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Title: Maternal consumption of piceatannol: a nutritional neuroprotective strategy against hypoxia-ischemia in rat neonates

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Abstract:

Hypoxia-ischemia (HI) remains a major cause of perinatal mortality and chronic disability in newborns worldwide (1-6 for 1,000 births) with a high risk of future motor, behavioral and neurological deficits. Keeping newborns under moderate hypothermia is the unique therapeutic approach but is not sufficiently successful as nearly 50% of infants do not respond to it. In a 7-day post-natal rat model of HI, we used pregnant and breastfeeding female nutritional supplementation with piceatannol (PIC), a polyphenol naturally found in berries, grapes and passion fruit, as a neuroprotective strategy. Maternal supplementation led to neuroprotection against neonate brain damage and reversed their sensorimotor deficits as well as cognitive impairments. Neuroprotection of *per os* maternal supplementation with PIC is a preventive strategy to counteract brain damage in pups induced by HI. This nutritional approach could easily be adopted as a preventive strategy in humans.

Highlights:

- Piceatannol ingestion decreases incidence of neonatal hypoxia-ischemia brain damage.
- Piceatannol maternal supplementation decreases severity of brain damage in pups.
- Piceatannol preserves motor and cognitive functions after neonatal hypoxia-ischemia.
- Maternal supplementation with piceatannol is neuroprotective in neonatal hypoxia-ischemia.

Keywords: Neuroprotection, Neonatal hypoxia-ischemia, Maternal supplementation, Piceatannol, Magnetic Resonance Imaging, Behavior.

1. Introduction

Hypoxia-ischemia (HI) remains a major cause of perinatal mortality and chronic disability in newborns worldwide (1-6/1,000 births) with a high risk of future motor, behavioral and neurological deficits (1,2). Despite this alarming socio-sanitary problem that affects both full-term and premature human neonates, the only commonly used therapy is based on controlled and moderate hypothermia. This unique therapeutic approach is not sufficiently successful as nearly 50% of infants do not respond to it (3). Therefore, it is imperative to develop new therapeutic strategies to reverse/prevent the sequalae of neonatal HI.

Accruing evidence indicates that polyphenols could play a neuroprotective role of in the context of HI, especially resveratrol (RSV) (4-7), but very little is known about its catecholic analog, piceatannol (PIC – 3,3',4,5'-trans-tetrahydroxystilbene), which occurs naturally in berries, grapes and passion fruit. PIC has greater bioavailability than RSV and its biological activity is much more effective (8).

HI induces brain deprivation of oxygen and the supply of the energetic substrate. In turn, this reduces ATP content and leads to a deficiency in ATP-dependent pumps and to cytotoxic edema. This cascade impacts the cerebral metabolism and causes neuronal death. While blood glucose is known to be the preferential substrate for the brain, lactate is increasingly thought to provide substantial brain energy fuel. This novel metabolic concept was first proposed by Pellerin and Magistretti in 1994 (9). As part of the astrocyte-neuron lactate shuttle (ANLS) as it is known, glucose taken from the circulation is first metabolized to lactate in the astrocytes, which are more glycolytic cells (10). Astrocytic lactate is then shuttled to the neurons, which are more oxidative cells, where it is converted into pyruvate and used as an energetic oxidative substrate. Interestingly, RSV has been shown to increase glycolysis (and therefore lactate production) in the brain, leading to a neuroprotective effect in a mouse model of middle cerebral artery occlusion (11).

The aim of the present work was to study the effect of a maternal supplementation with PIC on HI encephalopathy in rat neonates. For this purpose, we supplemented pregnant and breastfeeding females with PIC during the last week of gestation or during the last week of gestation and the first week of lactation. We used the classical Rice-Vannucci rat model (12) to induce HI brain injury in seven-day-old pups (P7). We evaluated neonatal brain damage and its evolution *in vivo* with magnetic resonance imaging (MRI). Brain lesions were characterized using diffusion-weighted imaging (DWI), a non-invasive technique. We evaluated the effects of PIC maternal supplementation on brain function in the same pups using motor and behavioral studies. No study has yet explored the possible neuroprotective role of PIC in the context of neonatal HI, especially with this original nutritional and transgenerational approach.

2. Results

2.1. Cerebral lesions induced by HI

DWI of P7-pup brains for sham and control (Ct) groups 3 h post-ligation is presented in Fig.1A. No brain injury was visible in the sham group. In the Ct group, HI induced brain damage (white arrow). In the Ct group, HI lesions involved 41 ± 3% of the total brain (Fig.1B). The severity of the HI edema was evaluated by ADC measurements (Fig.1C). In all structures, ADC was significantly reduced in the Ct group compared to the sham group.

2.2. Early neuroprotection with maternal PIC supplementation

DWI of P7-pup brains is presented Fig.2A. At P7 and P9, the volume of damaged brain (%, normalized to total brain volume) was measured (Fig.2B). Pups in the Ct group had the largest lesions compared to those in the picL and picGL groups (P7: 40.8 ± 2.9%, 20.8 ± 5.6%, and 15.9 ± 5.8%, for Ct, picL, and picGL groups, respectively; and P9: 20.3 ± 2.9%, 3.5 ± 1.8%, and 5.9 ± 2.9%, for Ct, picL, and picGL groups, respectively). The percentage of rats that had brain damage in each group is shown in Fig.2C. In the Ct group, all pups had brain lesions (100%) whereas only 75% and 55% had HI damage in the picL and picGL groups, respectively. Considering only the pups that underwent the initial brain lesion, we were still able to detect a statistical difference between the control and PIC groups (Fig.2D). ADC maps (Fig.2E) were acquired by DWI and ADC values (Fig.2F) were measured in the cortex, hippocampus and striatum at P7 and P9 to characterize the brain edema induced by HI. ADC measures the speed of water molecule diffusion in the extracellular environment in mm^2/s; the lower the value, the more severe the edema. At P9, ADC values in the Ct group were still much lower in all structures than those in the sham group,. In the picL and picGL groups, ADC values returned to those of the sham group. To confirm our data at the cellular level, we performed Nissl staining to evaluate neuronal death at P9 in the penumbra region (Table 1). In the cortical region, there was a 50% drop in the

number of neurons in the Ct group compared to the sham group, and only a 15% drop in the picL and picGL groups. In the striatum, while there was a 25% drop in the number of neurons in the Ct group compared to the sham group, picL and picGL groups had the same number of neurons than the sham group.

2.3. Long-term neuroprotective effect of maternal PIC supplementation

Angiography performed at P7 confirmed carotid ligation, while at P30 it revealed vascular recovery and rearrangement in the PIC groups (Fig.3A). At P30, a blood vessel gap in the ipsilateral hemisphere was observed (Fig.3A, white arrow) due to brain necrosis (blue arrow on the corresponding T2-weighted image). At P30, 67% of Ct rats still had brain damage, compared to 37 % and 29% in the picL and picGL groups, respectively (Fig.3B). The Ct group still had the highest lesion sizes compared to thePIC groups (P30 lesion sizes: 27.8 ± 7.9%, 3.0 ± 1.7% and, 2.4 ± 2.2% for Ct, picL and picGL groups, respectively). To assess the white matter reorganization, fractional anisotropy (FA) was measured at P30 (Fig.3C). FA values range from 0 (isotropic diffusion) to 1 (anisotropic diffusion) and allow white fiber arrangements to be assessed. A decrease in FA is generally attributed to a loss of neuronal fibers. For all brain structures considered, the Ct group had lower FA than the PIC groups. Since not enough lesions were detected at P30 in the picL and picGL groups, and because no significant difference was found between these two groups, data from the picL and picGL groups were pooled.

