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Forebrain Expression of Serine Racemase During Postnatal Development

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Abstract

N-methyl-D-aspartate receptors (NMDARs) are important for synaptogenesis, synaptic maturation and refinement during the early postnatal weeks after birth. Defective synapse formation or refinement underlie cognitive and emotional abnormalities in various neurodevelopmental disorders (NDDs), including schizophrenia (Sz) and autism spectrum disorder (ASD). Serine racemase (SR) is a neuronal enzyme that produces D-serine, a co-agonist required for full NMDAR activation. NMDAR hypofunction as a result of genetic SR elimination and reduced synaptic availability of D-serine reduces neuronal dendritic arborization and spine density. In adult mouse brain, the expression of SR parallels that of NMDARs across forebrain regions including the striatum, amygdala, hippocampus, and medial prefrontal cortex (mPFC). However, there have yet to be studies providing a detailed characterization of the spatial and temporal expression of SR during early periods of synaptogenesis. Here, we examined the postnatal expression of SR in cortical and subcortical brain regions important for learning, memory and emotional regulation, during the first four weeks after birth. Using dual-antigen immunofluorescence, we demonstrate that the number of SR+ neurons steadily increases with postnatal age across the mPFC, amygdala, hippocampus and striatum. We also identified differences in the rate of SR protein induction both across and within brain regions. Analyzing existing human *post-mortem* brain *in situ* data, there was a similar developmental mRNA expression profile of *SRR* and *GRIN1* (GluN1 subunit) from infancy through the first decade of life. Our findings further support a developmental role for D-serine mediated NMDAR activation regulating synaptogenesis and neural circuit refinement, which has important implications for the pathophysiology of Sz and other NDDs.

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Credit authorship contribution statement

Oluwarotimi Folorunso – Conceptualization, Methodology, Visualization, Writing, Original draft preparation, Methodology, Formal analysis; Theresa L. Harvey – Writing, Review and Editing, Methodology, Visualization, Formal analysis; Cristina Cruz – Methodology; Ellie Shahbo – Methodology; Ismail Ajjawi – Methodology; Stephanie Brown – Methodology, Review and Editing; Darrick T. Balu – Conceptualization, Supervision, Writing, Review and Editing.

Conflicts of interest

DTB served as a consultant for LifeSci Capital and received research support from Takeda Pharmaceuticals. All other authors report no biomedical financial interests or potential conflicts of interest.

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Keywords

N-methyl-D-aspartate receptor (NMDAR); Serine racemase; D-serine; GRIN1; Schizophrenia; Synaptogenesis

1. Introduction

N-methyl-D-aspartate receptor (NMDAR) activity is important for synaptic plasticity, learning, and memory (Collingridge et al., 2013). A distinctive feature of NMDAR activation is that in addition to glutamate, it requires the simultaneous binding of a co-agonist to the obligatory GluN1 subunit at the glycine modulatory site (GMS). During postnatal development, NMDARs play a role in synaptogenesis, synaptic maturation and refinement (Gambrill and Barria, 2011). Defective synapse formation or maturation underlie cognitive and emotional deficits associated with neurodevelopmental disorders (NDDs), including schizophrenia (Sz) and autism spectrum disorder (ASD) (Neniskyte and Gross, 2017). Although, D-serine was initially characterized as a gliotransmitter, it is now known that astroglial L-serine (Yang et al., 2010) is shuttled to neurons where it is converted to D-serine by the neuronal enzyme serine racemase (SR) (Neame et al., 2019; Wolosker et al., 2016). Furthermore, our laboratory and others have shown that SR is mainly expressed in the postsynaptic neurons, suggesting that D-serine is released in an autocrine fashion at the same spine where it locally regulates NMDARs activity (Lin et al., 2016; Wong et al., 2020). D-serine is functionally a more potent activator of synaptic NMDARs than glycine, and mounting evidence suggests that it serves as the major NMDAR co-agonist in limbic brain regions (Le Bail et al., 2015; Li et al., 2013; Papouin et al., 2012). As such, mice lacking D-serine either due to constitutive or neuronal specific genetic deletion of SR display NMDAR hypofunction, as well as impaired neuroplasticity and learning (Balu et al., 2013; Balu et al., 2016; Basu et al., 2009; Benneyworth et al., 2012; Perez et al., 2017).

Although SR expression parallels that of NMDARs across various regions including the striatum, amygdala, hippocampus, and medial prefrontal cortex (mPFC) in the adult brain (Balu et al., 2018; Basu et al., 2009; Miya et al., 2008), there has yet to be an extensive spatiotemporal characterization of SR expression across postnatal brain development. Early weeks of postnatal development are important for proper synaptic formation and refinement. In rodents, the formation of synapses begins during the first week after birth; axonal and dendritic processes, and cortical lamination peaks around postnatal day (PND) 10, while synaptogenesis and synaptic refinement peaks around PND14 and PND21-30 respectively (Farhy-Tselnicker and Allen, 2018). In humans, the peak density of synapses is around 1-4 years old (Verrall et al., 2010), which developmentally is approximately equivalent to PND 10 in mice.

