**

No differences in GAD67 protein levels were observed between WT and HRM. Moreover, CORT treatment did not affect GAD67 levels either in the FC (Supplementary Fig. S2) or DH (Supplementary Fig. S3).

PV protein levels were unchanged both in male and female DH (Supplementary Fig. S3) as well as in male FC (Supplementary Fig. S2). However, in female FC there was a significant genotype×treatment interaction (F(1,22)=7.2, p=0.014). CORT treatment caused a small, but significant decrease in PV levels in WT mice (p=0.047, Tukey’s test) but not in HRM (Supplementary Fig. S2).

**Corticosterone treatment does not affect GR levels in frontal cortex and hippocampus**

In the FC of male and female mice, GR levels were unchanged by CORT treatment and did not differ between WT and HRM (Supplementary Fig. S4). CORT treatment or genotype did not affect GR levels; however, there was a main effect of hippocampus pole and GR levels were about twice as high in DH than VH in males (F(1,48)=49.1, p<0.001) and in females (F(1,48)=25.2, p<0.001) (Supplementary Fig. S5).

**Discussion**

The main finding of this study was that CORT treatment in adolescence selectively down-regulated reelin and GluN2C levels in the FC and DH of male mice, but not female mice. These changes were not accompanied by parallel changes in PV, GAD67 or GR in the brain regions studied.

**Sex- and form-specific decreases in reelin levels after corticosterone treatment**

Stress triggers the activation of the HPA axis which leads to the increased secretion in the plasma of glucocorticoid hormones; cortisol in humans and CORT in rodents. Glucocorticoids can then reach the brain and bind to two types of receptors, the GR and the mineralocorticoid receptors (MR), which are localised in regions important for memory such as the hippocampus and the PFC (de Kloet et al., 2005; Joels et al., 2006). Our observations of a down-regulation of reelin in dorsal hippocampus and frontal cortex of mice treated with CORT may mimic effects normally evoked by activation of the HPA axis after stress exposure and the involvement of stress feedback loops in these two brain regions. Interestingly, we did not observe changes of reelin levels in the ventral hippocampus. Evidence suggests that the dorsal and ventral poles of the hippocampus are functionally different (Fanselow and Dong, 2010). The dorsal hippocampus is thought to be involved in learning and memory, whereas the ventral hippocampus is associated with fear and anxiety (Moser and Moser, 1998; Bannerman et al., 2004). Therefore, the differential effect of CORT in these sub-regions could have selective behavioural consequences, although further studies are needed to confirm this.

Some previous studies have investigated the sub-regional localisation of glucocorticoid receptors in the hippocampus. For example, Robertson and co-workers reported that GR expression is twice higher in dorsal hippocampus than ventral hippocampus and that MR expression is twice higher in ventral hippocampus than dorsal hippocampus of rats (Robertson et al., 2005). Our present study showed that the same ratio occurs for GR
in mice. Specifically, GR expression was about twice higher in dorsal hippocampus than ventral hippocampus independent of the sex, genotype or treatment. MRs have 10-fold higher affinity for CORT and are predominantly occupied when basal level of CORT are low, whereas GR are activated only when CORT levels are high such as after stress exposure (De Kloet et al., 1998). Lower expression of GRs in the ventral than dorsal hippocampus could explain the absence of CORT treatment effect on reelin in the ventral hippocampus. Supporting an implication of GR in the modulation of reelin protein levels, Gross et al. (2012) demonstrated that GRs colocalise in virtually all reelin-positive cells in the post-natal mouse hippocampus. Furthermore, GR have been reported to be expressed on hippocampal dendritic spines of rats (Jafari et al., 2012) and reelin was observed at the surface of these structures after secretion by GABAergic neurons (Rodriguez et al., 2000).

