Alterations of glutamatergic markers in the prefrontal cortex of serotonin transporter knockout rats: a developmental timeline.

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Abstract

The serotoninergic system plays a key role in environmental sensitivity, potentially through down-stream effects on the GABAergic and glutamatergic systems. We previously demonstrated that juvenile serotonin transporter knockout ($SERT^{-/-}$) rats, showing increased environmental sensitivity, exhibit a decreased GABA-mediated inhibitory tone in the cortex. Since the GABAergic and glutamatergic systems are tightly interconnected, we here analyzed glutamatergic markers in the prefrontal cortex of $SERT^{-/-}$ rats, from the early stages of life until adulthood. We found that SERT inactivation in pre-weaning, juvenile, and adult rats was associated with reduced expression of proteins essential for the glutamatergic synapses such as GluN1, PSD95, CDC42, and SEPT7. These lifelong molecular changes may destabilize glutamatergic signaling and possibly contribute to stress-sensitivity and vulnerability to stress-related disorders associated with SERT alteration.

1. Introduction

Serotonin (5-HT) is a neurotransmitter implicated in a plethora of physiological and behavioral functions lifelong. One key regulator of 5-HT signaling is the serotonin transporter (5-HTT in humans, SERT in rodents), which is responsible for the reuptake of 5-HT from the synaptic cleft. The polymorphic region in the promoter of the human 5-HTT gene (5-HTTLPR) leads to the formation of the (long (l) and short (s)) allelic variants, resulting in differences in the transcription and function of the transporter (Lesch 2005; Murphy and Lesch 2008; Lesch et al. 1996; Heils et al. 1997). The short variant is associated with neuroticism (Lesch 2005) and may enhance the vulnerability to develop depressive episodes upon stress exposure (Bleys et al. 2018).

Although the 5-HTTLPR does not exist in rodents, the behavioral phenotypes observed in animals with the deletion of the SERT gene are very similar to those of humans carrying the 5-HTTLPR s-allele (Caspi et al. 2010; Holmes et al. 2003; Homberg et al. 2016). The animals are well suitable to understand the potential neuronal mechanisms underlying such evolutionary conserved effect of the 5-HTT gene on behavior. We previously found that SERT$^{-/-}$ rats display reduced inhibitory control over excitatory neurons in the cortex at postnatal day (PND) 21 (infancy) (Miceli et al. 2017). This was associated with reduced expression of various GABAergic markers that we independently also found to be reduced in the prefrontal cortex from early life to adulthood (Guidotti et al. 2012; Calabrese et al. 2013). Moreover, according to the observation that brain function depends on an intricate balance between excitation and inhibition (Zafra et al. 1991), we recently reported that SERT$^{-/-}$ rats display increased expression of the glutamatergic GluN2A subunit and reduced expression of the GluN1 receptor subunit in the prefrontal cortex (Karel et al. 2018). These observations suggest that the glutamatergic system has been altered too in these animals, but in what way is not yet clear.

Here, we investigated markers that may be indicative of glutamatergic function in the prefrontal cortex. Since we previously observed that alterations in neuroplastic mechanisms as well as of GABAergic markers develop early in life (Calabrese et al. 2013), we assessed the glutamatergic markers across development. To this aim, we measured the protein and the gene expression levels of glutamatergic markers, namely NMDA mandatory subunit 1 (GluN1) and post-synaptic density protein 95 (PSD95) in the prefrontal cortex of pre-weaning, juvenile, and adult SERT$^{-/-}$ rats and their wild-type counterparts.

Furthermore, we focused on the cell division cycle 42 (CDC42) pathway, one of the molecular mechanisms activated by glutamatergic stimulation that is involved in the correct formation and functions of spines. In particular, we analyzed the levels of the CDC42 and of the septin 7 (SEPT7).

2. Experimental procedure

Animals

SERT knockout rats (SERT$^{-/-}$, Slc6a41Hubr) were generated on a Wistar background by ENU-induced mutagenesis (Smits et al. 2006). Male SERT$^{-/-}$ and SERT$^{+/+}$ rats were sacrificed by decapitation at PND 7 (pre-weaning stage; 4-10 animals per genotype), 21 (juvenile stage; 4-13 animals per genotype) and 100 (adult stage; 4-12 animals per genotype), according to our previous studies (Calabrese et al. 2013). Brains were immediately frozen on dry ice and prefrontal cortical subregions (cingulate cortex 1, cingulate cortex3, and infralimbic cortex), corresponding to plates 6-10 of the atlas of Paxinos and Watson, were dissected from 2-mm-thick frozen slices.