Accumulating evidence in humans and animal studies points to NMDAR hypofunction as a convergence point for the positive, negative and cognitive symptoms of schizophrenia (Sz) (Balu and Coyle, 2015; Bodkin et al., 2019; Lee and Zhou, 2019). Thus, we examined SR protein expression across limbic brain regions that are adversely affected in Sz and contribute to the cognitive, social and emotional symptoms of Sz. We show that there is a

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steady increase in the number of SR expressing neurons at the three time-points examined (PND8, 16, 25/29) in the amygdala, hippocampus, striatum and mPFC. Interestingly, we find that there is different temporal pattern of SR expression within various sub-regions of these areas. We see a similar developmental profile of *SRR* and *GRIN1* (obligatory NMDAR subunit) mRNA expression in human *post-mortem* brain at ages homologous to what we examined in mice.

2. Materials and methods

2.1 Mice

C57BL/6J male mice at postnatal day (PND) 8, 16 and 25/29 were used for all experiments. All animals were grouped housed in polycarbonate cages and maintained on a 12-hour light/dark cycle in a temperature (22C)- and humidity-controlled vivarium. PND 8,16 were removed from dam right before being perfused individually. Animals were given access to food and water ad libitum. The McLean Hospital Institutional Animal Care and Use Committee approved all animal procedures.

2.2. Immunofluorescence

Mice were deeply anesthetized with isoflurane and then intracardially perfused with cold 1x PBS (0.5M PB, NaCl, pH 7.4), followed by 4% paraformaldehyde (Electron Microscopy Sciences, Cat # 19202). Brains were cryopreserved in 30% sucrose/PBS at 4°C, sectioned at 30 μm using a Leica SM 2010R Microtome, and stored in a cryoprotectant solution (ethylene glycol, glycerol, 0.5M PB, NaCl, KCl, in dH_20) at -20°C. Sections were washed three times with 1x PBS, incubated in permeabilizing solution (0.3% Triton X-100 in PBS) for 30 minutes, followed by an hour incubation in blocking buffer (20% donkey serum, 1% BSA, 0.1% glycine, 0.1% lysine in 1x PBS). Sections were then incubated overnight at 4°C in primary antibodies in 5% donkey serum, 1% BSA, 0.1% glycine, and 0.1% lysine. The primary antibodies used in this study were: mouse anti-serine racemase (BD biosciences, cat #612052) and rabbit anti-NeuN (Abcam, cat #ab177487). Sections were washed three times with 1x PBS and incubated for an hour with Donkey anti-mouse 488 and Donkey anti-rabbit 568 antibodies in a 1x PBS solution containing 5% donkey serum, 1% BSA, 0.1% glycine, and 0.1% lysine. After secondary antibody incubation, tissues were washed three times with 1x PBS and mounted on Fisherbrand Superfrost Plus glass microscope slides. Finally, slides were cover slipped with ProLong Gold anti-fade medium (Invitrogen).

2.3. Quantification of SR+ neurons

The number of SR+ neurons was estimated using systematic random sampling with a standardized Optical Fractionator workflow in StereoInvestigator software (MBF Biosciences). At least six sections from each mouse (n=3; section interval = 6) were analyzed and matched from rostral to caudal positions. Cells were counted at 20x using exposure and image settings that were consistent across all sections from mPFC (bregma 2.96mm to 1.34mm), striatum (bregma 1.98mm to −2.18mm), and amygdala (bregma −0.82mm to −2.06mm). The threshold for SR+ cell detection was determined by initial visual analysis of the intensity and shape of the relevant fluorescent staining, compared against background 4′,6-diamidino-2-phenylindole (DAPI) staining. For the amygdala, we

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used a counting frame size of 75x75μm and a sampling grid size of 250x250μm. For the mPFC, we used a counting frame size of 125x125 μm and a sampling grid size of 350x350μm. For the striatum, we used a counting frame size of 75x75μm and a sampling grid size of 500x500μm. Images of CA1 region of the hippocampus (bregma −1.34mm to −1.8mm) (n = 3 mice; 2-3 dorsal sections/mouse) were collected on a Leica SP8 confocal microscope (40x objective). A region of interest was drawn covering the CA1 pyramidal layer in image J, cell counter plug-in was used to estimate the number of SR+ cells and NeuN+ cells, and the percentage of SR/NeuN neurons was determined. The average volume for each brain region did not significantly differ between animals (Supplementary Tables 1–4). Our sampling parameters also produced highly accurate cell population estimates across all brain regions (Gundersen coefficient of error values < 0.1; Supplementary Tables 1, 3, 4)

2.4. *In Situ* Hybridization Human mRNA expression

Colorimetric *in situ* hybridization (CISH) with digoxigenin was used to detected *SRR* and *GRIN1* human mRNA (Miller et al., 2014). *SRR* and *GRIN1* gene expression data was downloaded from the Allen Brain Atlas Developmental Transcriptome (Miller et al., 2014).

2.5. Statistical analysis

Statistics were calculated using Prism 8 (GraphPad Software, San Diego, CA, USA). One-way ANOVA with Tukey's post hoc test was used for multiple comparisons. Unpaired t-test was used to compare between regions at each time point. Data are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001. Supplementary Tables 5 and 6 show detailed results for all statistical comparisons.

