

Early Life Stress Stimulates Hippocampal Reelin Gene Expression in a Sex-Specific Manner: Evidence for Corticosterone-Mediated Action

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ABSTRACT: Early life stress predisposes to the development of psychiatric disorders. In this context the hippocampal formation is of particular interest, because it is affected by stress on the structural and cognitive level. Since little is known how early life stress is translated on the molecular level, we mimicked early life stress in mouse models and analyzed the expression of the glycoprotein Reelin, a master molecule for development and differentiation of the hippocampus. From postnatal day 1 (P1) to P14, mouse pups were subjected to one of the following treatments: nonhandling (NH), handling (H), maternal separation (MS), and early deprivation (ED) followed by immediate (P15) or delayed (P70) real time RT-PCR analysis of reelin mRNA expression. We show that at P15, reelin mRNA levels were significantly increased in male H and ED groups when compared with the NH group. In contrast, no stress-induced alterations of reelin mRNA expression were found in female animals. This sex difference in stress-mediated stimulation of reelin expression was maintained into adulthood, since at P70 intergroup differences were still found in male, but not in female mice. On the cellular level, however, we did not find any significant differences in cell densities of Reelin-immunolabeled neurons between treatment groups or sexes, but an overall reduction of Reelin-expressing neurons in the adult hippocampus when compared to P15. To address the question whether corticosterone mediates the stress-induced up-regulation of reelin gene expression, we used age-matched hippocampal slice cultures derived from male and female mouse pups. Quantitative determination of mRNA levels revealed that corticosterone treatment significantly up-regulated reelin mRNA expression in male, but not in female hippocampi. Taken together, these results show a sex-specific regulation of reelin gene expression by early life experience, most likely mediated by corticosterone. © 2010 Wiley Periodicals, Inc.

KEY WORDS: maternal separation; Cajal-Retzius cell; brain-derived neurotrophic factor; slice culture; development

INTRODUCTION

Recent evidence suggests that stress-induced hippocampal damage may play an important role in the etiology of depressive disorders

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(McEwen, 2004). Patients suffering from major depression or posttraumatic stress disorder have been reported to exhibit hippocampal volumetric loss (Bremner et al., 2000; Smith, 2005). In animal models, sustained exposure to stress is known to induce dendritic atrophy within the hippocampal CA subfields (Magarinos et al., 1996; Vyas et al., 2002) and to decrease neurogenesis in the dentate gyrus (Pham et al., 2003; Mirescu et al., 2004). The form and extent of stress-induced hippocampal damage is thought to depend upon the timing, type, duration, and frequency of the stressor (Pacak and Palkovits, 2001; Radley and Morrison, 2005). In fact, early life adverse experience is known to alter adult responses to stress (Ladd et al., 2000), thus contributing to the generation of individual differences in vulnerability, not only to stress but also to stress-related psychopathology (Heim et al., 2000; Heim and Nemeroff, 2002). Although it is evident that sustained stress, both in early life and adulthood, can adversely affect hippocampal structure and function (Magarinos et al., 1996; Vyas et al., 2002; Brunson et al., 2003; Buwalda et al., 2005), the underlying molecular mechanisms are largely unknown.

Early separation paradigms represent established animal models to induce early life stress and to study the immediate or long-term consequences of stress experience (Meaney, 2001; Pryce and Feldon, 2003). Depending on duration and type of stress the effect on the individual can differ greatly: Postnatal brief isolation of young rodents (“handling”/H) results in high open field exploration, enhanced learning and memory in adulthood. Longer repeated separation of the intact litter (“maternal separation”/MS), however, has negative effects such as reduced stress resistance, increased anxiety, and reduced spatial navigation learning (Pryce and Feldon, 2003). These differential responses may be mediated by differential regulation of genes that are important for neuronal development or plasticity.

In the present study we focused on a stress-related investigation of the glycoprotein Reelin, a crucial positional signal for layer formation and differentiation of cortical and hippocampal neurons (Rakic and Cavinness, 1995; Frotscher, 1998; Rice and Curran, 2001; Soriano and Del Rio, 2005; Förster et al., 2006; Cooper, 2008). Also in adulthood, the Reelin signaling cascade is of vital importance for synaptic functions that are essential for cognition and learning

(Herz and Chen, 2006). Accordingly, decreased Reelin expression has been found in psychiatric diseases such as schizophrenia and mood disorders (Impagnatiello et al., 1998; Fatemi, 2005) as well as in temporal lobe epilepsy (TLE) (Haas et al., 2002; Heinrich et al., 2006) implying that reduced Reelin levels entail structural and functional deficits in the brain.

With the concept that early life stress severely affects hippocampal structure and function, we used several maternal separation paradigms to study their potential impact on reelin expression in the mouse hippocampus. For the first time, we present evidence that early life stress stimulates reelin gene expression in a sex-specific manner and that glucocorticoids most likely mediate this process.

MATERIALS AND METHODS

Animals

Female multiparous C57Bl/6NCrl mice (Institute stocks) were bred at the central animal facility of the University Clinic Freiburg. Mice were housed in individual cages with food and water ad libitum and kept in a 12-h light-dark cycle at a room temperature of $22 \pm 1^\circ\text{C}$. Litters were culled on postnatal day 1 (P1). Litter size was standardized to seven pups (four females and three males or vice versa). If litters were smaller, only those with a minimum of five to six pups were included in the study. Due to a natural variation of litter size, the sample size was variable between treatment groups (see below). We randomly assigned whole litters to one of four rearing conditions from P1 to P14: nonhandling (NH), handling (H), maternal separation (MS), and early deprivation (ED). All animal procedures were carried out in accordance with the guidelines of the European Community's Council Directive of 24 November 1986 (86/609/EEC). Experiments were performed in agreement with the German law on the use of laboratory animals and approved by the Regierungspräsident Freiburg. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Separation Protocols

The handling and separation procedures were based on standardized protocols (Pryce and Feldon, 2003; Millstein and Holmes, 2007) and were performed during daytime (light cycle) from P1 until P14 as follows (Fig. 1a): NH group: during the whole experimental period, dam and pups were not disturbed except for a single change of the cage. H and MS: whole litters were removed from the dam by placing them in separate cages for 10 min (H) or 3 h (MS) daily. ED group: each pup was separated individually from mother and litter mates for 3 h daily by transfer into plastic containers lined with bedding. After separation, the pups were returned to their home cages, where they were reunited with the dam. To avoid additional stress on pups and dam the separation procedures were performed by the same person. The day after the end of the experimental period (P15) one half of the animals was sacrificed in the morning, the other

half was allowed to survive until P70 (Fig. 1b). In order to avoid confounding family group effects, subjects of each original litter were randomly assigned either to RT-PCR, morphological analysis, or to the two age groups. Detailed information about group sizes/experiment is specified below.

