Antidepressant Use during Pregnancy and Serotonin Transporter Genotype (SLC6A4) Affect Newborn Serum Reelin Levels

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Short title

Reelin changes after antenatal antidepressants

This study was undertaken to determine whether altered early serotonin signaling either via gestational serotonin reuptake inhibitor (SRI) exposure or genetic variations in the serotonin transporter promoter region (SLC6A4) alters levels of reelin, an important glycoprotein in neurodevelopment, in mothers and their neonates. Serum reelin protein expression was quantified by immunoblot from maternal and neonatal blood collected at delivery from women taking either an SRI during gestation or controls. SRI-exposed mothers had higher levels of one reelin fragment, while SRI-exposed neonates had lower total reelin levels, particularly in females and reelin levels differed with SLC6A4 genotype. Lower neonatal reelin levels predicted less time spent sleeping and more irritability during neonatal behavioral assessment on day 6 of life. Our results suggest that prenatal SRI exposure and the SLC6A4 genotype influences reelin protein expression in both the mother and newborn and that this may be reflected in neonatal behavior.

Keywords 5-12

Antenatal depression; selective serotonin reuptake inhibitors (SSRI); serotonin norepinephrine reuptake inhibitor (SNRI); development; gestation; infant; mood, Neurobehavioral Assessment of the Preterm Infant (NAPI).

Antenatal maternal treatment with serotonin reuptake inhibitors (SRI) antidepressants is increasingly used to manage mood disorders during pregnancy and this can affect the serotonin (5-HT) system (e.g. Field, 2010). SRIs readily cross the placenta (Kim, Riggs, Misri, Kent, Oberlander, Grunau, Fitzgerald & Rurak, 2006) and increase central serotonergic tone in the fetus and may influence physiological and behavioral outcome of the infant (Oberlander, Bonaguro, Misri, Papsdorf, Ross & Simpson, 2008; Oberlander, Gingrich & Ansorge, 2009; Oberlander, Papsdorf, Brain, Misri, Ross & Grunau, 2010) which is reflected in poor neonatal adaptation in the first days of life (Koren & Boucher, 2009) and in an increased risk for internalizing behaviors later in childhood (Misri, Reebye, Kendrick, Carter, Ryan, Grunau & Oberlander, 2006). However, there is a dearth of knowledge about the mechanisms underlying these effects. Animal studies show that early neonatal exposure to SRIs can disrupt the maturation and alter the composition of the 5-HT system (Maciag, Simpson, Coppinger, Lu, Wang, Lin & Paul, 2006), and a recent study in adult mice demonstrated that fluoxetine treatment can reverse the established state of neuronal maturation in hippocampal granule cells (Kobayashi, Ikeda, Sakai, Yamasaki, Haneda, Miyakawa, & Suzuki, 2010). Importantly, 5-HT acts as a neurotrophic factor during development (Homberg, Schubert & Gaspar, 2010) and altered early 5-HT signaling, secondary to early SRI exposure, may have multiple upstream and downstream developmental effects via multiple molecular factors (Capello, Bourke, Ritchie, Stowe, Newport, Nemeroff & Owens, 2011; Maciag et al., 2006; Weaver, Paul, Lin & Simpson, 2010).

One of the early targets of the in-growing 5-HT afferents during brain development is reelin-expressing Cajal-Retzius Cells, which are detectable in the human cortical marginal zone as early as gestational week five (Meyer, Perez-Garcia, Abraham & Caput, 2002) and express 5-

HT_3 receptors (Chameau, Inta, Vitalis, Monyer, Wadman & van Hooft, 2009). Reelin is an extracellular glycoprotein which plays an important role in the early growth and development of the mammalian cortex by triggering a signaling cascade which contributes to proper neuronal migration and positioning during cortical development (Tissir & Goffinet, 2003). A recent study by Chameau et al. (2009) has shown that 5-HT_3 receptors may directly or indirectly control the amount of reelin release from Cajal-Retzius cells and therewith mediate pyramidal neuron dendritic morphology. Further, injecting pregnant mice with a serotonergic agonist led to lower cortical reelin levels and altered cortical arrangements in neonatal pups (Janusonis, Gluncic & Rakic, 2004). Given these links between 5-HT system and reelin-releasing Cajal-Retzius cells, it is conceivable that disturbances in early serotonergic signaling secondary to prenatal SRI exposure will affect prenatal reelin levels (for review see: Tissir & Goffinet, 2003). Homozygous *Reeler* mice, a spontaneous mouse model deficient in reelin (encoded by *Reln*) (Falconer, 1951), present with inverse neocortical neuron arrangements and abnormal neuron migration and ataxic gait (Curran & D'Arcangelo, 1998), while heterozygous *Reeler* mice, which have 50% of normal levels of reelin (Smalheiser, Costa, Guidotti, Impagnatiello, Auta, Lacor, Kriho & Pappas, 2000), show learning impairments (Ammassari-Teule, Sgobio, Biamonte, Marrone, Mercuri & Keller, 2009; Brigman, Padukiewicz, Sutherland & Rothblat, 2006; Krueger, Howell, Hebert, Olausson, Taylor & Nairn, 2006), increased glucocorticoid-sensitivity (Lussier, Romay-Tallon, Kalynchuk & Caruncho, 2010), and sensorimotor gating deficits (Barr, Fish, Markou & Honer, 2008; Costa, Davis, Pesold, Tueting & Guidotti, 2002). Further altered reelin levels have been associated with multiple neuropsychiatric disorders over the last decade and may be a biomarker of certain diseases such as schizophrenia and autism (Fatemi, Earle & McMenomy, 2000; Fatemi, Kroll & Stary, 2001; Fatemi, Stary & Egan, 2002; Guidotti, Auta,

Davis, Di-Giorgi-Gerevini, Dwivedi, Grayson, Impagnatiello, Pandey, Pesold, Sharma, Uzunov & Costa, 2000). Together these studies suggest that reelin levels during development are sensitive to serotonergic or environmental influences reflected in brain maturation and neuronal structure (Janusonis et al., 2004). Disruptions in reelin levels during development may result in impaired cognitive or motor function and may increase the vulnerability for later neuropsychiatric disorders.

Critical to regulation of serotonin is the presynaptic membrane-bound serotonin transporter protein (5-HTT), the very target for serotonin reuptake inhibitor antidepressants (SRIs). The gene that codes for serotonin transporter on human chromosome 17q11 (SLC6A4) has been widely studied in relation to behavioral and neuropsychiatric disorders (Caspi, Hariri, Holmes, Uher & Moffitt, 2010; Homberg & Lesch, 2011). Variations in the gene encoding the serotonin transporter (5-HTTLPR, SLC6A4) consist of two alleles, a long (l) (16 repeats of 20-23 base pair sequence) and a short (s) (14 repeats) that affect gene expression (Heils, Teufel, Petri, Stober, Riederer, Bengel & Lesch, 1996; Lesch, Wolozin, Estler, Murphy & Riederer, 1993; Ramamoorthy, Bauman, Moore, Han, Yang-Feng, Chang, Ganapathy & Blakely, 1993). Variants in SLC6A4 have a substantial effect on the amount of serotonin available at the postsynaptic site, thereby influencing risk for cognitive and emotional disturbances (Canli & Lesch, 2007) and, not unexpectedly, the efficacy of SRIs (Gressier, Bouaziz, Verstuyft, Hardy, Becquemont & Corruble, 2009; Kim, Lim, Lee, Sohn, Kim, Hahn & Carroll, 2000). The short allele is associated with less transcription of SLC6A4 and approximately 50% reduction in serotonin reuptake compared with the long variant (Heils et al., 1996). The short allele has further been associated with reduced grey matter volume in limbic brain regions (Pezawas, Meyer-Lindenberg, Drabant, Verchinski, Munoz, Kolachana, Egan, Mattay, Hariri &

Weinberger, 2005), highlighting the importance of 5-HT and 5-HTT on neurodevelopment. Thus SRI-exposure *in utero* may have a differential impact on brain development of fetuses with the short vs. the long allele, possibly mediated through the impact of 5-HT on reelin.

