# Increased thin-spine density in frontal cortex pyramidal neurons in a genetic rat model of schizophrenia-relevant features

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**Abstract**

The cellular mechanisms altered during brain wiring leading to cognitive disturbances in neurodevelopmental disorders are still unknown. We have previously reported in a genetic animal model of schizophrenia-relevant behavioral features, the Roman-High Avoidance rat strain (RHA-I), altered cortical expression of neurodevelopmentally regulated synaptic markers. To further inquire into this phenotype, we looked at dendritic spines in cortical pyramidal neurons, as changes in spine density and morphology is one of the main processes taking place during adolescence. An HSV-viral vector carrying green fluorescent protein (GFP) was injected into the frontal cortex (FC) of a group of 11 RHA-I and 12 Roman-Low Avoidance (RLA-I) rats. GFP labelled dendrites from pyramidal cells were 3D reconstructed and number and types of spines quantified. We observed an increased spine density in the RHA-I corresponding to a larger fraction of immature thin spines, with no differences in the more mature stubby and mushroom spines. Glia cells, parvalbumin (PV) interneurons and surrounding perineuronal net (PNN) density are known to participate in regulating pyramidal neuron dendritic spine maturation. We determined by stereological-based quantification a significant higher number of GFAP-positive astrocytes in the FC of the RHA-I strain, while no difference in microglia (Iba1-positive cells). The number of inhibitory PV interneurons or PNN density, on the contrary, was unchanged. Results support our belief that the RHA-I strain presents a more immature FC, with some structural features like those observed during adolescence, adding construct validity to this strain as a genetic behavioral model of neurodevelopmental disorders.

**Keywords:** schizophrenia; neurodevelopmental disorders; behavioral animal models; synaptic maturation; brain development; prefrontal cortex

## 1. Introduction

The frontal cortex (FC) remains a focal point for understanding the neuropathology of neurodevelopmental disorders, and more specifically schizophrenia (van Aerde and Feldmeyer, 2015). This area is implicated in cognitive and executive processes that include decision making, attention and working memory (Szczepanski and Knight, 2014;Riga et al., 2014;Chudasama, 2011); all of which are altered in schizophrenia (Thai et al., 2019). At the molecular level, synaptic elements in the FC are dysregulated in individuals with schizophrenia (Coley and Gao, 2019;Focking et al., 2015;Goto et al., 2010), while at the cellular level, the excitatory-inhibitory cortical circuitry, consisting of pyramidal neurons and inhibitory parvalbumin-containing (PV) interneurons is disrupted in schizophrenia brains (Gonzalez-Burgos et al., 2015).

The inbred Roman High Avoidance (RHA-I) is a genetic rat strain presenting schizophrenia-relevant behavioral features (for review see (Giorgi et al., 2019)). This strain, when compared to its counterpart, the inbred Roman Low Avoidance (RLA-I) rat strain and outbred rat strains/stocks taken as external controls, displays consistent behavioral traits indicative of impaired executive function (Fernandez-Teruel et al., 2006;Martinez-Membrives et al., 2015;Oliveras et al., 2015;Lopez-Aumatell et al., 2009;Diaz-Moran et al., 2012;Merchan et al., 2018). Recently, we have demonstrated that the RHA-I strain possesses differences in expression levels of several synaptic markers associated with the neurodevelopmental time window of executive function maturation, suggesting a more immature FC in the RHA-I (Elfving et al., 2019). This is interesting as halted synaptic maturation has been associated with neurodevelopmental disorders (Catts et al., 2013;Selemon and Zecevic, 2015). We wanted to further inquire into this strain-specific synaptic phenotype by focusing on dendritic spine maturation.