Perfusion and Tissue Preparation

Mice were deeply anesthetized with a mixture of ketamine (200 mg/kg), xylazine (10 mg/kg), and acetylpromazine (0.2 mg/kg) followed by transcardial perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 10 min under hydrostatic pressure with a flow rate of 5 to 7 ml/min. The brains were removed from the skull, postfixed in the same fixative for 5 h at 4°C , rinsed in PB, and cut in coronal sections (50 μm) on a vibratome (Leica, VT100M).

Immunocytochemistry

Immunolabeling for Reelin was performed by a free-floating procedure described previously (Heinrich et al., 2006). Following several washing steps in 0.1 M phosphate-buffered saline (PBS), pH 7.2, tissue sections were pretreated in 0.25% Triton X-100 in PBS for 30 min followed by incubation in 10% normal serum and 0.25% Triton X-100 in PBS for 20 min. Incubation with a mouse anti-Reelin antibody (1:500; G10) was performed in the presence of 0.1% Triton X-100 and normal goat serum (1:100) for 5 h at room temperature followed by an overnight incubation at 4°C . After several washing steps the sections were incubated with biotinylated anti-mouse antibody (1:250; Vector Laboratories, Burlingame, CA) for 2 h followed by three washing steps in PBS. Tissue-bound antibodies were visualized using the Vectastain ABC Kit (Vector Laboratories) and reacted with 0.05% 3,3'-diaminobenzidine in PBS with 0.002% H_2O_2 . Sections were mounted on gelatin-coated slides, air-dried, dehydrated in graded ethanol, and coverslipped with HYPER-MOUNT (Shandon Inc., Pittsburgh, PA).

Double Immunolabeling

For double immunolabeling, vibratome sections of P15 male mice (NH group, $n = 6$, six sections/animal) were pretreated as described above. Incubation with the two primary antibodies, mouse monoclonal anti-Reelin G10 (1:1,000) and rabbit polyclonal antigluocorticoid receptor (GR-M20; 1:500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA), was performed simultaneously as described before (Heinrich et al., 2006). Sections were analyzed with a Zeiss Axioplan2 fluorescence microscope equipped with an ApoTome setting (Carl Zeiss AG, Göttingen, Germany). Individual images were captured using the AxioVision software (Carl Zeiss AG).

Cell Counts

Cell counts were performed in hippocampal sections of all four experimental groups (males and females separately) and both time points (P15 and P70) to determine the density of Reelin-immunolabeled cells. Numbers of animals were the fol-

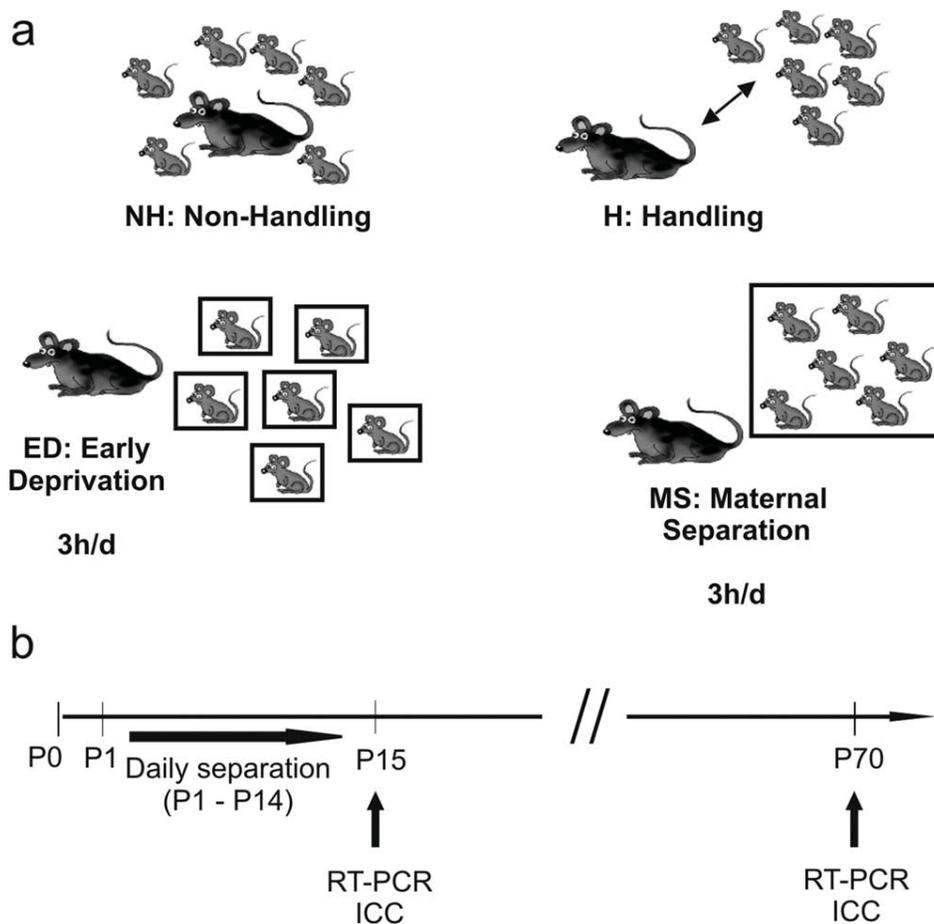


FIGURE 1. a. Group design and separation paradigms used in the present study. b. Schedule of the experimental design. P, postnatal day; RT-PCR, reverse transcription polymerase chain reaction; ICC, immunocytochemistry.

lowing: at P15: NH (n_f [female] = 4; n_m [male] = 4), H (n_f = 5; n_m = 5), ED (n_f = 5; n_m = 5), MS (n_f = 6; n_m = 6) and at P70: NH (n_f = 6; n_m = 5), H (n_f = 5; n_m = 5), ED (n_f = 5; n_m = 6), MS (n_f = 8; n_m = 5).

Cell density was determined using a 20 \times objective and the StereoInvestigator image analysis system (MicroBrightField Inc., Colchester, VT). The area of the hippocampus was divided in three regions of interest (ROI; CA1, CA3 + hilus, dentate gyrus) per section. All Reelin-immunolabeled cells were counted in these three ROIs/section. Cell density for each ROI/section was determined by calculating the number of cells/mm². Six representative coronal sections covering the entire septotemporal axis of the hippocampus were evaluated in this way. Cell densities were expressed as cell number/mm² and subsequently averaged for each ROI and each animal. Mean and SEM were calculated for each group followed by a two-factorial analysis of variance (see Statistical Analysis).

RNA Extraction and Reverse Transcription

Numbers of animals assigned for gene expression analysis were the following: at P15: NH (n_f = 10; n_m = 7), H (n_f =

8; n_m = 8), ED (n_f = 7; n_m = 6), MS (n_f = 8; n_m = 7) and at P70: NH (n_f = 9; n_m = 11), H (n_f = 10; n_m = 8), ED (n_f = 8; n_m = 12), MS (n_f = 5; n_m = 11).