2.4. Restoration of motor abilities and recovery of cognitive functions with maternal PIC supplementation

Early reflex tests were performed from P8 to P12 (Fig.4A and B). The Ct group had significantly the worst performances on the righting reflex test, compared to the sham, picL and picGL groups (Fig.4A). No difference was found between the sham group and the PIC groups. Similarly, the Ct

group took the longest to leave a 25-cm diameter circle during the locomotion test, whereas the PIC groups were the fastest (Fig.4B).

Sensorimotor deficits were also assessed at P24 using the mNSS (Fig.4C). This test allows the evaluation of sensory, motor and reflex performances. Scoring ranges between 0 (no impairment) to 18 (most severe impairment). The mNSS values of the Ct group were significantly higher compared to the picL and picGL groups (3.5 ± 0.7; 0.8 ± 0.3; 0.7 ± 0.4 for Ct, picL, and picGL groups, respectively). No difference was found between the sham and PIC groups.

The novel object recognition test was performed at P45 and a discrimination index was calculated (Fig.4D). No difference was found between the sham and PIC groups whereas pups in the Ct group clearly showed impairment in their long-term memory (discrimination index: 0.77 ± 0.04; 0.47 ± 0.01; 0.68 ± 0.01; 0.83 ± 0.01, for sham, Ct, picL, and picGL, respectively).

3. Discussion

In this study, we focused on PIC, a less well known and studied hydroxylated analog of RSV. Its bioavailability (8,13-15) is greater than that of RSV, but it has not been tested to date in the context of neonatal HI nor by a nutritional and transgenerational approach. Neuroprotection by maternal PIC supplementation was observed in HI pups as early as 3 h post-injury, thus demonstrating its short-term effect. Although MRI values in brain lesions (lesion size, ADC, FA) did not allow the picL and picGL groups to be discriminated, fewer pups in the picGL group developed cerebral damage, suggesting that the longer the PIC is administered, the better is the neuroprotection. While a spontaneous 50% decrease in lesion size was observed in the Ct group during the 48 h after the injury, the recovery was much higher in the PIC groups (90% and 85% reduction of brain lesions in picL and picGL groups, respectively).

In addition to measurements of brain lesion size, DWI provides insights into physical parameters that reflect edema severity (the lower the ADC, the greater the edema) and neuronal fiber integrity (the higher the FA, the more neuronal fibers are preserved). Indeed, the deficit in oxygen and nutrients occurring during HI induces a decrease in metabolism and thus in ATP content. ATP-dependent pumps are no longer efficient, which leads to an ionic disequilibrium and water influx. This cellular swelling is called cytotoxic edema and occurs very early after stroke. It is characterized by swelling of both astrocytes and neuronal dendrites (16,17). Typically, ADC values decrease within minutes of brain injury due to water movement restriction in this cytotoxic edema, a phenomenon that was observed in our study as soon as 3 h after ligation in all groups. During the 48 h after HI, ADC values follow a dynamic process and tend to recover, a phenomenon called pseudonormalization (18). In our study, we found a statistically significant increase in ADC values between P7 and P9 in all HI groups. However, the recovery was complete only in the PIC groups. Finally, neuronal death, which was evaluated at P9 by histology, confirmed our MRI results at the cellular level. Taken together, all these data

demonstrate that maternal PIC supplementation leads to the following (i) a reduction in the number of pups with brain lesions; (ii) a reduction in brain lesion size when brain lesions are present in PIC groups; (iii) less severe edema inside brain lesions.

The neuroprotective effects of PIC were still present at P30. While two thirds of control pups still had brain lesions, only one third of those in the PIC groups had brain damage. Moreover, in brain-damaged pups, FA were higher in all structures in the PIC groups compared to the Ct group, reflecting a higher density of neural fibers. Therefore, maternal PIC supplementation significantly improved the spatial distribution or rearrangement of white matter fiber bundles.

In behavioral terms, PIC supplementation restored motor functions. When sensorimotor functions were assessed in juvenile rats (P24) using the mNSS, the PIC groups had the best performances, reaching those of the sham group.

Among the deleterious effects induced by HI, cognitive impairment due to neuronal and white matter damage is often a consequence in injured neonates (19). HI rodent models also display memory deficits (7,20-22). In addition to showing anatomical improvements, our data revealed a neuroprotective role of maternal PIC supplementation on the hippocampal-dependent long-term memory of pups, which was completely restored. These results could be compared with those of Arteaga *et al*., which demonstrated that RSV improves the long-lasting cognitive deficits induced by HI (7).

This neuroprotective effect of maternal PIC supplementation could be due to its antioxidant properties. Although the antioxidant properties of RSV are well documented, the additional hydroxyl group in the stilbene ring B of PIC significantly increases its free radical scavenging activity as well as its bioavailability (13-15). However, the neuroprotective effect of PIC may be linked to modulation of the brain metabolism. Although no study has addressed the role of PIC in the regulation of cerebral metabolism under physiological or pathological conditions, a few have examined the role of RSV on this parameter (Fig.5). Under physiological conditions, neuronal

glutamate is released in the synaptic cleft and is recaptured and recycled by astrocytes into glutamine *via* the glutamine synthetase (GS) enzyme. The astrocytic uptake of glutamate is predominantly (~ 90%) mediated by the glutamate transporter type-1 (GLT-1) (23). Rat transient forebrain ischemia led to a decrease in GLT-1 expression that was reversed by RSV pre-HI i.p. injections for 3 weeks (24). After HI, a decrease in glutamate uptake by astrocytes was observed (25), leading to overstimulation of N-methyl-D-aspartate glutamate receptors and a disruption of ionic homeostasis, especially of Na^+ and Ca^{2+}. Glutamatergic excitotoxicity is a major cause of neuronal death in HI (26). In rat hippocampal slices, RSV (50 µM) treatment increased astrocytic glutamate uptake during oxidative injury induced by H_2O_2 (27). This stimulation of glutamate uptake is associated with an RSV (100 µM)-dependent increase in GS expression to facilitate the recycling of glutamate to glutamine (28). An increase in GS activity was also found in primary astrocyte cultures of P1 Wistar rats in the presence of RSV (10 µM) (29). In parallel, glutamate uptake was shown to increase astrocytic glycolysis and then the astrocytic production of lactate in primary cultures of mouse cortical astrocytes (9). As proposed in the ANLS model, astrocytic lactate is shuttled to neurons *via* monocarboxylate transporters and is used as a neuronal energy fuel. In the context of PIC treatment, the use of lactate by neurons might counteract the energy deficit induced by HI. Moreover, it might allow the reallocation of spared glucose to go through the phosphate pentose pathway, leading to the production of glutathione. Such a glutathione increase has been shown in the presence of RSV (100 µM), after an oxidative injury induced by H_2O_2 in rat hippocampal slices (28). Similarly, RSV (5 µM) pretreatment of neural stem cells undergoing oxygen-glucose deprivation/reoxygenation led to an increase in neuronal glutathione levels (30). Similarly, a decrease in reactive oxygen species (ROS) was measured in astrocyte cultures exposed to H_2O_2 oxidative stress in the presence of RSV (1 µM) (31), and in neural stem cell cultures (32). Since PIC is a homolog of RSV but with greater bioavailability, its neuroprotective effect found here could involve regulation of the brain metabolism, as with RSV.

This is the first demonstration of the neuroprotective role of PIC in HI. At P30, only one third of the pups whose mother had been supplemented with PIC still had lesions. However, the lesions represented < 3% of the total brain volume and motor and cognitive functions had been completely recovered. Furthermore, PIC was not administered intraperitoneally to the pups but was given to their mother *via* drinking water in the nutritional range. Since 50% of infants do not respond to hypothermia therapy (3), these preliminary results are extremely promising and may be of relevance for the clinical management of neonates suffering from HI.