3. Results

3.1 SR expression in mPFC

The mPFC is believed to be the rodent analogue of the dorsolateral prefrontal cortex in humans (Vertes, 2004), which plays a critical role in cognition and is dysfunctional in patients with Sz (Potkin et al., 2009). Two divisions of the mPFC, the infralimbic (IL) and prelimbic (PL) cortices, are heavily interconnected to other limbic structures such as the hippocampus, amygdala, and striatum. In the IL (Fig 1A–F) cortex at P8, only 6% of neurons were SR+ (Fig 1G). There was a modest, nonsignificant 17% increase in the number of SR+ neurons from PND8 to PND16. By PND 25/29, 45% of neurons were SR+, which was significantly more than the proportion of SR+ neurons observed at PNDs 8 and 16 (Fig 1G). The proportion of SR+ neurons in the prelimbic (PL) cortex increased from 15% at PND8 to 41% at PND16 and 46% at PND25/29 (Fig 1G; p = 0.051). At PNDs-8,16, and 25/29, there were no differences in the percentage of SR+ neurons between IL to PL (Fig 1H). There was no change in NeuN counts in the PL, however, in the IL, there was a significant increase in NeuN + counts comparing PND8 to PND25/29, and PND16 to PND25/29 (Fig 1I).

3.2 SR expression in the hippocampus

The hippocampus is important for episodic memory and spatial cognition and is well known to be dysfunctional in psychiatric disorders, such as in Sz (Guo et al., 2019). In the adult

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hippocampus, there is robust SR expression throughout the pyramidal cell layer (Basu et al., 2009; Miya et al., 2008). Given the importance of NMDAR function for LTP at the CA3-CA1 Schaffer collateral synapse, we examined here the temporal profile of SR expression in the pyramidal cell layer of the dorsal CA1 subfield (Fig 2A–G). Interestingly, at P8 only 11% of neurons were SR+. This proportion significantly increased to 52% at P16 and modestly increased to 62% by P25/29 (Fig 2G). There was a significant increase in NeuN + counts comparing PND8 to PND16, and PND8 to PND25/29 (Fig 2H).

3.3. SR expression in the amygdala

The amygdala is a central hub in the emotional learning circuit, integrating sensory information from both cortical and subcortical brain regions (Janak and Tye, 2015). Human *post-mortem* and neuroimaging studies have demonstrated amygdala dysfunction in subjects with schizophrenia (Berretta et al., 2007; Holt et al., 2006; Perlman et al., 2004; Reynolds, 1983). The amygdala as a whole is composed of many interconnected nuclei. Two of the best characterized nuclei are the basolateral amygdala (BLA) and central nucleus (CeA). The BLA is a cortical-like structure comprised mainly of excitatory glutamatergic principal cells, while the CeA is a ventrocaudal extension of the striatum and consists of local and projecting GABAergic neurons. The BLA had 12% of SR+ neurons at PND8, that significantly increased to 33% at PND16 and 56% at PND25/29 (Fig 3A–G). However, in the central CeA, approximately 25% of neurons expressed SR at PND8 and PND16, with that number doubling to 51% by PND 25/29 (Fig 3). At PND8, there was a higher percentage of SR+ neurons in the CeA compared to BLA, however, no difference was seen at PND 16 and PND 25/29 (Fig 3G). The maximum expression of SR was observed at PND25/29 in BLA(Balu et al., 2018). There was no change in total NeuN counts between the three timepoints (Fig 3I).

3.4. SR expression in the striatum

The striatum consists primarily of GABAergic medium spiny neurons that receive and send projections to other limbic brain regions. Different subdivisions of the striatum serve distinct roles in learning, with the dorsal striatum (*caudate putamen*; CP) mediating flexible action selection and automatic action, whereas the ventral striatum (*nucleus accumbens*; NAc) is thought to be critical for learning the value of stimuli (Balleine et al., 2009). The percentage of SR+ neurons in the CP significantly increased from 15% at PND8 to 40% at PND16. There was modest 54% increase in SR+ neurons between PND16 and PND25/29 (Fig 4G). In the NAc, 36% of neurons co-localized with SR at PND 8 and 44% at PND 16, but by PND 25/29 the percentage of SR+ neurons significantly increased to 61% (Fig 4A–G). At PND8 there was a significantly higher percentage of SR+ neurons in the NAc compared to the CP; however, there were no differences at PND 16 and PND 25/29. In both the NAc and CP, there were significant increases in the percentage of SR+ neurons comparing PND8 to PND25/29 (Fig 4G). There was no change in NeuN counts in the NAc, however, in the CP, there was a significant increase in NeuN + counts comparing PND8 to PND16, and PND8 to PND25/29 (Fig 4I).

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3.5 Expression profile of *SRR* and *GRIN1* in human cortical and subcortical brain regions.

Finally, in order to determine whether the developmental pattern and trajectory of SR expression is translationally relevant, we compared *SRR* and *GRIN1 in situ* mRNA expression data obtained from the Allen Human Brain Atlas at comparable ages to our mouse studies. Consistent with our findings, there was an increase in both *SRR* and *GRIN1* mRNA expression in the anterior cingulate cortex (ACC), ventrolateral prefrontal cortex (vlPFC), hippocampus, striatum and amygdala from 3.3 years (mouse PND8) to 11 years (mouse PND10-12) (Hagan, 2017) (Fig 5A–F).