Mice were deeply anesthetized with isoflurane (Abbott, Wiesbaden, Germany) and sacrificed by decapitation. The hippocampi were dissected at 4°C, homogenized, and total RNA was isolated using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). RNA integrity was determined using an electrophoresis Bioanalyzer (Agilent Technologies, Böblingen, Germany). RIN values were always in the range of 8 to 10 indicating very pure and intact RNA preparations. Reverse transcription (RT) was performed in 20 μ l reactions containing 1 μ g total RNA, 5 μ M random decamer primers, 500 μ M deoxyNTPs, 20 U RNase inhibitor, and 100 U of M-MLV reverse transcriptase (all from Ambion, Huntingdon, UK) for 60 min at 42°C following a standard protocol.

Real-Time Quantitative RT-PCR

Relative mRNA levels were determined by real time quantitative RT-PCR on a MyiQ Real Time PCR Detection System (Bio-Rad Laboratories, München, Germany) in the presence of

SYBR Green (ABgene, Hamburg, Germany) as described previously (Heinrich et al., 2006). The following mouse-specific primers (70 nM) were used: reelin: forward 5'-CCCAGCCCA GACAGACAGTT-3', reverse 5'-CCAGGTGATGCCATTGT TGA-3'; BDNF: forward 5'-GGGTCACAGCGGCAGATAAA-3', reverse 5'-GCCTTTGGATAACCGGGACTT-3'; glucocorticoid receptor: forward 5'-CAAAGGCGATAACCAGGATTCA-3', reverse 5'-AGGGCAAATGCCATGAGAAA-3'; S12: forward 5'-GGC ATAGCTGCTGGAGGTGTAA-3', reverse 5'-GGGCTTGGC GCTTGTCTAA-3'. Cycling conditions were as follows: 15 min at 95°C followed by 50 cycles of 15 s at 95°C and one min at 60°C. Monitoring the fluorescence signal, which is proportional to the amount of double-stranded product, yielded complete amplification profiles. Melting curves of the amplified products were used to control for specificity of the amplification reaction and indirectly also as a measure of RNA quality. From the amplification curves obtained, a threshold cycle number (Ct) was calculated, corresponding to the cycle number at which a user-defined fluorescence signal was reached. Differences in Ct values were used to calculate relative amounts of PCR product. Details of this relative quantification can be found at <http://docs.appliedbiosystems.com/pebi docs/04303859.pdf>. All quantifications were normalized to an established internal control for rodent brain (ribosomal S12 protein RNA, housekeeping gene), which was coamplified as described previously by others (Heinrich et al., 2006; Usarek et al., 2006; Nanda et al., 2008). For each group the results were given as mean \pm SEM.

Preparation of Organotypic Slice Cultures

Organotypic slice cultures from hippocampi of male or female mice pups were prepared using a method described earlier (Stoppini et al., 1991). In brief, mice pups (P7) were sacrificed, brains were removed and rapidly transferred to ice-cold preparation medium containing 74% Earle's minimum essential medium (MEM), 25% basal medium Eagle (BME), and 2 mM glutamine (all purchased from Gibco Invitrogen). Hippocampi were dissected, cut into 400 μ m slices using a McIlwain Tissue Chopper (Mickle Laboratory Engineering Co. Ltd., UK), placed onto 0.4 μ m cell culture membrane inserts (Millipore, Millipore, Bedford, MA) and transferred to a six-well plate containing 1 ml nutrition medium per well (46% MEM, 25% BME, 25% heat-inactivated horse serum supplemented with 0.65% glucose and 2 mM glutamine, pH 7.2). The slice cultures were incubated at 37°C and 5% CO₂ for 7 days, culture medium was changed three times a week.

Slice cultures were treated for 20 min with 0.1 or 1 μ M corticosterone (CS; Sigma Aldrich) according to Morsink et al. (2006) or with incubation medium (vehicle) and were subsequently replaced in incubation medium for 5 h. To determine the specificity of the CS response, we incubated a subset of slice cultures with the glucocorticoid receptor antagonist RU 38486 (0.5 μ M, Sigma Aldrich) for 20 min before and during CS treatment. For real time RT-PCR analysis, slice cultures were harvested and four slices/treatment and animal were pooled for subsequent RNA extraction and RT-PCR reaction

(see above). Total numbers of animals used for the gene expression analysis in slice cultures were the following: control ($n_f = 5$, $n_m = 10$), 0.1 μ M CS ($n_f = 5$, $n_m = 10$), 1 μ M CS ($n_f = 4$, $n_m = 10$), 0.1 μ M CS + RU 38486 ($n_f = 5$, $n_m = 5$), 1 μ M CS + RU 38486 ($n_f = 4$, $n_m = 4$).

For immunolabeling, hippocampal slice cultures were fixed in buffered 4% paraformaldehyde for 2 h at room temperature, washed several times in PB and resliced (20 μ m) on a sliding vibratome. Tissue sections were mounted on gelatin-coated slides and immunolabeling for Reelin was performed as described above. Detection was accomplished with a secondary antibody conjugated with Cy³ (1:200; Jackson ImmunoResearch).

Statistical Analysis

Data sets obtained from RT-PCR quantifications and cell counts were statistically analyzed with GraphPad Prism 4 (GraphPad Software, San Diego, CA). Intergroup comparisons between three or more groups in experiments with two age or sex groups were conducted with two-way ANOVAs (age \times separation or sex \times treatment of samples) followed by post hoc Bonferroni tests with a level of significance set at $P < 0.05$. Intergroup comparisons concerning only one age group were done via one-way ANOVAs (separation) followed by Tukey's multiple comparison tests also with a level of significance set at $P < 0.05$.

RESULTS

In order to investigate the effects of stress on hippocampal reelin gene expression we compared the influence of nonhandling (NH), handling (H), early deprivation (ED, individual isolation), and maternal separation (MS, isolation of the intact litter) during the first 2 weeks of life followed by immediate (at P15) or delayed analysis (at P70). These two time points were chosen to differentiate between immediate effects at P15 and potential stress-induced persistent changes at P70.

First we aimed at validating the applied stress paradigms by measuring hippocampal mRNA expression levels of brain-derived neurotrophic factor (BDNF) which previously has been shown to be influenced by environmental stimulation in rat hippocampus (Roceri et al., 2004; Lippmann et al., 2007; Nair et al., 2007).