The following study was undertaken to determine whether altered early serotonin signaling either via gestational SRI exposure or genetic variations in SLC6A4 alters reelin levels in serum from parturient women and their neonates, and whether newborn serum reelin levels can predict neonatal behavior. We hypothesize that maternal and neonatal reelin levels would be affected by gestational SRI-exposure and neonatal SLC6A4 genotype independent of maternal mood and that reduced reelin levels in neonates would be associated with altered neurobehavioral outcome.

METHODS

Subjects

With University of British Columbia Clinical Research Ethics Board and the Children's and Women's Health Centre of British Columbia Research Review Committee approval informed consent was obtained from the mother.

Pregnant women were recruited between October 2006 and October 2009 during their early second trimester from a Reproductive Mental Health Clinic, community midwife and family physician clinics in metropolitan Vancouver to participate in a larger prospective cohort study of the effects of prenatal psychotropic medication exposure on neonatal health. Mothers (n = 86) were included if they were either healthy (took no psychiatric medications (non-exposed)) or if they took one SRI antidepressant (paroxetine, fluoxetine, sertraline, venlafaxine, citalopram,

or escitalopram (exposed)). Three exposed mothers were also taking an anti-psychotic (quetiapine) during pregnancy. From this cohort, 19 maternal and newborn samples were not available, for technical reasons (inadequate serum sample or maternal and cord blood sample not obtained at delivery; SRI-exposed, n = 5; non-exposed, n = 14), which left 67 maternal samples at delivery (SRI-exposed, n= 25; non-exposed, n = 42). The mean prenatal SRI exposure was 255 days (see Table 1), with most mothers (23/25) taking the medication throughout their pregnancies. Maternal and neonatal demographic differences between those for whom we did have samples and those for whom we did not have samples were not significant. Further, demographic data and reelin levels did not differ between mothers on quetiapine or only SRIs. All SRI-treated mothers were already on antidepressant medications at the time of conception and had started the medication based on clinical need after a diagnosis of a mood disorder prior to the pregnancy.

Maternal Mood

Maternal mood was assessed at study enrolment (26-28 weeks) and at 36 weeks gestation (range 33-36 weeks). A clinician administered the Hamilton Rating Scale for Depression (HAM-D) to assess mood and measure the severity of depression (range 0-63). Higher scores are associated with higher levels of depression and we defined scores of ≥14 as moderate depression (Hamilton, 1960).

Blood Collection

At delivery blood samples were collected from the mothers and from the umbilical cord in Vacutainer tubes (Becton Dickinson and Company, Franklin Lakes, NJ) without additives, and

allowed to clot for 30 minutes. Samples were then centrifuged for 10 min at 3000g at 4°C and then the serum was separated and stored at - 80°C until analysis.

Western Blot Analysis

Western blot analysis was modified from Fatemi et al., Lugli et al. and Botella-Lopez et al. (Botella-Lopez, Burgaya, Gavin, Garcia-Ayllon, Gomez-Tortosa, Pena-Casanova, Urena, Del Rio, Blesa, Soriano & Saez-Valero, 2006; Fatemi et al., 2001; Lugli, Krueger, Davis, Persico, Keller & Smalheiser, 2003). Total protein levels in maternal and cord serum were determined via Bradford assay (Bio-Rad, Mississauga, OT, CAN) using bovine serum albumin as a standard. Each serum sample was adjusted for total protein content based on the Bradford assay before diluting it in dH_2O and SDS-Page sample buffer containing 10% β-ME and boiled for 3 min. Samples (60µg/lane) were then loaded in duplicates on to a 6% resolving gel with a 4% stocking gel and run with a control sample and prestained standards (Kaleidoscope ladder, Bio-Rad, Mississauga, OT, CAN) at 110V for 90 min at room temperature. Proteins were transferred at 40V at 4ºC for 18 hours to nitrocellulose membranes (Bio-Rad, Mississauga, OT, CAN). Membranes were transiently stained with Ponceau S (Sigma-Aldrich), and then blocked in 5% non fat dry milk for 90 min. Membranes were washed 3x for 10 min in 1X Tris-buffered saline + 0.05% Tween 20 between each step. Blots were incubated in 1:1000 Mouse-Anti-Reelin antibody 142 (Millipore, Temecula, CA, USA) in 5% bovine serum albumin for 75min, followed by incubation in the secondary antibody in 5% milk for 90 min (1:400, donkey-anti-mouse IgG, AP-conjugated, Millipore, Temecula, CA, USA). Lumi-Phos WB Chemiluminescent substrate (Thermo Scientific, Rockford, IL, USA) was applied and then the membranes were exposed and densities were analyzed using GeneSnap and GeneTools software (Syngene, Frederick, MD,

USA). *In vivo*, reelin has two cleavage sites, resulting in several fragments. The antibody used in this study (clone 142) binds to the N-terminal of the protein, thus staining two of these fragments and the full length protein, resulting in up to three visible bands after western blotting. The 420kDa band reflects the unprocessed full length reelin protein, the other two (310 and 180kDa) represent the proteolytic fragments of reelin (Fatemi et al., 2001). Densities were corrected for loading volume using Ponceau staining as described before (Romero-Calvo, Ocon, Martinez-Moya, Suarez, Zarzuelo, Martinez-Augustin & de Medina, 2010) and differences between membrane backgrounds were corrected using the control sample. A random sample was loaded at different concentrations (60, 120 and 180µg/lane) to ascertain that volume differences could be detected with densitometry (Supplemental Figure S1).

[Figure 1 here]

SLC6A4 Genotyping

Genomic DNA was extracted from maternal blood at delivery and newborn cord venous blood using the Flexigene DNA Blood Kit (Qiagen, Valencia, California). Polymerase chain reaction (PCR) was performed as previously described (Oberlander et al., 2010). Briefly, PCR was carried out with oligonucleotide primers flanking the polymorphism (corresponding to the nucleotide positions -1416 to -1397 (stpr5, 5′-GGCGTTGCCGCTCTGAATGC) and -910 to -888 (stpr3, 5′-GAGGGACTGAGCTGGACAACCAC) of the 5′-flanking regulatory region of *SLC6A4* to generate a 484- (*s* short allele) or 528-bp (*l* long allele) PCR product. PCR amplification was carried out in a final volume of 30 µl with 50 ng of genomic DNA, 2.5 mM deoxyribonucleotides (dGTP/7-deaza-2′-dGTP = l/l), 0.1 µg of sense and antisense primers, 10 mM tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 1 U of Taq DNA polymerase.

Annealing was carried out at 61°C for 30 s, extension at 72°C for 1 min, and denaturation at 95°C for 30 s for 35 cycles. As a quality control, 5% of samples were randomly chosen and re-tested; their genotypes were consistent with previous results.

Neonatal Neurobehavioral Assessments

The Neurobehavioral Assessment of the Preterm Infant (NAPI) was used as a standardized, non-stressful neurobehavioral assessment to sample neonatal behavioral state regulation (Korner, Brown, Thom & Constantinou, 2000; Korner, Constantinou, Dimiceli, Brown, Jr. & Thom, 1991). The NAPI was developed for preterm infants, but has been validated for the use with full-terms (Hyman, Snider, Majnemer & Mazer, 2005). During a home visit on Day 6 of life, a qualified physiotherapist performed this 30 minutes assessment during a morning session approximately 1.5 hours between feedings. A total of 71 items are scored; 22 of these are used to derive 7 a priori cluster scores, converted to standardized scores (see Table 2). Higher scores (except for 'Percent asleep') indicate better neonatal maturity and neurobehavioral control.