4. Experimental procedures

4.1. Study Design

The experimental design is presented in Fig.6. All animal procedures were conducted in accordance with the Animal Experimentation Guidelines of the European Communities Council Directive of November 24, 1986 (86/609/EEC). Protocols met the ethical guidelines of the French Ministry of Agriculture and Forests and were approved by the local ethics committees (9476). Sample size was determined based on a previous study (33). Only rats with no perfusion defect in the left hemisphere (confirmed by perfusion and angiographic MRI) were excluded from the study. Analyses were blindly performed by two separate experimenters (intra-experimenter variability < 1%; inter-experimenter variability < 5%).

4.2. Animals

Pregnant Wistar RJ-HAN females (Janvier Laboratories, France) were received at day 15 of gestation and were kept on a 12:12 hours light:dark cycle with food (A03) and water *ad libitum*.

4.3. Model of hypoxic-ischemic brain injury

Cerebral HI was performed as described previously (12,33). Briefly, P7 pups were anesthetized with isoflurane (4% for induction and 1.5% for maintenance) and a midline neck incision was made under local anesthesia (lidocaine 0.5%). The left common carotid artery was permanently ligated with a 7-0 silk thread. After recovery (30 min), pups were then placed for 2 h in a humidified hypoxic chamber (8% oxygen, 92% nitrogen – 33°C) (Intensive Care Unit Warmer, Harvard Apparatus, France). During hypoxia, sham-operated pups were kept separated from the dam in a heated atmosphere (33°C). After HI, pups were returned to their heated pad until the first imaging, performed 30 min later (3 h after the end of the ligation) then at P9, and P30.

4.4. Experimental groups and drug administration

Four experimental groups were established according to the maternal supplementation. Maternal drinking water alone for sham (n = 15) and control groups (Ct group, n = 18) was supplemented with a realistic nutritional amount of PIC (corresponding to one passion fruit/day for a pregnant woman) during the first week of lactation for the picL group (n = 8) or during the last week of gestation plus the first week of lactation for the picGL group (n = 11) (Tokyo Chemical Industry Co, France). PIC stock solution was prepared by dissolving 100 mg of PIC in 2 ml absolute ethanol. Four µl of the PIC stock solution were added in 200 ml of drinking water, an amount equivalent to 0.15 mg/kg/day since a pregnant Wistar rat female is drinking 50 ml/day. Each final PIC solution was prepared and changed everyday. Drinking bottles were enwrapped in aluminum foil to avoid PIC oxidation.

4.5. Nissl staining

P9 pups (n=3 per group) were perfused transcardially with PBS followed by 4% paraformaldehyde (PFA) in 0.1 M PBS (pH 7.2-7.4), for 10 min, under isoflurane (4.5 %). Brains were removed and post-fixed for 24 h, at 4°C, in PFA. Fixed brains were cryoprotected by sucrose immersion (48 h in a 20% sucrose solution, followed by 24h in a 30% solution, 4°C) and then frozen in liquid N_2 vapor. The 16 µm-thick brain sections were stained with 0.5% cresyl violet (Sigma-Aldrich, France) for 5 min and washed in distilled water for 10 s. Sections were dehydrated in 95 and 100% ethanol (2 min), defatted in xylene and mounted on coverslips in Depex mounting medium (Sigma Aldrich, Germany). Brain sections were observed using an optical microscope (Leica STP6000). ImageJ was used to count nuclei.

4.6. Acquisition of *in vivo* longitudinal MRI

Pup *in vivo* MRI was performed on a horizontal 4.7T Biospec 47/50 system (Bruker, Ettlingen, Germany) equipped with a 6-cm BG6 gradient system (1000 mT/m, Δ = 20 ms, δ = 4 ms). For MRI, pups were anesthetized with isoflurane (4% induction, 1.5% maintenance), and respiration was monitored by a ventral pressure sensor. Warmed water circulating in the gradient coil system was used to maintain body temperature. Measurements were performed with Paravision 6.0.1 (Bruker BioSpin, Karlsruhe, Germany). Anatomical T2-weighted images of the brain were obtained using a Rapid Acquisition with Relaxation Enhancement (RARE) sequence (20 axial slices, 0.7 mm thick, echo time (TE) 50 ms, repeating time (TR) 3000 ms, total duration 4 min 48 s). Brain lesions were assessed by magnetic resonance diffusion tensor imaging (DTI: 20 axial slices of 0.7 mm, b-value 1347 s/mm^2, TE 24 ms, TR 2 s, 30 directions, total duration 17 min 04 s). Magnetic resonance angiography was used to image the cerebral blood system (Time-of-Flight (TOF)-3D-Fast low angle shot (FLASH): axial slice, 20 mm thick, TE 2.2 ms, TR 13 ms, Flip angle 15°, total duration 10 min 59 s).

4.7. MRI Analysis

The extent of the lesion was determined from the signal of the apparent diffusion coefficient (ADC) maps of DWI. Only lesion sizes higher than 1% were considered. ADC maps and fractional anisotropy (FA) maps were computed using the Paravision 6.0.1 diffusion tensor calculation module. For each rat, a region of interest (ROI) was manually drawn on each section to encompass the injured area and determine the global brain area to quantify the lesion size (relative to the entire brain size). ROIs were manually determined in the lesion area (cortex, hippocampus, striatum) to evaluate the variations in ADC at P7 and P9. FA was determined at P30 in the ipsilateral cortex, hippocampus and striatum.

4.8. Behavioral tests

4.8.1. Early reflexes (6)

Righting reflex (P8, P10, P12)

Pups were placed in a supine position and the time required to turn over on all four paws and touch the surface was recorded. Each pup was given three trials and the mean time to perform the reflex was calculated.

Locomotion aptitude (P8, P10, P12)

Pups were placed in the middle of a 25-cm diameter circle. Time to leave the circle was measured.

4.8.2. Modified neurological severity score (mNSS) (P24)

Neurological functions were assessed using the mNSS (34), which is composed of motor (muscle status), sensory (tactile and proprioceptive), beam balance, reflexes (pinna, corneal and startle) tests. Each rat was graded on the mNSS scale (0: no impairment – 1-6: middle impairment – 7-12: moderate impairment – 13-18: severe impairment).

4.8.3. Novel object recognition (P45) (7)

Pup long-term memory was assessed using the novel object recognition test. After a habituation period in the open field on day 1, pups were further allowed to freely explore two identical objects placed in opposite corners of the arena for 10 min on the next two days. On the last day (day 4), one of the old objects was replaced by a different one and pups were allowed to freely explore the objects for 10 min. Duration of object exploration was measured. The discrimination index ([time with new object minus time with familiar object]/[time with new object plus time with familiar object]) was defined as the parameter for evaluation.

4.9. Statistical Analysis

Statistical analysis and graphs were performed using GraphPad Prism 7.00 software. All data were expressed as mean ± standard error of the mean (s.e.m). Number of pups is stated for each experiment within figure legends. The statistical significance of the differences between multiple groups was determined using One-Way ANOVA and Fisher's LSD post-hoc test. Statistical significance was defined as $P < 0.05$.

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4.11. Conflict of Interest

Authors declare that they have no conflict of interest.