Handling and Early Deprivation Significantly Stimulate BDNF mRNA Expression in the Mouse Hippocampus

When BDNF mRNA expression was quantified at P15 by real time RT-PCR analysis, we observed a significant treatment effect [two-way ANOVA (sex \times treatment) [$F_{(3,0)} = 5.51$; $P = 0.0023$]. Compared with the NH group, we found a significant elevation of BDNF mRNA levels after ED in females ($P < 0.05$) and after H in males ($P < 0.05$). The sex of the

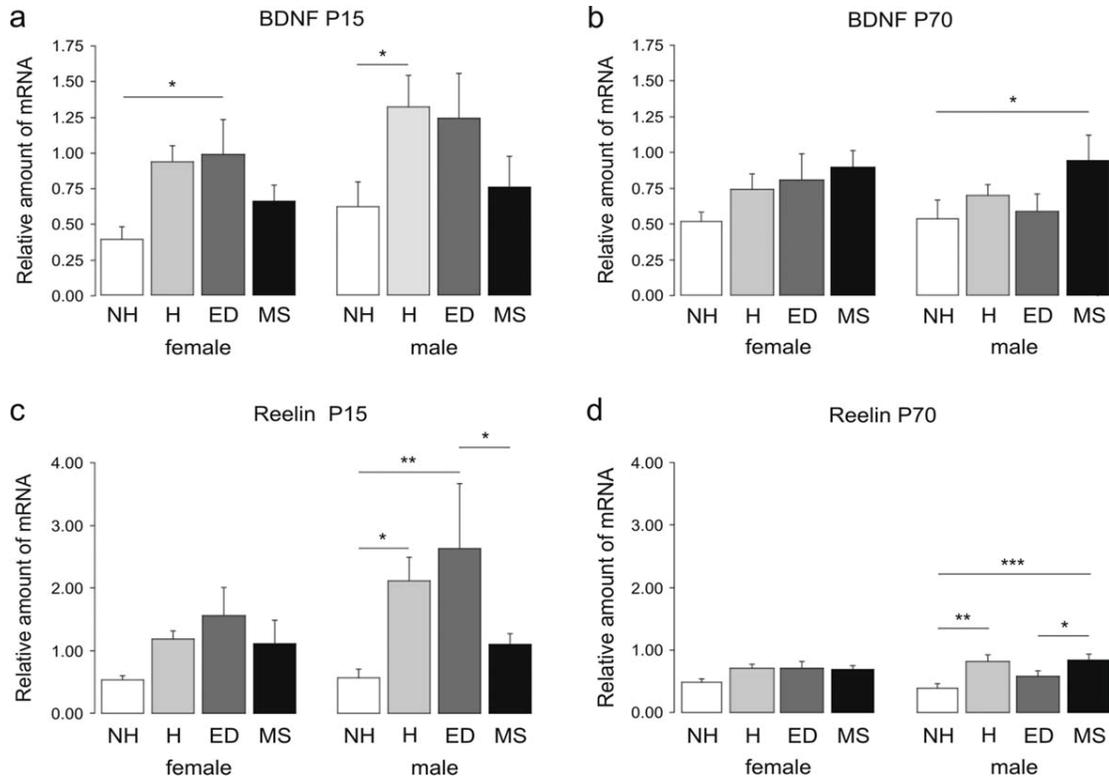


FIGURE 2. Quantitative evaluation of BDNF and reelin mRNA expression in the hippocampus after NH, H, ED and MS. Relative BDNF (a and b) and reelin (c and d) mRNA levels were determined by real time RT-PCR in extracts from isolated hippocampi at P15 (a and c) and P70 (b and d). Measurements were performed in duplicates, and relative mRNA levels were normalized with S12 RNA as an internal standard. Mean values (\pm SEM) are shown. Statistical analysis included two-factor-ANOVAs (age \times separation or sex \times separation) followed by post hoc Bonferroni tests with a level of significance set at $P < 0.05$. a. P15. When compared with NH, ED resulted in a significant increase of BDNF mRNA expression in female mice, whereas male mice responded

with a significant up-regulation after handling ($P < 0.05$). b. P70. Only MS stimulated BDNF mRNA expression significantly in males ($P < 0.05$), but not in females. c. P15. No significant differences in reelin mRNA expression were found in female mice after the different treatments. In contrast, significant up-regulation of reelin mRNA expression was found in male animals after handling ($P < 0.05$) and early deprivation ($P < 0.01$) in comparison to NH. d. P70. As at P15, no treatment effect was found in females. In males, however, up-regulation of reelin mRNA expression was observed in the H group ($P < 0.01$) and in MS ($P < 0.001$) when compared to NH, indicating long-term effects of the stress paradigms.

animals did not affect the results [two-way ANOVA (sex \times treatment) $F_{(1,0)} = 5.51$; $P = 0.0720$] (Fig. 2a).

In the adult stage, comparable BDNF mRNA levels were observed in all groups except for a slight, but significant, increase in the male MS group ($P < 0.05$) (Fig. 2b). No significant treatment effect was found at P70 [two-way ANOVA (sex \times treatment) $F_{(3,0)} = 2.63$; $P = 0.0575$].

These results show that in mice we were able to reproduce the stress-induced short-term up-regulation of BDNF mRNA expression reported previously by others in rats (Roceri et al., 2004; Nair et al., 2007). Thus, our experimental set-up was suitable to investigate the effect of early experience on the expression of reelin mRNA.

Early Experience Stimulates Reelin mRNA Expression in a Sex-Dependent Fashion

Next, quantitative real-time RT-PCR expression analysis for reelin mRNA was performed in the same cDNA samples used

for BDNF mRNA measurements. At P15 analysis of variance revealed significant, stress-induced alterations in the amount of reelin mRNA [two-way ANOVA (sex \times treatment) $F_{(3,0)} = 5.85$; $P = 0.0016$]. This effect was also observed in the adult stage [two-way ANOVA (sex \times treatment) $F_{(3,0)} = 6.22$; $P = 0.0009$] (Figs. 2c,d).

At P15, female and male hippocampi of the NH group exhibited comparable low levels of reelin mRNA. The various treatments, however, affected males and females differently: While female mice did not show any significant stress-induced changes of reelin mRNA expression, male mice responded to H ($P < 0.05$) and ED ($P < 0.01$) with a significant and strong up-regulation of reelin mRNA expression when compared with the NH group. Remarkably, ED led to a higher reelin mRNA expression, not only in comparison to NH, but also to MS ($P < 0.05$) (Fig. 2c).

