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Selective reduction in the expression of type-1 metabotropic glutamate receptors in the hippocampus of adult rats born by caesarean section

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Abstract

Perinatal hypoxia causes long-term neurobiological consequences, including alterations in mechanisms of activity-dependent synaptic plasticity and cognitive dysfunction. Changes in neurotransmitter receptors have been associated with these alterations, but little is known on how early hypoxia influences the expression and function of metabotropic glutamate (mGlu) receptors in adult life. This is an important issue because mGlu receptors are implicated in mechanisms of synaptic plasticity. Here, we examined the expression of mGlu1, mGlu5, and mGlu2/3 receptor subtypes in the hippocampus, nucleus accumbens, prefrontal cortex, and dorsal striatum in 6-month old Wistar rats (a) born by vaginal delivery; (b) born by caesarean section; and (c) born by caesarean section followed by 20 min of asphyxia. Unexpectedly, we found a large reduction of mGlu1α protein levels in the hippocampus of rats born by caesarean section regardless of the presence of asphyxia. No changes in mGlu1α receptor protein levels were found in the other brain regions. Levels of mGlu5 and mGlu2/3 receptors and levels of GluA2/3 and GluN1 subunits of AMPA and NMDA receptors did not differ among the three groups of rats in any brain region. These results are consistent with previous findings showing that changes in mGlu1 receptors occur within the epigenetic programming caused by early-life events.

KEYWORDS

anoxia, caesarean delivery, hippocampus, mGlu receptors

1 | INTRODUCTION

Obstetric complications including foetal and perinatal hypoxia/anoxia are significant risk factors for neurocognitive disorders, such as attention deficit hyperactivity disorder (ADHD), autism and schizophrenia (Curran et al., 2016; O'Neill et al., 2016; Perna & Cooper, 2012). The damage is proportional to the duration and severity of the hypoxic episode, ranging from true encephalopathy with moderate-to-severe neonatal symptoms (Sarnat & Sarnat, 1976) to mild or absent pathological signs at birth which, however, become predisposing factors for the central nervous system (CNS) disorders later in life (de Haan

et al., 2006; Marriott et al., 2017). A number of neurodevelopmental sequelae of perinatal hypoxia have been documented in humans (McNeil et al., 2000), including cytoarchitectural disruptions in the medial prefrontal cortex and hippocampus (Arnold et al., 1991; Marriott et al., 2017; McNeil et al., 2000).

Most of the studies on the outcome of perinatal hypoxia have focused on changes in monoaminergic transmission in brain regions that are involved in the pathophysiology of psychiatric disorders, such as the nucleus accumbens and prefrontal cortex (Decker et al., 2018; El-Khodor & Boksa, 1997; Sullivan & Brake, 2003). However, a growing body of evidence suggests that changes in the expression and/or activity of ionotropic glutamate receptors might contribute to the outcome of perinatal hypoxia. For example, early postnatal hypoxia down-regulates the expression of the Ca^{2+}-impermeable GluA2 subunit of AMPA receptors in the neocortex and hippocampus, an effect that might contribute to the pathophysiology of perinatal hypoxia-induced seizures (Sanchez et al., 2001). Perinatal hypoxia also causes abnormalities in the interaction between postsynaptic density protein-95 (PSD-95) and NMDA receptors in the hippocampal CA1 region. The resulting dysfunction of NMDA receptors might contribute to the increased seizure susceptibility and the long-lasting memory deficit associated with perinatal hypoxia (Chen et al., 2006, 2007). More recent findings demonstrate that prenatal hypoxia causes a reduced expression of GluN1, GluN2A and GluN2B NMDA receptor subunits associated with an impairment of the Wnt/β-catenin signalling in the hippocampus of offspring at adolescence (Wei et al., 2016), an evidence that highlights the disruptive effects of prenatal hypoxia on neurodevelopment.