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Table 1

	Sham	Ct	picL	picGL
cortex	**100.0 ± 2.4 #**	**49.2 ±3.8 ***	**85.1 ± 2.1**	**85.9 ± 2.2**
striatum	**100.0 ± 3.4**	**74.2 ± 1.6 ***	**96.4 ± 3.9**	**101.0 ± 4.1**

*Neuronal death was evaluated in the penumbra region (both cortical and striatal regions) on histological cryosections from P9 pups after Nissl staining. Data are presented as percentage of the number of neurons found in the sham group. * Significant difference from sham, picL and picGL, # Significant difference from Ct, picL and picGL, $p \leq 0.0002$, one-way analysis of variance (ANOVA) with Fisher's LSD post-hoc test, n = 3 per group).*

Figure legends:

Fig.1 Anatomical characterization of neonate brain injuries following HI episode. *(**A**) Typical diffusion-weighted images of P7 pup brains of sham and Ct groups. Images were obtained at 4.7 T, 3 h after injury. Damage appeared as a hyposignal (white arrow). (**B**) Quantification of lesion volumes for Ct group (%, normalized to total brain volume). (**C**) Ipsilateral ADC values in mm^2/s for sham and Ct groups in cortex, hippocampus and striatum. Results are mean values ± SEM. * Significant difference between two groups (**** $p < 0.0001$), one-way analysis of variance (ANOVA) with Fisher's LSD post-hoc test; n = 6 sham pups, n = 18 Ct pups).*

Fig.2 Short-term effect of PIC maternal supplementation on pup brain damage induced by HI. *(**A**) Typical diffusion-weighted images of P7-pup brain of Ct, picL and picGL groups. Images were obtained at 4.7 T, 3 h after injury. (**B**) Lesion sizes (%, normalized to total brain volume) for all pups at P7 and P9. (**C**) Percentage of P7 pups with more than 1% of brain lesion for Ct, picL and picGL groups, 3 h after injury. (**D**) Lesion sizes only for pups that had initial lesions in Ct, picL and picGL groups at P7 and P9. (**E**) ADC maps for Ct, picL and picGL P7 pups, presented on a color scale from purple (ADC = 0) to red (highest ADC values). (**F**) Ipsilateral ADC values for sham, Ct, picL and picGL groups, at P7 and P9. Results are mean values ± SEM. * Significant difference between two groups (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$), one-way analysis of variance (ANOVA) with Fisher's LSD post-hoc tests; (**A**) and (**B**) P7: Ct, n = 18; picL, n = 8; picGL, n = 11; P9: Ct, n = 19; picL, n = 8; picGL, n = 7 and P7: picL, n = 6; picGL, n = 6; P9: picL, n = 6; picGL, n = 6 for lesion size. (**C**) with a value only if lesion is present in the brain area.*

Fig.3 Long-term effect of PIC maternal supplementation on pup brain damage induced by HI. *(**A**) Typical angiographic images of P7- and P30-pup brains and typical anatomical T2-weighted images of P30-pup brains for Ct, picL and picGL groups. White arrow: blood vessel*

*gap in the ipsilateral hemisphere due to brain necrosis (blue arrow on the corresponding T2-weighted image). (**B**) Representation of the percentage of pups with more than 1% of brain lesion for Ct, picL and picGL groups, at P30 and lesion sizes for Ct, picL and picGL groups at P30. (**C**) FA measurement at P30 in ipsilateral white matter in cortex, hippocampus and striatum in Ct and PIC groups (picL and picGL data pooled). *Significant difference between two groups (* $p < 0.05$, ** $p < 0.01$) one-way analysis of variance (ANOVA) with Fisher's LSD post-hoc test; Ct, n = 6; picL, n = 8; picGL, n = 7 (for FA measurements, value was obtained only if lesion was present in the brain area).*

Fig.4 Sensorimotor behavioral tests and long-term memory. *Performances for the righting reflex (**A**) and locomotion test (**B**), between P8 and P12, for sham, Ct, picL and picGL groups. * Significantly different from sham group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$); # significantly different from picL group (# $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$); • significantly different from Ct group (• $p < 0.05$, •• $p < 0.01$, ••• $p < 0.001$, •••• $p < 0.0001$). (**C**) mNSS score at P24 for sham, Ct, picL and picGL groups. * Significant difference between two groups (*** $p < 0.001$). (**D**) Performances for the novel object recognition test were assessed at P45 for sham, Ct, picL and picGL groups. The discrimination index was calculated using the following equation: [time in new object – time in familiar object]/[time in new object + time in familiar object]. * Significant difference between two groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Results are mean values ± SEM. One-way analysis of variance (ANOVA) with Fisher's LSD post-hoc test; $n \geq 6$ pups.*

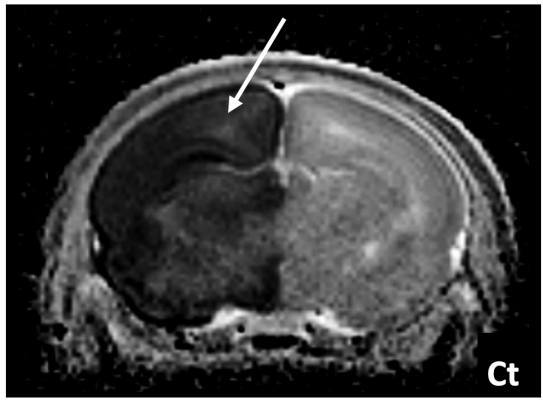
Fig.5 Schematic representation of effects of resveratrol on brain metabolism. *Red dashed arrows connect discussed point with the respective published work. Briefly, PIC is thought to act like RSV, which reverses the decrease in glutamate transporter type-1 (GLT-1) induced by HI [24] and increases the astrocytic uptake of glutamate [27]. This stimulation of glutamate uptake is associated with an increase in glutamine synthetase (GS) activity to facilitate the recycling of*

glutamate into glutamine [28,29]. In parallel, glutamate uptake increases astrocytic glycolysis and then the astrocytic production of lactate [9]. This astrocytic lactate is shuttled to neurons where it is used as energy fuel. RSV also leads to an increase in glutathione production [27,30] and a decrease in ROS [32].

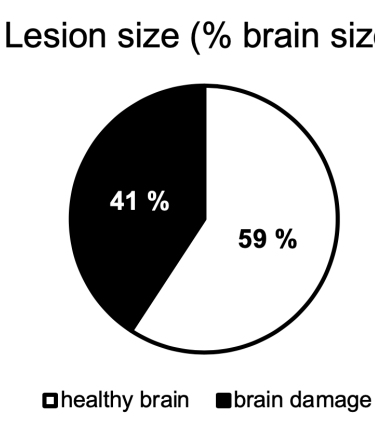
Fig.6 Scheme of experimental design. *Four groups were studied: sham group, Ct group, picL group: pups whose mother was supplemented with PIC (0.15 mg/kg/day) during the first week of lactation and a picGL group whose mother was supplemented with PIC during the last week of gestation and the first week of lactation. Ct, picL, picGL groups were exposed to HI procedure. HI brain damage was monitored by MRI (at P7, P9 and P30). Effect of maternal supplementation on HI pup sensorimotor functions was assessed with early reflexes test (from P8 to P12) and mNSS (P24). Long-term memory of pups was assessed with the novel object recognition test (P45).*