Dendritic spines are a major characteristic of pyramidal neurons. These are small protrusions receiving inputs from axonal terminals and have a significant role in regulating neuronal excitability and inter-neuronal communication (Rochefort and Konnerth, 2012;Glausier and Lewis, 2013). Dendritic spine alterations have been reported in multiple cortical areas and specifically on cortical pyramidal neurons in schizophrenia brains (Glantz and Lewis, 2000;Glausier and Lewis, 2013;Konopaske et al., 2014). Dendritic spines dynamically change their shape depending on the short- or long-term plasticity at the synapse (Bourne and Harris, 2007;Holtmaat et al., 2005;Kasai et al., 2003;Kasai et al., 2010). The smaller the spine, the more motile and unstable, with small spines being considered “learning spines” (Kasai et al., 2010). Animal studies show that the density of spines is significantly higher during early adulthood and

is progressively reduced during adulthood via pruning (Tjia et al., 2017;Arnsten, 2011;Silva-Gomez et al., 2003;Holtmaat et al., 2005). Also, thin spines predominate in the human adolescent brain (Holtmaat et al., 2005). During adulthood, thin spines transition to mushroom spines, which are more stable in shape and density (Glausier and Lewis, 2013;Bourne and Harris, 2007). Therefore, spine density and morphology can reflect neuronal development, plasticity and connectivity (Glausier and Lewis, 2013;Kasai et al., 2003;Tjia et al., 2017). An over-representation of small, more unstable spines and/or a decreased proportion of large, more stable spines in the FC is likely causing impaired cognitive functions (Kasai et al., 2010;Kasai et al., 2003). Dendritic spine alterations in cortical pyramidal neurons have consistently been reported in postmortem studies of humans with schizophrenia and related to the cognitive symptoms of the disorder (Glantz and Lewis, 2000;Hill et al., 2006;Black et al., 2004).

Excitatory pyramidal cells receive inhibitory input from PV interneurons, a subtype of GABAergic interneurons (Gonzalez-Burgos et al., 2015). PV projections are important for spine formation in cortical pyramidal neurons (Yin et al., 2013). Glial cells also play an essential role in synaptic regulation (Bernstein et al., 2009). The major type of glia cells, astrocytes, are main components of the "tripartite synapse" (Santello et al., 2019), and as such are involved in synaptic function, plasticity and maturation. Changes in astrocyte number in the PFC have been linked to several mental disorders, including schizophrenia (Lima et al., 2014;Schnieder and Dwork, 2011). Microglia are also involved in synaptic maturation (Mallya et al., 2019). A third important component that regulates synaptic maturation is the perineuronal nets, which are a part of the extracellular matrix that wraps around neuronal structures, especially for PV interneurons (Dzyubenko et al., 2016). Perineuronal nets help shape synaptic networks during development, and participate in synaptic pruning as well as in stabilizing dendritic spines in the adult brain (de et al., 2013).

In the present study we carried out the characterization of the FC of the Roman rats regarding their pyramidal neuron dendritic spine density and morphology. We further looked at cellular components known to be associated with their regulation. Hereby we expect to further characterize the synaptic alterations previously observed in the FC of the RHA-I rat strain. This will help us narrow the gap in the understanding of how specific neurobiological traits are linked to behaviors associated with schizophrenia-like behavior.

## 2. Materials and methods

### *2.1. Animals*

All animals included in this study were male RHA-I and RLA-I rats. Prior to the experiment the animals were housed in pairs of the same strain in macrolon cages (35 x 25 x 15 cm) under controlled conditions of light (12h light-dark cycle), temperature (22±2°C) and humidity (50-70%) with food and water *ad libitum*.

### *2.2. Spine density analysis*

For this analysis, 11 male RHA-I rats and 12 male RLA-I rats, aged approximately 4 months, were used. All surgical procedures were approved by the Universitat Autònoma de Barcelona Animal Care Committee and followed the guidelines of the European Commission on Animal Care. An HSV-GFP viral vector (produced by the Viral Core Facility at the McGovern Institute at MIT) was stereotaxically injected into the frontal cortex according to standard methods (for a more detailed protocol see (Holloway et al., 2016)). Rats were anesthetized with inhalatory isofluorane (4% for induction and 2.5-3% for maintenance) during the surgery. The virus was delivered bilaterally with a Hamilton syringe at a rate of 0.1 µL/min for a total volume of 0.5 µL on each side. The following coordinates were used: +1.6 mm rostrocaudal, −3.8 mm dorsoventral, +/-2.6 mm mediolateral from bregma (relative to dura) with a 10° lateral angle (Paxinos G and Watson C, 1998).