4. Discussion

Our results show the spatiotemporal expression of SR during early postnatal murine development in both cortical and subcortical regions, providing further evidence for the neuronal localization of SR under physiological conditions, where it is important for NMDAR function and LTP (Balu et al., 2013; Balu et al., 2014; Miya et al., 2008; Neame et al., 2019; Perez et al., 2017; Wolosker et al., 2016; Wong et al., 2020). We show that the increase in SR expressing neurons temporally coincides with the development of functional glutamatergic synapses and resembles *GRIN1* and *SRR* in humans at homologous periods of development. We show that the dorsal mPFC (PrL) has a higher number of SR expressing neurons at an earlier time point than the ventral mPFC (IL). In the striatum, we show that the NAc has a higher number of SR expressing neurons at PND8 and maintains this level at PND16, while in the CP there is a two-fold increase in the number of SR expressing neurons from PND8 to PND16. We see a difference in the percentage of SR expressing neurons between the two major amygdalar nuclei, as the CeA had a higher number of SR expressing neurons at PND8 compared to BLA, and the number of SR expressing neurons in BLA increased two-fold between PND8 and PND16.

NMDAR activity is a positive modulator of neuronal dendritic morphology and spine formation (Tada and Sheng, 2006). One of the most consistent *post-mortem* observations in Sz is a reduction in the dendritic complexity and spine density of cortical excitatory neurons (Glausier and Lewis, 2013; Konopaske et al., 2014; MacDonald et al., 2019; Sweet et al., 2009). In adult constitutive SRKO mice, we have shown that selectively reducing D-serine mediated NMDAR activation negatively impacts the dendritic morphology of excitatory cortical and hippocampal neurons (Balu et al., 2012; Balu et al., 2013; DeVito et al., 2011), while D-serine administration positively modulates dendritic plasticity *in vivo* and *in vitro* (Balu and Coyle, 2014; Lin et al., 2016). Conditional SR elimination in forebrain neurons starting at 8 weeks of age (Benneyworth et al., 2012), reduced dendritic branching and spine density of cortical excitatory neurons (Balu and Coyle, 2014). Our findings suggest that D-serine mediated NMDAR activation at earlier postnatal timepoints could be important for neuronal dendritic plasticity across development and brain regions. For example, deletion of neocortical GluN1 on pyramidal neurons during early postnatal development led to disruption of NMDAR-dependent transmission at PND9 and reduced dendritic spine density at PND 15 and PND 20 (Ultanir et al., 2007).

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NMDAR hypofunction as a result of D-serine elimination is also implicated in the cognitive and learning impairments observed in neuropsychiatric and substance abuse disorders. Constitutive SRKO mice have impairments in memory and cognition that depend on the hippocampus and mPFC (Balu et al., 2013; Balu et al., 2016; Balu et al., 2018; Basu et al., 2009; DeVito et al., 2011). These mice also show a decrease in sensitivity to the rewarding effects of cocaine and an inability to extinguish amphetamine conditioned hyperactivity, which were associated with altered striatal plasticity (Benneyworth and Coyle, 2012; Puhl et al., 2019). D-serine also facilitates the extinction of fear memories (Balu et al., 2018; Matsuda et al., 2010; Singewald et al., 2015) and drug seeking behavior (Hammond et al., 2013), which require proper NMDAR function across multiple cortico-limbic brain regions, particularly within the amygdala, mPFC, hippocampus, and striatum (Fenster et al., 2018). Interestingly, recent work has shown that restoration of GluN1 expression using the cre-recombinase system during development or adulthood in mice with congenital deletion of GluN1 rescued some cognitive function, suggesting that NMDAR controls plasticity of cognitive neuronal circuit well into adulthood (Mielnik et al., 2019). Thus, eliminating SR with spatiotemporal specificity will be important for elucidating how D-serine mediated NMDAR activation during postnatal development affects behaviors relevant to the pathophysiology of various neuropsychiatric disorders.

Dysfunction of parvalbumin inhibitory interneurons in corticolimbic circuits has been proposed to contribute to the pathophysiology of neuropsychiatric disorders, including schizophrenia (Ruden et al., 2020). In particular, studies have shown that GluN1 ablation on corticolimbic PV interneurons during early postnatal development, but not adulthood, produces brain and behavioral abnormalities consistent with Sz (Belforte et al., 2010; Korotkova et al., 2010; Nakazawa and Sapkota, 2020). It was recently shown that early postnatal GluN1 ablation in corticolimbic interneurons results in a functional disconnection of the ventral hippocampal-mPFC pathway (Alvarez et al., 2020). In addition, numerous pharmacologic and genetic models of NMDAR hypofunction, including the constitutive SRKO mice, display a reduced number of cortical PV interneurons (Cadinu et al., 2018; Steullet et al., 2017). Thus, future studies examining how SR deletion specifically from GABAergic interneurons during early postnatal timepoints affects neuroplasticity and behavior will provide important insights into both normal brain development and disease.