A

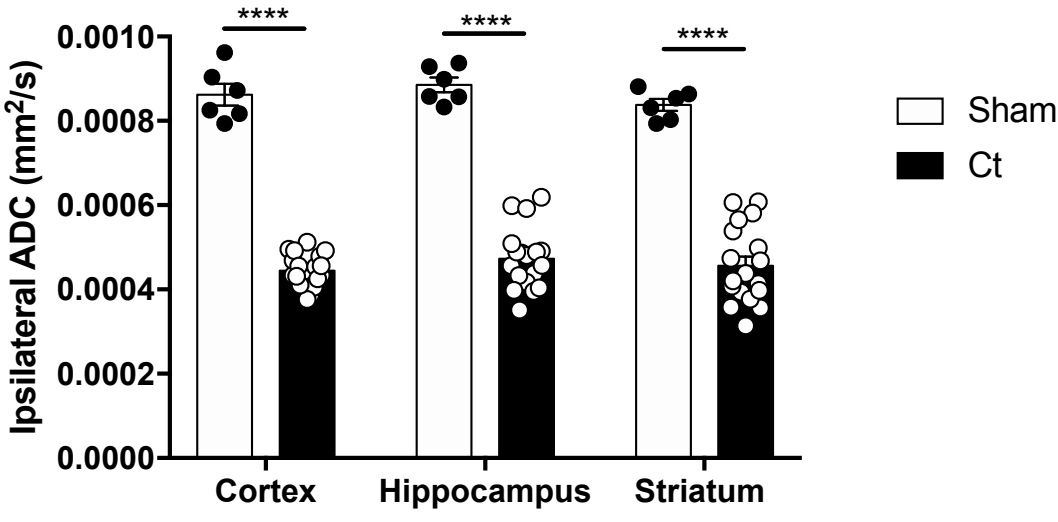

Sham


Ct

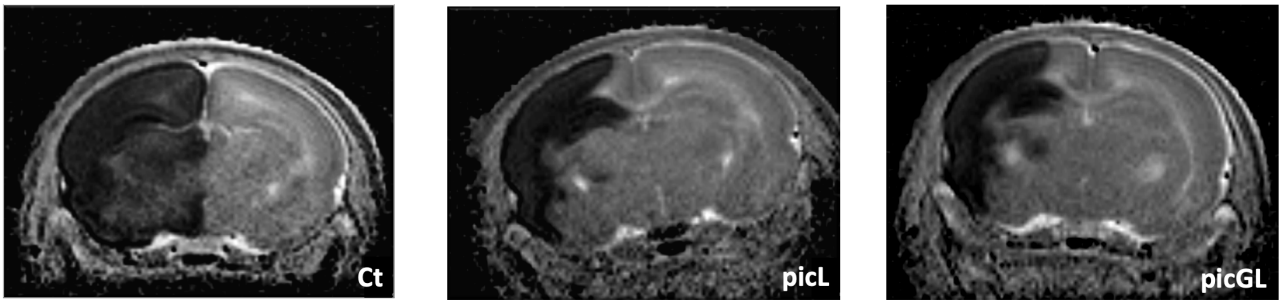
B


Lesion size (% brain size)
41 %
59 %
healthy brain
brain damage

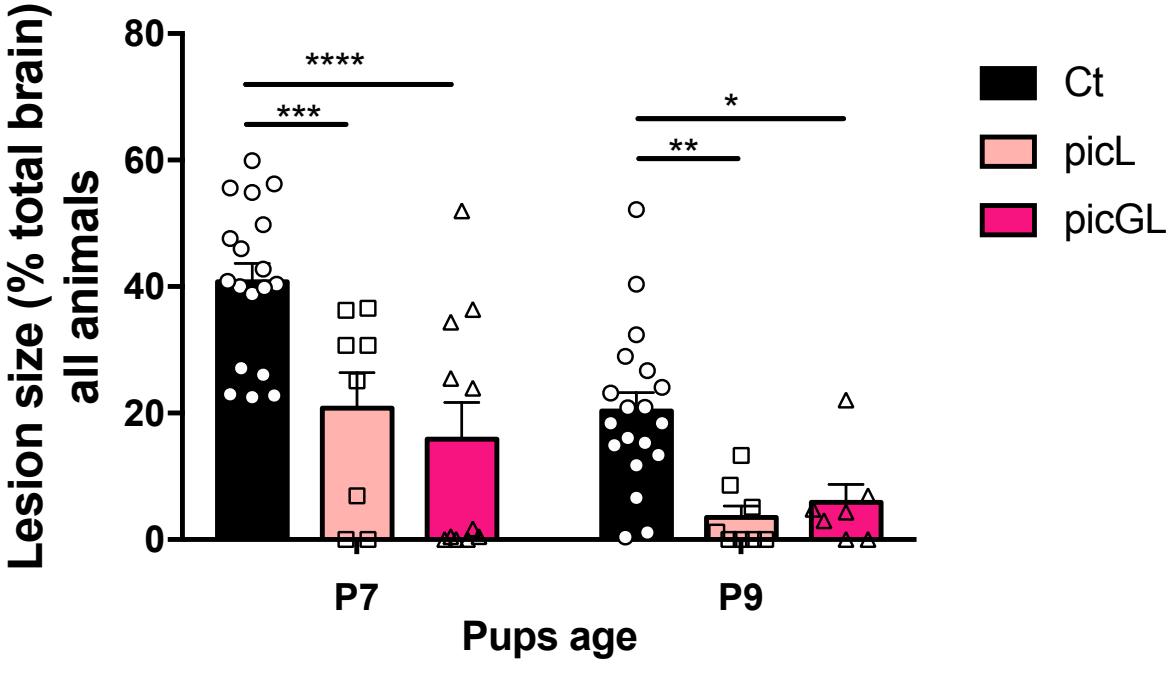
C


Ipsilateral ADC (mm²/s)
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
****
****
****
Cortex
Hippocampus
Striatum
Sham
Ct