Three days after surgery animals were deeply anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg) and perfused transcardially with 4% PFA in PBS. Brains were removed, immersion-fixed in 4% PFA in PBS at 4°C for 24h and stored in 30% sucrose for at least 48h. Brains were sectioned in 40 µm thick coronal sections using a vibratome (Leica VT1000S). The free-floating coronal brain sections were washed with PBS and incubated in 10% normal goat serum with 0.1% Triton X-100 in PBS for 60 min at 4°C followed by overnight incubation with the anti-GFP primary antibody (Invitrogen A-11122, 1:1000). The sections were rinsed in PBS for 3 x 10 min and incubated for 1 hour with Alexa Fluor-488 conjugated goat anti-rabbit antibody (ThermoFisher Sciencific A-11034, 1:2000). After washing sections three times with PBS they were mounted on glass slides and examined by confocal fluorescence microscopy (Zeiss LSM700, Carl Zeiss).

For the spine analysis, apical dendritic segments 50–150 µm away from the soma were randomly chosen from HSV infected neurons expressing GFP. Images were taken from pyramidal neurons, characterized by their triangular shape, in frontal cortical layers II/III. The following requirements had to be fulfilled for a dendritic segment to be analyzed: (i) the segment had to

be filled with the GFP signal completely, (ii) the segment had to be at least 50 µm from the soma and (iii) the segment should not overlap with other dendritic branches. Dendritic segments were 3D-imaged using a 63× lens (numerical aperture 1.46; Carl Zeiss) and a zoom of 2.0. Pixel size was 0.03 µm in the xy plane and the step size was 0.01 µm in the z plane. Images were taken with a resolution of 1024 × 1024, the pixel dwell time was 1.27 µm/s and the line average were set to 1.

We analyzed an average of 2-3 dendrites per neuron from a total of 8-10 neurons per animal. For quantitative analysis of spine size and shape, NeuronStudio was used with the rayburst algorithm described previously (Wearne et al., 2005). NeuronStudio classifies spines as stubby, thin or mushroom on the basis of the following parameters: (i) aspect ratio, (ii) head-to-neck ratio and (iii) head diameter. The counting was done by an investigator blinded to the two groups of rats.

### *2.3. Stereological quantification of parvalbumin neurons and glia cells*

A new cohort of 12 RHA-I and 11 RLA-I were perfused with 4% PFA. Following extraction, the brains were post-fixed in 4% PFA and 30% sucrose and then frozen in isopentane and sectioned into 80 µm thick coronal sections. Following systematic random sampling principles (Gundersen and Jensen, 1987), sections were collected in six parallel series through the most frontal part of the brain until Bregma level +0.02 mm. The free-floating 80 µm sections were rinsed for 2 x 10 min in PBS, incubated for 30 min in 3% $H_2O_2$ in PBS and rinsed in distilled water ($dH_2O$) for 3 x 5 minutes. The samples hereafter underwent demasking for 30 minutes at 90-95°C using 10% Target Retrieval Solution in PBS followed by 20 minutes at room temperature (Agilent (Dako), Santa Clara, CA, USA; pH 9 (S 2367) or pH 6 (S 1699)). After washing sections in PBS with 1% Triton X-100 (PBS-TX) 3 x 10 min and subsequently incubated for 60 min in PBS with 10% fetal calf serum (FCS), they were incubated at 4°C for 2-3 days with either a monoclonal mouse anti-PV antibody (Sigma P3088, 1:10,000), monoclonal rabbit anti-GFAP antibody (Abcam ab68428, 1:1000) or monoclonal rabbit anti-Iba 1 antibody (Abcam ab178846, 1:5000) diluted in 10% FCS/PBS. Sections were then washed in PBS-TX 3 x 10 min at RT and incubated for 2-3 days at 4°C with the secondary EnVision horseradish peroxidase (HRP) anti-mouse/anti-rabbit antibody (Agilent (DAKO), Santa Clara, CA, USA) diluted 1:10 in PBS-TX. Finally, sections were transferred to PBS, washed 5 x 10 min, and pre-incubated for 7 min in a solution of 0.01%/0.05% diaminobenzidine (DAB, Sigma Aldrich, St. Louis, MO, USA) in PBS and then for 10 min after addition of 30% $H_2O_2$. Sections were mounted and counterstained with cresyl violet (Sigma Aldrich) solution and coverslipped using Pertex glue (Histolab, Göteborg, Sweden).