No studies have been performed on the long-lasting effects of perinatal hypoxia on the expression and function of metabotropic glutamate (mGlu) receptors, although these receptors play a fundamental role in the regulation of synaptic transmission and in mechanisms of activity-dependent synaptic plasticity (reviewed by Nicoletti et al., 2011). mGlu receptors form a family of eight subtypes, divided into three groups on the basis of their amino acid sequence, pharmacological profile and G-protein coupling. Group-I mGlu receptors (mGlu1 and mGlu5 receptors) are coupled to $G_{q/11}$ proteins, and their activation stimulates polyphosphoinositide hydrolysis with ensuing formation of inositol-1,4,4-trisphosphate and diacylglycerol. Group-II (mGlu2, mGlu3) and group-III (mGlu4, mGlu6, mGlu7, mGlu8) subtypes are coupled to $G_{i/o}$, and their activation inhibits adenylyl cyclase activity and modulates the activity of Ca^{2+} and K^{+} channels (reviewed by Nicoletti et al., 2011).

To examine whether perinatal hypoxia leads to long-term changes in the expression of mGlu receptors we exposed rat foetuses in utero at term to global asphyxia after caesarean section (as described in Venerosi et al., 2006). This model mimics the complications associated with preterm delivery, where the entire organism is exposed to hypoxia and responds with adaptive changes that involve the cardiovascular and respiratory systems, the endocrine axis and the CNS. In this same experimental model, long term effects on behaviour associated with asphyxia were reported up to 6–8 months after birth in the offspring (Bonsignore et al., 2006; Cirulli et al., 2003; Venerosi et al., 2006). An advantage of this experimental protocol is that it allows one to dissecting the effect of asphyxia from the effect of the caesarean section (C-section) itself. Recent epidemiological evidence associated C-section mode of delivery with increased risk of adverse health effects in the new born, including neurodevelopmental disorders such as autism spectrum disorder (Curran et al., 2015). Recent experimental studies investigating short term effects of C-section in a mouse model of preterm birth demonstrated slight transient alterations in neuronal morphology and mild deficits in communicative behaviours. On this basis, the authors excluded significant effects of C-section per se on brain developmental processes (Chiesa et al., 2019). However, previous rat studies reported long-term effects of C-section on AMPA, NMDA and kainate receptors, distinct from the effects of hypoxia (El Khodor & Boksa, 2003). As a mode of delivery is increasingly studied as an effect modifier of perinatal risk factors (Kenkel, 2021), our study aimed at verifying the long term effects of either hypoxia or C-section on a class of receptors that plays a fundamental role in brain and behaviour plasticity with potential repercussion on CNS developmental trajectory.

We were surprised to find that the caesarean section by itself, independently of asphyxia, led to a robust and selective down-regulation of mGlu1 receptors in the hippocampus of the adult offspring.

2 | MATERIALS AND METHODS

2.1 | Animals and breeding procedures

Wistar rats were purchased from Harlan and kept in an air-conditioned room (housing room) at 21 ± 1°C and 60 ± 10% relative humidity, with a white/red light cycle (white light on from 8:30 a.m. to 8:30 p.m.). Pellet food and tap water were continuously available. One male and two virgin females were housed in Plexiglas cages, with the male being removed after 24 hr. Ten days later, pregnant females were individually housed until gestation day 22, when the caesarean section (C- section) was carried out. Rats were delivered by C-section and asphyxiated according to methods described by Venerosi et al. (2006).

2.2 | Induction of intrauterine asphyxia

The experimental design of this study was depicted in Figure 1. Pregnant rats on gestation day 22 (expected day of birth)