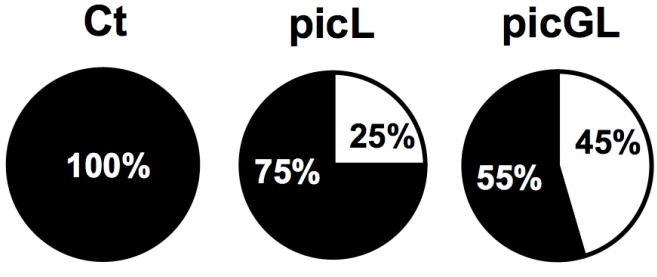
A



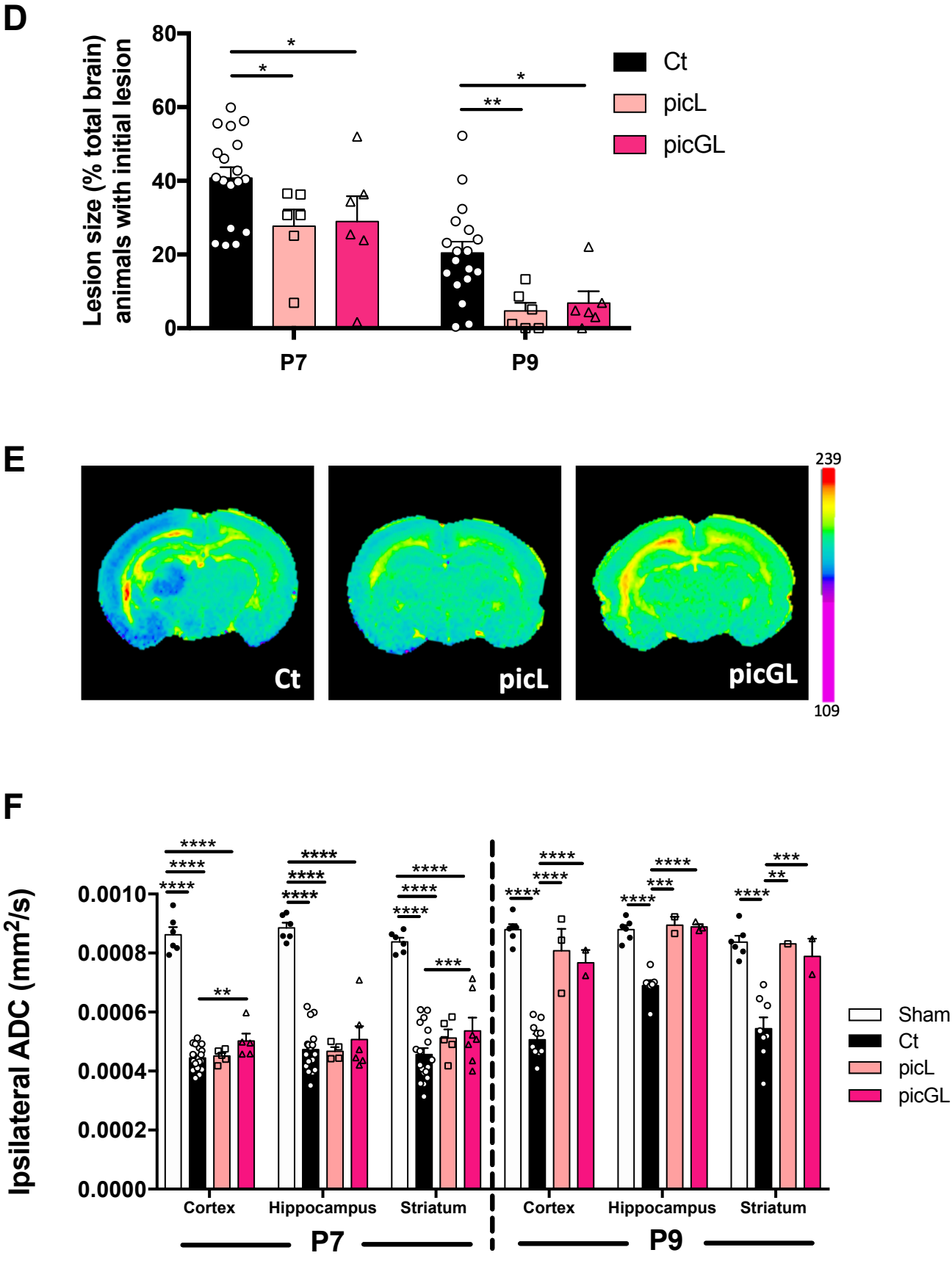
B

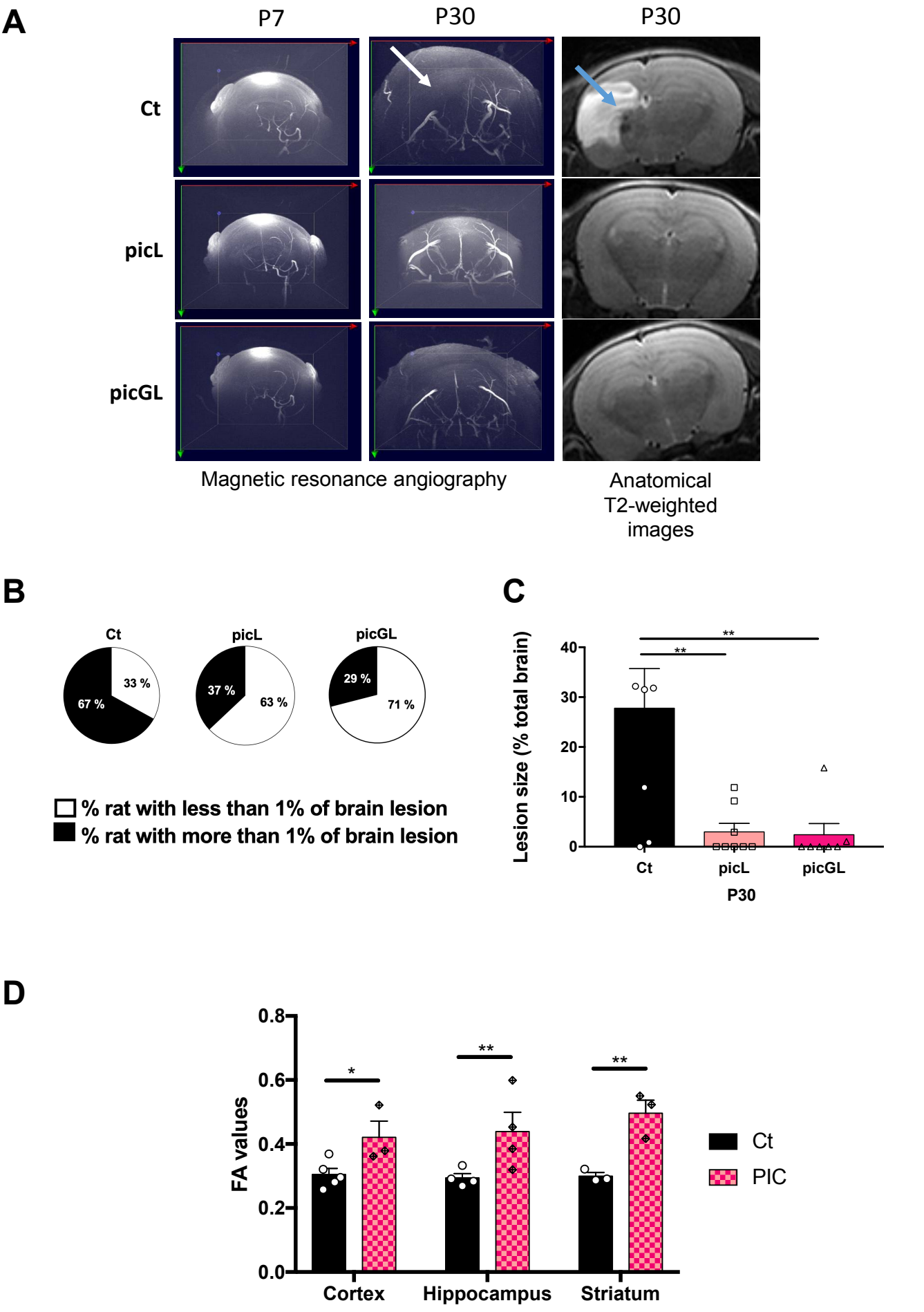


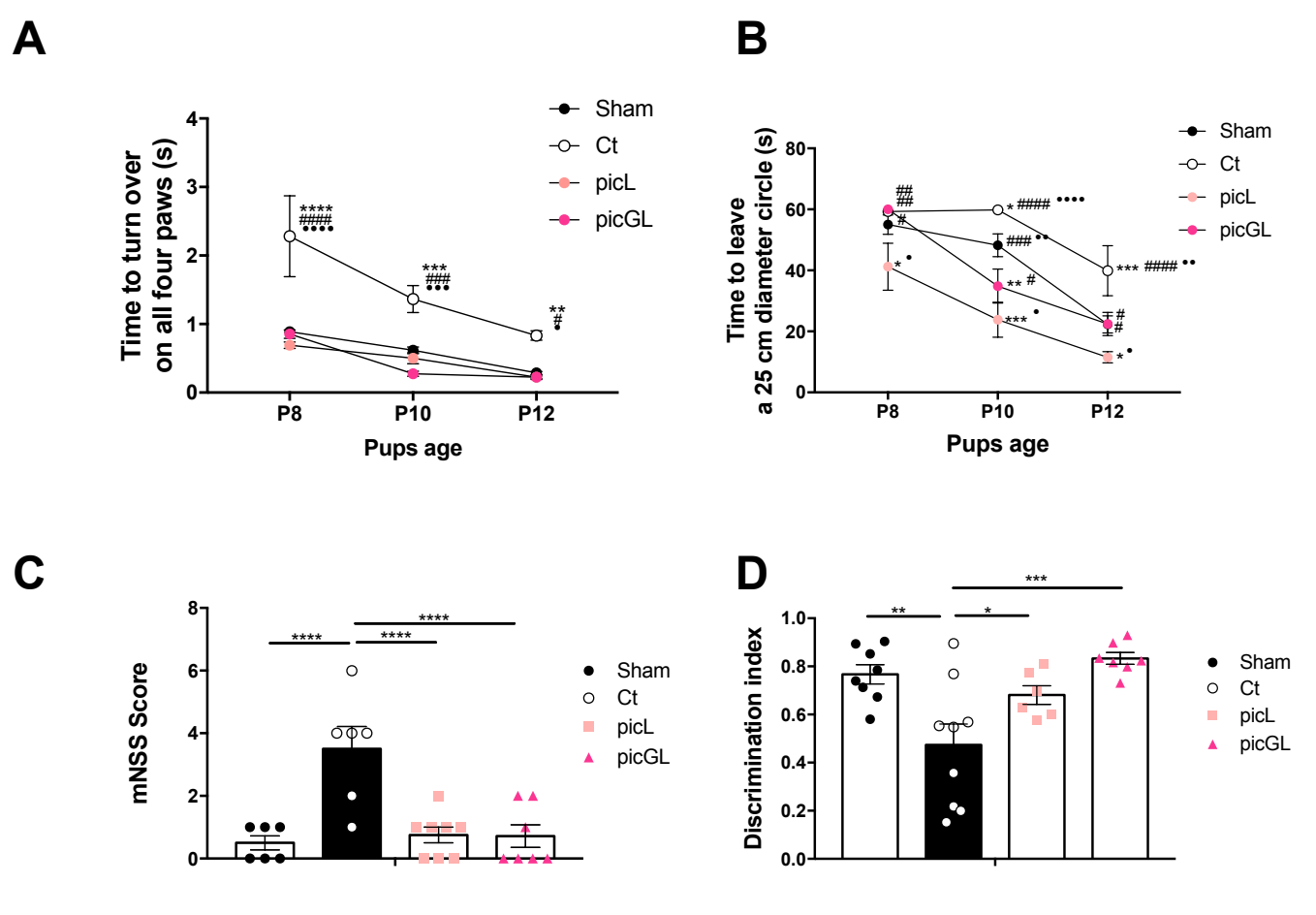
C

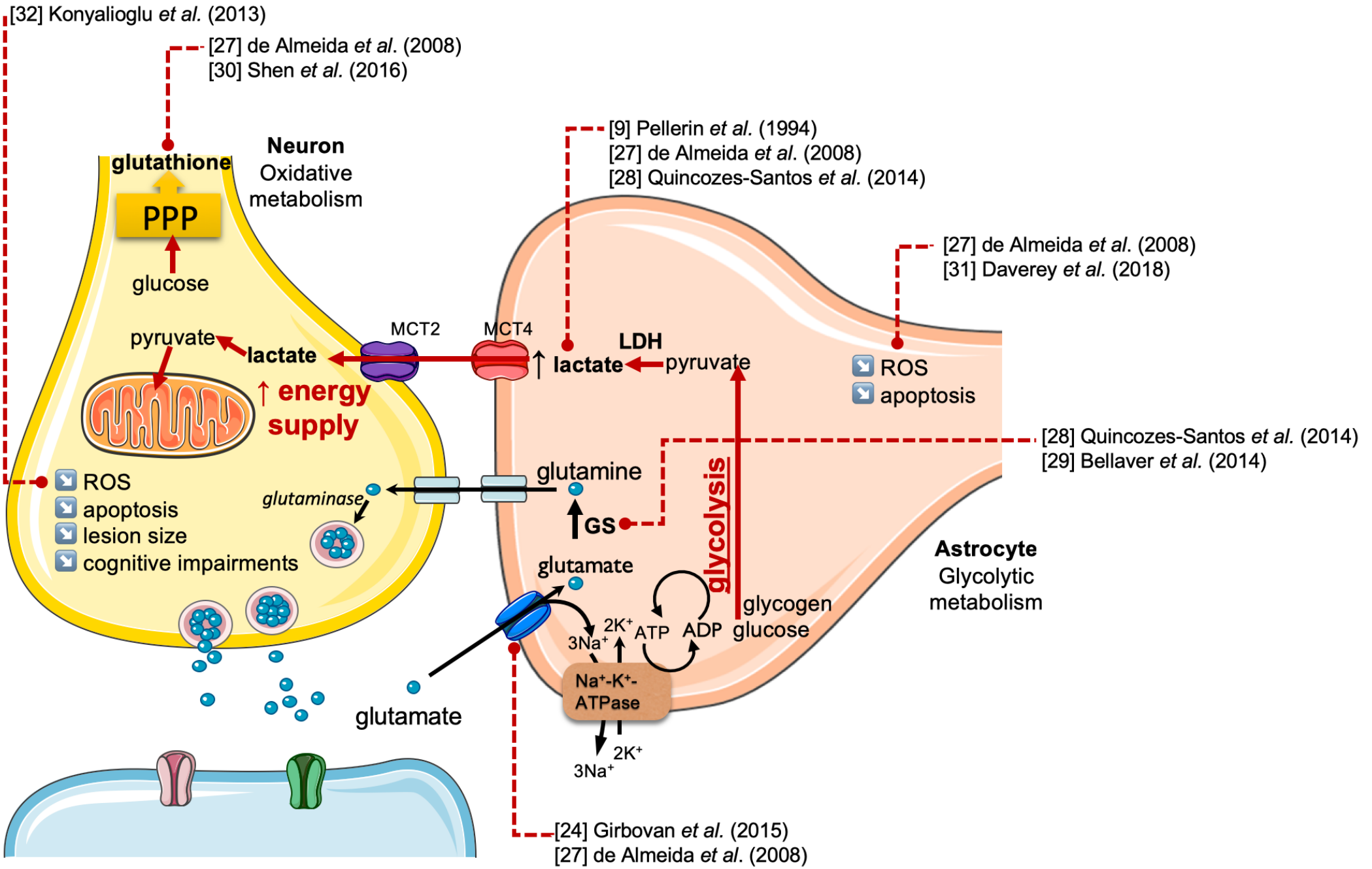


% rat with less than 1% of brain lesion
% rat with more than 1% of brain lesion


D
Lesion size (% total brain) animals with initial lesion
80
60
40
20
0
*
*
*
**
Ct
picL
picGL
P7
P9
E
Ct
picL
picGL
239
109
F
Ipsilateral ADC (mm²/s)
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
Cortex
Hippocampus
Striatum
P7
P9
Sham
Ct
picL
picGL


A
P7
P30
P30
Ct
picL
picGL
Magnetic resonance angiography
Anatomical T2-weighted images
B
Ct
picL
picGL
33 %
67 %
37 %
63 %
29 %
71 %
% rat with less than 1% of brain lesion
% rat with more than 1% of brain lesion
C
Lesion size (% total brain)
40
30
20
10
0
**
**
Ct
picL
picGL
P30
D
FA values
0.8
0.6
0.4
0.2
0.0
*
**
**
Cortex
Hippocampus
Striatum
Ct
PIC