A striking feature of our result is the sex-specificity of reelin down-regulation which occurred mainly in males. Evidence suggests that the sex of the animals influences HPA axis function (McCormick and Mathews, 2007). Notably, sex hormones can influence GR expression. For example, oestrogen decreases GR expression and function (Burgess and Handa, 1992; Krishnan et al., 2001) whereas testosterone increases GR levels in the hippocampus of rats in response to stress (Viau et al., 1996). Since our study, similar to previous work (Romeo et al., 2012), did not show any sex difference in GR levels in the hippocampus and the frontal cortex, it seems more likely that the sex effect of CORT treatment that we observed is mediated by mechanisms other than GR levels. For example, in plasma, most of the circulating CORT is bound to the glucocorticoid-binding globulin (CBG), a glycoprotein which regulates the tissue distribution of free CORT (Lewis et al., 2005) and serum levels of this protein are higher in females than males in humans (Fernandez-Real et al., 2002), rats (Romero Mdel et al., 2013) and mice (Jones et al., 1998). Estradiol treatment increases CBG production in liver of intact as well as adrenalectomised male rats (Feldman et al., 1979). It could therefore be speculated that higher CBG levels could have prevented some of the effects of CORT treatment in female mice.

Alternatively, estrogens can also exert direct effects on reelin levels. Indeed, estradiol treatment has been shown to increase protein levels of full-length reelin and its amino-terminal fragments in mouse cerebellum and organotypic slice cultures of early post-natal mouse hippocampus (Biamonte et al., 2009; Bender et al., 2010). In our model, oestrogen could stimulate reelin production as a compensatory mechanism in response to a CORT-induced down-regulation. Such a mechanism could involve direct oestrogen receptor (ER) ERα/GR cross-talk as previously described (Cvoro et al., 2011). Interestingly, ERα expression has been demonstrated in rats CR, different types of GABAergic neurons, and on dendritic spines where reelin is expressed (Nakamura and McEwen, 2005; Bender et al., 2010).

Conversely, testosterone can render males more susceptible to CORT-induced reelin down-regulation. Indeed testosterone has been shown to reduce reelin immunoreactivity in the high vocal centre of male starlings treated with testosterone (Absil et al., 2003), although this has not been studied in mice.

The approximately 50% decrease of reelin protein and RNA levels in hippocampus and PFC of patients with schizophrenia has been postulated to be caused by epigenetic modulation rather than genetic mutations (Levenson et al., 2008). Therefore, in order to mimic this reduction of reelin levels in schizophrenia, we included HRM where we expected a stronger effect of CORT

### Table 1

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<th>Male</th>
<th>Female</th>
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<tbody>
<tr>
<td></td>
<td>FC</td>
<td>DH</td>
</tr>
<tr>
<td>N-R6/FL</td>
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<td>WT+water</td>
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<tr>
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<td>3.513±0.213</td>
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Data are mean±S.E.M for 4–7 mice per group.
treatment against their 50% reduction of normal reelin gene expression. However, we did not observe any genotype interaction with the effect of CORT in any brain region. It is possible that, since HRM do not exhibit any...
with schizophrenia depending on the timing of their reelin down-regulation.

In apparent contrast to our results, one previous study found a decrease of reelin-positive cells in the subgranular zone and the hilus of CORT-treated HRM but not in WT (Lussier et al., 2011). This could be explained by the differences in the protocol. Notably, CORT treatment was administrated subcutaneously as daily injection of 5, 10 or 20 mg/Kg for 21 d, whereas in the current study it was via the drinking water. Moreover, we assessed protein levels by Western blot, whereas the previous work focused on reelin immunoreactivity. Future studies will be needed to ascertain whether the reelin down-regulation we observed reflects a decrease of reelin secretion or of the number of reelin expressing cells.