Statistical Analyses

Maternal and newborn characteristics were analyzed for group differences using one-way analysis of variance (ANOVA). One way ANOVAs were also performed to examine if delivery type (Caesarean section, vaginal) influenced reelin levels in mother or cord serum. Given the range of maternal mood scores in both groups and previous literature showing an impact of maternal mood on infant outcome (Oberlander et al., 2010; Oberlander, Weinberg, Papsdorf, Grunau, Misri & Devlin, 2008) and an association of depression and reelin levels, (Fatemi et al.,

2001; Lussier et al., 2010) we used maternal mood as a continuous covariate in all following analyses. We analyzed maternal reelin levels using a repeated measures analysis of covariance (ANCOVA) with band (180, 310, 420kDa) as the within-subject factor and group (non-exposed, exposed) as the between-subject factor, controlling for prenatal maternal mood (HAM-D score at 27 and 36 weeks).

Newborn reelin levels were analyzed using a repeated measures ANCOVA with bands (180, 310, 420kDa) as the within-subject factor and group (non-exposed, exposed) and sex (male, female) as the between-subject factors correcting for maternal prenatal mood (HAM-D scores at 36 weeks), and gestational age at birth, as reelin levels change during development (Meyer et al., 2002). All post-hoc comparisons utilized Newman-Keuls. Using separate models, maternal and neonatal reelin levels were analyzed with the maternal or neonatal genotype (long/long vs. > 1 short allele) and group as the between-subject factors; including the co-factors (i.e. HAM-D scores and/or gestational age). Similarly, we used multivariate models of covariance to detect differences in neurobehavioral outcomes (NAPI cluster scores) associated with prenatal SRI exposure or genotype adjusting for total reelin levels, maternal mood (HAMD 36 weeks), time of assessment and gestational age.

RESULTS

Maternal and Newborn Characteristics

Maternal and neonatal demographic characteristics are presented in Table 1 and 2. SRI-exposed mothers had significantly higher mood scores on the HAM-D scale during the third ($p=0.04$), but not second trimester ($p=0.09$). Seven exposed mothers displayed mood scores

indicative of moderate depression (i.e. HAM_D score ≥14), despite the SRI medication. In addition, it is important to note, that some mothers in the non-exposed group also scored high on the depression scale (i.e. HAM-D score ≥14, n=6). Therefore maternal reelin levels were also analyzed based on the presence of depression (i.e. grouped into: depressed, non-depressed). Neonates from SRI-exposed mothers were born at a lower gestational age ($p=0.005$) and had lower Apgar scores at 1 min ($p<0.001$) but not at 5min ($p=0.20$) after delivery.

Reelin Protein Level: Qualitative Outcomes

Reelin is expressed in 3 bands at 420, 310 and 180kDa in maternal serum from delivery, which is in line with previous reports in adult men and women (Botella-Lopez et al., 2006; Fatemi et al., 2001), however, in half the cases (59%) the 420kDa band (reflecting the unprocessed full length reelin protein) was not detectable. There were no significant differences between unexposed or exposed mothers ($p=0.11$) or between the long/long or >1short allele genotype ($p=0.75$) mothers with regards to who expressed the band and who did not. For umbilical cord serum, only a few cases (16%) displayed all three bands while most had only the 310kDa and 180kDa band. Again, there was no significant difference based on exposure ($p=0.16$), infant genotype ($p=0.09$) or sex ($p=0.10$). The 310kDa band was the most prevalent in both maternal and umbilical cord serum (Figure 1). As expected, reelin expression increased with increasing sample volume (Figure S1), but higher volumes did not change the appearance or distribution of the individual bands (i.e. the 420kDa was still not detectable even with higher sample volumes).

Maternal Reelin Levels

There was a significant group by band interaction effect ($F(2, 118) = 3.68$, $p = 0.03$), controlling for maternal mood. SRI-treated mothers had significantly higher expression levels of the 310kDa reelin band ($p = 0.047$, partial $\eta^2 = 0.065$), but not the 420kDa ($p = 0.29$) or 180kDa band ($p = 0.64$) compared to non-exposed mothers (Figure 2). There was no overall group effect ($p = 0.21$) or significant effect of the maternal mood scores as covariates (all p's > 0.05). However, to confirm that the observed effect was not due to the depression in some non-exposed women, we also analyzed the data using a repeated-measures ANOVA with group (non-exposed, exposed) and depression (absent or present (HAM ≥ 14)) as between-subject factors. As we observed earlier, there was a group by band interaction effect ($F(2, 118) = 4.46$, $p = 0.016$) but no significant main or interaction effects of depression (all p's > 0.05), further indicating that reelin level changes were due to SRI exposure and not mood. Delivery method (vaginal or caesarean) had no significant effect on total maternal serum reelin levels ($p = 0.99$).

Analyzing the maternal SLC6A4 genotype revealed a significant interaction effect of reelin bands, SRI exposure and genotype ($F(2,112) = 3.2$, $p = 0.05$). Further analysis indicated that only the 310kDa band showed a significant group effect as before with SRI-exposed mothers having greater reelin expression ($p = 0.007$), and a trend for an interaction effect of group and genotype ($p = 0.067$, partial $\eta^2 = 0.059$), with exposed mothers carrying the l/l allele having higher expression levels of the 310kDa band than exposed mothers carrying >1 short allele (Table 3).

[Figure 2 here]

Neonatal Reelin Levels

SRI exposure ($F(1, 57) = 4.22$, $p = 0.044$) and sex ($F(1, 57) = 4.62$, $p = 0.036$) influenced reelin levels in umbilical cord serum, controlling for maternal prenatal mood and

gestational age, with a trend for an interaction between exposure and sex ($F(1, 57) = 3.77$, $p = 0.057$). Because the 420kDa band was only expressed by 16% of the infants, we repeated the analysis using only the 310kDa and 180kDa bands and observed a significant interaction between exposure and sex ($F(1,57) = 4.3$, $p = 0.043$), a main effect of sex ($F(1,57) = 4.12$; $p = 0.046$) and a trend for exposure ($F(1,57) = 3.68$; $p = 0.06$). SRI-exposed female neonates had significantly lower reelin levels (310 + 180kDa) than non-exposed female neonates ($p = 0.002$; partial $\eta^2 = 0.254$, Figure 3). SRI-exposure accounted for ~25% of the overall variance in reelin expression in female neonates. There were no significant differences between exposed and non-exposed male neonates ($p = 0.66$). We also found a significant effect of gestational age ($F(1,57) = 5.10$, $p = 0.028$, $\eta^2 = 0.082$), with increasing gestational age at birth being associated with lower total reelin levels (Figure 4), but no significant effect of prenatal maternal mood and no significant main or interaction effect for the different bands (all p's > 0.05). Newborn reelin levels were not associated with maternal reelin levels, maternal alcohol use, maternal age, mode of delivery or Apgar scores (1min and 5 min) (all p's > 0.05).

Neonatal reelin levels varied with infant SLC6A4 genotype ($F(1, 55) = 4.39$, $p = 0.041$, partial $\eta^2 = 0.074$) and there was a main effect for SRI exposure ($F(1, 55) = 0.24$, $p = 0.015$), but no significant interaction of exposure and genotype ($p = 0.63$) or with individual bands (p's > 0.45) was observed (Figure 5). Infants with > 1 short allele were observed to have higher reelin levels than infants with both long alleles and SRI exposed infants had lower reelin levels compared to non-exposed infants as reported above.