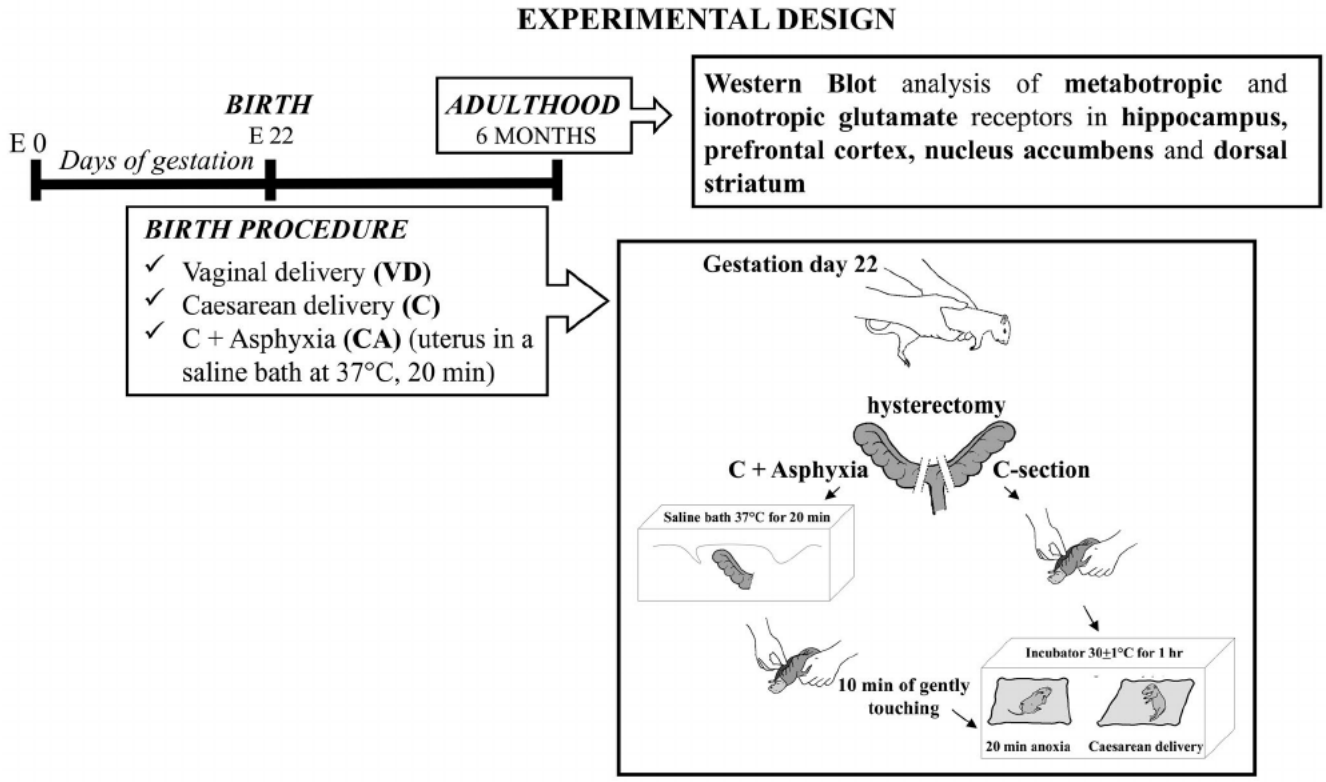

FIGURE 1 Experimental design of the study

were decapitated and the entire uterus was quickly removed. Immediately after hysterectomy, 50% of the foetuses were removed from the uterine horns and included in the group of caesarean-delivered without asphyxia (C-section group). The uterine horns, still containing the remaining foetuses, were placed into a saline bath at 37°C, pups were removed after 20 min (group of C-section +asphyxia) and stimulated to breath by gently touching them around the oral and genital areas for approximately 10 min. Pups were then maintained for 1 hr in an incubator at 30 ± 1°C (ElmedGinevri 0GB 1,000) until fostered by surrogate mothers that had given birth to healthy litters within the preceding 24 hr. Other pups born by vaginal delivery were removed within 12 hr after birth from donor mothers and fostered to surrogate mothers and included in the control group of vaginally delivered (VD) rats. To minimize differential rearing effects, one male and one female pup from the groups of C-section, C-section +asphyxia, and VD (six pups per dam, for a total of nine litters) were assigned to each foster dam. After weaning (postnatal day 21), animals were housed three per cage and left undisturbed until 6 months of age. Only male rats were used for the present study. All efforts were made to minimize the number of animals used and to alleviate their discomfort. All experimental procedures were performed in conformity with the Italian (D.L. 26/2014) and European Union Directive (2010/63/EU) on the protection of animals used for scientific purposes. Experiments were approved by the Italian Ministry of Health.