At P70 male, but not female mice, still exhibited slightly elevated levels of reelin mRNA in the H ($P < 0.01$) and MS ($P < 0.0001$) groups which reached significance relative to NH

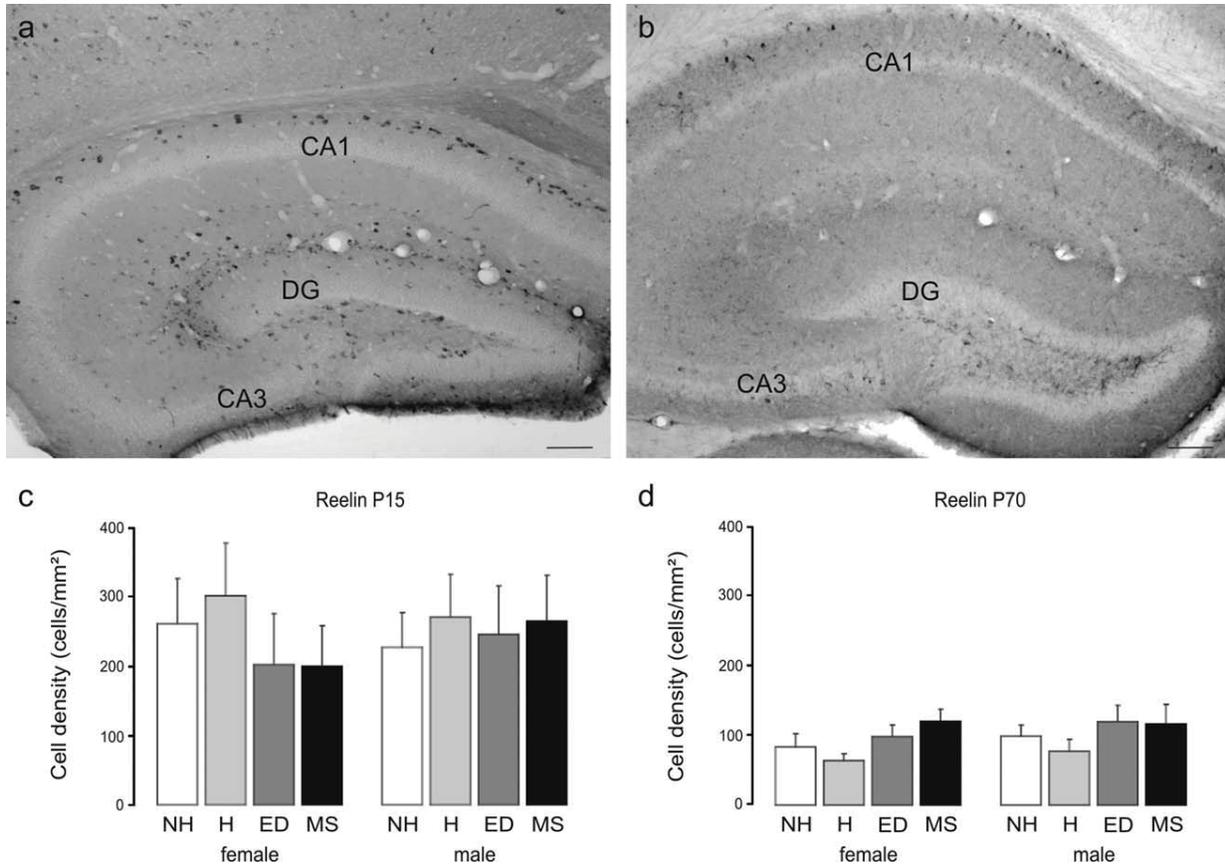


FIGURE 3. Density of Reelin-immunopositive neurons in the hippocampus of all experimental groups (NH, H, ED, MS) at P15 (a and c) and P70 (b and d). a and b. Representative tissue sections of P15 (a) and P70 (b) hippocampi immunostained for Reelin. Scale bars: 200 μ m. DG, dentate gyrus; CA, cornu ammonis. c and d. Histograms showing mean densities of Reelin-immunopositive neurons/mm² in the four

experimental groups at P15 (c) and P70 (d). No significant differences in cell densities were observed between experimental groups at both time points and both sexes (c and d). Cell densities were significantly [two-way ANOVA (age \times treatment)] reduced at P70 (c) when compared with P15 (d) both in male [$F_{(1,0)} = 19.31$; $P < 0.0001$] and female mice [$F_{(1,0)} = 20.89$; $P < 0.0001$].

(Fig. 2d). In addition, MS led to stimulation of reelin expression in male mice when compared with ED ($P < 0.05$).

These results show that the basal levels of reelin mRNA are similar in hippocampi of male and female mice. Both sexes respond to environmental stimuli with an up-regulation of reelin gene expression, but female mice are much less responsive. In addition, the data show that environmental influence in the early postnatal period leads to a long-term elevation of reelin expression only in males indicating a sex-difference in the regulation of reelin gene expression.

Density of Reelin-Synthesizing Cells in Mouse Hippocampus is not Altered by Stressful Experience

To address the question whether the observed changes in reelin mRNA expression were accompanied by alterations in the density of Reelin-expressing cells, we performed immunocytochemistry for Reelin on tissue sections of hippocampus immediately after the environmental manipulation at P15 and in the adult stage (P70). Cell densities were first determined in

three ROIs (dentate gyrus, CA1, CA3 + hilus), separately. At both time points and in both sexes the dentate gyrus showed the highest density of Reelin-positive cells when compared with CA1 and CA3 + hilus. Statistical analysis [two-way ANOVA (ROI \times treatment)] revealed, however, that there was no interaction between area and treatment in P15 female [$F_{(6,0)} = 0.63$; $P = 0.7020$], P15 male [$F_{(6,0)} = 0.64$; $P = 0.7006$], P70 female [$F_{(6,0)} = 0.63$; $P = 0.7052$], and P70 male animals [$F_{(6,0)} = 1.14$; $P = 0.3535$]. Therefore we decided to pool cell densities of all three ROIs/section (Fig. 3).

At both time points no significant differences in cell densities between the experimental groups were observed in female and male hippocampi when compared with NH (Figs. 3c,d). However, a significant age effect [two-way ANOVA (age \times treatment)] was found, since the density of Reelin-positive neurons was significantly reduced in male [$F_{(1,0)} = 19.31$; $P < 0.0001$] and female mice [$F_{(1,0)} = 20.89$; $P < 0.0001$] at P70 when compared with P15.

These results indicate that the population of Reelin-synthesizing neurons remains stable during stressful events in early life, but undergoes an age-dependent reduction in cell density,

independent of experimental manipulation or sex. Moreover, these data show that male and female hippocampi have similar densities of Reelin-expressing neurons and hence the strong stimulation of reelin mRNA expression in males must result from increased mRNA synthesis in individual cells.

Expression of Glucocorticoid Receptor mRNA and Colocalization of GR With Reelin

Glucocorticoid receptors (GR) are important mediators of the stress response (Weaver et al., 2004; de Kloet et al., 2005) and are strongly expressed in the hippocampus (Sarrieu et al., 1988; O'Donnell et al., 1994) where they mediate the effects of the stress hormone corticosterone.

In order to test whether GR expression was affected in the treatment groups, we performed real time RT-PCR analysis for glucocorticoid receptor mRNA levels in the P15 group of mice (Fig. 4a), which had shown the stress-related up-regulation of reelin gene expression (Fig. 2).

Quantitative analysis of GR expression levels revealed a significant treatment effect [two-way ANOVA (sex \times treatment) $F_{(3,0)} = 3.01$; $P = 0.0380$]. Female and male hippocampi of the NH group exhibited comparable low GR mRNA levels. While female mice did not show any significant stress-induced changes of GR expression, male mice responded to H with a significant and strong up-regulation of GR gene expression when compared to the NH ($P < 0.01$) and to the MS group ($P < 0.05$) (Fig. 4a).