[Figure 3-5 here]

Neonatal Behavioral Outcome

Higher neonatal reelin in umbilical cord serum was associated with increased percentage of sleep during the standardized neurobehavioral assessment (NAPI) on day 6 of life ($F(1, 46) = 5.61$; $p = 0.023$, partial $\eta^2 = 0.13$) and lower irritability scores ($F(1, 46) = 4.61$, $p = 0.038$, partial $\eta^2 = 0.11$; Figure 6). Further, the infant's genotype was significantly associated with motor development/ vigor ($F(1, 46) = 10.83$, $p = 0.002$, partial $\eta^2 = 0.22$), with alertness and orientation ($F(1,46) = 5.11$, $p = 0.03$, partial $\eta^2 = 0.12$), and with irritability ($F(1, 46) = 7.91$, $p = 0.008$, partial $\eta^2 = 0.17$), with carriers of > 1 short allele having higher motor development/vigor and irritability scores, but lower alertness and orientation scores (Table 4). SRI exposure was not a significant predictor of any of the neonatal behaviors (cf. Table 2) and there were no significant associations with neonatal behavior for any of the other co-factors.

[Figure 6 here]

DISCUSSION

In this study we report the impact of altered serotonin signaling on maternal and neonatal peripheral reelin levels associated with gestational SRI antidepressant use and genetic variations for SLC6A4. We observed significantly lower reelin levels in umbilical cord serum in SRI-exposed newborns compared to non-exposed newborns, particularly in SRI-exposed females, even after controlling for maternal prenatal mood and gestational age at birth. Moreover, lower neonatal reelin levels were associated with reduced percentage of time spent sleeping and increased irritability during a neonatal behavioral assessment. Importantly, mothers treated with an SRI antidepressant during pregnancy showed a significant increase in the 310kDa reelin fragment and there was no association between maternal and umbilical cord reelin levels, thus

making it not likely that cord levels were a mere reflection of maternal levels. Furthermore we showed that infants with > 1 short allele of SLC6A4 had higher reelin levels, while in mothers a different pattern was seen with SRI-exposed mothers carrying the l/l allele having higher levels of the 310kDa reelin fragment. To our knowledge, this is the first demonstration that reelin can be measured in umbilical cord serum and that reelin levels are associated with *in utero* SRI exposure and the SLC6A4 genetic variation and that reelin levels may predict behavioral maturity.

In the current study, SRI-exposed neonates were born at a lower gestational age compared to non-exposed neonates, as has been previously reported (Oberlander, Warburton, Misri, Aghajanian & Hertzman, 2006; Wisner, Sit, Hanusa, Moses-Kolko, Bogen, Hunker, Perel, Jones-Ivy, Bodnar & Singer, 2009). Further, consistent with previous literature showing that reelin levels decrease after the fetal phase of brain development (Ikeda & Terashima, 1997; Meyer et al., 2002) gestational age at birth was negatively associated with umbilical cord reelin. Thus, the younger age in the SRI-exposed group should have suggested higher, rather than the observed lower, reelin levels for these newborns which strengthens our findings of a significant association of SRI exposure with reelin levels. Animal studies have shown an association between the serotonergic system and reelin levels (Chameau et al., 2009; Janusonis et al., 2004; Lakatosova, Celec, Schmidtova, Kubranska, Durdiakova & Ostatnikova, 2011) and one study found lower cortical and blood serum reelin levels in pups exposed to increased maternal serotonergic tone (Janusonis et al., 2004). Therefore, it is conceivable, that a SRI-induced increase in serotonergic tone in the fetus (Oberlander et al., 2009) may accelerate or shorten key neurodevelopmental processes, which in turn may be reflected in lower reelin levels at birth. Similarly, higher reelin levels may reflect less neurobehavioral maturity, as illustrated by our

observation that higher reelin levels were associated with lower levels of irritability and increased levels of sleep. Considering that the percentage of time spend sleeping decreases with gestational age, while higher irritability reflects more maturity (Korner & Constantinou, 2001), our findings suggest that more mature infants may have lower reelin levels. However, this needs further study as the infant's SLC6A4 genotype was also significantly associated with a number of neurobehavioral indices reflecting maturity, but in opposite directions. This underlines the importance of the role early serotonin signaling may play in neurodevelopment, but also suggests that there may be complex developmental patterns or interactions between genotype, SRI-exposure and the impact of serotonin.

Our results revealed that SRI-exposed girls, but not boys, had lower reelin levels compared to non-exposed controls. Some studies reported a female-specific association between the reelin gene and schizophrenia (Liu, Chen, McGrath, Wolyniec, Fallin, Nestadt, Liang, Pulver, Valle & Avramopoulos, 2010; Shifman, Johannesson, Bronstein, Chen, Collier, Craddock, Kendler, Li, O'Donovan, O'Neill, Owen, Walsh, Weinberger, Sun, Flint & Darvasi, 2008) or bipolar disorder (Goes, Willour, Zandi, Belmonte, MacKinnon, Mondimore, Schweizer, DePaulo, Jr., Gershon, McMahon & Potash, 2010), but findings to date are sparse. It is clear from these studies and our finding however that sex may be an important factor to consider when examining the association of reelin and risks for mental health disorders.

In addition, our results also revealed a significant effect of the infant SLC6A4 genotype on reelin expression with infants carrying either one or both short alleles having higher umbilical cord serum levels of reelin than those infants with the l/l allele. Interestingly, for mothers we saw that reelin levels were affected by maternal SLC6A4 genotype in an opposing pattern, with l/l allele carrying SRI-exposed mothers having higher serum levels of the 310 kDa fragment of

reelin. These results should however be interpreted with caution due to our relatively small sample size and the limited number of carriers of two long alleles in our sample, which also prevented us from further investigating the effect of gender in our analyses of the genotype. More studies are needed to confirm the link between the SLC6A4 polymorphisms, the associated altered expression levels of the 5 HT-transporter, and reelin levels and to explore potential mechanisms explaining this relationship.

We found for the first time that pregnant women taking SRIs had higher serum levels of the 310kDa reelin fragment than controls at the time of delivery. Our findings are partly consistent with results from Fatemi et al., (2001) who reported for a small sample of medicated male and female adults with depression that serum levels of the 310kDa reelin fragment were slightly increased and levels of the 180kDa fragment decreased. Both studies suggest that peripheral levels of reelin may be altered in patients with depression on mood-elevating medications, and further research needs to elucidate whether reelin is involved in the neuropathology of depression or if the observed alterations in reelin levels are associated with the antidepressant treatment. Interestingly, SRI exposure had opposite effects on reelin levels in mothers and newborns, which may be explained by the different function of reelin during adulthood and development. For adults, reelin has been associated with synaptic plasticity, neurogenesis and repair processes (Forster, Bock, Herz, Chai, Frotscher & Zhao, 2010; Kobold, Grundmann, Piscaglia, Eisenbach, Neubauer, Steffgen, Ramadori & Knittel, 2002; Rogers & Weeber, 2008), while during development it serves as a neurotrophic factor and thus its release or expression levels may be triggered and regulated by different mechanisms compared to adulthood. Further, in adults, reelin has been identified in liver, chromaffin cells, blood and the brain (Samama & Boehm, 2005; Smalheiser et al., 2000), while during development, more

organs seem to express reelin temporarily (D'Arcangelo, Miao, Chen, Soares, Morgan & Curran, 1995; Samama & Boehm, 2005; Smalheiser et al., 2000) and it remains to be studied what contributes to the pool of reelin in cord or neonatal blood (Botella-Lopez et al., 2006). In addition, further studies are needed to identify links between peripheral and central reelin levels as it is unclear currently in how far the peripheral levels are representative of cortical levels. It is clear however, that reelin levels measured in serum are associated with neuropsychiatric disorders such as schizophrenia, mood disorders or autism (Fatemi et al., 2001; Fatemi et al., 2002), and in the current study we have shown that peripheral reelin levels are reduced in SRI-exposed neonate girls.