2.3 | Western blot analysis

Six-month old male rats were killed by decapitation, the brains were rapidly removed, chilled on ice, and sectioned freehand as described in detail by Glowinski and Iversen (1966).

Briefly, the prefrontal cortex was dissected from the most rostral coronal section (2.5 mm thick). Subsequently, a coronal section (2.5 mm thick) was made at the level of the optic chiasma (which passes through the anterior commissure) and the dorsal striatum (between the lateral ventricles and the corpus callosum) and the nucleus accumbens (from the medial and basal structures under the dorsal striatum) were dissected. The hippocampus was gently separated from the remaining caudal part of the brain. The hippocampus, prefrontal cortex, nucleus accumbens and dorsal striatum were stored frozen at −80°C. On the day of the experiment, tissue was homogenized at 4°C with a polytron in 500 µl of 100 mM Tris buffer, containing phenylmethylsulfonyl fluoride 1 mM, leupeptin 10 µg/ml and aprotinin 10 µg/ml pH 7.2. Protein concentrations were determined using the Bradford protein assay. Seventy micrograms of proteins were suspended in SDS-bromophenol blue reducing buffer containing 40 mM dithiothreitol. Protein extracts were separated on 8% SDS-polyacrylamide gels (Amersham Bioscience), and after electrophoresis (ProteanIIXi, Biorad) proteins were transferred to nitrocellulose in 35 mM Tris, 192 mM glycine and 20%

methanol at 450 mA for 4 hr. After transfer, blots were incubated in a blocking solution containing Tris-buffered saline (TBS), 0.5% (w/v) Tween-20, 1% (w/v) non-fat milk and 1% (w/v) bovine serum albumin. Afterwards, blots were incubated overnight with polyclonal anti-mGlu1α (Millipore, 07-617, 1:500), anti-mGlu5 (Millipore, AB5675, 1:1000), anti-mGlu2/3 (Millipore, 06-676, 1:250), anti-GluN1 (Millipore, ABN241, 1:1000), GluA2/3 (Millipore, 07-598, 1:1000) or monoclonal anti-β-actin (Sigma, A5316, 1:1000) antibodies at 4°C in blocking solution. After incubation with the primary antibody, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibodies (1:10,000 and 1:5,000, respectively; Amersham Bioscience) for 1 hr at room temperature. Immunoreactive bands were visualized with an enhanced chemiluminescence system (Amersham Biosciences). After immunoblotting, digitized images of immunoreactive bands were acquired and the area of immunoreactivity corresponding to each band was measured using the Image-J computer program.

2.4 | Statistical analysis

Results were analysed by one-way ANOVA with a mode of delivery as between factor. ANOVA was performed separately for each CNS area considered (hippocampus, prefrontal cortex, nucleus accumbens and dorsal striatum). The ANOVA analyses were followed by Fisher's LSD *post-hoc* comparisons. Statistical significance was set at $p<0.05$. All data are expressed as mean ± *SEM*.

3 | RESULTS

Immunoblots of mGlu1α receptors showed one band at 140 kDa corresponding to receptor monomers. No receptor dimers could be detected under our experimental conditions (Figure 2a). mGlu1α receptor protein levels were largely reduced in the hippocampus of adult rats delivered by C-section or by C-section followed by asphyxia, as compared to vaginally delivered control rats. Hippocampal mGlu1α protein levels did not differ between rats delivered by C-section without and with asphyxia (Figure 2 and Table 1 for OD values). No changes in mGlu1α receptor protein levels were found in the prefrontal cortex, nucleus accumbens, and dorsal striatum after C-section or C-section +asphyxia (Table 1).