Finally, D-serine is the primary co-agonist for NMDAR-dependent synaptic plasticity (e.g. long-term potentiation and depression) in multiple adult forebrain regions, including hippocampus (Balu et al., 2013; Le Bail et al., 2015; Mothet et al., 2000; Papouin et al., 2012), amygdala (Li et al., 2013), and NaC (Curcio et al., 2013; Liu et al., 2016). Biochemical evidence from *ex vivo* and *in vitro* studies provides evidence for a postsynaptic localization of SR (Balan et al., 2009; Lin et al., 2016; Ma et al., 2014). Using a immunohistochemistry and a single-cell approach to genetically eliminate SR, we recently demonstrated a cell-autonomous role for postsynaptic D-serine in regulating function at hippocampal CA3-CA1 synapses during postnatal development and adulthood (Wong et al., 2020). It will be useful to determine whether D-serine plays an integral role in regulating NMDAR-dependent plasticity across other forebrain regions during postnatal development.

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5. Conclusions

Reductions of D-serine-dependent NMDAR activity have been postulated to contribute to pathophysiological mechanisms governing several neuropsychiatric disorders, including schizophrenia and post-traumatic stress disorder (Balu, 2016; Inoue et al., 2018; Wolosker and Balu, 2020). The spatiotemporal expression of SR, which mimics NMDAR expression, highlights the possibility that D-serine mediated NMDAR activation plays a role in synapse formation and temporal wiring of circuits involved in cognitive, social and emotional behaviors. Future studies will define the transcriptional regulatory mechanisms responsible for the spatiotemporal expression pattern of SR in both excitatory and inhibitory neurons, as well as determine whether SR+ neurons map onto specific circuits that connect limbic brain regions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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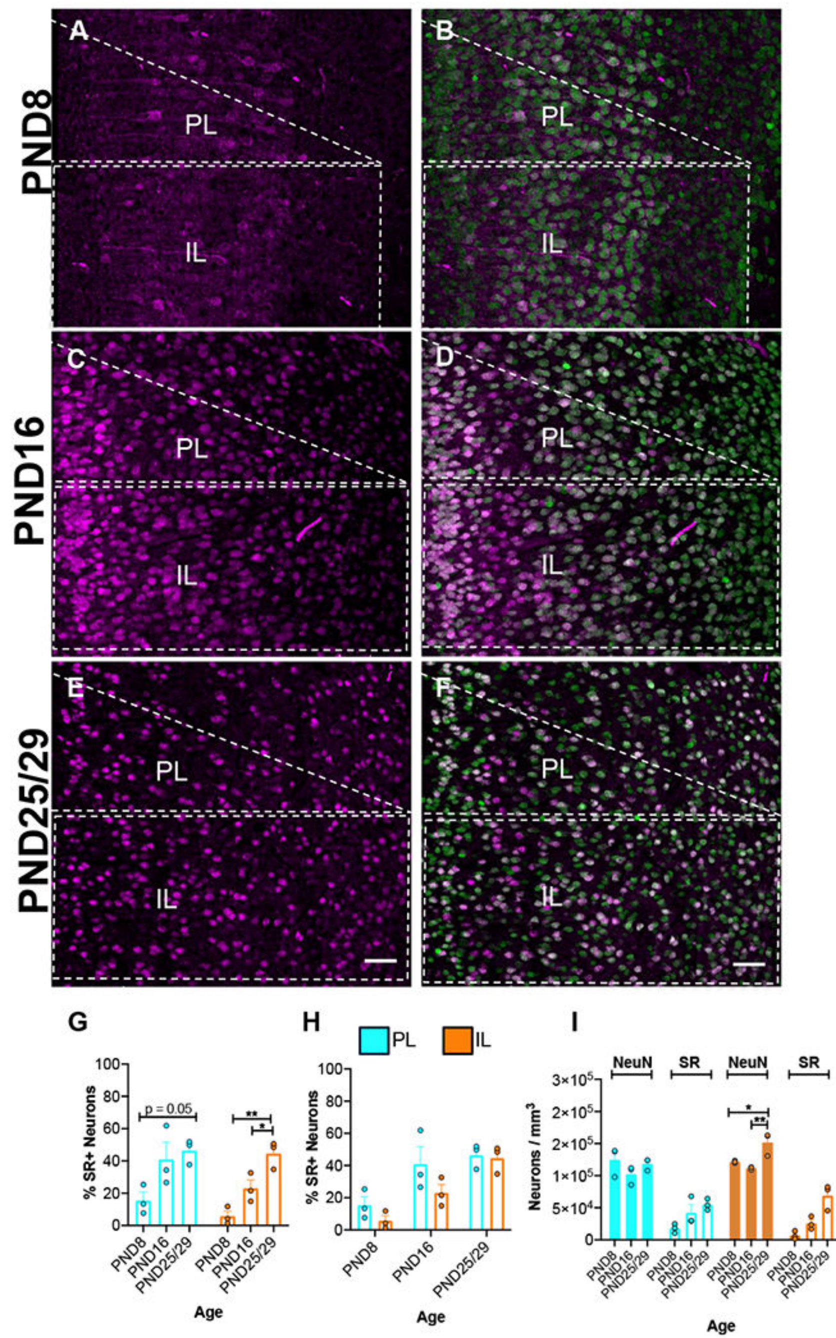

Figure 1.

Quantification of serine racemase expressing neurons in prefrontal cortex regions across postnatal development.