In order to investigate whether Reelin-synthesizing cells are potentially responsive to glucocorticoids we performed double immunolabeling for Reelin and GR on tissue sections of P15 mouse hippocampus. Reelin-immunolabeled cells were prominent in stratum oriens, stratum lacunosum moleculare, whereas GR was ubiquitously expressed in the whole hippocampal formation (Figs. 4b,c). At higher magnification we observed that virtually every Reelin-positive cell displayed nuclear GR labeling (Figs. 4d-i). In these cells GR immunolabeling was confined to the nucleus, whereas Reelin immunoreactivity was seen in the surrounding cytoplasm. These data indicate that in the P15 mouse hippocampus all Reelin-producing neurons, irre-

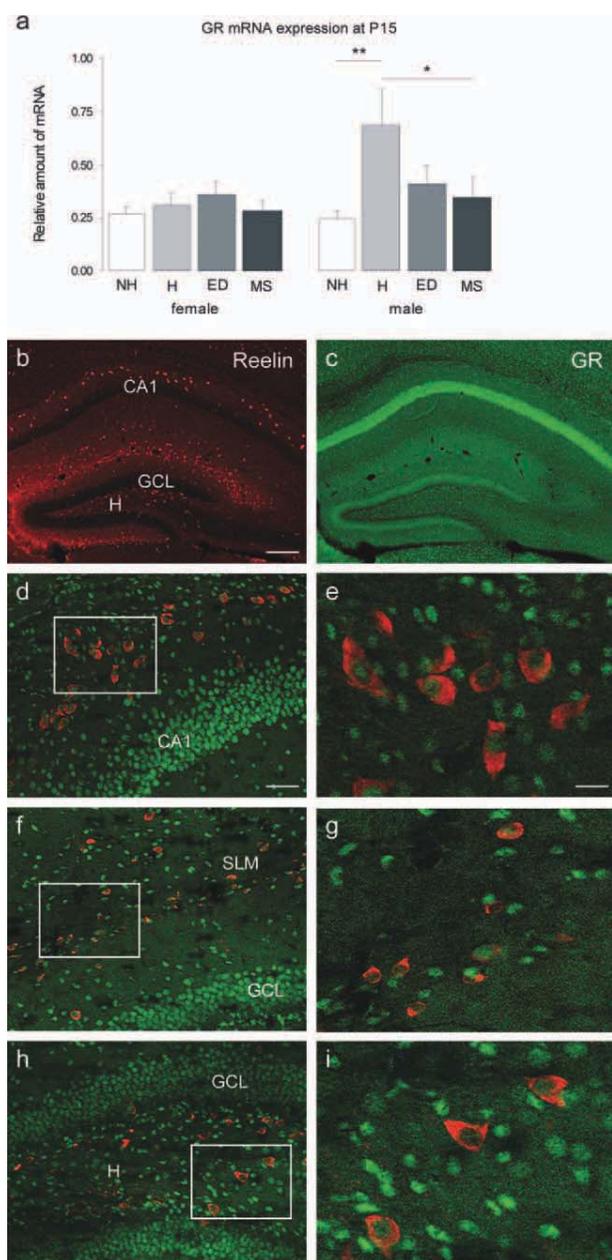


FIGURE 4. Quantification of glucocorticoid receptor expression and colocalization with Reelin in the postnatal mouse hippocampus. **a.** Histograms showing hippocampal glucocorticoid receptor (GR) mRNA levels in female and male animals of the four experimental groups (NH, H, ED, MS). Relative mRNA levels were determined by real time RT-PCR in extracts from isolated hippocampi at P15. Measurements were performed in duplicates, and relative mRNA levels were normalized with S12 RNA as an internal standard. Mean values (\pm SEM) are shown. Female mice exhibited similar levels of GR expression in all experimental groups, whereas in males a significant up-regulation of GR mRNA expression was observed in response to H when compared with NH and MS. Statistical analysis revealed a clear treatment effect [two-way ANOVA (sex \times treatment) $F_{(3,0)} = 3.01$; $P = 0.0380$]. **b–i.** Double immunolabeling for Reelin (red) and GR (green) at P15. Representative hippocampal tissue sections are shown. **b.** Immunolabeling for Reelin. Many Reelin-positive cells can be seen in stratum oriens of CA1, in stratum lacunosum moleculare and in the hilus. Scale bar: 200 μ m (also valid for **c**). **c.** Immunolabeling for GR. Prominent immunostaining for GR is visible in CA1 pyramidal cells and granule cells of the dentate gyrus. Weaker stained cells can better be recognized at higher magnification shown in **d–i**. **d–i.** Overlay of Reelin (red) and GR (green) immunolabeling. **d, f, h.** Double immunolabeled cells with cytoplasmatic Reelin and nuclear GR staining are prominent in stratum oriens (**d** and **e**), in stratum lacunosum moleculare (**f** and **g**), and the hilus (**h** and **i**). Framed areas are shown in high magnification in **e, g, and i**. Note that virtually every Reelin-positive cell contains a GR-positive nucleus. Besides, many GR single-labeled cells are present throughout the hippocampus. Scale bars: **d, f, h:** 50 μ m; **e, g, i:** 15 μ m. CA1, cornu ammonis; GCL, granule cell layer; SLM, stratum lacunosum moleculare; **h,** hilus.

spective of their functional specification, are potential targets of corticosterone action.

Corticosterone Stimulates Reelin mRNA Expression in Hippocampal Slice Cultures

To test whether corticosterone affects reelin gene expression in the hippocampus, we used hippocampal slice cultures obtained from male and female P7 mice and kept them in culture for 8 days in analogy to the P15 time point in vivo. These cultures exhibited many Reelin-expressing neurons as shown by immunolabeling (Fig. 5a).

Treatment with corticosterone (CS; 0.1 μ M and 1 μ M) or with CS plus RU 38486 was performed for 20 min followed by reincubation in medium for 5 h. Subsequently, reelin mRNA expression was quantified by real time RT-PCR analysis. Under control conditions, reelin mRNA levels were similar in organotypic slice cultures from female and male mice. CS treatment, however, elicited a significant, dose-dependent increase of reelin mRNA expression only in slice cultures from male, but not from female animals (Fig. 5b). This effect was specifically mediated by the GR, since preincubation with the GR antagonist RU 38486 abolished the CS-stimulated increase of reelin mRNA (Fig. 5b), showing a male-specific CS-mediated regulation of reelin gene expression.

DISCUSSION

The main results of this study are: Early life experience stimulates reelin gene expression in a sex-specific manner, since only male mice respond to handling or early deprivation with an up-regulation of reelin mRNA synthesis, which persists into adulthood. In addition, we show that corticosterone (CS) specifically induces reelin mRNA expression in slice cultures obtained from male, but not female mouse pups, indicating that CS mediates the induction of reelin gene expression observed after H and ED in vivo.

Expression of BDNF mRNA is Regulated by Environmental Stimuli in Postnatal Mouse Hippocampus

In the present study we used four early rearing conditions to investigate the consequences of environmental influences on the expression of BDNF and reelin mRNAs in mouse hippocampus under consideration of age and sex of the subjects. In contrast to previous studies that often applied only one rearing condition, we used a broad experimental set-up which allowed us to compare the consequences of four early rearing conditions on the genes of interest. Moreover, we used mice as experimental animals being of particular interest for behavioral studies of transgenic or knockout mice, because so far the majority of studies concerning early separation paradigms were performed in rats.

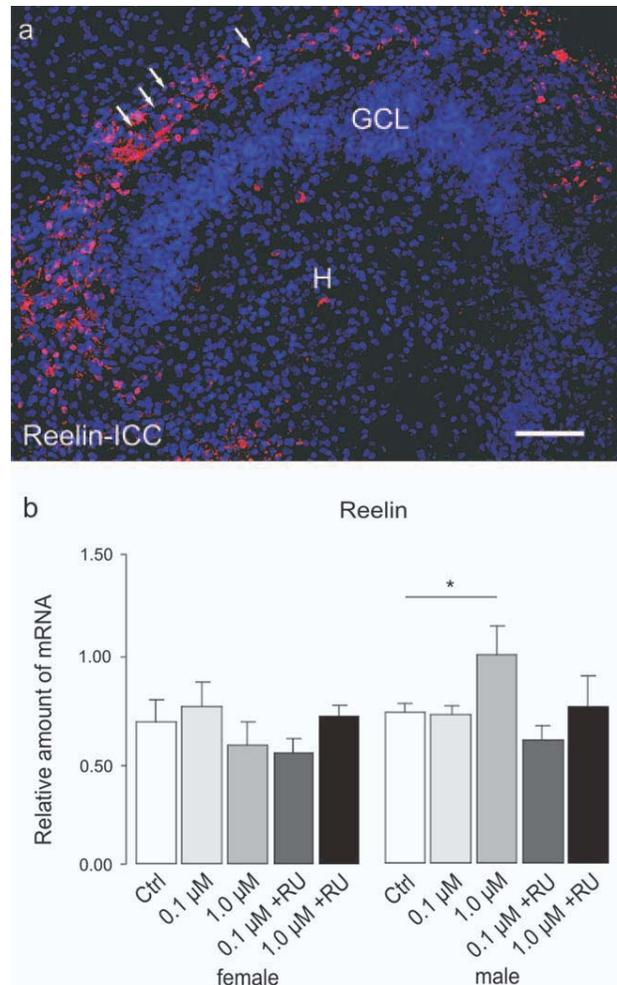


FIGURE 5. Corticosterone treatment increases reelin gene expression in hippocampal slice cultures from male mice. **a.** Representative section of an organotypic hippocampal slice culture (OHSC) immunolabeled for Reelin. OHSC were prepared from P7 mouse pups and were incubated for seven days in vitro. Many Reelin-immunopositive neurons (red) are located along the hippocampal fissure (arrows) and in the hilus. Tissue section was counterstained with DAPI (blue). GCL, granule cell layer; H, hilus. Scale bar: 100 μ m. **b.** Histograms showing the relative levels of reelin mRNA after treatment with corticosterone (CS) or CS plus GR antagonist RU 38486. Reelin mRNA levels were determined 5 h after treatment in extracts from hippocampal slice cultures by real time RT-PCR as described in Methods. Application of 1 μ M, but not 0.1 μ M CS significantly stimulated reelin mRNA expression in slice cultures from males, but not females. Combined application of CS and RU 38486 abolished this effect.

We have chosen to use BDNF as control gene for our separation protocols, since earlier reports have shown that BDNF expression is regulated by separation paradigms in the rodent hippocampus (e.g., Roceri et al., 2004; Lippmann et al., 2007; Nair et al., 2007). However, none of the published studies compared in parallel four experimental conditions, sex differences and immediate versus long-term effects in mice. We found

that female and male mice responded to H and ED with a short-term stimulation of BDNF mRNA synthesis relative to NH, but we found no effects in adulthood. Similar results have been obtained in rats showing an immediate, but not persistent, increase in hippocampal BDNF mRNA levels after MS (Roceri et al., 2004; Nair et al., 2007). Hence we reproduced in mice that BDNF gene expression is sensitive to environmental influence and thus validated our experimental paradigm for further experiments.

Stimulation of Reelin Gene Expression by Early Experience is Sex-Dependent

The definition of appropriate control conditions for separation experiments has been a matter of controversy (for review see Meaney, 2001; Pryce and Feldon, 2003). NH has been used as control group in many studies, but some researchers consider NH as an experimental condition which lacks external stimulation necessary for proper brain development (Meaney, 2001; Pryce and Feldon, 2003). H represents a condition which mimics best the situation in the wild, where short and frequent periods of absence from the litter are common during food gathering of the dam (Pryce and Feldon, 2003). In contrast, MS has been shown to be harmful, since repeated and extended separation from the mother is a stressful experience for the pups and changes their stress response in adulthood (Plotsky and Meaney, 1993; Liu et al., 2000; Pryce and Feldon, 2003). ED, which involves deprivation of not only the mother but also the littermates for an extended period of time, paradoxically has been found to have "positive" effects, since adult ED rats show enhanced cognitive abilities when compared with normal rearing conditions (Pryce and Feldon, 2003).

We decided to use the NH condition as reference, since others have used NH as reference group in many rat studies (Pryce and Feldon, 2003; Ruedi-Bettschen et al., 2006). Moreover, we observed lowest reelin mRNA levels in the NH group independent of sex and age, representing basal reelin expression in the hippocampus. In contrast, after postnatal separation we found striking differences in the response of males and females: H and ED led to a significant up-regulation of reelin mRNA synthesis in male hippocampus relative to NH and this enhanced mRNA synthesis persisted to some degree into adulthood. In particular, in the MS group reelin mRNA levels were maintained. This could be due to a slower turnover or longer half-life of reelin mRNA in this particular group when compared with H or ED. On the cellular level, *in situ* hybridization experiments did not reveal differences in reelin mRNA expression pattern between MS and the other experimental groups. Both interneurons and surviving Cajal-Retzius cells were positive for reelin mRNA (Haas, unpublished data) indicating that only subtle expression differences exist between groups and that those can only be detected by more sensitive real-time PCR measurements. Along this line, it remains open whether the increased mRNA expression levels found at P15 in male H, ED, and MS groups are translated into protein.

Female mice, however, showed only slight and not significant changes of reelin mRNA levels indicating that male mice are more sensitive to early environmental influences. Interestingly H and ED had both strong stimulating effects on reelin gene expression when analyzed at P15, which is in line with the observations of Pryce and Feldon (2003) who showed in rats that H and ED have similar stimulation effects in terms of enhanced cognitive functions and reduced anxiety in adulthood. MS, in turn, which is not considered as a strong stimulus according to Meaney (2001) did not result in significant enhancement of reelin mRNA expression when compared with NH.

An important result of our study is that only male mice responded to environmental manipulation with a short- and long-term up-regulation of reelin mRNA synthesis indicating a sexual dimorphism in the regulation of reelin gene expression and implying that male mice are more responsive to environmental stimulation than female mice. Although nonhandled male and female mice show the same levels of reelin mRNA and the same density of Reelin-expressing neurons, they up-regulate and maintain reelin mRNA synthesis with different intensity. In line with our results are recent studies showing that postnatal separation increased anxiety-like behavior and stress-reactivity in adulthood in male C57Bl/6 mice, but not in female mice (Romeo et al., 2003; Venerosi et al., 2003; Macri and Laviola, 2004; Parfitt et al., 2004). Moreover, sex differences with respect to stimulation of Reelin synthesis have been found in heterozygous male, but not female, *reeler* mice, which responded to 17 β -estradiol with increased Reelin expression in the cerebellum (Biamonte et al., 2009). In addition, Ognibene et al. (2007) noticed sex differences in emotional and communicative behavior of male and female infant *reeler* mice after MS.

We did not find sex differences on the cellular level, since average densities of Reelin-immunopositive neurons were similar in all experimental groups and in both sexes. As we did not determine total cell numbers of Reelin-expressing neurons in the entire hippocampus, but instead counted cell densities in representative sections at all levels of the longitudinal axis, we have to interpret our findings with care. Nevertheless, the unchanged cell densities indicate that the increased reelin mRNA expression in males was likely due to stimulated expression in individual hippocampal neurons and that the applied separation paradigms did not cause cell death of Reelin-synthesizing neurons in the P15 hippocampus. In the adult stage, however, the density of Reelin-expressing cells was strongly reduced in all experimental groups and in both sexes showing that the maturation-dependent decline of hippocampal reelin expression (Alcantara et al., 1998; Drakew et al., 1998; Haas et al., 2000) is independent of sex and treatment.

A recent study showed a transient increase of Reelin protein levels and of Reelin-synthesizing neurons in layer 1 of the medial prefrontal cortex of juvenile rats reared in social isolation for 35 days after weaning at P25 (Cassidy et al., 2010). In contrast to our study, Cassidy et al. (2010) did not clarify whether the observed changes in Reelin-positive cell numbers were due to changes in expression rate or to cell death of Reelin-synthesizing neurons, and these authors did not differentiate between sexes.

Glucocorticoids as Potential Mediators of Reelin mRNA Up-Regulation in Males

Separation in the early postnatal period has been shown to increase corticosterone levels in mice pups (Schmidt et al., 2002), indicating that glucocorticoids and the glucocorticoid receptor (GR) are mediating the stress response in young mice (de Kloet et al., 2005; Weaver et al., 2006; Oitzl et al., 2010). Upon stressful events the hypothalamic-pituitary-adrenal axis (hypothalamo-pituitary-adrenal, HPA) is activated, adrenal glucocorticoids are released and target limbic brain areas, in particular the hippocampus, where glucocorticoid receptors are abundantly expressed (for review see Brunson et al., 2003; see also Allen Brain Atlas [Allen Brain Atlas Resources [Internet]. Seattle (WA): Allen Institute for Brain Science. ©2009. Available from: <http://www.brain-map.org/>]). Here we show by quantitative PCR analysis that at P15 GR mRNA is expressed with similar abundance in hippocampi of all experimental groups and in both sexes. Only male, handled mice exhibited a significant increase in GR mRNA levels.

In addition, we show by double immunolabeling that GR colocalizes with Reelin in virtually all Reelin-synthesizing neurons in mouse P15 hippocampus indicating that Reelin-expressing neurons are potentially responsive to glucocorticoids. Considering the differential responsiveness of female and male mice to the applied separation procedures we directly tested the impact of the stress hormone corticosterone (CS) on reelin gene expression in hippocampal slice cultures prepared from male and female mouse pups. This *in vitro* approach excluded endogenous hormonal influence via the circulation. We found that corticosterone significantly increased reelin mRNA levels within hours in hippocampi from male, but not female mice. The applied CS treatment regime has been previously established by Morsink et al. (2006) to analyze gene expression patterns regulated by acute activation of hippocampal GR. In that study three waves of gene expression were observed in a time frame of 1 to 5 h, which is exactly the time range in which we found up-regulation of reelin mRNA expression in response to CS treatment. Interestingly Lussier et al. (2009) reported recently that systemic and repeated application of CS for 21 days in adult rats caused a reduction of Reelin-immunolabeled neurons in the subgranular zone of the dentate gyrus and in CA1 stratum lacunosum indicating selective responsiveness and vulnerability of these neurons to stress hormones. Physical restraint stress or handling applied for three weeks, however, did not affect cell numbers. These results confirm our observation that Reelin-synthesizing cells are responsive to CS and that environmental stress does not affect cell numbers of Reelin-synthesizing cells in the hippocampus.

Corticosteroids modulate gene transcription either via binding of GR homodimers to a hormone response element in the DNA or via protein-protein interactions of GR monomers with transcription factors such as NF- κ B (Karst et al., 2000). The mouse *reelin* promoter has been partially characterized and has been shown to contain consensus sites for the binding of transcription factors such as SP1 and AP2 (Royaux et al.,

1997). Whether the *reelin* promoter contains a GR-responsive element is presently unknown. Therefore the exact mechanism leading to corticosterone-mediated induction of the *reelin* gene remains open.

What is the Functional Significance of Reelin Gene Induction by Environmental Experience?

The extracellular matrix protein Reelin is very important for proper hippocampal development: Reelin regulates positioning, dendritic maturation and synaptogenesis of hippocampal neurons (Del Rio et al., 1997; Tissir and Goffinet, 2003; Zhao et al., 2004; Niu et al., 2004, 2008). In turn, stressful events during early postnatal life have been shown to severely affect dendritic differentiation, spine formation, and hippocampal function (Magarinos et al., 1996; Vyas et al., 2002; Brunson et al., 2003; Buwalda et al., 2005). Although we did not explicitly analyze dendritic morphology or spine density in our four experimental groups, it is very likely that they are affected by the applied separation paradigms. Hence it is tempting to speculate that the stress-induced up-regulation of the reelin mRNA expression in male mice represents a compensatory reaction to protect the hippocampus against stress-induced dendritic damage.

One has to be cautious with interpretation of results obtained in preclinical animal models with respect to their relevance for the understanding of psychiatric diseases. Yet, it is well documented that women show a higher incidence of stress-related psychiatric disorders and differences in stress reactivity have been implicated in this disparity (Marcus et al., 2005; Ter Horst et al., 2009). Hence it is well conceivable that males have developed adaptive mechanisms to protect themselves against stress-induced cellular damage. The up-regulation of reelin expression mediated by stress hormones described in our mouse study may also hold true in humans and represent a protective mechanism exclusively found in men.

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