Expression of mGlu5 and mGlu2/3 receptors was unchanged in rats born by C-section or C-section +asphyxia in all brain regions examined (Table 1).

Because changes in the expression of ionotropic glutamate receptors have been reported in the hippocampus and prefrontal cortex of animals subjected to perinatal hypoxia (see Introduction and References therein), we also examined the expression of the GluN1 subunit of NMDA receptors and the GluA2/3 subunit of AMPA receptors. None of the two receptor subunits showed significant changes in any brain regions (Table 2).

Thus, at least under our experimental conditions, caesarean delivery independently of asphyxia caused a large and selective down-regulation of mGlu1α receptors in the hippocampus with no changes in the expression of other mGlu or ionotropic glutamate receptors.

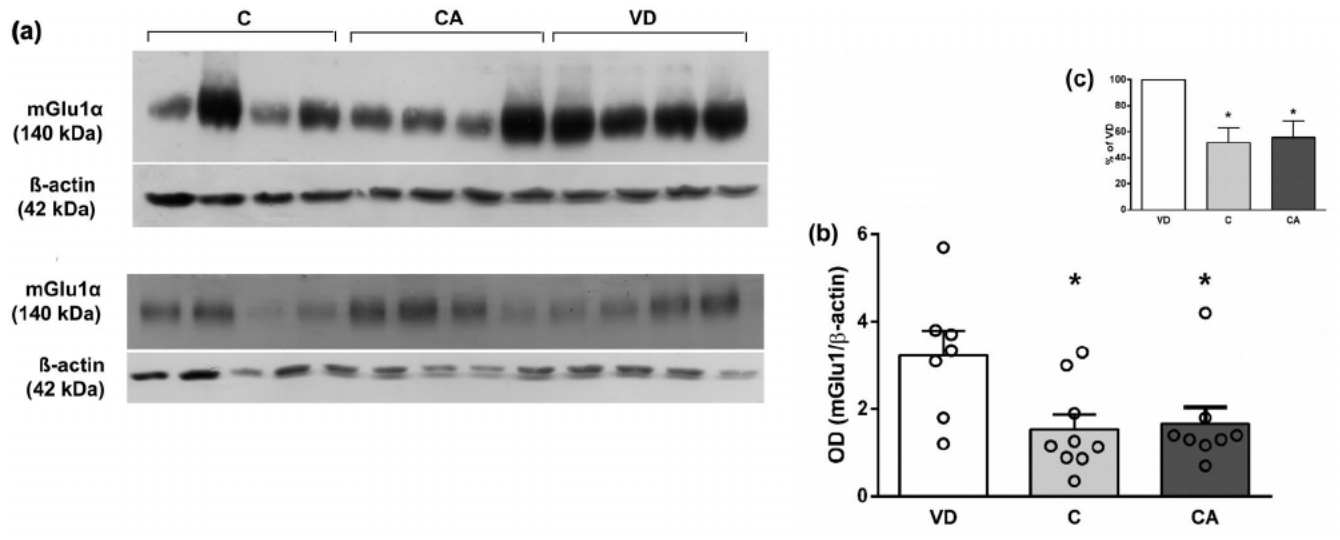

FIGURE 2 (a) Western blot analysis of mGlu1α receptor in the hippocampus of rats vaginally delivered (VD, $n = 7$), delivered by caesarean section without asphyxia (C, $n = 9$) and by caesarean section with asphyxia (CA, $n = 8$). Each lane shows the receptor expression of individual animals from the three groups. (b) Densitometric analysis, of mGlu1α receptor expression, is expressed as optical density (OD). (c) Densitometric analysis is expressed as percentage of VD. Data shown as mean + *SEM*. ANOVA: $F_{2,21} = 4.86$; Fisher's LSD *post-hoc*, *$p < .05$ versus VD

TABLE 1 Densitometric analysis, expressed as optical density (OD), of mGlu1, mGlu5 and mGlu2/3 receptor expression in the hippocampus, prefrontal cortex, nucleus accumbens and dorsal striatum of rats vaginally delivered (VD, $n = 7$), delivered by caesarean section without asphyxia (C, $n = 9$) and by caesarean section with asphyxia (CA, $n = 8$)