All procedures used in this study were compliant with the rules and principles of the 2010/63/EU Directive, were approved by the Committee for Animal Experiments of the Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands, and all efforts were made to minimalize animal suffering and to reduce the number of rats used.

RNA preparation and gene expression analyses

Total RNA was extracted, and samples were processed for real-time polymerase chain reaction (PCR) by a TaqMan qRT–PCR instrument (CFX384 real-time system, Bio-Rad Laboratories S.r.l.), as previously described (Calabrese et al. 2013). The relative target gene expression was calculated by the comparative cycle threshold (Ct) method. Primer sequences used were purchased from Eurofins MWG-Operon. GluN1: forward primer (FP): TCATCTCTAGCCAGGTCTACG and reverse primer (RP): CAGAGTAGATGGACATTCGGG; PSD95: FP: CAAGAAATACCGCTACCAAGATG and RP: CCCTCTGTTCCATTCACCTG; CDC42: FP: AAGGCTGTCAAGTATGTGGAG and RP: GCTCTGGAGATGCGTTCATAG; SEPT7: FP: AAGAAGGTGGCGTTCAGTTG and RP: CGTCTGTTCACTCGAGATTCTG.

Protein extraction and western blot analysis.

The protein levels of GluN1, PSD95 CDC42 and SEPT7 were investigated in the crude synaptosomal fraction, extracted from the rats prefrontal cortex as previously reported (Calabrese et al. 2013). 10 μg of proteins was run on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Blots were blocked and incubated at room temperature with the primary antibodies (Genetex; GluN1: 1:1000,

Invitrogen; PSD95: 1:4000, Cell Signaling; CDC42: 1:1000, Cell Signaling; SEPT7: 1:500) and then for 1 h at room temperature with the corresponding secondary antibody (GluN1: 1:3000, anti-mouse; PSD95: 1:8000, anti-rabbit; CDC42: 1:1000, anti-rabbit; SEPT7: 1:2000, anti-rabbit). Immunocomplexes were visualized using the Western Lightning Plus ECL (PerkinElmer) and Chemidoc MP imaging system (Bio-Rad Laboratories).

Statistical analyses

The effects of age (PND7, PND21, and PND100) and genotype ($SERT^{+/+}$and $SERT^{-/-}$) were analyzed using the two-way analysis of variance (ANOVA) followed, when appropriate, by Fisher's Protected Least Significant Difference (PLSD). Data were correlated using Pearson correlation analysis. Results were considered statistically significant with p values <0.05. Data are presented as means $\pm$ standard error (SEM). For graphic clarity, $SERT^{+/+}$/PND7 is set at 100%.

3. Results

In line with our previous results (Karel et al. 2018), we observed a significant effect of genotype ($F_{1,52}$=9.739, p<0.01) and of age ($F_{1,52}$=19.420, p<0.001) on GluN1 gene expression (Fig.1A). Indeed, even if the changes observed across ages were similar for the two genotypes, the mRNA levels of the mandatory subunit of the NMDA receptor were significantly reduced in SERT$^{-/-}$ compared to SERT$^{+/+}$ rats from PND21 (-38%, p<0.05 vs SERT$^{+/+}$) and PND100 (-14%, p<0.05 vs SERT$^{+/+}$). In line, its protein levels (Fig.1C) were significantly affected by genotype ($F_{1,32}$=10.093, p<0.01) and by age ($F_{1,32}$=24.964, p<0.001). In particular, we found a significant downregulation at PND21 (-39%, p<0.01 vs SERT$^{+/+}$) and at PND100 (-47%, p<0.05 vs SERT$^{+/+}$). Moreover, we found a significant effect of genotype ($F_{1,57}$ = 5.630, p<0.05) and of age ($F_{1,57}$ = 10.006, p<0.001) for Psd95 mRNA levels (Fig. 1B), that were decreased only at PND21 (-26%; p<0.05 vs SERT$^{+/+}$). PSD95 protein levels (Fig. 1D) were influenced by genotype ($F_{1,34}$ = 8.480, p<0.01) and by age ($F_{1,34}$ = 66.069, p<0.001), with a reduction from PND21 (PND21: -26%, p<0.05 vs SERT$^{+/+}$) to PND100 (-30%, p<0.01 vs SERT$^{+/+}$).