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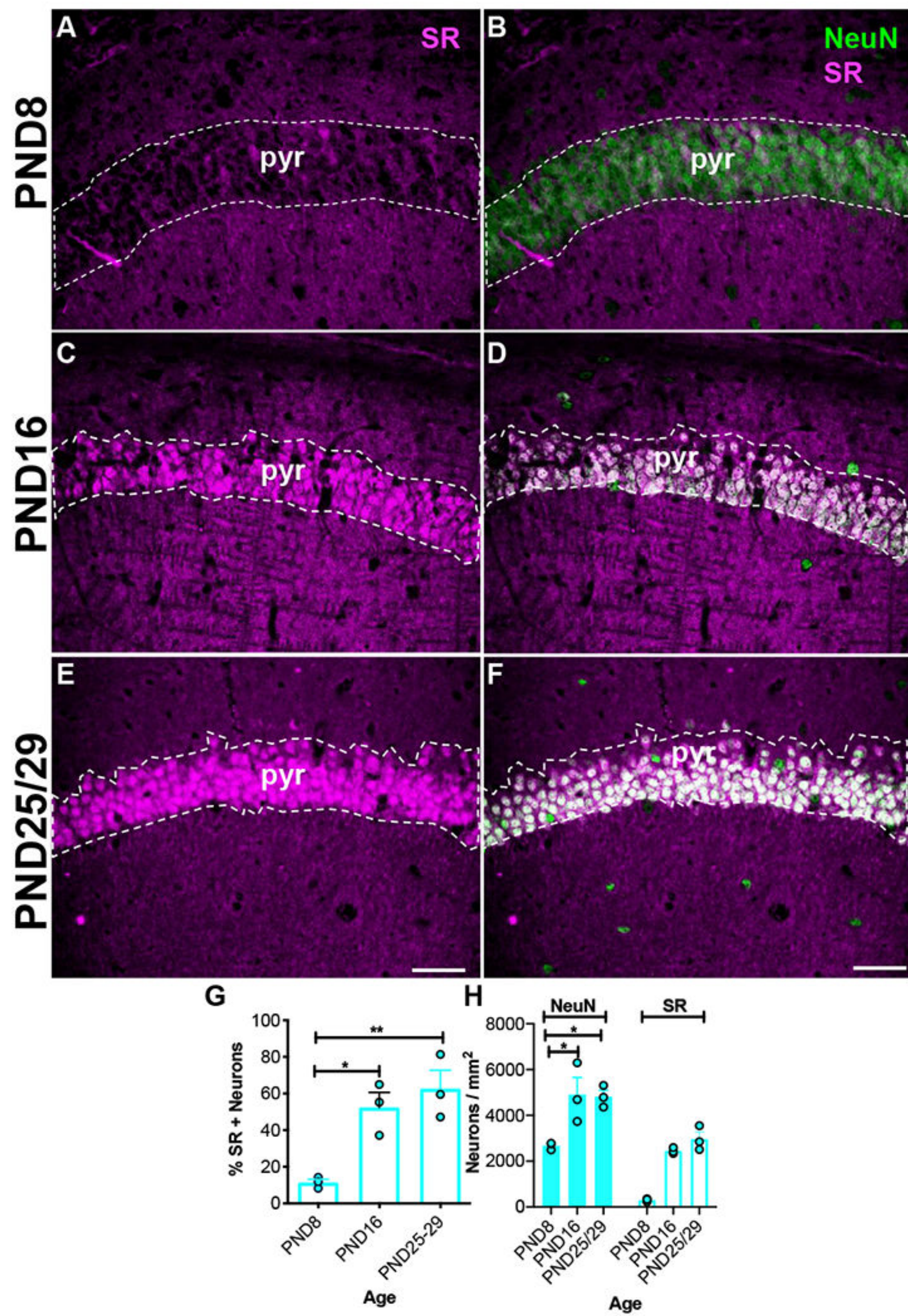

Figure 2.

Serine racemase expressing neurons in hippocampus across postnatal development.