To our knowledge, this is the first report of measuring reelin levels in serum from mothers at delivery and from umbilical cord. Fifty percent of the mothers and over 80% of the infants did not express the band resembling the full length reelin protein (420kDa). Others have reported only traces of the 180-kDa band in serum of adults (Smalheiser et al., 2000). One possible explanation for the differences in abundance of the three bands in different tissues and studies is that there may be individual or activity-dependent differences in the splitting of reelin at its two in vivo cleavage sites. For example, during pregnancy or delivery, reelin might be cleaved at higher rates than in normal adults due to altered enzymatic activities during these periods, which would result in lower expression of the uncleaved full length protein (i.e. the 420kDa band). This may also explain why we saw a significant effect for just one of the reelin fragments in the mothers (310kDa), while for the infants the significant effects were found independent of the individual fragments (i.e. total reelin levels). The physiological significance of the cleavage *in vivo* and the respective functions of the resulting individual fragments are not completely understood yet, but evidence is emerging that both the full length protein as well as

the processing of reelin into fragments are functionally important (Jossin, Gui & Goffinet, 2007; Jossin, Ignatova, Hiesberger, Herz, Lambert de & Goffinet, 2004; Kohno, Kohno, Nakano, Suzuki, Ishii, Tagami, Baba & Hattori, 2009). For instance, Jossin et al. (2004) reported that the central fragment of Reelin is critical to its function during cortical plate development, while others have shown that the N-terminal region of reelin regulates postnatal dendritic maturation of cortical pyramidal neurons (Chameau et al., 2009). In contrast, Kohno et al., (2009) demonstrated that cleavage at the N-terminal of Reelin practically abolishes its signaling activity *in vitro*.

CONCLUSION

Early brain maturation is a delicate process that is very sensitive to changes in neurochemistry. The current study represents the first step in determining whether differences in reelin levels during development in humans can be detected and future research will show whether reelin may be used as a biomarker for the prediction of altered brain development of at risk populations. A recent study suggests an increased risk for autism after in utero SRI exposure (Croen, Grether, Yoshida, Odouli & Hendrick, 2011) and autism in turn has been linked to reduced reelin levels and impairments in the reelin signaling system (Fatemi, 2002; Fatemi, Snow, Stary, Araghi-Niknam, Reutiman, Lee, Brooks & Pearce, 2005). Thus, more research is needed to determine how certain genetic phenotypes, or developmental influences such as SRI exposure, impact reelin and its receptors and if this will increase vulnerabilities for neuropsychiatric disorders such as schizophrenia or autism.

Notes

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Conflict of Interest

The authors have nothing to declare.

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Table 1. ***Maternal Characteristics***

	Non-exposed (n = 42)	**Exposed (n = 25)**	**P-value**
Maternal age at delivery, mean ± SD, years	34.4 (± 5.2)	33.6 (±4.8)	0.50
Maternal years of education, mean ± SD, years	18.3 (± 3.4)	17.8 (±3.9)	0.56
Maternal mood (HAM-D score, mean ± SD)[a]			
2nd trimester (26-28 weeks)	7.5 (±6.2)	10.3 (±7.0)	0.09
3rd trimester (33-36 weeks)	7.4 (±6.0)	10.5 (±4.8)	0.04*
Delivery type (vaginal/cesarean), n	32/10	19/6	0.98
Maternal SLC6A4 genotype, (l/l / > 1 s) n[b]	14/28	7/17	0.73
SRI dose (median (range)), mg			
Paroxetine (n=3)	n.a.	30 (10-40)	
Fluoxetine (n=3)	n.a.	60 (20-80)	
Sertraline (n=4)	n.a.	125 (38-200)	
Venlafaxine (n=9)	n.a.	150 (75-225)	
Citalopram (n=5)	n.a.	40 (40-50)	
Escitalopram (n=1)	n.a.	50	
Days of SRI exposure, mean ± SD	n.a.	255 (± 49)	

[a] Due to some missing data for the maternal questionnaires some of the presented means are based on lower n's (for 36 weeks mood scores: n = 40 for non-exposed and n = 23 for exposed group).

[b] DNA sample from one SRI-exposed mother was unavailable for genotyping

Table 2. ***Newborn Characteristics and Neurobehavioral Assessment***

	Non-exposed (n = 42)	**Exposed (n = 25)**	**P-value**
Neonatal characteristics			
Gestational age at birth, mean ± SD, weeks	39.8 (±1.5)	38.7 (±1.5)	0.005*
Birth weight, mean ± SD, g	3528.3 (±449)	3308.2 (±551)	0.08
Sex, % male	47.6	40	0.57
Apgar scores, mean ± SD			
1min	8.7 (±0.6)	7.1 (±1.9)	<0.001*
5min	9.0 (±0.3)	8.9 (±0.4)	0.20
Infant SLC6A4 genotype (l/l / >1 s), n[a]	15/26	7/17	0.54
Neurobehavioral Assessment of the Preterm Infants (NAPI), mean ± SD[b]			
Motor development/vigor	66.8 (±12.5)	71.9 (±12.1)	0.14
Scarf sign	62.9 (±19.4)	61.8 (±19.3)	0.84
Popliteal angle	44.8 (± 31.2)	50.7 (±24.3)	0.44
Alertness/Orientation	68.5 (±16.3)	61.1 (±24.6)	0.17
Irritability	68.5 (±18.7)	60.8 (±23.2)	0.16
Cry quality	68.6 (±32.3)	59.1 (±33.2)	0.29
Percent asleep	11.5 (±15.3)	10.2 (±12.1)	0.74

[a] DNA samples from one SRI-exposed and one non-exposed infant were unavailable for genotyping

[b] Due to some missing data for the neurobehavioral assessment the presented means for the NAPI are based on lower n: non-exposed (n = 35-36) exposed (n = 22-24).

Table 3. ***Maternal reelin levels by SRI exposure and serotonin transporter genotype***

SRI-exposure	**Non-exposed**		**Exposed**	
SLC6A4 Genotype	Long/long (n= 13)	>1 short (n=27)	Long/long (n=6)	>1 short (n=16)
420kDa fragment	0.62 (±0.77)	0.63 (±0.69)	0.50 (±0.84)	0.38 (±0.62)
310kDa fragment	5.12 (±1.08)	5.18 (±1.15)	6.73 (±2.30)	5.46 (±1.03)
180kDa fragment	4.38 (±0.52)	3.93(±0.75)	3.89 (±0.66)	3.85 (±0.61)

Table 3 displays the relative reelin expression of each band (fragment) ± standard deviation for the maternal reelin levels for carries of both long alleles (long/long) of the SLC6A4 variation or > 1short allele separately for either control (non-exposed) or SRI-exposed mothers. There was an interaction effect of bands, group and genotype ($F(2,112) = 3.2$, $p = 0.05$), which seems to be driven by higher levels of the 310kDa fragment in exposed mothers.

Table 4: ***NAPI behavior and infant SLC6A4 genotype***

	Long/long (n= 17)	>1 short (n=30)
Motor development/vigor	62.5 (± 12.8)	72.5 (± 11.5) *
Scarf sign	54.9 (± 20.2)	66.7 (±19.6)
Popliteal angle	43.1 (± 28.3)	48.9 (± 28.7)
Alertness/Orientation	75.8 (± 9.5)	64.2 (±19.2) *
Irritability	57.7 (±18.2)	70.6 (± 18.4) *
Cry quality	55.9 (± 34.8)	65.0 (± 32.6)
Percent asleep	10.5 (± 12.9)	10.2 (± 14.6)

* Indicates a significant association of infant genotype and NAPI behavior in a multivariate analysis controlling for maternal mood (HAM-D at 36 weeks), gestational age at birth, SRI-exposure and total reelin levels. Higher NAPI scores (except for 'Percent asleep') indicate better neonatal maturity and neurobehavioral control.