	Mean of mGluRs/β-actin (OD) ± *SEM*		
	VD	**C**	**CA**
Hippocampus			
mGluR1	2.98 ± 0.54	1.54 ± 0.33*	1.66 ± 0.38*
mGluR5	1.13 ± 0.06	1.05 ± 0.04	1.29 ± 0.27
mGluR2/3	1.79 ± 0.30	2.30 ± 0.35	2.29 ± 0.36
Prefrontal cortex			
mGluR1	1.20 ± 0.05	1.35 ± 0.14	1.24 ± 0.16
mGluR5	1.63 ± 0.09	1.75 ± 0.18	1.53 ± 0.07
mGluR2/3	1.01 ± 0.15	1.22 ± 0.16	1.29 ± 0.13
Nucleus accumbens			
mGluR1	1.18 ± 0.10	1.19 ± 0.10	1.50 ± 0.27
mGluR5	3.19 ± 0.19	3.50 ± 0.22	2.94 ± 0.17
mGluR2/3	2.30 ± 0.18	2.06 ± 0.39	2.86 ± 0.43
Dorsal striatum			
mGluR1	1.12 ± 0.17	1.16 ± 0.09	1.06 ± 0.17
mGluR5	1.44 ± 0.10	1.85 ± 0.59	1.80 ± 0.09
mGluR2/3	2.02 ± 0.09	2.27 ± 0.25	2.10 ± 0.07

Note: Data are shown as mean ± *SEM*. ANOVA: $F_{2,21} = 4.86$; Fisher's LSD *post-hoc*, *$p < .05$ versus VD.

TABLE 2 Densitometric analysis, expressed as optical density (OD), of GluN1, GluA2/3 receptor expression in the hippocampus, prefrontal cortex, nucleus accumbens and dorsal striatum of rats vaginally delivered (VD, $n = 7$), delivered by caesarean section without asphyxia (C, $n = 9$) and by caesarean section with asphyxia (CA, $n = 8$)

	Mean of AMPA and NMDA receptors subunit/β-actin (OD) ± *SEM*		
	VD	**C**	**CA**
Hippocampus			
GluN1	1.66 ± 0.32	1.67 ± 0.08	1.86 ± 0.32
GluA2/3	2.77 ± 0.50	2.55 ± 0.20	3.32 ± 0.62
Prefrontal cortex			
GluN1	0.73 ± 0.02	0.64 ± 0.10	0.75 ± 0.09
GluA2/3	2.29 ± 0.31	2.03 ± 0.22	2.34 ± 0.23
Nucleus accumbens			
GluN1	0.61 ± 0.08	0.50 ± 0.11	0.68 ± 0.12
GluA2/3	1.29 ± 0.11	1.39 ± 0.10	1.32 ± 0.09
Dorsal striatum			
GluN1	1.90 ± 0.23	1.70 ± 0.27	2.01 ± 0.20
GluA2/3	3.00 ± 0.30	3.51 ± 0.20	3.33 ± 0.22

Note: Data are shown as mean ± *SEM*. ANOVA: not significant.

4 | DISCUSSION

Our study was originally designed to examine the long-term effects of perinatal hypoxia on the expression of mGlu receptors. Neither hypoxia nor mode of delivery was found to change the levels of mGlu5 and mGlu2/3 receptors and levels of GluA2/3 and GluN1 subunits of AMPA and NMDA receptors in any brain region in adult rats (for a review on NMDA see Smaga et al., 2019; for a review on AMPA see Diering & Huganir, 2018; for a review on mGlu see Nicoletti et al., 2011). However, unexpectedly we found that caesarean delivery by itself, rather than hypoxia, caused a selective, long-lasting and region-specific down-regulation of mGlu1α receptors in the hippocampus. A previous study (El-Khodor et al., 2004) found that adult rats born by C-section showed increases in AMPA receptor binding in nucleus accumbens shell, NMDA receptor binding in the cingulate cortex and kainate receptor binding in the hippocampal CA1 region. Due to the well-known functional interaction between mGluR1, NMDA receptors and AMPA receptors (Lüsche & Huber, 2010), the possibility that these changes contribute to alterations in mGlu1 receptors in the hippocampus has to be taken into account. However, the selectivity of the effect here reported is not so surprising if considering the key role of the mGlu1 in brain development programming.