The number of PV, GFAP and Iba1 positive cells was estimated by the optical fractionator stereological method by use of the NewCAST software (Visiopharm, Hoersholm, Denmark). The counting procedure was performed bilaterally in the mPFC, which includes the infralimbic cortex (IL), prelimbic cortex (PrL) and cingulate cortex (Cgl). The area was delineated using NewCAST software (Visiopharm, Hoersholm, Denmark) following the Paxinos & Watson rat brain atlas ($5^{th}$ edition). The most frontal section included was at Bregma +5.16 mm and the last section at Bregma +2.52 mm (Fig. 1). The counting was performed by an investigator blinded to the two groups of rats.

### *2.4. Perineuronal net screening*

A new cohort of 7 male RHA-I and 8 male RLA-I rats were sedated with 5% inhaled isoflurane and euthanized by decapitation at approximately 8 months of age, 3 weeks after they had completed a series of behavioral tests (Sanchez-Gonzalez et al., 2019). Their brains were dissected, frozen in liquid nitrogen and stored at -80°C.

The brains were sectioned into 10 µm coronal sections and collected on glass slides that were kept at -80°C. The brain sections were fixed by submerging them in -20°C acetone for 10 minutes followed by 10 minutes air drying at RT. The sections were washed in PBS for 2 x 5 minutes and incubated in 0.3% $H_2O_2$ for 10 minutes followed by rinsing in PBS for 2 x 5 minutes. The sections were then incubated at RT for 1 hour with lectin from Wisteria floribunda (WFA) biotin conjugate (Sigma-Aldrich L1516, 1:200) diluted in PBS followed by 2 x 5 minutes in PBS. Hereafter, the sections were incubated for 1 hour at RT with Streptavidin-HRP (abcam ab7403, 1:500) diluted in PBS and rinsed in PBS for 2 x 5 minutes. Sections were then incubated in DAB + $H_2O_2$ (25 µg DAB + 25 µl $H_2O_2$ in 50 ml PBS) for 15 minutes followed by 5 minutes in distilled water. Sections were counterstained with Mayer's hematoxylin and coverslipped using Pertex glue (Histolab, Göteborg, Sweden).

For the image analysis, 10 images of neurons with WFA staining of their perineuronal nets were acquired from the mPFC of each of the 15 brains using an Olympus BX50 microscope (Olympus, Ballerup, Denmark) and a 40X oil immersion objective with a numerical aperture (NA) of 1.0. Images on screen were visualized by a Basler camera (Basler, Ahrensburg, Germany) with a final on-screen magnification of 925X. In FIJI (ImageJ) the option "Color deconvolution" with the vector H DAB chosen as the stain was used on the images. The image option showing only the DAB staining was then used to measure the "Mean grey value" by drawing around randomly selected neurons manually. A mean staining intensity was then calculated for all the neurons in

each of the 15 brains. The analysis was performed by an investigator blinded to the two groups of rats.