To assess spine development, we focused subsequent measures on the CDC42 pathway. As shown in figure 2A, Cdc42 mRNA levels were significantly affected by genotype ($F_{1,53}$ = 4.519, p<0.05) and by age ($F_{1,53}$ = 5.201, p<0.01) with a genotype x age interaction ($F_{1,53}$ = 8.740, p<0.01). Indeed, CDC42 gene expression was up-regulated at early life stages in SERT$^{-/-}$ rats (PND7: +40%, p<0.01 vs SERT$^{+/+}$; PND21: +33%, p<0.05 vs SERT$^{+/+}$) and down-regulated in adult SERT$^{-/-}$ rats (-21%, p<0.05 vs SERT$^{+/+}$). On the contrary, CDC42 protein levels were reduced at all the ages investigated (PND7: -46%, p<0.05 vs SERT$^{+/+}$; PND21: -51%, p<0.01 vs SERT$^{+/+}$; PND100; -33%; p<0.05 vs SERT$^{+/+}$) (Fig. 2C) as suggested by the significant effect of genotype ($F_{1,24}$ = 18.429, p<0.001), of the age ($F_{1,24}$ = 5.781, p<0.05). We furthermore found that the gene expression of Sept7, the most abundant septin in rat brain postsynaptic density fraction (Peng et al. 2004), was influenced by age ($F_{1,51}$ = 17.573, p<0.001) with a genotype x age interaction ($F_{1,51}$ = 4.137, p<0.05). Accordingly, its mRNA levels were significantly reduced in SERT$^{-/-}$ rats at PND7 (-28%, p<0.05 vs SERT$^{+/+}$) (Fig. 2C) and at PND100 (-20%, p<0.05 vs SERT), while SEPT7 protein levels were decreased at PND7 (-51%, p<0.05 vs SERT$^{+/+}$) and at PND21 (-40%, p<0.05 vs SERT$^{+/}$) (Fig. 2D) as indicated by the significant effect of age ($F_{1,20}$ = 14.582, p<0.001).

Interestingly, correlation analysis across all ages revealed significant positive correlations between Sept7 and both GluN1 and Psd95 ($p<0.05$) (Fig. 3 A, C, E, G), and CDC42 and GluN1 protein levels ($p<0.05$) (Fig. 3F).

4. Discussion

In this study, we found that perturbation of the serotonergic system affected the expression of fundamental markers of the postsynaptic density of the glutamatergic synapse, such as GluN1 and PSD95, that were down-regulated starting from the first stages of life. These effects were associated with the reduction of CDC42 and SEPT7 protein levels, which followed the same temporal profile. These findings point to a lifelong down-regulation of components of glutamatergic synapses in the prefrontal cortex of SERT$^{-/-}$ rats.

Interestingly, for almost all markers considered, the temporal expression profile is similar for the two genotypes, with the mutant rats starting from lower levels (except for the CDC42 gene expression). Accordingly, we found a positive correlation between Sept7 and GluN1 and Psd95, while CDC42 significantly correlated only with GluN1 at protein level.

Moreover, we found a peak around PND21, with a decrease in the later age that may be in line with the structural reorganization taking place during developmental pruning and stabilization of spines and synapses (Nietzer et al. 2011).

We previously observed that the lack of SERT is associated with GABAergic dysfunctions from the first phases of life (Calabrese et al. 2013) and it has been demonstrated that, while GABA acts as inhibitory neurotransmitter in the mature brain, it plays a key role in the formation of excitatory synapses during development (Oh et al. 2016). On these bases, it is plausible that this developmental process contributes to the lifelong alterations observed in the present study. More specifically, the glutamatergic molecular abnormalities we observed here may represent a compensatory change in response to the increased glutamatergic tone found in young SERT$^{-/-}$ rats, showing a reduced inhibitory control over excitatory neurons (Miceli et al. 2017).

The decreases in CDC42 and SEPT7 may alter the trafficking for and from the spines, thus contributing to alterations in spine formation. Actually, we found for CDC42 an apparent discrepancy between SERT$^{-/-}$ and SERT$^{+/+}$ rats at PND7 and PND21, which might be due to an attempt of the system to up-regulate the mRNA levels in order to counteract the lack of proteins.