A great body of data indicates that mGlu1 receptors are specifically sensitive to early environmental influences. Notably, Michael Meaney and his associates have found that variations in postnatal maternal care induced epigenetic changes in mGlu1 receptor expression in the hippocampus, in the absence of effects on mGluR5, with the offspring of low pup licking/grooming mothers showing a lower expression of mGlu1 receptors, associated with reduced histone acetylation at the *Grm1* gene promoter, as compared to high pup licking/grooming mothers (Bagot et al., 2012). Our findings suggest that the mode of delivery is able to specifically modulate mGlu1 receptors as postnatal maternal care. As a matter of fact, labour and vaginal delivery are powerful triggers of several physiological systems (cardiovascular, respiratory, sensory) in the new born, which must rapidly adapt to the external environment. In a recent controlled study in mice (Castillo-Ruiz et al., 2018) vaginally-born offspring exhibited an acute decrease in cell death across the brain that was absent in C-section–born mice. This led the authors to speculate on the protective role of vaginal birth in the long term, possibly mediated by several factors, including cytokine release, oxytocin surge and appropriate microbial colonisation. Specifically, vaginal delivery promotes the physiological surge of various cytokines and/or their receptors that are implicated in neonatal immunity through different mechanisms and such increase is significantly higher in vaginal delivery than in C-section

(Malamitsi-Puchner et al., 2005). The increase of inflammatory mediators has a physiological role possibly promoting activation and maturation of microglial cells in crucial brain regions such as the hippocampus (McAdams & Juul, 2012). Furthermore, it has to be considered that rats in the C-section and C-section with asphyxia groups have in common the absence of transit through vaginal microbiota of the dam, in contrast with what experienced by the vaginally delivered group. Differences in microbial colonisation at birth could have effects on the brain and more specifically on hippocampal development. Moreover, gut microbiota appears to regulate mouse behaviour through glucocorticoid receptor pathway genes in the hippocampus (Luo et al., 2018). Finally, parturition is associated with a massive release of oxytocin in the maternal bloodstream that reaches the foetus. Oxytocin surge during vaginal birth is able to promote GABA switch in the neonatal mouse brain, a crucial maturational event, which can influence the life course trajectory (typical versus pathological), as shown by Tyzio et al. (2006) (for reviews see Kenkel, 2021; Zinni et al., 2018).

Noteworthy, in the hippocampus, mGlu1α receptors are predominantly expressed by somatostatin-positive GABAergic interneurons of the CA1 stratum oriens-alveus, which control the excitability and synchronisation of pyramidal neurons (Baude et al., 1993; Ferraguti et al., 2004; Freund & Buzsáki, 1996). mGlu1 receptors may mediate a multifacet regulation of hippocampal activity-dependent synaptic plasticity. Tonegawa and his associates were the first to demonstrate that mice lacking mGlu1 receptors show an impairment of hippocampal long-term potentiation (LTP) and context-dependent associative learning (Aiba et al., 1994).