A
Time to turn over on all four paws (s)
Sham
Ct
picL
picGL
P8
P10
P12
Pups age
B
Time to leave a 25 cm diameter circle (s)
Sham
Ct
picL
picGL
P8
P10
P12
Pups age
C
mNSS Score
Sham
Ct
picL
picGL
D
Discrimination index
Sham
Ct
picL
picGL


[32] Konyalioglu et al. (2013)
[27] de Almeida et al. (2008)
[30] Shen et al. (2016)
glutathione
Neuron
Oxidative
metabolism
PPP
glucose
pyruvate
lactate
MCT2
MCT4
[9] Pellerin et al. (1994)
[27] de Almeida et al. (2008)
[28] Quincozes-Santos et al. (2014)
LDH
lactate
pyruvate
[27] de Almeida et al. (2008)
[31] Daverey et al. (2018)
ROS
apoptosis
↑ energy
supply
[28] Quincozes-Santos et al. (2014)
[29] Bellaver et al. (2014)
glutamine
glutaminase
GS
glycolysis
ROS
apoptosis
lesion size
cognitive impairments
glutamate
Astrocyte
Glycolytic
metabolism
glycogen
glucose
3Na+
2K+
ATP
ADP
Na+-K+-
ATPase
2K+
3Na+
glutamate
[24] Girbovan et al. (2015)
[27] de Almeida et al. (2008)