### *2. 5. Statistical analysis*

All the analyses were performed with the Statistical Package for Social Science software (SPSS) or GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). For the analyses of total spine density, cell numbers and perineuronal nets we performed unpaired Student's t-test for independent groups. For the analysis of the dendritic spine types we performed repeated measures ANOVA with the strain as between-subject factor and the three types of spines as within-subject factors followed by Sidak's post-hoc analysis. Before the analysis, all data was tested for outliers by the Grubb's test, and no statistically significant outliers were identified. The data passed the test for normality, and the F-test to compare variances showed no significant difference between the variances in all cases. P-values equal to or less than 0.05 were considered statistically significant.

## 3. Results

### *3.1. Increased thin dendritic spine density in the RHA-I*

A homogenous distribution of HSV-GFP positive pyramidal neurons (characterized by their triangular shape) was observed in cortical layers II/III (Fig. 2A). Labelled dendrites possessed stubby, thin and mushroom spines, as illustrated in Fig. 2B. For the analysis of dendritic spine quantification, we created the variable "density" for the total number of spines and for each of the three types of spines (stubby, thin or mushroom). Density was calculated as the number of spines per µm of the labelled dendrite. There was a significantly higher number of total spines in the RHA-I when compared to the RLA-I (df=21, p=0.014) (Fig. 3A). We then performed a repeated measures ANOVA which revealed an interaction "type x strain" effect ($F(2,42)=6.091$, $p=0.048$), "spine type" ($F(1.030, 21.63)=68.96$, $p<0.0001$) and a "strain" effect ($F(1,21)=7.102$, $p=0.014$). There was no "dendrite" effect ($F(21,42)=0.727$, $p=0.78$). Sidak's post-hoc analysis revealed a significant effect of the "thin" type of spines (df=19.91, p=0.04) (Fig. 3B).

### *3.2. No difference in number of parvalbumin-positive interneurons*

PV immunopositive interneurons were evenly distributed across the FC (Fig. 4A). Only PV-positive cell bodies were quantified. Total number of PV-positive neurons was estimated by stereology in RLA-I (n=11) and RHA-I (n=12) (Table 1). When comparing both groups, no difference in total number of PV interneurons was found (df=21, p=0.7) (Fig.5A).

### *3.3. Increase number of astrocyte and no difference in microglia*

After immunostaining the same twenty-three brains for GFAP, we performed an initial blinded screening and found a clear group-specific distinction (Fig. 4C,D). Based on this, we reasoned that five brains per group for the stereological quantification would give us enough power. Total number of GFAP positive cells were stereologically estimated (Table 2) and when statistically comparing both groups, we obtained a significantly higher number of GFAP reactive astroglia cells in the mPFC of the RHA-I (df=8, p=0.01) (Fig 5B). We then immunostained parallel sections of the same ten brains for Iba-1 (Fig. 4E, F) and performed a stereological quantification for the estimation of total microglia numbers (Table 3). When statistically comparing the groups, we found no significant differences in microglia (df=8, p=0.58) (Fig.5C).

### *3.4. No difference in perineuronal nets density*

We stained a new set of rat brains (7 RHA-I and 8 RLA-I) for Wisteria floribunda agglutinin (WFA) as a marker of perineuronal nets. Looking at the mPFC, we could detect the classical staining around the neuronal cell body and initial dendritic segments (Fig. 6A). However, when using densitometry to quantify the staining intensity, we did not find a significant difference between the RLA-I and RHA-I rats (Fig. 6B) ($df=13$, $p=0.58$).

## 4. Discussion

In this study we found differences in synaptic density between the Roman rat strains, with the RHA-I presenting an increased number on cortical pyramidal neurons of thin dendritic spines, which are considered more immature (Holtmaat et al., 2005). This is accompanied by an increased number of astrocytes. These results further support our idea of a more immature synaptic state in the FC of the RHA-I strain (Elfving et al., 2019). This was not accompanied by differences in the number of microglia and PV interneurons, or in perineuronal net density surrounding the neurons.

PFC-related deficits have previously been described for the RHA-I rats. When compared to their RLA-I counterparts, they present decreased cortical activity (Meyza et al., 2009;Tapias-Espinosa et al., 2019), reduced prefrontal cortex volume (Rio-Alamos et al., 2019;Sanchez-Gonzalez et al., 2019) and neurotransmitter receptor alterations (Klein et al., 2014;Fomsgaard et al., 2018). Unbalanced dendritic spine dynamics in this strain, shown here by an increased number of immature dendritic spines, may be underlying this FC dysfunction. There is consensus that a more immature, impaired cortical function and connectivity is one of the main features of schizophrenia and is associated with the cognitive deficits associated with this disorder (Chari et al., 2019;Pu et al., 2019;Hagihara et al., 2014). The RHA-I present many of these cognitive deviations, such as greater impulsive behavior (Klein et al., 2014), latent inhibition deficits (Fernandez-Teruel et al., 2006) and impaired spatial working memory and reference learning/memory (Martinez-Membrives et al., 2015;Oliveras et al., 2016;Oliveras et al., 2015). This may point to an altered maturation state of the FC in the RHA-I.

Supporting the observations of the present study, we have previously reported increased expression levels of the NMDA receptor subunit GluN2B in the FC of the RHA-I rats, while no differences in AMPA receptor subunits were observed (Elfving et al., 2019). Thin spines, like the ones observed in the RHA-I strain, express NMDA but not AMPA receptors (Kasai et al., 2010;Kasai et al., 2003). In contrast, AMPA receptors are mostly associated with large spines, represented by the more stable stubby and mushroom types (Glausier and Lewis, 2013;Kasai et al., 2003). the GluN2B receptor is associated with spine density regulation (Liu et al., 2017) and with “silent synapses” (Han and Heinemann, 2013). Thin spines are considered “silent synapses” since they are transient and are involved in rapid plasticity (Glausier and Lewis, 2013;Kasai et al., 2010). Therefore, our previous observation of an increased cortical expression of GluN2B in the RHA-I corresponds very well with an increased number of thin spines observed in this study, and further supports the general idea of an involvement of this receptor in synaptic regulation (Shen

et al., 2011). Also observed in the RHA-I was the upregulation in FC of other known regulators of dendritic spine morphogenesis (Elfving et al., 2019), such as the scaffolding protein Homer 1 (Sala et al., 2001) and the neurotrophin BDNF (Tyler and Pozzo-Miller, 2003). Homer 1 exerts an activity-dependent negative control on the density and size of dendritic spines (Sala et al., 2003) and BDNF regulates Homer 1 expression (Ji et al., 2010).

We know that the RHA-I rats have a stop codon in their DNA that results in the absence of the glutamate metabotropic receptor 2 (mGluR2) protein (Wood et al., 2017;Fomsgaard et al., 2018). The absence of mGluR2 could be directly related to their more immature dendritic spine phenotype. Expression of this receptor is regulated during postnatal brain development, specifically during time windows of synaptic maturation (Meguro et al., 1999;Defagot et al., 2002;Catania et al., 1994). The mGluR2 is involved in long term depression and spine elimination (Ramiro-Cortes and Israely, 2013). Hence, it would be expected that absence of mGluR2 would be reflected in increased spine density as seen here. The mGluR2 is also important for astroglia sensing of glutamatergic tonus (Tang and Kalivas, 2003). Astrocytes participate in the stabilization (Bernardinelli et al., 2014) and maturation of excitatory synapses (Reemst et al., 2016) and thus the increased number of astrocytes observed in the PFC of the RHA-I rats could be associated with the dendritic spine alterations in this strain. The increase in astrocytes may be a compensatory mechanism to help stabilize a larger number of thin spines in the RHA-I strain, but their inability to sense glutamate levels, due to lack og mGluR2 may also be leading to increased astroglia release of D-serine, a co-agonist of the NMDA receptor (Meunier et al., 2017), and by that to overstimulation of the GluN2B containing receptors, maintaining the spines in a more “immature” state. Dysfunction of astroglia during brain development has indeed been linked to neurodevelopmental disorders (Reemst et al., 2016). This idea is sustained by the fact that we only observed alterations in astroglia and not microglia cells. Microglia is involved in synaptic pruning and synaptic elimination (Mallya et al., 2019). The RHA-I shows increased and not reduced number of dendritic spines which corresponds well with no increase in microglia cells.

According to our observations, the increased dendritic spine density in pyramidal neurons is not a consequence of differences in number of PV-containing GABAergic neurons in the RHA-I, as we found no significant strain differences in numbers of interneurons in the FC. This does not exclude that there may be differences in number of synaptic contacts between PV and pyramidal neurons. In schizophrenia brains it is reported lower PV mRNA expression and density of PV-immunoreactive puncta while intact number of PV interneurons (Chung et al., 2016;Glausier et

al., 2014;Enwright et al., 2016;Beasley et al., 2002). It has been suggested that PV interneuron immaturity may be the underlying cause of PV-neuron dysfunction in schizophrenia (Hagihara et al., 2014). PV interneurons express *ErbB4*, the receptor for the trophic factor neuregulin 1 (*Nrg1*) (Yin et al., 2013;Agarwal et al., 2014). *Nrg1* increases spine density in pyramidal neurons through the *ErbB4* (Yin et al., 2013;Cahill et al., 2013). Unbalanced *Nrg1/ErbB4* signaling leads to dendritic spine destabilization and glutamatergic hypofunction (Li et al., 2007). Interestingly, there is increased expression levels of *Nrg1* in the PFC of the RHA-I rats (Elfving et al., 2019), which could explain the increased spine density observed. It could be of interest to investigate whether the RHA-I strain also show PV neuron specific *ErbB4* dysfunction. *ErbB4* signaling is required for attention (Tan et al., 2018), and as attentional deficits are one of the main behavioral features of the RHA-I (Klein et al., 2014;Oliveras et al., 2015) it would be no surprise if there was an impaired *Nrg1/ErbB4* signaling in this strain.

As expected, there was no apparent difference in the amount of perineuronal nets in the FC between the two rat strains. Perineuronal nets are primarily associated with PV-interneurons (Shi et al., 2019) and as we did not see differences in number of PV-neurons between the strains, it is no surprise to observe no differences in perineuronal nets. We cannot exclude that small differences were overseen. The perineuronal net density changes reported in schizophrenia brains are very small (Alcaide et al., 2019). Nevertheless, it seems that the apparent immature state of the PFC of the RHA-I rats regarding increased number of thin dendritic spines and astrocytes are not attributed to a significant alteration in perineuronal nets.

To conclude, the present findings add further construct validity of the RHA-I rat strain as a neurodevelopmental genetic animal model. Our observations point to a more immature endophenotype of the FC in this strain that may undelay their neurobehavioral profile. Similar neurobiological features are observed during adolescence.

## 5. Tables

**Table 1. Precision of estimates – Parvalbumin-IR in mPFC**. Numerical estimation of the mean number of Parvalbumin (PV) immune-positive neurons in the mPFC of RHA-I and RLA-I rats.

| | Mean (N) | Range (N) | Mean ($Q^-$) | Range ($Q^-$) | Mean (P) | Range (P) | CE (N) | CV (N) |
|---|---|---|---|---|---|---|---|---|
| **RHA-I (n=12)** | 70035 | [31009-106729] | 189 | [83-297] | 191 | [138-231] | 0.079 | 0.318 |
| **RLA-I (n=11)** | 73642 | [34146-109226] | 199 | [90-284] | 215 | [165-238] | 0.077 | 0.323 |

CE: Coefficient of error; CV: Coefficient of variation; IR: Immunoreactivity; mPFC: Medial prefrontal cortex; N: Total number of cells; P: Number of frames counted; $Q^-$: Number of cells counted

**Table 2. Precision of estimates – GFAP-IR in mPFC.** Numerical estimation of the mean number of GFAP immune-positive astrocytes in the mPFC of RHA-I and RLA-I rats**.**

| | Mean (N) | Range (N) | Mean ($Q^-$) | Range ($Q^-$) | Mean (P) | Range (P) | CE (N) | CV (N) |
|---|---|---|---|---|---|---|---|---|
| **RHA-I (n=5)** | 96368 | [46301-144128] | 126 | [61-201] | 149 | [143-173] | 0.099 | 0.439 |
| **RLA-I (n=5)** | 30339 | [15504-50814] | 41 | [21-68] | 153 | [144-166] | 0.176 | 0.484 |

CE: Coefficient of error; CV: Coefficient of variation; GFAP: Glial fibrillary acidic protein; IR: Immunoreactivity; mPFC: Medial prefrontal cortex; N: Total number of cells; P: Number of frames counted; $Q^-$: Number of cells counted

**Table 3. Precision of estimates – Iba1-IR in mPFC.** Numerical estimation of the mean number of Iba1 immune-positive microglia in the mPFC of RHA-I and RLA-I rats**.**

| | Mean (N) | Range (N) | Mean ($Q^-$) | Range ($Q^-$) | Mean (P) | Range (P) | CE (N) | CV (N) |
|---|---|---|---|---|---|---|---|---|
| **RHA-I (n=5)** | 79430 | [69525-99260] | 137 | [122-169] | 69 | [61-79] | 0.087 | 0.158 |
| **RLA-I (n=5)** | 74417 | [55655-88059] | 133 | [104-156] | 59 | [49-66] | 0.089 | 0.197 |

CE: Coefficient of error; CV: Coefficient of variation; Iba1: Ionized calcium-binding adapter molecule 1; IR: Immunoreactivity; mPFC: Medial prefrontal cortex; N: Total number of cells; P: Number of frames counted; $Q^-$: Number of cells counted

## 6. Figure Legends

**Figure 1. Brain section diagrams.** Coronal brain section diagrams of the medial prefrontal cortex from which sections were sampled (highlighted area) comprising the infralimbic cortex, prelimbic cortex and cingulate cortex. Ranging from Bregma 5.16 mm (most anterior) to Bregma 2.52 mm (most posterior). Brain section diagrams are modified from Paxinos & Watson rat brain atlas (5th edition).

**Figure 2. A. Representative HSV-GFP injections**. Left panel: field of the site of infection at 10x magnification. Right panel: detail of an infected pyramidal neuron at 63x magnification. **B.** Representative 3D reconstructions. Dendrite and dendritic spines from a pyramidal neuron in the PFC of a RHA-I rat (left panel) and a RLA-I rat (right panel). The white arrows indicate thin spines in panel A and mushroom spines in panel B.

**Figure 3. Spine densities.** Mean +SEM of spine density. **A.** Total spine density. **B.** Spine density for each of the different types of spines: stubby, thin and mushroom spines. Spine density was calculated from the number of quantified dendritic spines in a constant length of 50 µm of 2-3 dendrites per neuron from a total of 8-10 neurons per animal in a sample of n=11 RHA-I and n=12 RLA-I, *p<0.05.

**Figure 4**. **Representative images of parvalbumin and glial cells in FC**. **A.** Parvalbumin cells distributed in cortical layers and **B** two PV interneurons at higher magnification. Astrocytes in an RHA-I (**C** ) and in a RLA-I (**D)** rat. The same is shown for microglial cells, in an RHA-I (**E**) and RLA-I (**F**) rat.

**Figure 5**. **Total cell numbers in FC**. Mean ± SEM of the total number of cells. **A.** Parvalbumin interneuron number estimation (n=11+12), **B.** Astrocytes number estimation (n=5+5), **C.** Microglia number estimation (n=5+5) RHA-I/RLA-I, ** p ≤ 0.01.

**Figure 6**. **Perineuronal nets in FC**. **A.** Representative images of the perineuronal nets in the FC in a RHA-I rat. **B.** Mean ± SEM of the mean WFA intensities in the PFC of the RLA-I/RHA-I rats (n=8+7).

## 7. References


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