Pharmacological blockade of mGlu1 receptors with the orthosteric antagonist, LY367385, impairs both induction and late phases of LTP and long-term depression (LTD) at Schaffer collateral-CA1 synapses in hippocampal slices (Neyman & Manahan-Vaughan, 2008) and impairs both LTP induction in the dentate gyrus and reference memory in freely moving rats (Naie & Manahan-Vaughan, 2005). The large down-regulation of mGlu1 receptors we have found in the hippocampus of rats born by caesarean section suggests that these animals may have deficits in hippocampal-dependent synaptic plasticity; and this hypothesis is supported by the evidence that low maternal care induced a reduction of both mGlu1 receptors and 3,5-dihydroxyphenylglycine-induced LTD in the hippocampus of the offspring (Bagot et al., 2012). No gross behavioural changes were found so far in adult rats or mice born by caesarean section (including responses to stress or novelty, locomotor and anxiety behaviour) (Boksa et al., 1998; Chiesa et al., 2019), with the exception of enhanced dopamine-mediated motor behaviour (Vaillancourt & Boksa, 1998) and alterations in social interaction during adolescence (Venerosi et al., 2006). Specifically, the alterations described in C-section rats are remarkable as they involve social anxiety considered a marker for the onset of neuropsychiatric disorders (Rapoport et al., 2005). Given the implication of Group I mGlu receptors in the physiology of stress and emotional processing (Peterlik et al., 2016), our present findings suggest that the mild behavioural changes described to date in C-section rats involve long lasting alteration in mGlu1 receptors functional role. To our knowledge, no specific studies on the long term effects of C-section on hippocampal-dependent behaviour or hippocampal-dependent synaptic plasticity have been performed so far. The only changes reported to date refer to early transient underdevelopment of dendritic arbour, associated to very mild variation of ultrasound vocalisation at postnatal day 9 (Chiesa et al., 2019, 2020). The present findings suggest that the long term consequences of mode of delivery require further studies as they might have also potential implications for investigating the etiopathogenesis of other CNS disorders.

For example, changes in two schizophrenia-related genes, Csmd1 and Mmp16 that encode for CUB and Sushi multiple domains 1 and metallopeptodase 16, respectively, were recently found in the hippocampus and striatum of rats born by caesarean section (Paparelli et al., 2017) and loss-of-function mutations of the Grm1 gene encoding for the mGlu1 receptor have been associated with schizophrenia (Cho et al., 2014; Garcia-Barrantes et al., 2015). Whether changes in these genes are interrelated is an interesting question that warrants further investigations.

We acknowledge that the present study presents some limitations, namely the lack of information on gene expression levels and the missing correlation with hippocampal-related functional outcome. Further studies should include the female sex, for assessment of the sex-dependent vulnerability of the effects as well as the evaluation of the mGlu receptors at an earlier time point along development. However, our findings, though still preliminary, open a research perspective on the role played by early experiences in vulnerability to later diseases. A large body of evidence indicates that mGlu1 receptors shape the vulnerability of hippocampal neurons to hypoxic/ischemic insults. mGlu1 receptor antagonists are neuroprotective in hippocampal slices exposed to oxygen-glucose deprivation and attenuate hippocampal neuronal death in models of transient global ischemia by enhancing GABAergic transmission (Pellegrini-Giampietro, 2003). mGlu1 receptors also play a key role in mechanisms of ischemic pre-conditioning and post-conditioning in the hippocampus (Scartabelli et al., 2008; Werner et al., 2007). It will be interesting to examine whether caesarean delivery leads to long-lasting changes in the vulnerability of hippocampal neurons to transient global ischemia or other insults causing excitotoxic neuronal death.

In conclusion, despite the original hypothesis of the present study, we do not find hypoxia effects on glutamate metabotropic receptors, but a highly selective and region-specific

down-regulation of mGlu1α receptors without changing the expression of other mGlu or ionotropic glutamate receptors in rats undergoing caesarean delivery. This result strengthens the hypothesis that the mGlu1 receptor in the hippocampus might be a sensitive target for events occurring early in life and that could play a role in epigenetic programming (Bagot et al., 2012; Tribe et al., 2018).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

PERMISSION TO REPRODUCE MATERIAL FROM OTHER SOURCES

This manuscript does not contain a previously published table or figure.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS

ARZ, AV, GC, and GSA performed experiments; ARZ, PC, and FN analysed data; ARZ, PC, AV, GC and FN design research and wrote the manuscript.

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