NMDAR activity regulates morphological changes in dendritic spines and the PSD95 protein family is fundamental for the localization and clustering of receptors at the postsynaptic membrane (Wenthold et al. 2003). Hence, the reduction of GluN1 and PSD95 are suggestive of alterations in glutamatergic spine

formation. While morphological analyses are needed to confirm this hypothesis, Nietzer and collaborators already showed a reduction in spine density in the prefrontal cortex of SERT$^{-/-}$ mice (Nietzer et al. 2011). Together with previous studies, our results suggest that perturbations in 5-HT homeostasis during development lead to stable modifications of the glutamatergic synapse.

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Figure legends

Fig.1 Developmental analysis of GluN1 and PSD95 gene and protein expression changes in SERT$^{-/-}$ rats. GluN1 (A-C) and PSD95 (B-D) mRNA and protein levels were measured in the prefrontal cortex of SERT$^{-/-}$ and SERT$^{+/+}$ rats at postnatal days (PND) 7, 21 and 100. Data, expressed as the percentage of SERT$^{+/+}$/PND7 animals (set at 100%), represent the mean ± SEM of at least 4 independent determinations. *p<0.05, **p<0.01 vs SERT$^{+/+}$/same age (Two-way ANOVA with PLSD).

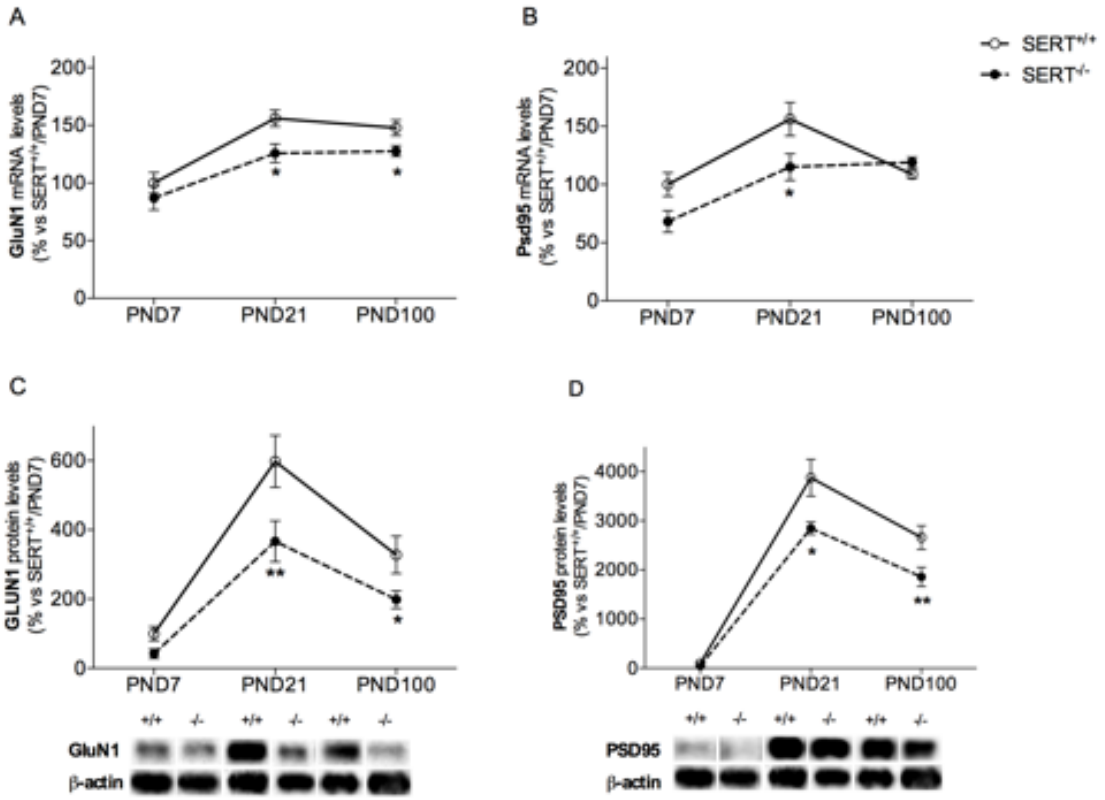


Fig.2 Developmental analysis of CDC42 and SEPT7 gene and protein expression changes in SERT$^{-/-}$ rats. CDC42 (A-C) and SEPT7 (B-D) mRNA and protein levels were measured in the prefrontal cortex of SERT$^{-/-}$ and SERT$^{+/+}$ rats at postnatal days (PND) 7, 21 and 100. Data, expressed as the percentage of SERT$^{+/+}$/PND7 animals (set at 100%), represent the mean ± SEM of at least 4 independent determinations. *p<0.05, **p<0.01 vs SERT$^{+/+}$/same age (Two-way ANOVA with PLSD).