Figures Legends

Figure 1

Representative example of western blot staining for reelin in maternal serum (at delivery) and umbilical cord serum.

All samples were run in duplicates. The 420kDa band was not consistently expressed in umbilical cord serum. #: sample ID

Figure 2

Maternal reelin serum levels at delivery.

SRI-exposed mothers had higher levels of the 310kDa band compared to non-exposed mothers. * $p<0.05$

Figure 3

Reelin protein expression in umbilical cord serum of non-exposed or SRI-exposed newborns.

Total reelin was lower in SRI-exposed girls compared to non-exposed girls or boys. There was no significant difference between the different fragments (bands) of the reelin protein and as the 420kDa band was not consistently expressed only the cumulative 310kDa and 180kDa levels are presented here. *$p<0.05$

Figure 4

Reelin expression in relation to gestational age at birth for non-exposed and SRI-exposed newborns.

Figure 5

Reelin expression (adjusted for HAM-D and gestational age at birth ±S.E.M.) in infants carrying the long or >1 short allele of the SLC6A4 variation.

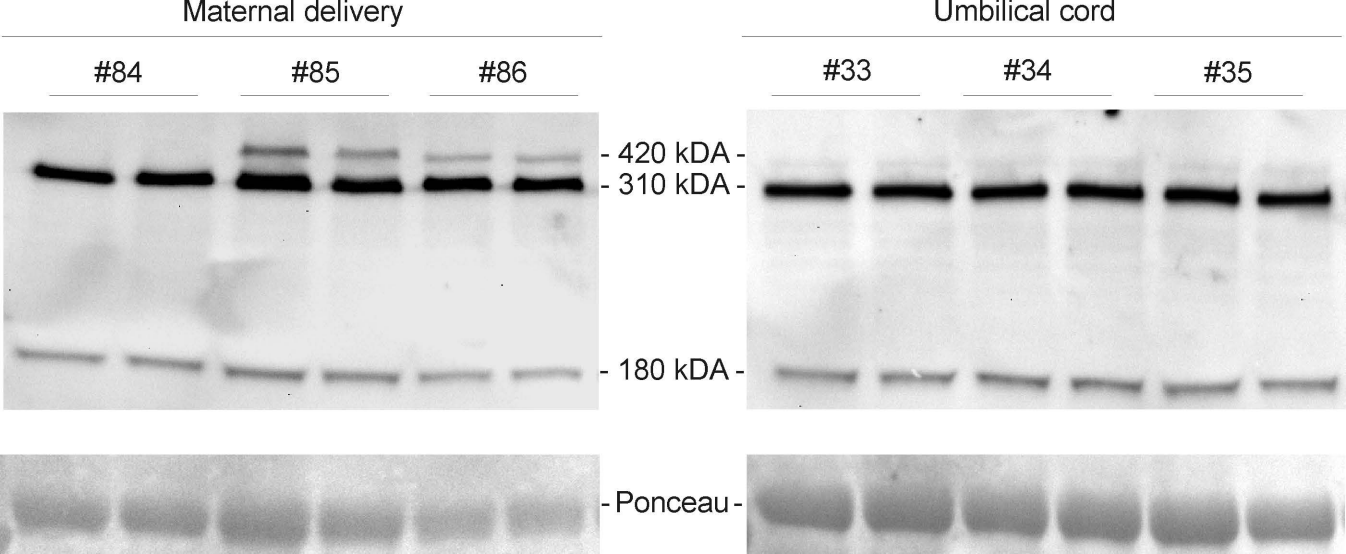
[#] Infants exposed to SRIs in utero had lower reelin levels compared to non-exposed and (*) infants with at least one short allele (>1 short) had higher reelin levels compared to carriers of both long alleles.