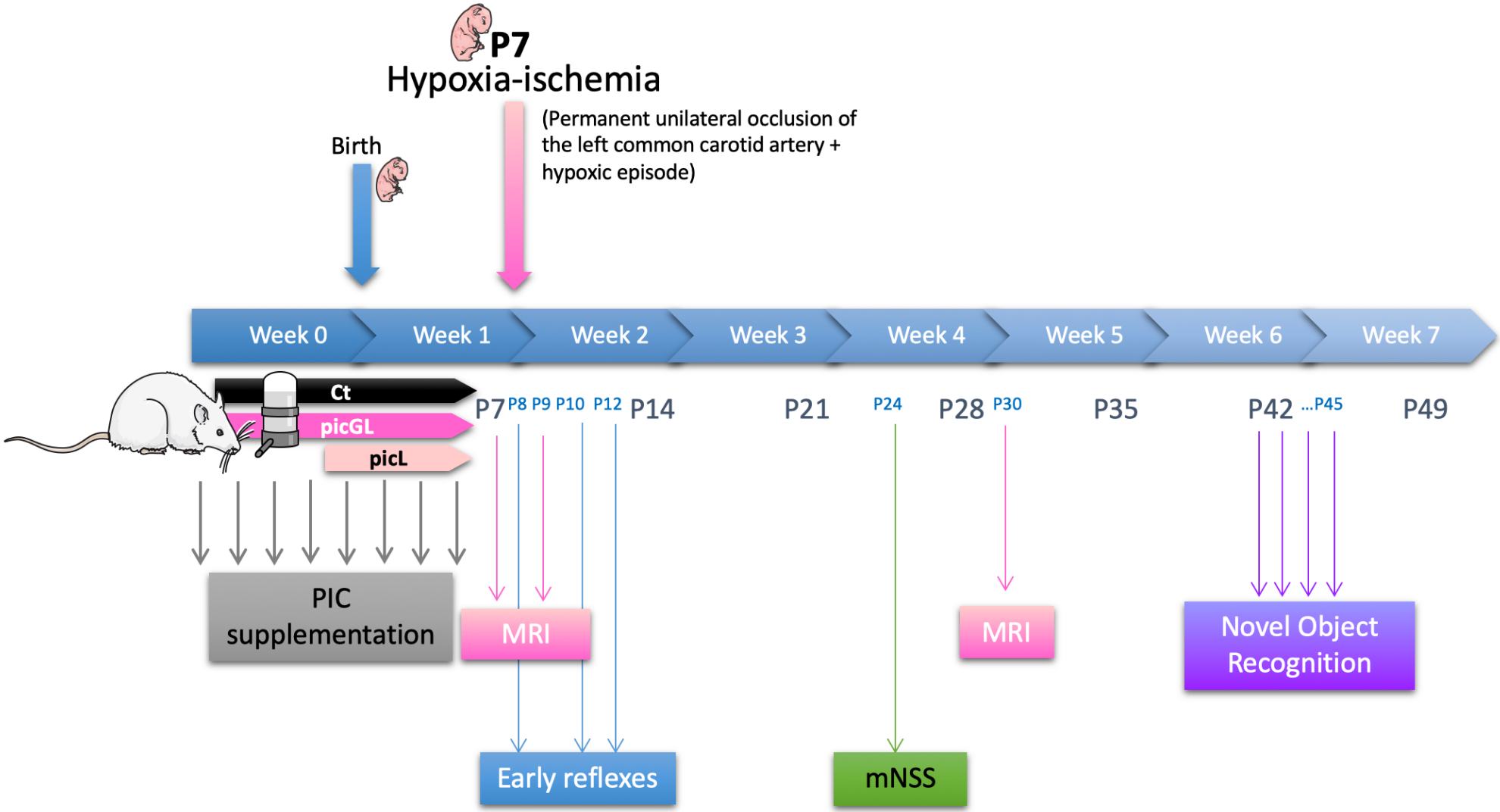
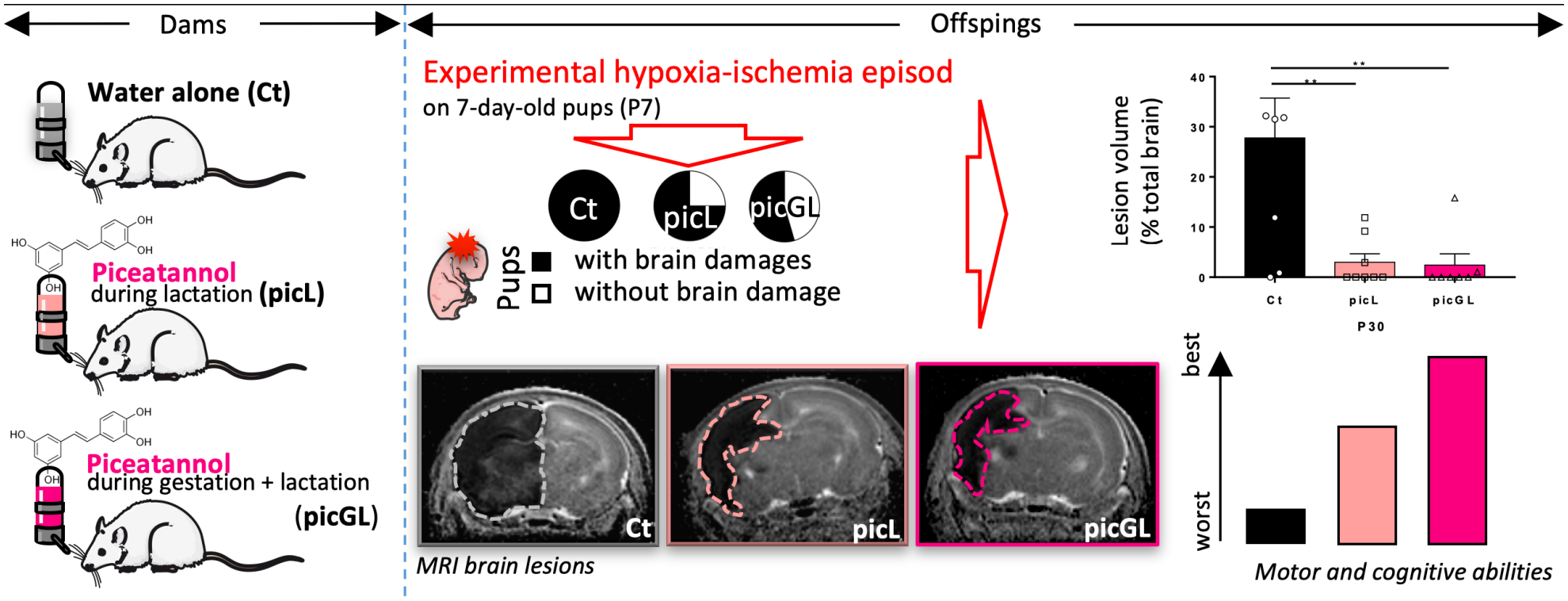

P7
Hypoxia-ischemia
(Permanent unilateral occlusion of the left common carotid artery + hypoxic episode)
Birth
Week 0
Week 1
Week 2
Week 3
Week 4
Week 5
Week 6
Week 7
Ct
picGL
picL
P7 P8 P9 P10 P12 P14
P21
P24
P28 P30
P35
P42 ...P45
P49
PIC supplementation
MRI
MRI
Novel Object Recognition
Early reflexes
mNSS

Table 1

	Sham	Ct	picL	picGL
cortex	**100.0 ± 2.4 #**	**49.2 ±3.8 ***	**85.1 ± 2.1**	**85.9 ± 2.2**
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*Neuronal death was evaluated in the penumbra region (both cortical and striatal regions) on histological cryosections from P9 pups after Nissl staining. Data are presented as percentage of the number of neurons found in the sham group. * Significant difference from sham, picL and picGL, # Significant difference from Ct, picL and picGL, $p \leq 0.0002$, one-way analysis of variance (ANOVA) with Fisher's LSD post-hoc test, n = 3 per group).*


Dams
Offspings
Water alone (Ct)
Piceatannol
during lactation (picL)
Piceatannol
during gestation + lactation
(picGL)
Experimental hypoxia-ischemia episod
on 7-day-old pups (P7)
Ct
picL
picGL
Pups
with brain damages
without brain damage
Lesion volume
(% total brain)
40
30
20
10
0
**
**
Ct
picL
picGL
P30
Ct
picL
picGL
MRI brain lesions
best
worst
Motor and cognitive abilities