Finally, the observed CORT-induced reelin down-regulation in males appeared to be fragment-specific. Although levels of full-length reelin and N-R2 were significantly down-regulated in the frontal cortex of males treated with CORT, we observed a significant decrease only for N-R6 and N-R2 in the dorsal hippocampus. These results suggest that the CORT effect is post-translational. Since the ratios of N-R6/FL and N-R2/FL are similar between the different groups, CORT treatment does not appear to affect reelin cleavage per se, but most likely the availability or degradation of the affected fragments. Previous studies have shown that, upon secretion by GABAergic neurons, reelin protein is cleaved at the N-terminus and C-terminus by an unidentified metalloproteinase and protease, respectively (Lambert de Rouvroit et al., 1999). The location and timing of this cleavage as well as its role in the reelin signalling remain unclear. It has been suggested that reelin is anchored in the extracellular matrix and that its central domain, critical for the binding to ApoER2 and VLDLR, is released by cleavage (Jossin et al., 2007). Alternatively, it has been suggested that reelin dimerisation is essential for its biological activity and is controlled by the N-terminal region, which does not interact with these receptors but is essential for reelin signalling (Utsunomiya-Tate et al., 2000; Kubo et al., 2002). In addition, after binding reelin is internalised by endocytosis and degraded by lysosome. It has been found that the N-R2 fragment is produced in the endosome following reelin binding at ApoER2 and re-secreted in the extracellular space (D’Arcangelo et al., 1999; Morimura et al., 2005; Hibi and Hattori, 2009). Thus the fate of reelin and its different fragments from secretion to internalisation by neurones remains unclear.

Although the significance of the form-specific regulation of reelin that we observed remains to be elucidated, it is noteworthy that such specific modulation has also been found in blood of patients with schizophrenia and major depression (Fatemi et al., 2001), in maternal serum and umbilical cord blood, in brains of patients with Alzheimer’s disease (AD) and β-amyloid precursor protein (APP)-overexpressing mutant mice (Botella-Lopez et al., 2006, 2010) and in rats after treatment with chronic psychotropic drugs (Fatemi et al., 2009). This suggests a role for differential reelin cleavage within the brain.

**Down-regulation of reelin levels by corticosterone treatment is accompanied by selective NMDAR subunit down-regulation**

We found that the down-regulation of reelin in male dorsal hippocampus and frontal cortex of CORT-treated mice was accompanied by selective down-regulation of the GluN2C subunit of NMDAR. Several NMDAR subunits have been identified GluN1, GluN2 (A, B and C) and GluN3 (A and B). Functional NMDARs are heterotetramers containing two obligatory GluN1 subunits and two regulatory subunits, usually GluN2. Importantly, the GluN2 subunits determine the electrophysiological and pharmacological properties of the NMDAR channels (Cull-Candy and Leszkiewicz, 2004; Paolelli, 2011). Studies have shown that reelin can modulate NMDAR subunit composition by acting on GluN2A and GluN2B subunits. Notably, reelin is required for the developmental switch from GluN2B- to GluN2A-containing NMDARs during hippocampal maturation (Sinagra et al., 2005). Reelin also controls NMDAR subunit composition by regulating NMDAR surface trafficking notably by controlling GluN2B subunit mobility (Groc et al., 2007). It has also been shown that activation of Scr tyrosine kinase family by reelin binding to its receptor increases the phosphorylation of GluN2A and GluN2B (Beffert et al., 2005). Important for the present results, it has been shown that GluN2C can be phosphorylated by protein kinase B (PKB) (Chen and Roche, 2009), which itself can be activated by the phosphatidylinositol-3-kinase (PI3K), which in turn can be activated by reelin (Bock et al., 2003). Thus, reelin down-regulation could decrease PI3K/PKB-mediated phosphorylation of GluN2C resulting in its down-regulation.

GluN2C expression is first seen postnatally and is specifically enriched in the cerebellum, suggesting an important role for motor control. However, it is also expressed in certain neurones of the cerebral cortex and the hippocampus (Monyer et al., 1994). Moreover, the study of the NR2C subunit-β-galactosidase knock-in mouse showed that higher expression of GluN2C is found in the dorsal hippocampus (Karavanova et al., 2007). Interestingly, GluN2C knockout mice present with deficits in learning and memory and fear acquisition. These behavioural impairments are relevant to schizophrenia and involve notably the hippocampus and the frontal cortex (Hillman et al., 2011). Thus, changes in GluN2C subunit expression in the present study could result in altered learning and memory and fear conditioning, although further experiments will need to be done to confirm this. Chen and Roche (2009) furthermore showed that GluN2C expression, unlike GluN2A and GluN2B, prevented NMDAR-induced toxicity in cerebellar granule
neurons. It is then possible that GluN2C subunits exert the same role in the hippocampus and the frontal cortex despite relatively low expression.