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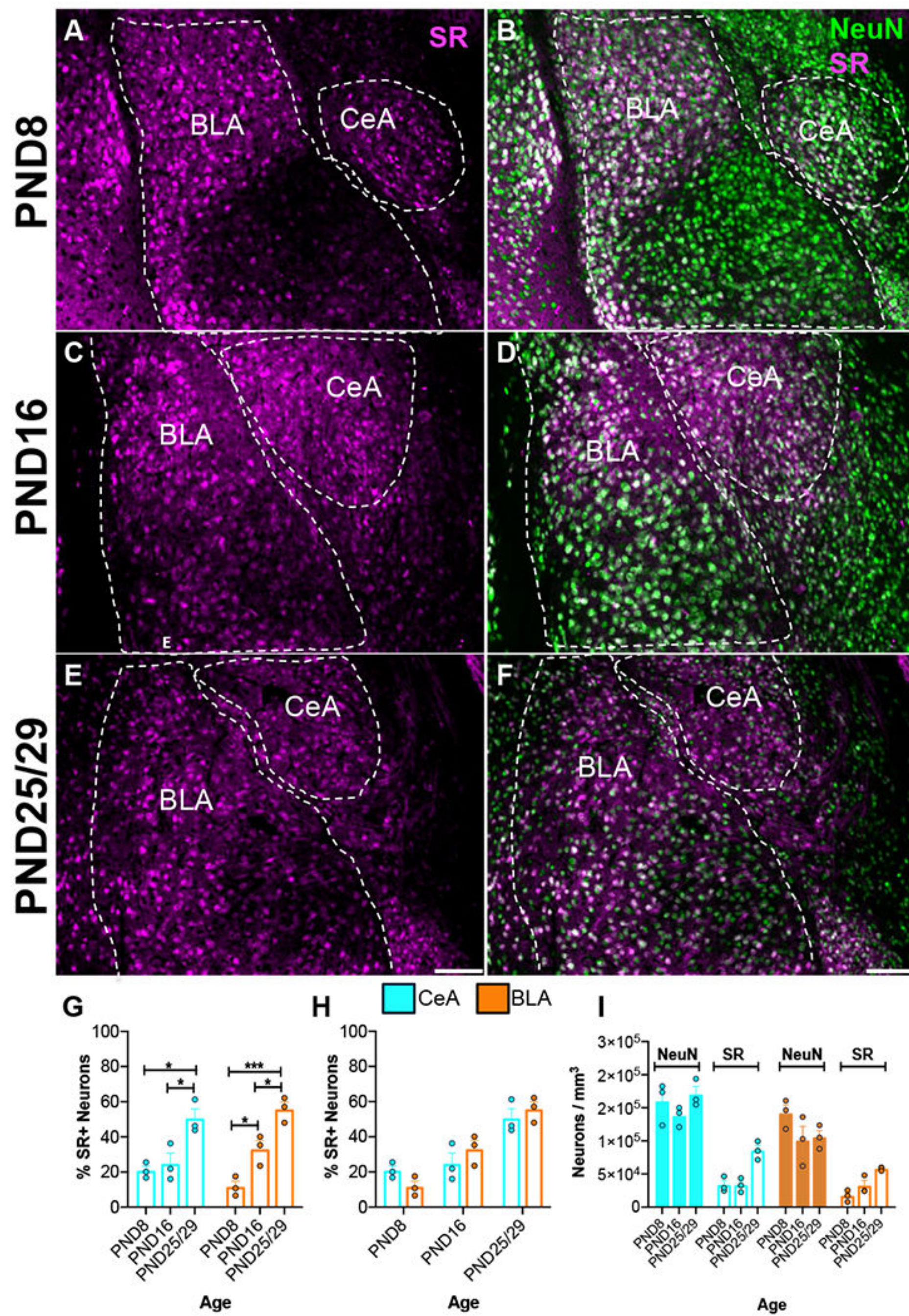

Figure 3.

Quantification of serine racemase expressing neurons in amygdala regions across postnatal development.

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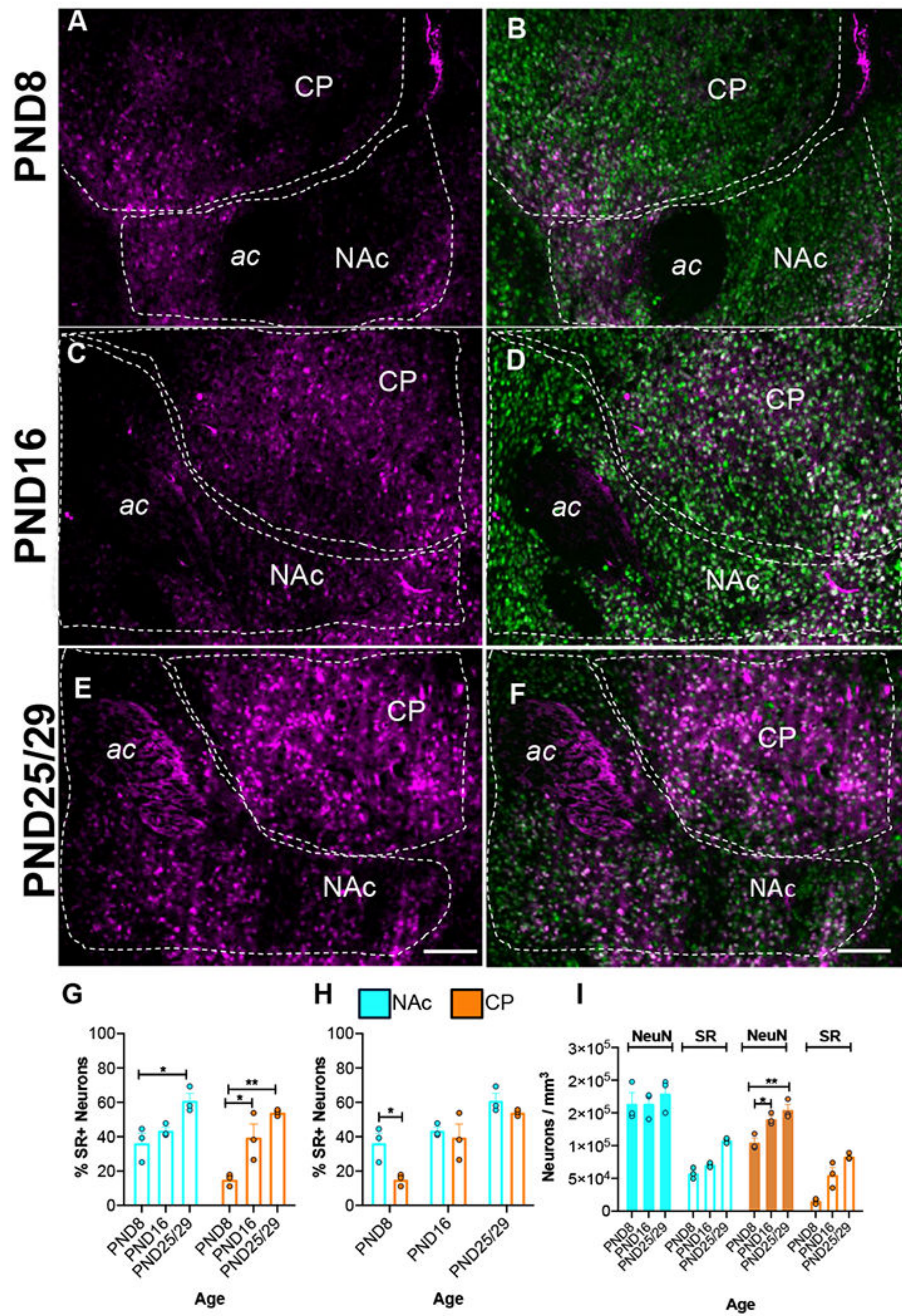