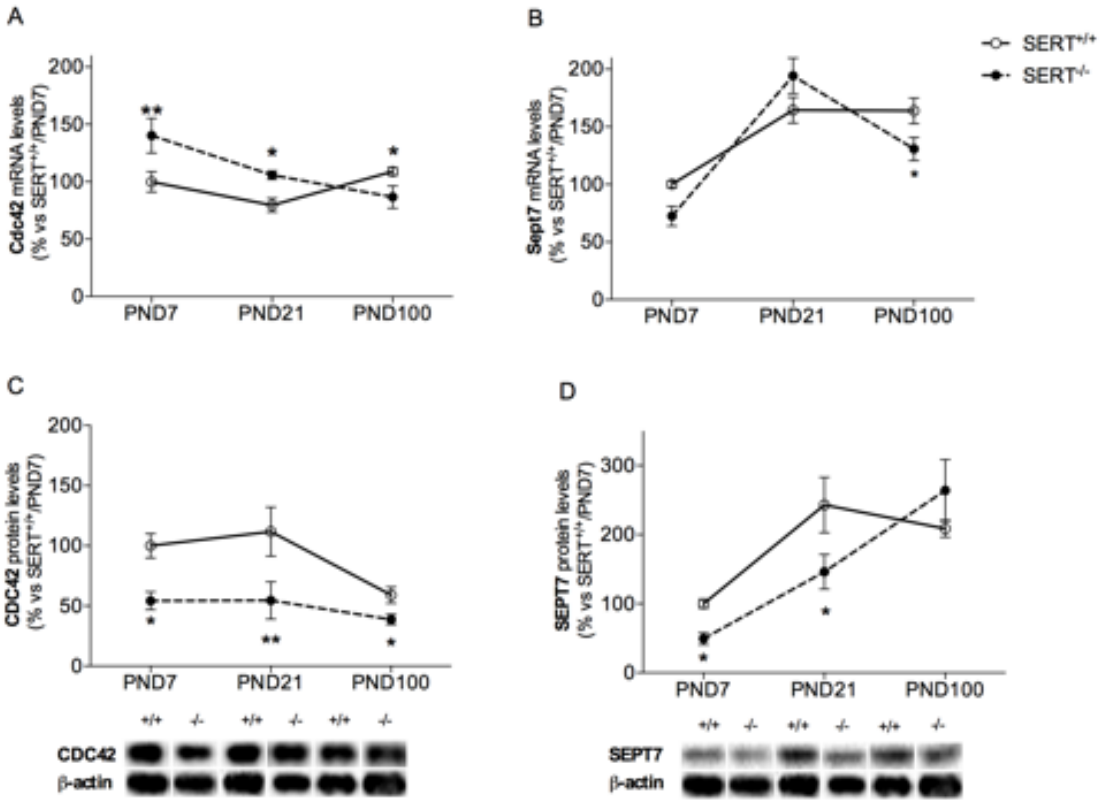
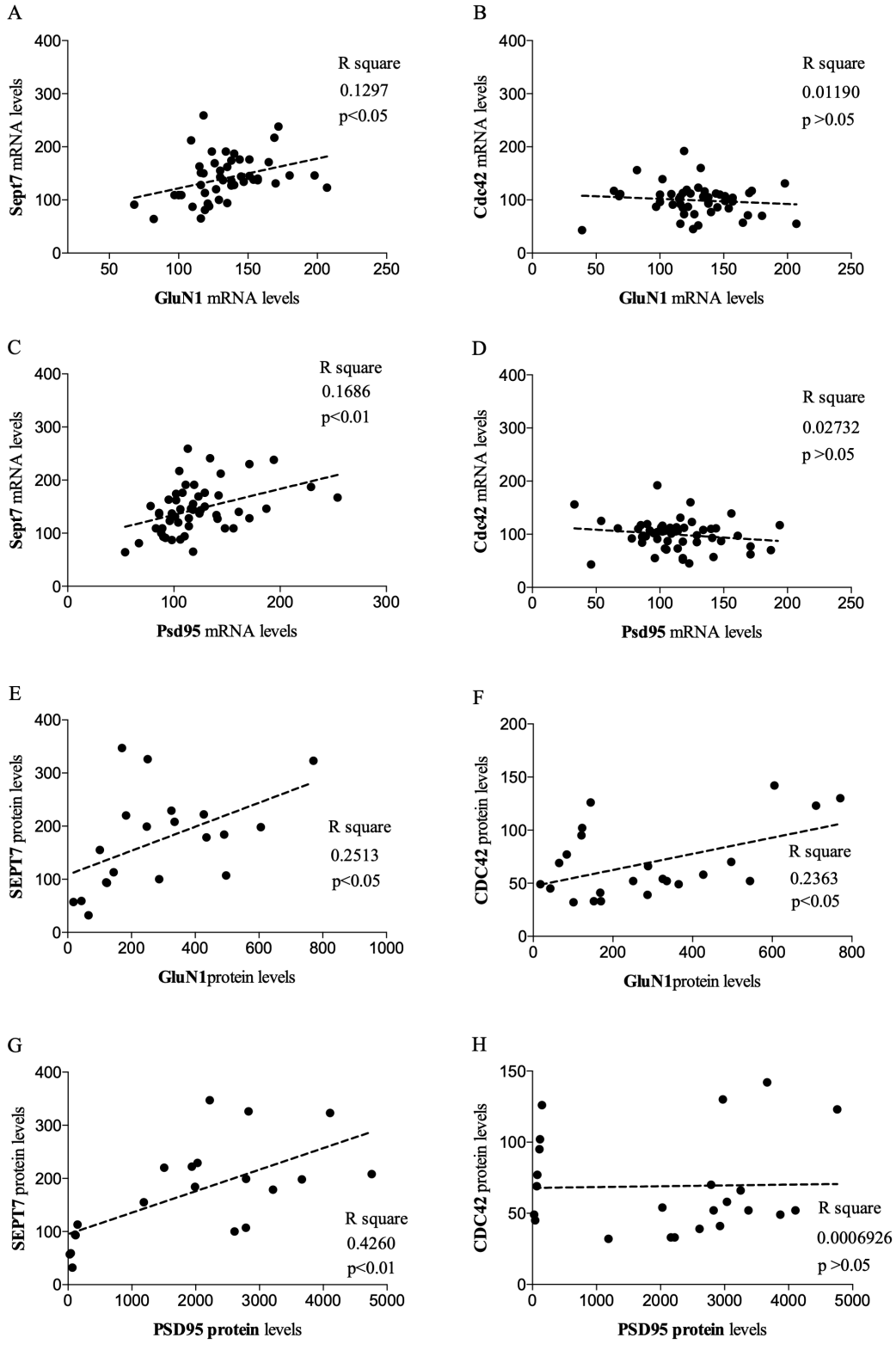


Fig.3. Correlations between Sept7 and GluN1 mRNA and protein levels (A, E), Sept7 and Psd95 mRNA and protein levels (C, G), Cdc42 and GluN1 mRNA and protein levels (B, F), Cdc42 and Psd95 mRNA and protein levels (D, H). Data are expressed as scatterplots, with a line indicating the coefficient of the correlation between the individual data points.



Author's contributions

FC, JRH and MAR and were responsible for the study concept and design.

PB performed and analyzed the molecular analysis.

Data analysis and interpretation were done by PB, FC, JRH and MAR.

PB drafted the manuscript and FC and JRH critically revised the manuscript.

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Compliance and Ethical standards:

Conflict of interest: M.A.R. has received compensation as speaker/consultant from Lundbeck, Otzuka, Sumitomo Dainippon Pharma and Sunovion, and he has received research grants from Lundbeck, Sumitomo Dainippon Pharma and Sunovion.

F.C., P.B., J.R.H. have nothing to declare.

Ethical approval: All procedures used in this study were compliant with the rules and principles of the 2010/63/EU Directive, were approved by the Committee for Animal Experiments of the Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands.