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Perinatal Asphyxia Reduces the Number of Reelin Neurons in the Prelimbic Cortex and Deteriorates Social Interaction in Rats

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Key Words
Perinatal asphyxia · Social interaction · Schizophrenia · Reelin

Abstract
Obstetrical complications of perinatal asphyxia (PA) can often induce lesions that, in the long-term, manifest as schizophrenia. A deterioration of the medial prefrontal cortex (mPFC) and a reduction in the number of GABAergic neurons are commonly observed in the pathophysiology of schizophrenia. In this study, we investigated the link between PA, reelin and calbindin diminution and psychiatric diseases that involve social interaction deficits. This was achieved by observing the effect of 19 min of asphyxia on both subpopulations of GABAergic neurons. PA was produced by water immersion of fetus-containing uterus horns removed by cesarean section from ready-to-deliver rats. PA generated a significant and specific decrease in the number of reelin-secreting neurons in mPFC layer VI [$F(2, 6) = 8.716$, $p = 0.016$; PA vs. vaginal controls (VC), $p = 0.03$, and PA vs. cesarean controls (CC), $p = 0.022$]. This reduction reached approximately 60% on average. Changes in the percentage of reelin neurons including all the cortex layers did not achieve a significant outcome but a trend: CC % 10.61 ± 1.34; PA % 8.64 ± 1.71 [$F(2, 6) = 1.299$, $p = 0.33$]. In the case of calbindin, there was a significant decrease in cell density in the PA group [2-way repeated-measures ANOVA, $F(1, 4) = 13.03$, $p = 0.0226$]. The multiple-comparisons test showed significant differences in the superficial aspect of layer II (Sidak test for multiple comparisons CC vs. PA at 200 µm: $p = 0.003$). A small, but significant difference could be seen when the distance from the pia mater to the start of layer VI was analyzed (CC mean ± SEM = 768.9 ± 8.382; PA mean ± SEM = 669.3 ± 17.75; $p = 0.036$). Rats exposed to PA showed deterioration in social interactions, which manifested as a decrease in play soliciting. In this model, which involved severe/moderate asphyxia, we did not find significant changes in locomotive activity or anxiety indicators in the open field task. The loss of reelin neurons could be conducive to the shrinkage of the prelimbic cortex through the reduction in neuropil and the deterioration of the function of this structure.

Introduction

Obstetric complications have been linked to a wide variety of psychiatric disorders [1, 2]. These complications include perinatal asphyxia (PA), a deficient oxygen sup-

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ply to the fetus during or immediately before or after birth. Nerve tissue has a special susceptibility to hypoxia, resulting in neurological damage normally affecting half of the surviving individuals. Well-established consequences of PA include: attention deficit hyperactivity disorder, epilepsy, mental retardation and cerebral palsy or spasticity which manifests in the short and long term [3, 4]. Most of this deficits have been replicated in animal models including rodents and nonhuman primates [5–7]. Furthermore, retrospective clinical studies indicate that obstetrical complications in general, and PA in particular, could also induce lesions that manifest as schizophrenia in later life [8–10]. There is an increasing amount of evidence indicating that schizophrenia is the result of multiple factors that include genetics, neurodevelopment and psychological and social processes [11, 12]. In fact, it is known that the probability of developing schizophrenia after PA increases 5-fold [13, 14]. Considering all of the above, some authors propose that PA in rodents could be a valuable animal model for studying some aspects that appear in schizophrenia [15, 16].

The pathophysiology of schizophrenia includes decreases in reelin and a strong deterioration of the medial prefrontal cortex (mPFC) [17]. Reelin is a large secreted protein of the extracellular matrix. During development, reelin is crucial for the correct cytoarchitecture of laminated brain structures and is produced by the Cajal-Retzius neurons, mainly located in layer I. After birth, most of these cells degenerate, but reelin expression persists in the postnatal and adult brain. Reelin is also involved in synaptic plasticity in the adult where it is secreted by GABAergic neurons [18, 19]. Interestingly, PA has been shown to decrease reelin expression in the hippocampus in mice [20].

The PFC participates in many higher brain functions, including mnemonic processes, attention and action planning, decision-taking, behavioral inhibition, control of emotional signals and social interaction and play [21, 22].

It is also known that social interaction is one of the most susceptible behaviors to damage in the central nervous system [23], and its deterioration is one of the first symptoms of schizophrenia and other psychiatric diseases, such as autism [24]. In schizophrenia, impairments in social interaction are included in the negative symptoms alongside affective flattening, apathy and anhedonia [25]. These symptoms are usually prior to the onset of positive symptoms [26, 27].

Considering all this evidence, it seems possible that there is a link between PA, reelin diminution and psychiatric diseases that involve social interaction deficits. In this direction, we have decided to analyze the effect of PA upon the pattern of immunolabeled reelin and calbindin neurons in the prelimbic cortex of the rat. We also analyzed the effect of PA on social behavior, anxiety and locomotor activity. This was achieved by observing the effect of 19 min of PA in 2 behavioral paradigms, the open field task (OF) and the social interaction task (SI).

Methods

Animals

PA was induced using a noninvasive model of hypoxia-ischemia, as described [28] in Sprague-Dawley albino rats, with sanitary certification from the animal facility of our institution. The animal model and care were approved by the CICUAL (Comité Institucional para el Uso y Cuidado de Animales de Laboratorio, resolution No. 2079/07) and followed the principles presented in the 'Guidelines for the Use of Animals in Neuroscience Research' by the Society for Neuroscience. Appropriate proceedings were performed to minimize the number of animals used and their suffering/discomfort.

Animals were kept at 24°C, with light/dark cycles of 12/12 h and food and water ad libitum. The pregnant rats were killed by decapitation and immediately hysterectomized after their first pup was delivered vaginally. Full-term fetuses, still inside the uterus, were subjected to asphyxia performed by transient immersion of both uterine horns in a water bath for 19 min at 37°C (i.e. PA). After asphyxia, or immediately after hysterectomy in the case of cesarean control (CC) pups, the uterine horns were opened. The pups were removed and stimulated to take their first breath. The pups were then placed for recovery and given to a surrogate mother. To avoid hormonal variations, only male rats were included in this study. Pups were weaned on postnatal day 25 and male siblings were housed together.

A total of 33 rats, all of them 30 days old, were separated into 3 groups, the vaginal control (VC) group, i.e. pups delivered by natural vaginal birth, the CC group and the PA group. For each experimental group, the offspring of 4 dams were used in order to minimize possible confounders produced by siblings.

Behavioral Studies

To decrease anxiety levels during the tests, the animals were handled for 2 or 3 min daily for 5 days. The images of the OF and SI experiments were taken with a Logitech high-resolution camera connected to a laptop. The arena is an 84 × 84 cm wooden box painted with waterproof lacquer and divided into 9 quadrants. The box was carefully cleaned with 70% alcohol between experiments. The room was maintained under dim light (10 lux).

Open Field. Animals were given a single trial and their behavior was recorded for 5 min [29–31]. The following parameters were measured: entries and time spent in the central quadrant and total quadrant crossings.

Social Interaction Studies. After the OF, the rat was removed from the arena and housed for 24 h in isolation (n = 10 per group or n = 11 in the case of play soliciting). A control rat of the same sex and age, which was not familiar with the experimental rat, was

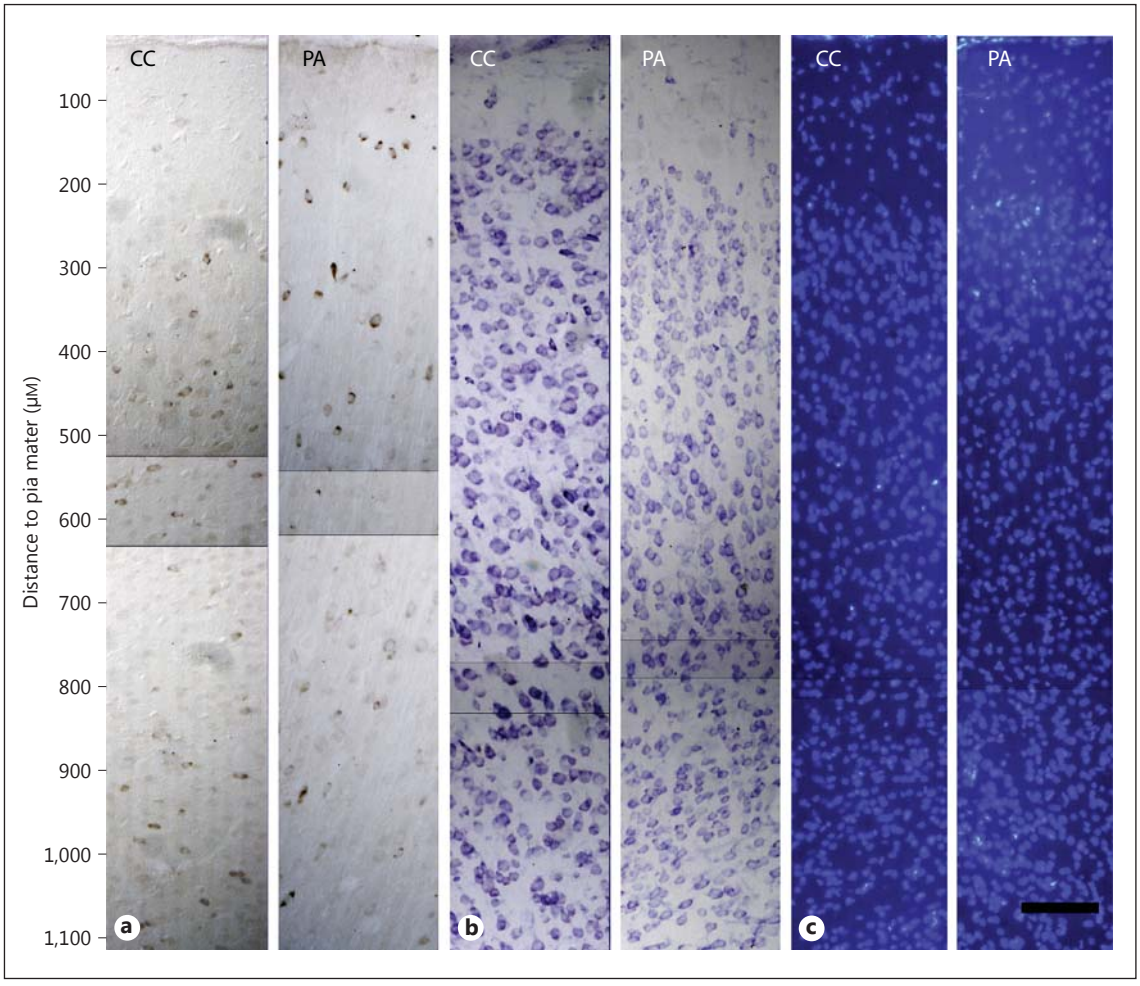

Fig. 1. Immunolabeling of reelin (**a**), cresyl violet stain (**b**) and DAPI stain (**c**) in the prelimbic cortex of the rat. PA = 19 min of asphyxia 37°. Scale bar = 100 μm.

subjected to the same conditions. The control rat's back was marked and the 2 rats were placed in the box at the same time. Their behavior was recorded for 15 min. Only the experimental rat's behavior was measured.

Parameters Studied in the Experiments. (1) Total number of social interactions. (2) Play soliciting, i.e. pouncing, the experimental rat touches the other rat's neck with its snout and then performs a rubbing motion on it. This behavior represents the vast majority of play soliciting. (3) Bites/pulls, i.e. the experimental rat bites and/or pulls the other rat (its tail, ears, back, etc.). (4) Play behavior, i.e. pinning (the rat rotated 180° about its longitudinal axis) and boxing/fighting. (4) Exploratory behavior, i.e. anogenital sniffing and fecal depositions (boli). See Trezza et al. [32] for more information about these parameters.

Behavioral Analysis and Statistics

All behavioral counts were performed by a skilled observer blinded to treatment. In all cases, parametric (1-way ANOVA, Tukey's test for multiple comparisons) and nonparametric tests (the Kruskal-Wallis and Dunn's test for multiple comparisons) were performed to confirm the results; in each case, we report the appropriate statistic parameter. All the data were tested for normality with the D'Agostino test. Only boli counts did not pass the normality test.

Histology and Reelin Immunohistochemistry

After the SI (1:30 h), 3 random rats (n = 3 by group) were anaesthetized with an i.p. overdose (2 ml) of chloral hydrate 15% and perfused with 50 ml of saline solution followed by 300 ml of fixative (4% paraformaldehyde) at room temperature.

After perfusion, the brain was carefully removed and then stored for <2 days in phosphate-buffered saline (PBS, pH 7.4).

Coronal tissue sections, 40-μm-thick, were cut on a vibratome and stored in antifreeze solution (PBS, ethylene glycol 30%, glycerol 30%) at –20°C until being processed by the indirect immunofluorescence technique of Coons [65]. The free-floating sections were rinsed in 0.1 M PBS and preincubated in 0.1 M PBS containing 0.3% Triton X-100 and 1% goat serum at room temperature. The

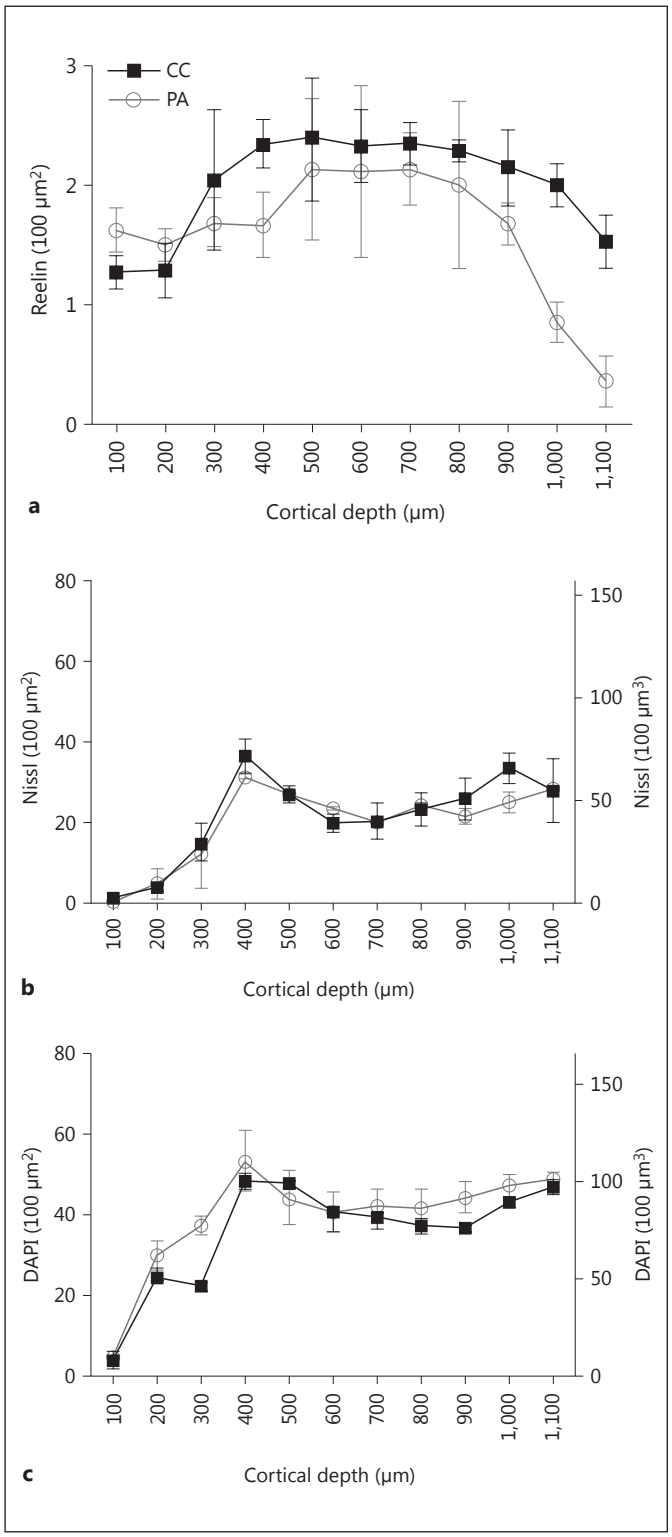

Fig. 2. Comparison of densities of labeled cells by 100 µm² (and a 3-dimensional approximation on the right side; Abercrombie's correction factor, DAPI = 0.82, Nissl = 0.78) in function of the depth of the prelimbic cortex (n = 3 per group). **a** Reelin immunostain. **b** Cresyl violet. **c** DAPI.

incubation with the primary antisera (1:1,000; monoclonal mouse anti-reelin, Abcam, or anti-calbindin, Leica) was carried for 3–4 days in multiwells at 4°C. Subsequently, the sections were washed and then incubated for 120 min with biotinylated antibody, goat anti-mouse (1:1,000, Vector Laboratories). After 3 washes, the sections were incubated for 1 h in Vectastain Elite ABC solution (Vector Laboratories). Sections were then washed and immersed in a solution of 0.05 mg/ml 3,3′-diaminobenzidine, containing 1 µl/ml H_2O_2 and nickel until color development (in all cases, for <10 min). The reaction was stopped by 2 rinses in distilled H_2O. Staining appeared as a brown/black precipitate in the soma and proximal dendrites. The sections were then mounted on gelatin-coated slides.

The Nissl sections were stained with 0.75% cresyl violet solution at pH 2–3. Nissl and reelin sections were dehydrated with graded alcohols (70, 95 and 100%), placed in xylene and coverslipped using Canada balsam. DAPI-stained sections were mounted with glycerol.

Microphotographs of the prelimbic PFC were taken by transmission microscopy (Axiophot, Zeiss, Germany) with a ×10 objective. Panoramic montages were made including all layers of the cortex until a depth of 1,100 µm (±1.10 lateral axis). Within each experimental subject, 3 sections at different levels between 3.8 and 3 in the anteroposterior axis were analyzed. The white matter was used as a landmark and the photomicrographs were taken between the 2.5 and 5 dorsoventral axes approximately as estimated in Paxinos and Watson [66]. Three photomicrographs covered most of the analyzed structure; these were randomly assigned (but without repeating) to each of the 3 sections from each rat.

Cell Counts

Manual counting was performed using ImageJ software, which automatically stores the number and position of cells. Two dissectors were used perpendicular to the surface of the cortex, and the cells in contact with one of the dissectors were not counted. The volumetric densities could be slightly overestimated as they were extrapolated from 2-dimensional sections. It is not expected that this generated any significant difference between the experimental groups. To overcome this issue, we applied Abercrombie's correction factor (DAPI-stained nuclei size = 8.33 (median) factor = 0.82, Nissl-stained cell size = 11.14 (median) factor = 0.78). Layer VI was analyzed twice and in the second analysis, the differences generated by the atrophy of the cortex were taken into consideration. We paid special attention to not include white matter in the measurement. Despite these changes, similar results were obtained.

Histological results were reported using 2-way ANOVA with repeated measures. One-way ANOVA was used in the case of the analysis restricted to layer VI followed by Tukey's test for multiple comparisons. In all cases, Excel 2010 and GraphPad Prism version 6.01 were used for statistical analysis.

Results

PA generated a significant and specific decrease in the number of reelin-secreting neurons in the mPFC layer VI [$F(2, 6) = 8.716$, $p = 0.016$; PA vs. VC $p = 0.03$, PA vs. CC, $p = 0.022$] (fig. 3). This reduction, on average, reached ap-

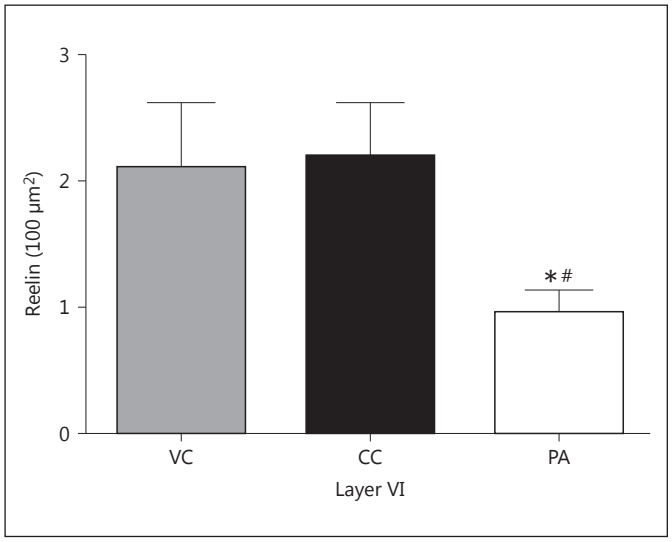

Fig. 3. Comparison of densities of labeled cells with reelin in layer VI of the prelimbic cortex of the VC, CC and PA (19 min of asphyxia 37°) groups (n = 3 per group). F(2, 6) = 8.716, p = 0.016; PA vs.VC, * p = 0.03, PA vs. CC, # p = 0.022.

proximately 60%. Changes in the percentage of reelin neurons including all the cortex layers did not achieve a significant outcome but a trend; CC % 10.61 ± 1.34; PA % 8.64 ± 1.71 [F(2, 6) = 1.299, p = 0.33] (fig. 1, 2).

In the case of the calbindin immunostain, the reduction in the number of neurons produced by the asphyxia yielded significant differences [2-way repeated-measures ANOVA, F(1, 4) = 13.03, p = 0.0226] (fig. 4). The multiple-comparisons test showed significant differences in the superficial aspect of layer II (Sidak test for multiple comparisons CC vs. PA at 200 µm, p = 0.003). The distribution of calbindin cells in the mPFC did not show the characteristic band in layer II observed in the other cortices. Instead, we found some isolated groups of neurons in layer II and scattered cells in the other layers.

The reduction of reelin neurons in layer VI and of calbindin neurons in layer II was not accompanied by a reduction in counts of neurons or cells in general (fig. 2, 3). The estimated percentage of neurons stained with cresyl violet (containing Nissl bodies) versus DAPI produced similar results in both groups (CC 50% ± 4.42; PA 56.4% ± 4.27).

A small but significant difference (12%) could be seen when the distance from the pia mater to the start of layer VI was analyzed (CC mean ± SEM = 768.9 ± 8.382; PA mean ± SEM = 669.3 ± 17.75; p = 0.036).

No differences were observed between the VC and CC with regard to the distribution of reelin and calbindin, the morphology and other cytoarchitectonical measurements. Layer V was clearly visible with Nissl as a band of neurons with large and strongly stained pyramidal cells. In opposition to layer VI, the end of layer V presented low dorsoventral variability on the distance to the pia mater.

The behavioral outcomes in the OF were similar for all groups analyzed and only the number of fecal depositions presented significant differences. The locomotive activity expressed as the number of quadrant crossings was consistent in all groups [F(2, 27) = 1.618; p = 0.2169]. Similar results were observed for the number of entries in the central square [F(2, 27) = 0.19; p = 0.9] and the time that the animals remained there [F(2, 27) = 0.89; p = 0.45]. Thus, there were no substantial differences in locomotive activity or in anxiety-like estimators.

The number of boli exhibited a significant effect (Kruskal-Wallis test 16.24, p = 0.001). The multiple-comparisons test indicated that the number of boli for CC groups was significantly higher than the number obtained for PA (p = 0.045) groups (fig. 6). The number of boli was also analyzed in the Social interaction, yielding very different results. There was also a significant main effect (Kruskal-Wallis test 13.73, p = 0.003) but the number of boli was significantly lower in the case of the VC and CC groups compared to the PA group (p = 0.012 and p = 0.027, respectively). In order to compare these results, it is important to note that there was a decrease in the total number of fecal depositions in the SI experiment compared to in the OF experiment; in the SI, the VC group had a mean of 0.0458 boli/min/rat (SE = 0.028) compared to a mean of 0.350 boli/min/rat (SE = 0.1329) in the OF.

There was a significant decrease in play soliciting produced by the PA [F(2, 30) = 4.391, p = 0.021; PA vs. VC, p = 0.046; PA vs. CC, p = 0.034] (fig. 5).

No other effect of asphyxia yielded significant differences besides boli and play soliciting in the SI.

Surprisingly, there were some behavioral differences between the CV and CC groups. In the OF, there was a significant increase in the number of boli in the CC group compared with the VC group (p = 0.012). Furthermore, in the boxing play behavior, there was a significant reduction in the CC group that persisted in the PA group [ANOVA F(2, 27) = 4.214, p = 0.0114, multiple-comparisons VC vs. CC, p = 0.0188 and VC vs. PA, p = 0.0132]. The other parameters analyzed, including pinning, anogenital sniffing and grooming, did not yield significant differences.

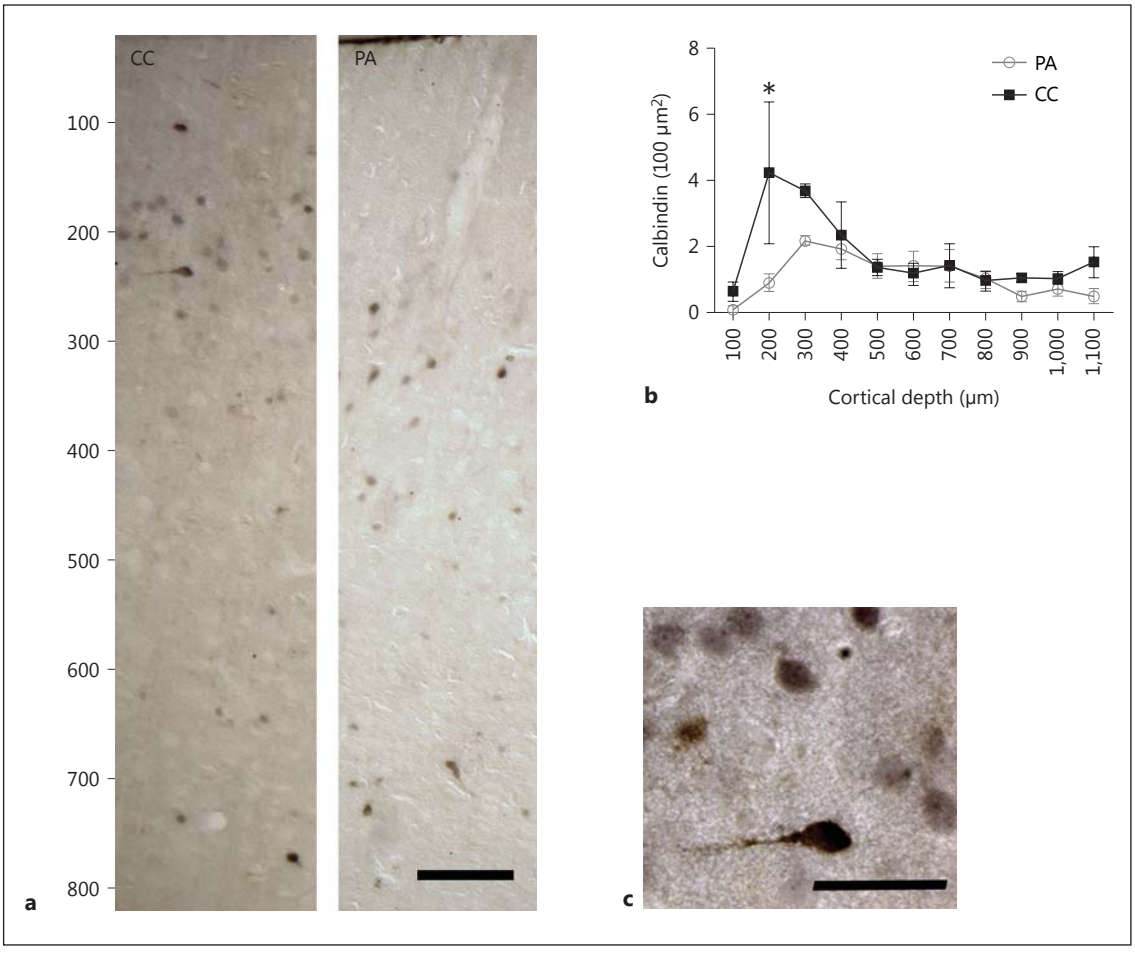

Fig. 4. a Detection of calbnindin by immunohistochemistry in CC and PA groups. Scale bar = 100 μm. **b** Cell density by 100 μm^2 from the pia mater (depth 0) to layer VI * Significant differences from normoxic control; 2-way repeated measures ANOVA F(1, 4) = 13.03, * p = 0.022 and Sidak test for multiple comparisons CC vs. PA at 200 μm, p = 0.003; n = 3 per group. **c** Magnification of calbindin stain. Scale bar = 50 μm.

Discussion

In this work, we observed that rats exposed to PA showed deterioration in play soliciting, an increase in boli, one estimator of anxiety-like behavior during social interaction and a reduction in the immunolabeling of 2 subpopopulations of GABAergic neurons, i.e. reelin and calbindin. These reductions are layer-specific. The reduction in calbindin was exhibited in layer II and the reduction in reelin in layer VI.

It has been reported that PA produces shrinkage of the hippocampus, corpus callosum, cerebellum and neocortex [35, 36]. In addition, myelination deficits, alterations in glial cells and reduced axonal density [37–39] have been found. In the work of Van de Berg et al. [36], the atrophy in the mPFC was reported as a tendency. Our work corroborates that the perinatal hypoxia produces cortical shrinkage in this structure in the young adult rat. There was no significant reduction in the number of cells or neuronal populations. There was, however, a trend to an increase in their density. Therefore, the level of cellular loss did not seem to contribute to the shrinkage, which was probably generated by the loss of neuropil, although a decrease in cellular size could have contributed to this phenomenon [40]. The loss of neuropil could also explain the paradoxical trend for an increase in cell densities.

The mechanism involved in reelin reduction could be related to the preferential vulnerability of GABAergic neurons to excitotoxicity during the perinatal period [41]. It has been described that PA generates a reduction

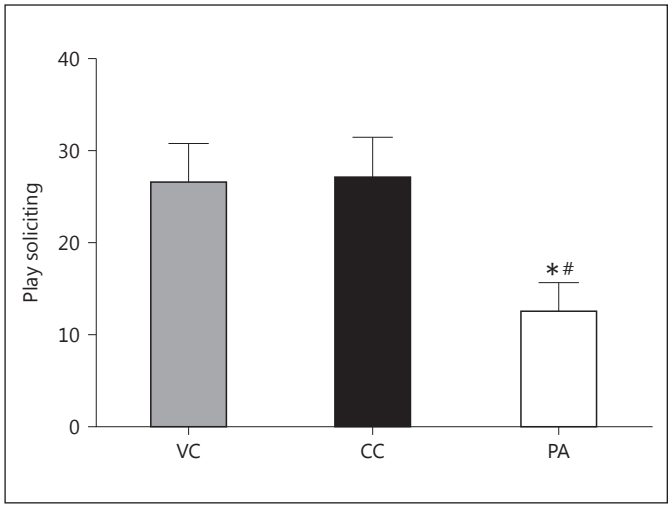

Fig. 5. PA caused a decrease in play soliciting in the PA group in the SI. ANOVA followed by Tukey's test for multiple comparisons (PA vs. VC, * p = 0.046; PA vs. CC, # p = 0.034; n = 11 per group).

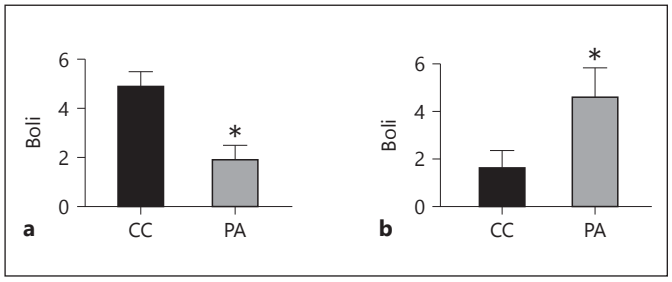

Fig. 6. The number of fecal depositions (boli) in the OF (**a**) and SI (**b**) after 24 h of isolation. In the OF, the boli counts were significantly higher in the CC group than in the PA group; in the SI, the result was the opposite. Dunn's test * p = 0.003 (OF), * p = 0.027 (SI). The bar indicates the standard error (n = 10 per group).

in GABAergic cells in the cortex [42] and striatum [41]. During the perinatal period, the Cajal-Retzius cells disappear and a subpopulation of GABAergic neurons starts to secrete reelin around the second postnatal week [43]. In the adult, almost all cells expressing reelin also show positive staining for GAD65 and/or GAD67 in the hippocampus [18] and cortex [44]. It is also known that reelin neurons express calretinin [45]. At least 80% of adult calretinin neurons are generated during the fetal period [46], but the generation and migration of GABAergic neurons continues after birth [47].

Calbindin is expressed in a subpopulation of GABAergic neurons that do not overlap with reelin/calretinin neurons [48]. Most calbindin neurons migrate to the cortex during fetal development but, as in the case of calrretinin, it has been reported that they retain neurogenesis in the adult [49].

There are 2 ways in which PA could reduce the number of GABAergic neurons: by inducing cell death or by impairing their production. Excitotoxic processes are different in newborns compared to adults. In the neonate, the Cl- currents are still not reversed and GABAergic transmission is excitatory [50]. Moreover, glutamatergic transmission is mostly silent and the few active synapses seem to make contact with the interneurons [51]. Thus, excitotoxicity may involve GABAergic neurons; we hypothesize that it may even be mediated by them. In our view, the most probable cause of the decrease in both subpopulations of GABAergic neurons is exitotoxic cell death, but a deterioration of the migration/differentiation or loss of GABAergic neural progenitors [52] cannot be discarded, especially in the case of the reelin neurons.

The increase in neuronal density could mask the loss of GABAergic neurons that represent only 16% of neurons in this structure [53]. If there is a relationship between shrinkage and reelin reduction, it is more likely that the decrease in reelin will produce shrinkage, not vice versa.

Postmortem studies of schizophrenic patients consistently show a decrease in the expression of GABAergic biomarkers such as parvalbumin and GAD67 [54, 55].

The mPFC is an important area involved in social interaction behavior. Therefore, it seems possible that the reduction in reelin and the reduction of the thickness of the cortex may be involved in behavioral deterioration. However, there could be alternative explanations for social interaction deterioration, bearing in mind that there are other morphological alterations generated by asphyxia [56]. Schizophrenia exhibits very striking coincidences with PA, which generates susceptibilty to the disease, a decrease in the number of reelin secreting neurons [17, 57] and a deterioration in social interaction. Moreover, the atrophy without general cell loss is similar to the postmortem alteration observed in schizophrenia [58].

In the OF, there were no significant differences in locomotor activity or anxiety-like indicators; these were very consistent between groups. Alterations in these factors could modify the counts of social interaction due to response competition [59]. Therefore, the social interaction deficits in the PA rats were not produced by alterations in locomotor activity or anxiety levels, besides those that could be produced by the SI itself. We observed an increase in fecal depositions in the CC group compared

to the VC group. Increased fecal deposition in rats is one of the best indicators of an increase in emotionality, and stress could justify that increment. The OF is widely used in the study of anxiety, since this test evaluates the response of animals to a moderately stressful situation such as exposure to a broad new environment and separation from the rest of its cage mates (review [60]). We attribute the increase in fecal depositions in the CC group to elevated levels of emotionality caused by the separation from their cage mates, compared to the control group. This is supported by the fact that the increase in fecal depositions was reversed by introducing another rat into the arena in the SI experiment. In the case of the asphyctic rats, we saw the opposite, a decrease of fecal depositions produced by PA in the OF that could be related to a diminished anxiety response to separation from their cage mates. We also saw a relative increase in fecal depositions in the SI. This could indicate that the asphyctic rats find being alone relatively more comfortable than interacting with unknown cage mates. We also observed a behavioral difference in boxing between VC and CC groups. No histomorphological differences were observed between these 2 control groups. Perhaps the stress of passing through the vaginal canal, or lactation and contact with the mother immediately after birth could generate these differences.

Previous work has shown that both the PA and cesarean section produce alterations in the social interaction patterns of rats [61, 62]. In the study by Venerosi et al. [62], an increase in exploratory behavior in CC animals was found, while play soliciting remained constant in all groups. There are several possible explanations for this apparent discrepancy: (1) differences in the CC group, since we allowed the mother to deliver 1 pup before the start of the experiment in order to use nonpremature rats, (2) we used a slightly lower level of asphyxia and (3) in Materials and Methods in Venerosi et al. [62], there was no mention of pouncing whereas in our work it represented most of the play soliciting. Since pouncing involves rubbing the tip of the snout on the other rat's neck, it could be misinterpreted as an exploratory behavior.

In the study by Laviola et al. [61], asphyxia was performed 24 h after birth. We believed that confirmation of their results with our model was important because the effects of PA can vary depending on whether the asphyxia occurs before or after the first breath. Despite this, their results were very similar to ours, even after taking into account that their rats were 15 days old.

The behavioral paradigm of social interaction is commonly used to validate animal models of psychiatric disorders that encompass social deficits, especially those related to autism and schizophrenia (review [63]). It is well known that play behavior generates a reward that is conducive to future social interactions [64] and is crucial for correct social development [65]. Moreover, social isolation has been reported to decrease PFC synaptic spines [66]. The inhibition of the PFC generates a pattern of social interaction characterized by low play soliciting [22]. At the same time, several studies propose PA as a model of schizophrenia, focusing on the positive symptoms [15, 16]. Our work adds an important aspect; PA deteriorates play soliciting, an important form of social interaction. We support the hypothesis that the decrease in play soliciting in the PA group could have the same physiological origin as the negative symptoms of schizophrenia. PA damages calbindin and reelin neurons, which could be conducive to shrinkage of the cortex by reduction of neuropil and deterioration of the cortical function.

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Disclosure Statement

There were no conflicts of interest.

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