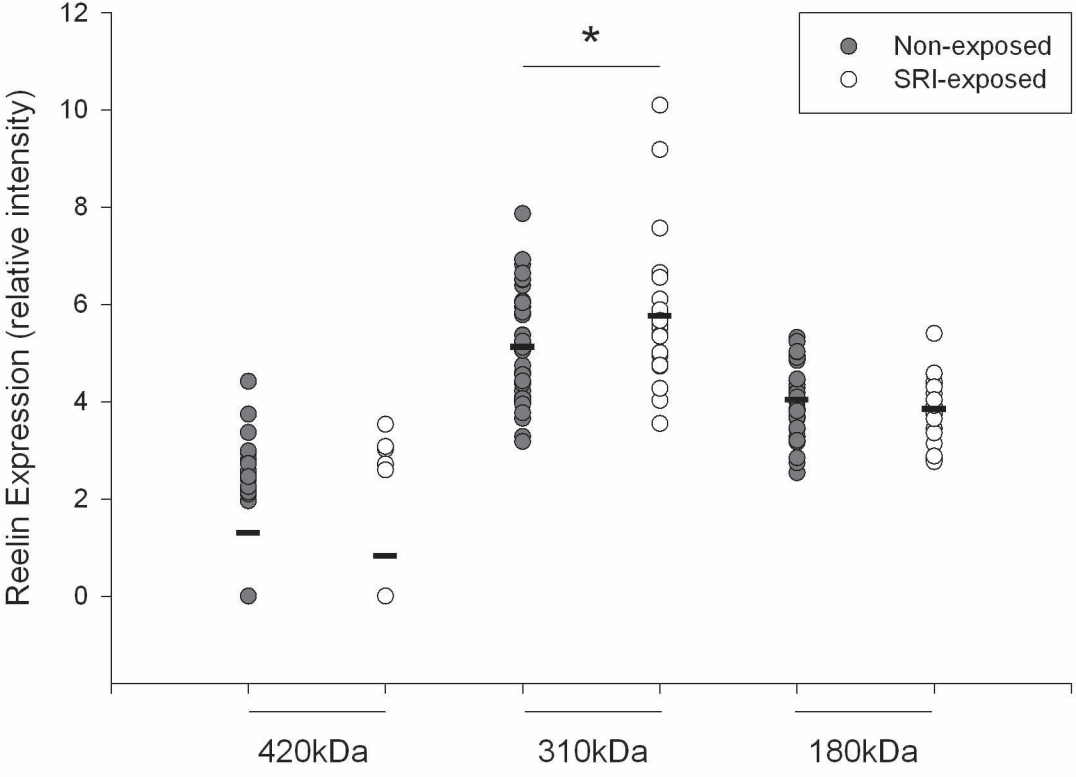
Figure 6

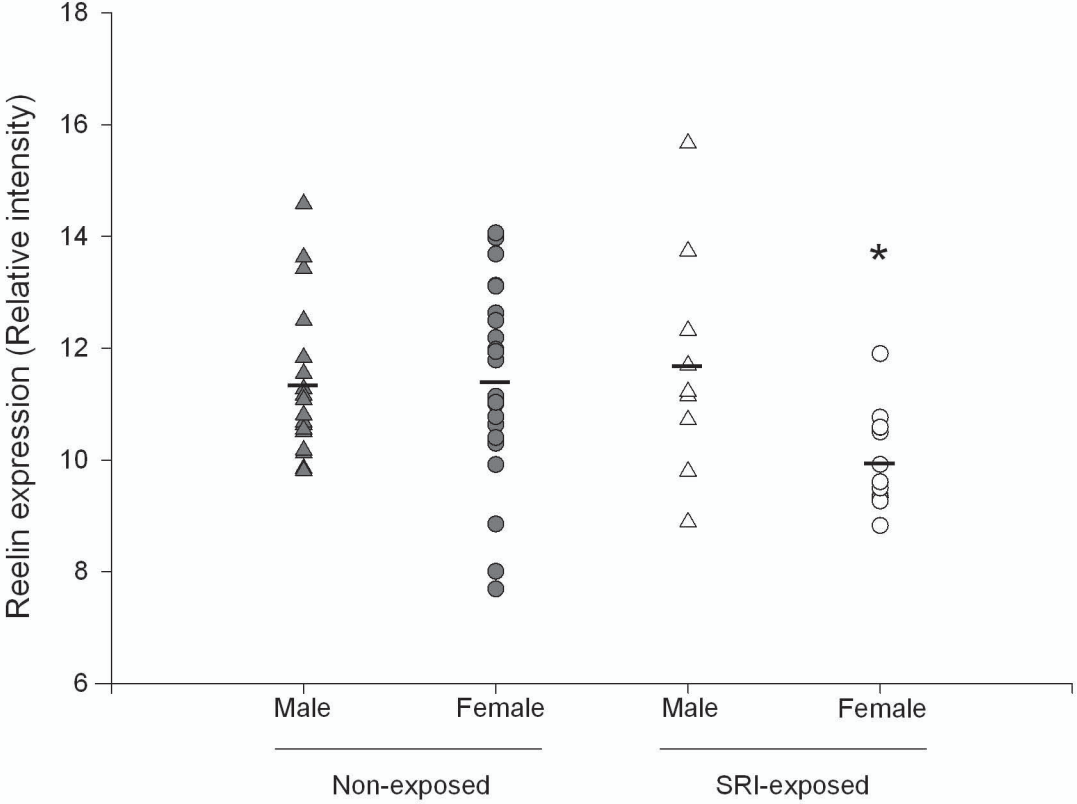
Reelin expression was significantly correlated with percentage of time spent sleeping (partial correlation coefficient r = 0.35) during the neonatal behavioral assessment (NAPI) on day 6 (A) and with NAPI irritability scores (partial correlation coefficient r = -0.37; B).

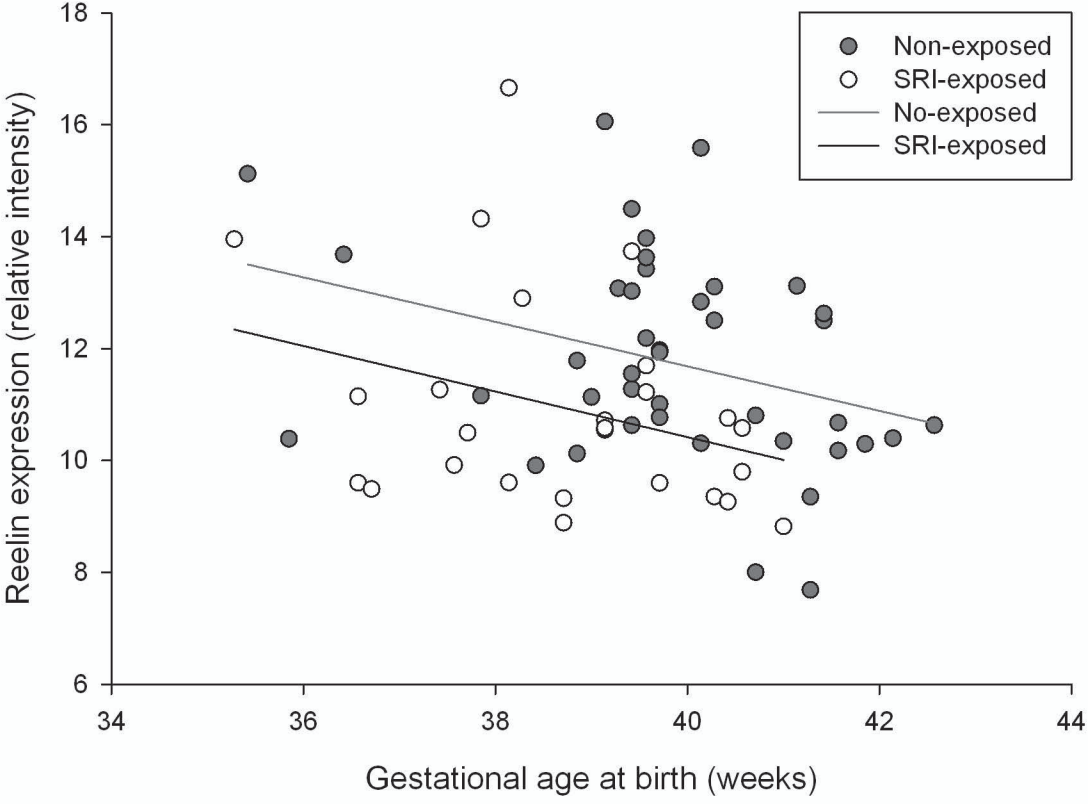
Supplemental Figure S1

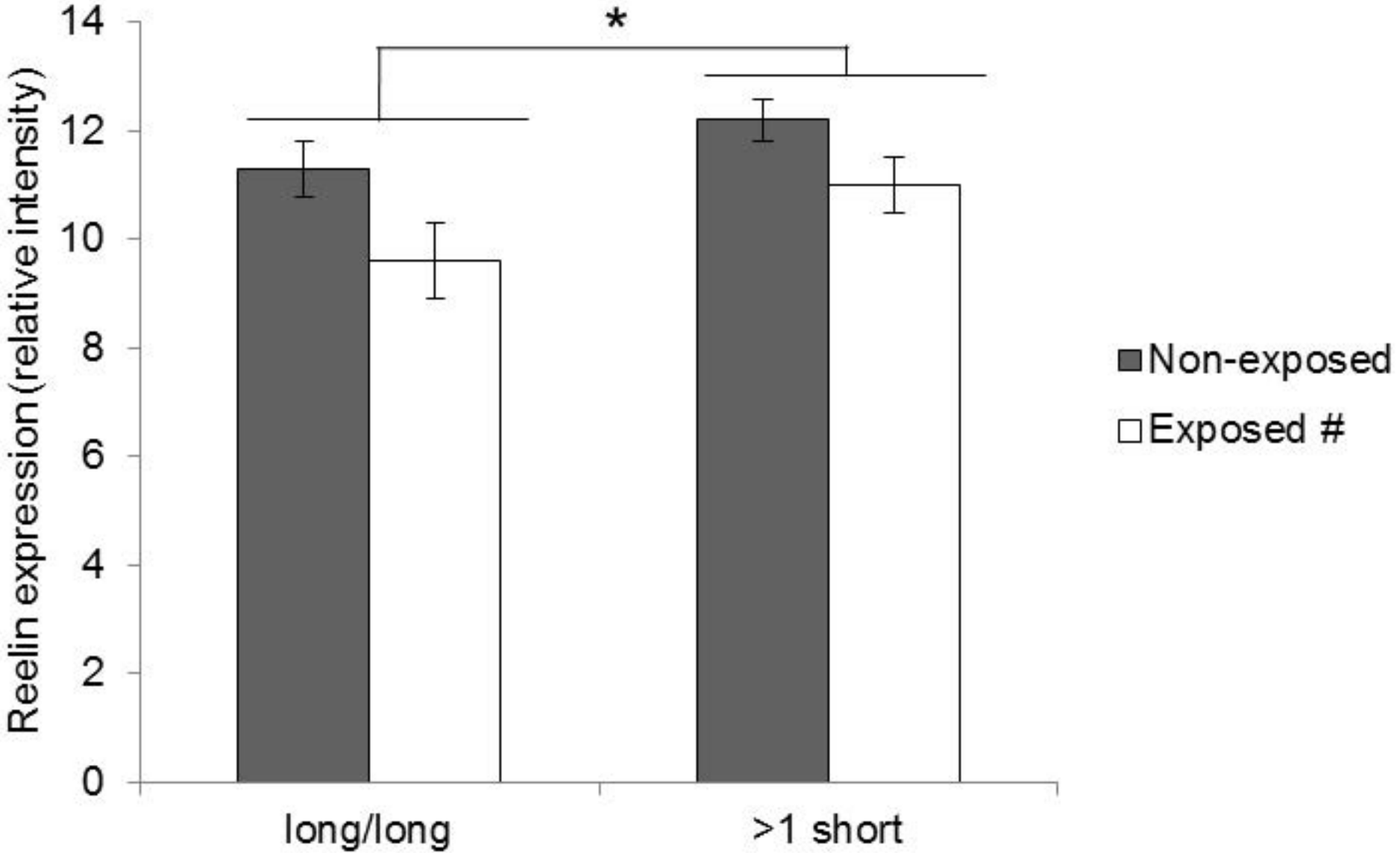
Reelin and Ponceau absorbance increases with increasing volume loaded per lane. A: Reelin staining (top) and Ponceau staining (bottom) of the same 3 columns with either 60, 120, or 180μg of protein loaded. B: Ponceau and reelin absorbance increase simultaneously with higher protein volumes and correlate significantly. Therefore, Ponceau bands can be used as a volume reference for the amount loaded per lane.