Figure 4.

Quantification of serine racemase expressing neurons in striatal regions across postnatal development

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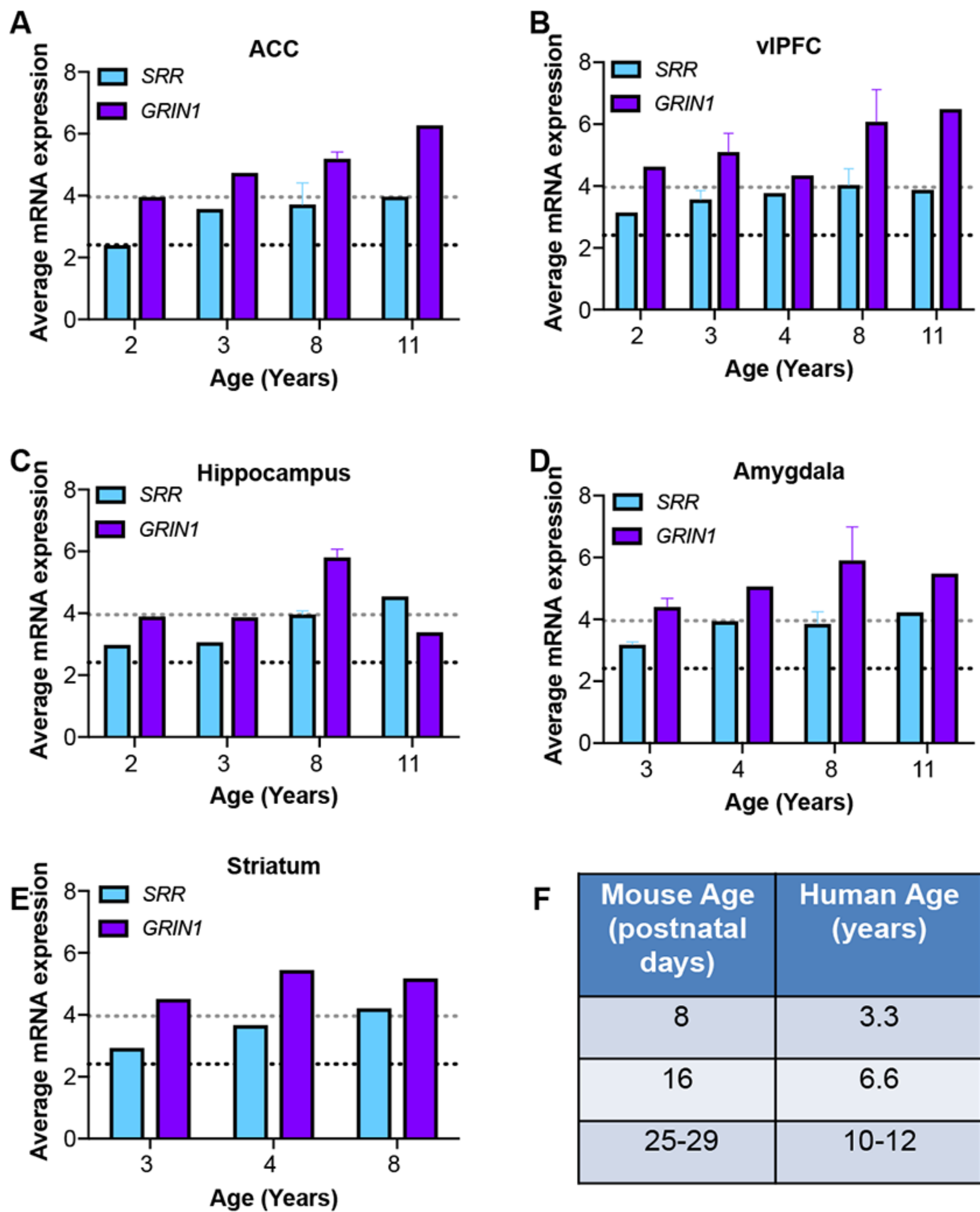

Mouse Age (postnatal days)	Human Age (years)
8	3.3
16	6.6
25-29	10-12

Figure 5.
Expression profile of SRR and GRIN1 in the human developing brain.