It should be noted that a direct effect of chronic corticosterone on GluN2C expression cannot be excluded. Indeed, CORT treatment and stress exposure have previously been shown to modulate NMDAR subunits composition (Tse et al., 2012). Analysis of NMDAR subunits in schizophrenia brains is inconsistent between studies; however, the few studies assessing GluN2C subunit levels reported a decrease similar to our observations. GluN2C mRNA was decreased in prefrontal cortex of patients with schizophrenia but not with bipolar disorder or depression (Akbarian et al., 1996; Beneyto and Meador-Woodruff, 2008). Moreover, decreased GluN2C gene expression was found in the right cerebellum of schizophrenics and controls carrying a neuregulin-1 risk variant for schizophrenia (Schmitt et al., 2010).

In a previous study, we reported differential expression of NMDAR subunits in frontal cortex between WT and HRM (van den Buuse et al., 2012), i.e. an increase of GluN1 and a decrease of GluN2C in HRM males and females in comparison with their WT littermates that was not observed in the present study. These apparent discrepancies could be explained by the fact that in our previous study the mice underwent extensive behavioural characterisation including drug challenges which could have influenced the NMDAR results. In the present study, we used a cohort of mice which did not undergo behavioural tests and then did not receive any drugs injections.

Down-regulation of reelin levels by corticosterone treatment is not accompanied by changes in PV or GAD67 levels

NMDAR hypofunction in schizophrenia is postulated to occur mainly in PV-positive neurons, a subset of GABAergic interneurons, and to be responsible for down-regulation of their density (Nakazawa et al., 2012). Since GluN2C is expressed in the PV-positive interneurons of the FC of rats (Xi et al., 2009), we assessed PV and GAD67 levels to determine if GluN2C down-regulation could have an impact on the GABAergic neurons. We found that GluN2C down-regulation was not accompanied by changes in both GABAergic neurons markers. Previous studies described a decrease in the density of PV- and GAD67-containing neurons mainly in some layers of CA1 and CA2 regions of the hippocampus of female HRM (Nullmeier et al., 2011) and a reduction of the number of GAD67-immunopositive neurons in some areas of the cortex of male HRM (Liu et al., 2001). Two other studies found a decrease in GAD67 protein level, one in the frontal cortex of HRM of unspecified sex and age (Pillai and Mahadik, 2008), another in both frontal cortex and hippocampus of 14-day-old and 30-day-old male HRM (Kutiyananawalla et al., 2012). Contrary to these previous studies, we did not find a decrease in PV and GAD67 in HRM compared to WT. The previously reported localised down-regulations could be too small to be detected by WB in our study or arise from the age differences of the animals.

Interestingly, in the frontal cortex CORT treatment induced a down-regulation of PV only in females WT and not female HRM. The fact that WT males were not affected by CORT suggests a role for female sex hormones, particularly oestrogen, since they can influence the HPA axis as mentioned above. The lack of effect of CORT on PV levels in female HRM may be related to altered oestrogen production in HRM compared to WT controls. For example, Biamonte et al. (2009) showed that the level of 17 alpha-estradiol was higher in the cerebella of 15-day-old HRM females compared to WT. These results suggest a different hormonal profile for female HRM which could protect them from PV CORT-induced down-regulation.

Conclusion

In summary, these results show that stress, simulated here by CORT treatment, causes long-lasting and selective reductions of reelin and NMDAR subunit levels in male FC and DH. This sex-specific down-regulation in regions implicated in schizophrenia suggests an interaction of reelin and NMDAR in the effects of stress in the illness.

Supplementary material

For supplementary material accompanying this paper, visit http://dx.doi.org/10.1017/S1461145714000121.

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Statement of Interest

None.

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