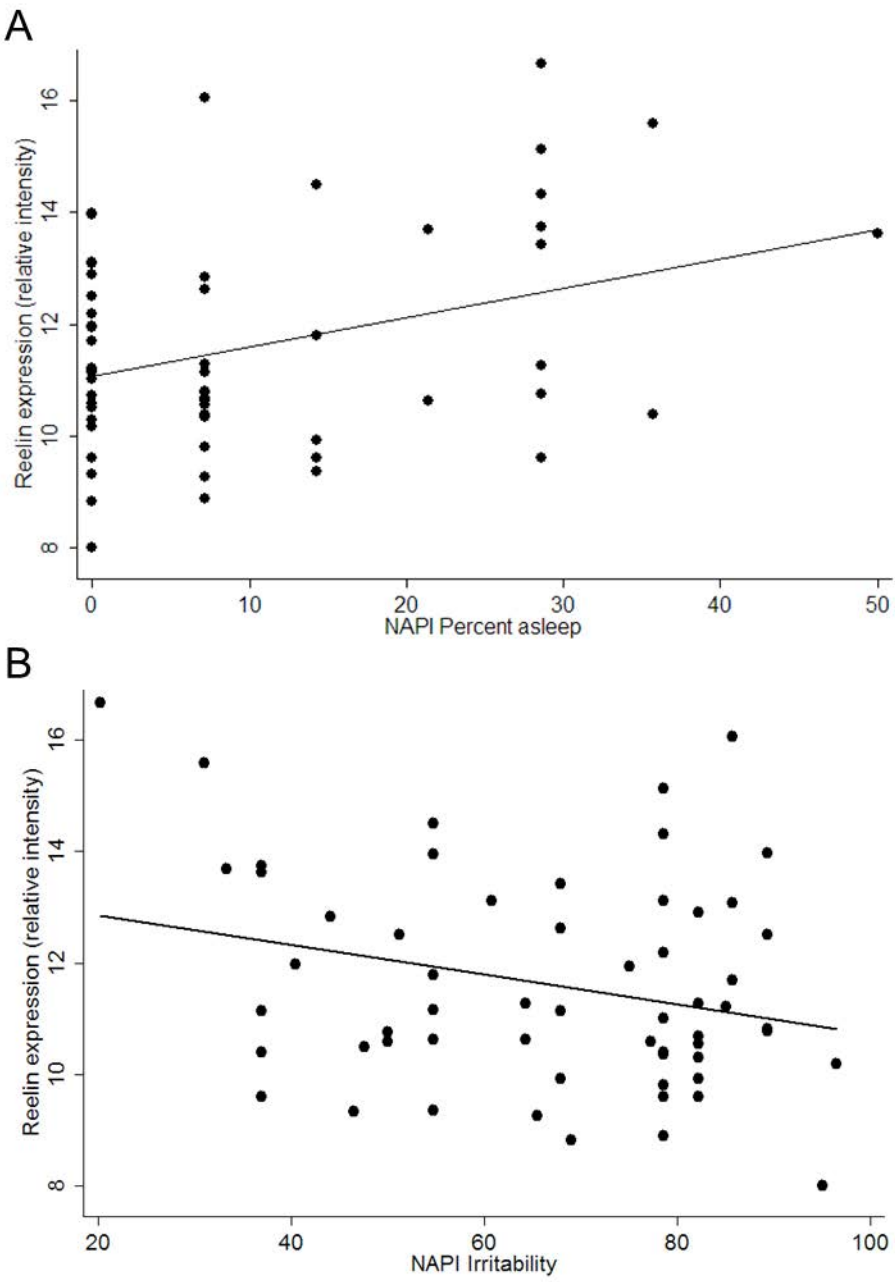

Maternal delivery
Umbilical cord
#84
#85
#86
#33
#34
#35
- 420 kDA -
- 310 kDA -
- 180 kDA -
- Ponceau -


Reelin Expression (relative intensity)
12
10
8
6
4
2
0
*
Non-exposed
SRI-exposed
420kDa
310kDa
180kDa

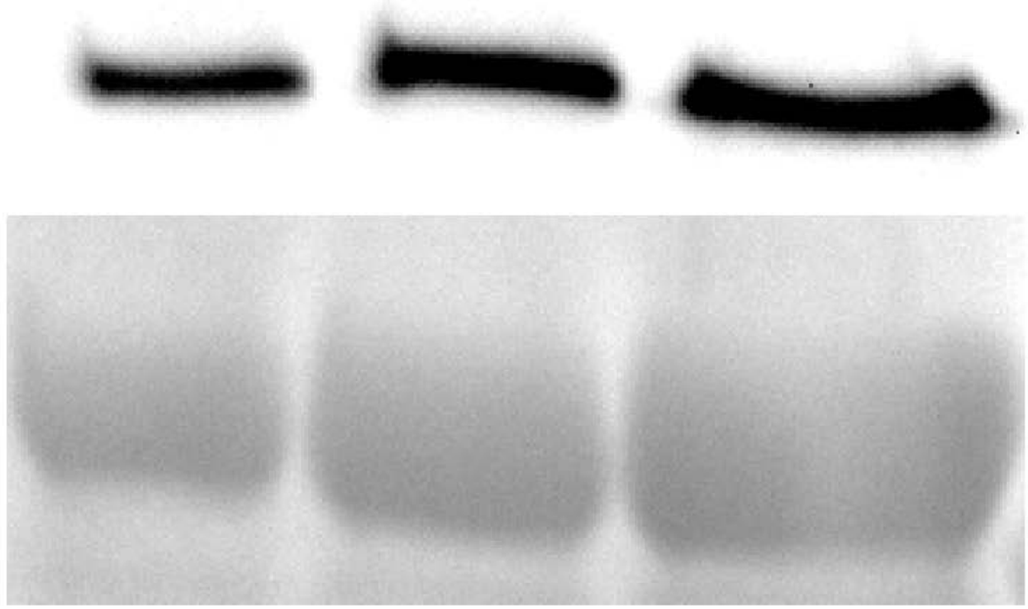

18
16
14
12
10
8
6
Reelin expression (Relative intensity)
*
Male
Female
Male
Female
Non-exposed
SRI-exposed


Non-exposed
SRI-exposed
No-exposed
SRI-exposed
Reelin expression (relative intensity)
Gestational age at birth (weeks)
18
16
14
12
10
8
6
34
36
38
40
42
44


*
14
12
10
8
6
4
2
0
Reelin expression (relative intensity)
long/long
>1 short
Non-exposed
Exposed #


A
Reelin expression (relative intensity)
8
10
12
14
16
0
10
20
30
40
50
NAPI Percent asleep
B
Reelin expression (relative intensity)
8
10
12
14
16
20
40
60
80
100
NAPI Irritability

A



310 kDa Reelin band

~300 kDa Ponceau band

60 120 180

Protein volume loaded [µg]

B