## BASIC SCIENCE ARTICLE

# Long-term neurological effects of neonatal caffeine treatment in a rabbit model of preterm birth

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**BACKGROUND:** Neonatal caffeine treatment might affect brain development. Long-term studies show conflicting results on brain-related outcomes. Herein we aimed to investigate the long-term effects of neonatal caffeine administration in a rabbit model of preterm birth.
**METHODS:** Preterm (born day 29) and term (day 32) pups were raised by wet nurses and allocated to treatment with saline or caffeine for 7 or 17 days. At pre-puberty, neurobehavioral tests were performed and brains were harvested for immunostaining of neurons, synapses, myelin, and astrocytes.
**RESULTS:** Survival was lower in preterm saline pups than in controls, whereas caffeine-treated preterm pups did not differ from term control pups. Preterm saline pups covered less distance compared to controls and were more likely to stay in the peripheral zone of the open field. Corresponding differences were not seen in preterm caffeine pups. Preterm animals had lower neuron density compared to controls, which was not influenced by caffeine treatment. Synaptic density, astrocytes, and myelin were not different between groups.
**CONCLUSION:** Caffeine appeared to be safe. All preterm rabbits had lower neuron density but anxious behavior seen in preterm saline rabbits was not seen in caffeine-treated preterm pups.

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## INTRODUCTION

Premature infants are at risk for apnea of prematurity (AOP), a developmental disorder where the lack of central respiratory control leads to temporary cessation of breathing for 15–20 s leading to bradycardia and desaturation. Incidence of AOP is inversely correlated with gestational age and present in most infants born before 30 weeks.[1–4] Besides the immaturity of the respiratory system (both centrally and peripherally), there is also evidence suggesting that central nervous inflammation might play a role.[5] Methyl-xanthines are one of the most widespread drugs in neonatal medicine and have a proven efficiency for reducing the frequency of apnea and decreasing the need for mechanical ventilation.[6]

Caffeine has replaced other methylxanthines, i.e., theophylline and aminophylline, due to its wider therapeutic index. Although these drugs were already used for >40 years, the CAP trial in 2006 was the first report proving their safety by demonstrating that caffeine did not harm short-term neonatal neurological outcome.[7] In further follow-up of this study, it was shown that neonates treated with caffeine had a dramatic reduction in the rate of cerebral palsy and a better neurological outcome at 18 months.[8–10] This beneficial effect was, however, less pronounced at school age.

Through its action on the adenosine receptor (AR), caffeine stimulates respiration by increasing respiratory drive, enhancing diaphragmatic contractility, and increasing sensitivity to carbon dioxide.[11] AR has four different subtypes (A1AR, A2AAR, A2BAR, and A3AR), which are all antagonized by caffeine.[12,13] In the brain, mainly subtypes A1AR (hippocampus) and A2AAR (striatum) are found, for which caffeine has a high affinity.[14–16] In the central nervous system, caffeine has anti-inflammatory properties and can provide neuroprotective effects by countering the effects of adenosine, e.g., by antagonizing the excitatory A2A receptor.[15,17] In case of hypoxia and ischemia, levels of adenosine increase rapidly during hypoxia/ischemia and have been shown to alter normal oligodendrocyte development, necessary for myelin formation.[18] Doyle et al. demonstrated that neonates treated with caffeine had better myelin microstructure.[19] However, these findings were not seen in 11-year-old children; moreover, there were signs of a slower growth of the corpus callosum in caffeine-treated subjects.[20]

Animal studies on the effects of caffeine on brain-related outcomes show conflicting results. Some studies found increased dendritic length and arborization,[21] increased myelination,[22] decreased inflammation,[23] and attenuation of deficits in spatial memory. Other studies have found no effect on developing white or gray matter,[24] and some studies found a negative impact with reduced brain weight,[25] decreased cell proliferation,[26] induced apoptosis,[27] and delayed cerebral myelin synthesis.[28]

Therefore, we aimed to investigate the long-term effects of neonatal caffeine administration in a rabbit model of preterm (PT) birth.

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## MATERIALS AND METHODS

### Handling of rabbit pups
The animal protocol was approved by the Swedish animal Ethics Committee in Lund. The study included 52 PT rabbit pups from 8 litters. A half-breed between the New Zealand White and Lop was used. The PT rabbit pups were delivered by cesarean section at gestational day 29 (term 32 days) after the does were anesthetized with intravenous Propofol (5 mg/kg). After birth, the pups were dried vigorously and placed in a closed infant incubator, marked for identification with a subcutaneous microchip (SweVet Piab Mikrochip, 1.4 × 8 mm, Sjöbo, Sweden) and randomized to either study group (Caffeine citrate, Peyona, Chiesi Farmaceutici) or control group (vehicle solution). Randomization was performed within each litter (www.random.org). The PT pups were handfed once at 2 h of age with 70 mL/kg of bovine colostrum (Whole Colostrum, ColoDan, Denmark) using a 3.5 French feeding tube. Thereafter, the PT pups were randomized and placed with a wet nurse together with two term pups from her own offspring. A wet nurse is a rabbit doe who has undergone a term vaginal delivery on the previous day where all but two of her own pups are replaced with PT rabbit pups delivered by cesarean section. The PT rabbit pups were housed and fed by the wet nurse until they were fully weaned, which occurred at 5 weeks of age. Study end point was between postnatal day (PND) 80 and 84, after finalized neurobehavioral testing.

### Caffeine/vehicle administration
Study medication, caffeine or vehicle solution, was administered daily through an oro-gastric 3.5 French feeding tube. Caffeine (Peyona 20 mg/mL, Chiesi Farmaceutici, Parma, Italy) was diluted 1/20 with NaCl 9 mg/mL. On PND 1, the pups received a loading dose of 20 mg/kg, i.e., 20 mL/kg, and thereafter a daily maintenance dose of 10 mg/kg. Vehicle solution was NaCL 9 mg/mL with a loading dose of 20 mL/kg and a maintenance dose of 10 mL/kg. This treatment was continued until postconceptional day 39 for term or, for PT pups, day 36. Caffeine pups were at that moment randomized to cessation or continuation of caffeine treatment, corresponding to 7 and 17 days of treatment, respectively. This resulted in 5 groups: term saline 7d (controls), term caffeine 7d, PT saline 7d, PT caffeine 7d, and PT caffeine 17d. Rabbit pups were weighed daily for 17 days, thereafter once weekly.

### Behavioral tests
Between PND 70 and 84, behavior was assessed as previously described.[29] All tests were performed in a square arena (140 cm × 140 cm) surrounded by 80-cm high plastic walls. Three different tests were used: the open field (OF) test, the dark–light box (Dark box) test, and the object recognition task (ORT) with different intervals. Tests were performed between 9 a.m. and 4 p.m. on different days in the following order: OF; Dark box; ORT habituation; ORT 30 min inter-trial interval; ORT 300 min; ORT 24 h; OF; Dark box. In between each rabbit, the arena was cleaned with a 10% ethanol solution to erase olfactory clues.

*OF test.* Each rabbit was individually taken from their cage and wrapped in a cloth to shield them from light. They were placed in the arena in one of the corners. The testing area was subdivided into three zones: the central square "center", the outer rim "peripheral zone" and in between the "intermediate zone." We evaluated the delay before they left the starting area, time spent in each area, and the total distance covered during the test. Furthermore, we analyzed secondary anxiety markers such as time to leave the start area, freezing, rearing, biting, and urinating/defecating.

*Dark–light box test.* The rabbit was placed in a plastic non-transparent box (45 cm length (*L*), 64 cm width (*W*), 40 cm height

(*H*)) with a small opening (10 × 10 cm) in one of the walls facing the center of the arena. The time to escape the dark box was recorded. The test was canceled if the rabbit did not leave the box within 6 min.

*Object recognition task.* Rabbits were taken out of their cage and placed in a smaller cage for 5 min (42 cm *L*, 19 cm *W*, 27 cm *H*). Each rabbit was then introduced into the OF in one of the corners. During the familiarization phase (T1; 5 min), two similar objects were placed inside the arena, 60 cm apart and 40 cm from each corner, which the rabbit could explore and interact with freely. The inter-trial interval between familiarization and testing increased each day in length: 30 min, 300 min, and 24 h. During the test phase (T2; 5 min), one of the objects was replaced by a novel object. All objects were different on each testing day. To habituate the rabbits to the experimental settings, the first two sessions were considered as part of the training. Exploration of the objects was defined as sniffing or touching while pointing toward either object within a distance of <2 cm. Exploration indices "E1" and "E2" were calculated as the total time the objects were explored during T1 and T2, respectively. For T2, the discrimination index was calculated as (D1/E2) where "D1" reflects the difference in time exploring the old and novel object. Rabbits were excluded from the analysis in case of no exploration of any object during T1 or T2.

*Behavioral analysis.* For all tests, the position of the animal was registered automatically by a PC through a video camera installed 250 cm above the field. The images were analyzed using the Video Tracking Software (SMART, Panlab SL, Barcelona, Spain). Authors were blinded during data acquisition and analysis.

### Tissue collection
After neurobehavioral testing, rabbits were anesthetized with intramuscular ketamine (35 mg/kg) and xylazin (6 mg/kg) and transcardially perfused with 0.9% saline and heparin (100 U/mL) followed by perfusion fixation with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4). The brain was removed and further underwent immersion fixation in 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4).

The brain tissue was dehydrated in a graded ethanol serial (70–99.99%) ended with Xylene (100%) and were then immersed in paraffin and embedded in paraffin blocks. Sections (4 μm) were made on a rotating microtome (Microm HM 360, Microm International GmbH, Walldorf, Germany), and sections were collected on microscopic slides (SuperFrost Plus, Thermo Scientific/Gerhard Menzel B.V. & Co., Braunschweig, Germany).

### Immunohistochemistry
One coronal section was made each 100 μm from the moment the corpus callosum, hippocampus, thalamus, amygdala, and hypothalamus were seen in the same plain. Paraffin sections were de-paraffinized and rehydrated (in Xylene followed by a graded ethanol serial) and rinsed in 0.1 M phosphate-buffered saline (PBS, pH 7.4). Sections were then incubated in PBS containing 0.05% Triton X-100 (TX) and 1% bovine serum albumin (BSA) for 30 min at room temperature (RT).

Sections were incubated with monoclonal mouse antibodies against anti-Neu-N (1:150, catnr MAB377, Millipore, Ternecula, CA, USA), synaptophysin (1:100, clone Sy38, ab8049, Abcam, Cambridge, UK), or myelin basic protein (MBP; 1:75, clone SMI94, catnr 836504, BioLegend, San Diego, CA, USA) or with polyclonal chicken antibodies against glial fibrillary acidic protein (GFAP; 1:1200, catnr ab4674, Abcam, Cambridge, UK) for 16 h at 4 °C. Antibodies were diluted with the PBS-TX-BSA solution. The labeling was visualized using secondary polymer horseradish peroxidase (HRP)-conjugated antibodies against mouse IgG (1:1, catnr K4001, DAKO, DK) or against chicken IgY (1:750, Jackson IR,

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West Grove, PA, USA) for 30 min at RT. The HRP conjugation was reacted in a PBS solution containing di-aminobenzidine (DAB, 0.5 mg/ml) and hydrogen peroxidase (0.1%) for 10 min at RT. As antibody specificity controls, the incubation of primary antibodies was excluded from the labeling protocol of adjacent sections. Following the HRP–DAB reaction, sections were rinsed in PBS, dehydrated (graded ethanol serial ended with 100% Xylene) and mounted (Pertex, Histolab, Gothenburg, Sweden) and cover-slipped. All incubations were performed in moisture chamber, and sections were rinsed with PBS containing TX (0.05%) between incubations.

### Image acquisition and quantification
Sections were slide scanned to obtain digital images (Hamamatsu, NanozoomerS60, Hamamatsu, Japan).

On each slide, the following brain areas were manually annotated in the slide-scanned images as regions of interest (ROI) for subsequent evaluation: amygdala, caudate nucleus, corpus callosum, internal capsule, and hippocampus with subregions CA1 and dentate gyrus. Neu-N-positive neurons, GFAP-positive cells and fibers, and synaptophysin-positive neuronal fiber structures were analyzed. MBP immunolabeling was evaluated in the corpus callosum and internal capsule.

For each ROI, the DAB brown color was obtained from the digital image (×40) using the color deconvolution module in Fiji/ImageJ (http://fiji.sc) and converted to 8 bit. Neu-N-positive cells were enumerated in ImageJ by the default auto-threshold segmentation method and watershed separation after initial smoothing. Small objects were excluded (objects <100 μm$^2$ were excluded to remove debris and only counted the actual cells) and the number of identified cells were divided by the overall area for neuron density assessment. ROIs with GFAP, synaptophysin, and MBP labeling were quantified using a fixed threshold and the relative area (positive area/overall area) and the optical density (OD = log(max intensity/mean intensity)) were calculated. Enumeration of GFAP-positive cells were performed in QuPath.[30] The resulting mean of left and right regions was reported and used for statistical analysis. For neuron density, 2 sections were taken 400 μm apart. Authors were blinded during sectioning, staining, acquisition, and analysis.

### Statistical analysis
Analysis was performed by comparing each group to the reference group (controls). Mann–Whitney and Wilcoxon's signed-rank test, respectively, were used for independent and paired continuous variables. The comparisons were based on individual baseline characteristics, i.e., birth weight, weight at testing, and sex. In the ORT, one-tailed $T$ test was used for comparison of D2 scores with chance level (i.e., D2 = 0). Statistical significance was set at $p = 0.05$. Results are presented as mean (standard deviation) or median (interquartile range). The analysis was done using Graph Pad Prism 7 for Windows (GraphPad Software, La Jolla, CA, USA, www.graphpad.com).

## RESULTS
### Survival and growth
Fifty-two PT pups were obtained by C-section from 8 rabbit does. In the 7 foster mothers, 14 vaginally delivered term pups were kept with their mother. Randomization to treatment resulted in 31 PT caffeine, 21 PT saline, 7 term caffeine, and 7 term saline (control) pups. At PND 7, 9 out of 17 remaining PT caffeine pups were randomized to prolonged treatment (17 days).

Birth weights were significantly lower in the PT pups compared with controls ($37.6 \pm 1.0$ vs $57.7 \pm 2.3$ g; $p < 0.0001$), and this difference was still present when only survivors were analyzed. Neonatal growth curves displayed a delay in weight gain in the PT pups in the first days of life but afterwards they displayed similar growth to term pups, which they caught up with by PND 72 (Fig. 1). Treatment groups did not differ in weight or weight gain in either term or PT pups.

Survival was lower in the PT saline group compared with controls (33% vs 83%; $p = 0.03$) but not between caffeine-treated pups and controls (55% vs 83%; $p = 0.12$) (Fig. 2). Duration of caffeine treatment in PT pups was not related to survival.
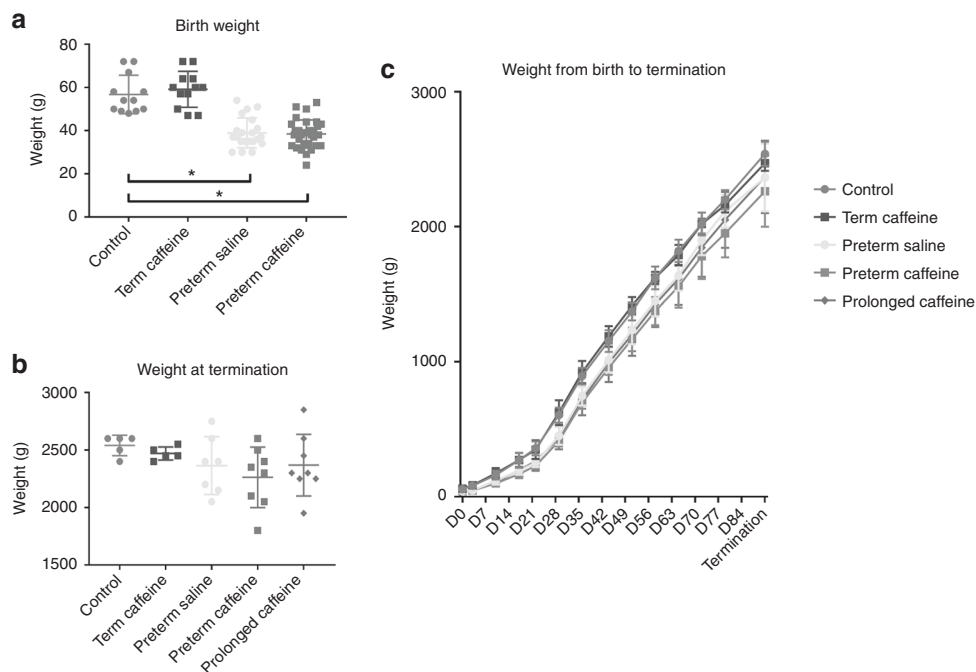


**Fig. 1 Growth from birth until termination.** On the left, body weights of rabbits at birth (**a**) and at termination (**b**). On the right, growth curves are displayed (**c**). *$p < 0.05$.

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Neurodevelopmental testing

At PND 70, 5 control, 5 term caffeine, 7 PT saline, 8 PT caffeine, and 8 prolonged caffeine rabbits underwent neurobehavioral testing. Both sexes were equally present (16 females vs 17 males). When rabbits were placed in the OF, PT saline-treated rabbits covered less distance compared to controls (11.5 m [0.9–7.5] vs 16.2 m [4.1–19.6]; $p = 0.048$). Furthermore, they were more likely to remain in the peripheral zone with their body in contact with the wall (97.7% [83.4–100%] vs 60.5% [34.8–92.4%]; $p = 0.029$). The time duration for exploring the center showed a similar trend; however, this was not significant. Also secondary anxiety treatments were not significant different between groups (Fig. 3). Caffeine-treated pups did not show this difference compared to controls (10.9 m [3.8–15.6] vs 16.2 m [4.1–19.6]; $p = 0.6$) (Fig. 3).
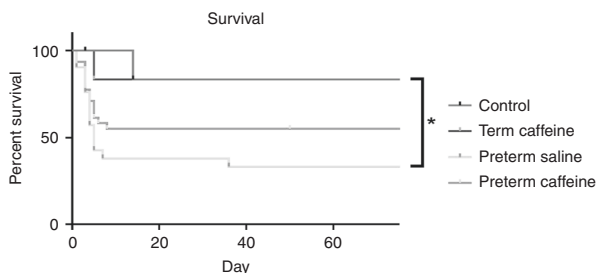


**Fig. 2 Survival curves of the different treatment groups.** $*p < 0.05$.

The prolonged treatment group and the term caffeine group had similar outcomes to controls. Adjustment for sex, genetic mother, or wet nurse had no influence on traveled distance or behavior in the OF.

Evaluation of rabbits in the dark box did not reveal any significant differences between groups. Rabbits in all treatment groups were as likely to leave the dark box compared to controls and the time to leave was similar.

In the ORT test, all groups displayed similar exploratory behavior with comparable exploration times on all testing days. When assessing the ORT, all groups showed recognition of the old and the new object in the 30 min, 300 min, and 24 h interval without differences between treatment groups (Table 1).

After all tests were performed, we re-evaluated rabbits in the OF and dark box. At this time, no differences could be seen between any groups in the OF in any of the parameters.

Immunohistochemistry and quantification

On histologic assessment PT saline pups showed lower neuron density in the hippocampus compared to controls (278 [264–285] vs 297 [288–318] neurons/mm$^2$; $p = 0.01$). A similar finding was found in the PT caffeine pups (273 [261–289] vs 297 [288–318] neurons/mm$^2$; $p = 0.02$) (Fig. 4, Supplementary Fig. S1). There was no difference between controls and the term caffeine or prolonged caffeine group. Although whole hippocampal regions showed differences, analyzed subregions of the hippocampus (i.e., CA1 and dentate gyrus) showed no significant differences. In the
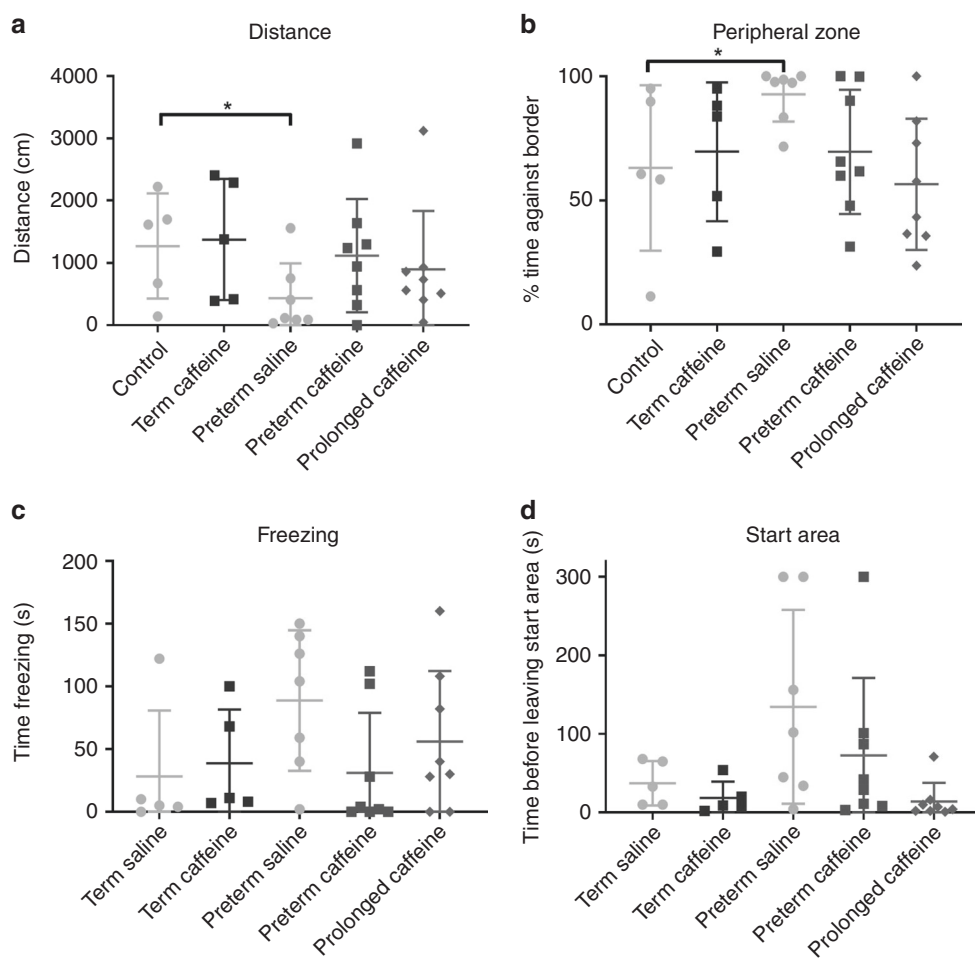


**Fig. 3 Behavior of the different groups in the open field.** Average distance traveled in the open field (**a**). The percentage of time the rabbit was spending in the peripheral 15 cm area (**b**). Secondary anxiety markers time spent freezing (**c**) and the time rabbits take before leaving the starting area (**d**). $*p < 0.05$.

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**Table 1.** Exploration times and novel object recognition ratios in the NORT.

|  | Control | Term caffeine | Preterm saline | Preterm caffeine | Prolonged caffeine |
|---|---|---|---|---|---|
| Exploration times |  |  |  |  |  |
| 30 min (s) | 14 [1–23] | 3 [1–13] | 4 [2–18] | 3 [0–22] | 5 [0–6] |
| 300 min (s) | 25 [5–48] | 9 [2–27] | 12 [6–36] | 4 [0–28] | 14 [7–27] |
| 24 h (s) | 19 [5–29] | 16 [11–35] | 18 [0–28] | 19 [7–29] | 10 [2–42] |
| Novel object recognition ratios |  |  |  |  |  |
| 30 min | 0.1 [−0.3–0.6] | 0 [−0.3–0.2] | −0.3 [−1–1] | −0.3 [0.8–0.2] | 0.8 [−0.6–1] |
| 300 min | 0.2 [0.1–0.5] | 0 [0–0] | 0.5 [0.3–0.9] | 0.2 [−0.8–0.9] | 0.4 [0–0.7] |
| 24 h | 0.1 [−0.2–0.4] | 0.7 [0.2–0.9] | 0.4 [0.1–0.5] | 0.3 [−0.3–0.5] | 0.2 [−0.2–0.4] |

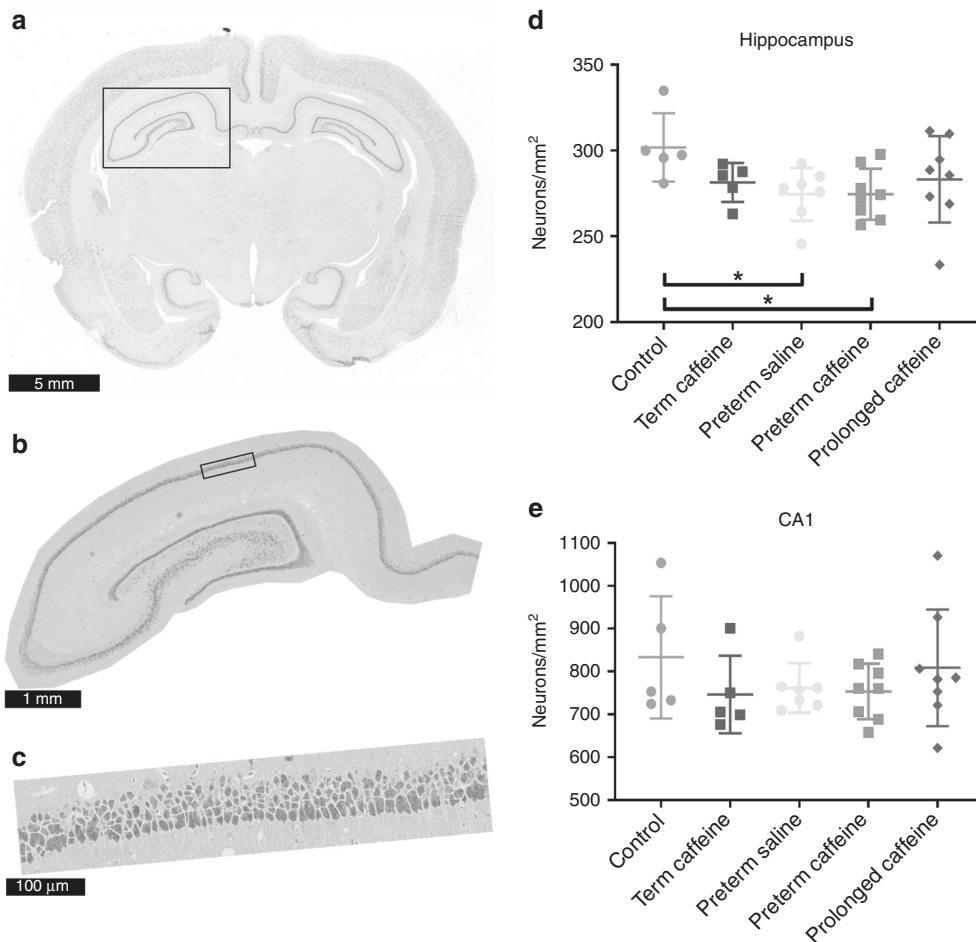Data are displayed as median with interquartile ranges



**Fig. 4 Neu-N stain of a rabbit brain with DAB, background stain is hematoxylin.** Overview of the entire brain (**a**) with magnification of the hippocampus (**b**) and the CA1 region (**c**) where segmented cells are outlined in yellow. On the right, graph with neuron density in the hippocampus (**d**) and CA1 region (**e**). *$p < 0.05$.

amygdala and caudate nucleus, there were no significant differences between the treatment groups. On evaluation of synaptic density, no statistically based differences were obtained, however, with a trend toward lower values in the amygdala of the PT saline animals compared to controls (0.38 [0.31–0.41] vs 0.47 [0.34–0.56]; $p = 0.4$; Fig. 5). On evaluation of myelin formation, the density of immunoreactive myelin fibers was equal between term and PT animals in all groups (corpus callosum PT saline vs controls: 406 [359–538] $\times 10^3$ vs 470 [378–571] $\times 10^3$; $p = 0.4$; Supplementary Fig. S2). No differences between groups were observed in the

number of GFAP-positive cells (hippocampus PT saline vs controls: 694 [495–1483] vs 929 [645–1124]; $p = 0.7$) or GFAP-positive fibers in all ROIs (hippocampus PT saline vs controls: 0.09 [0.05–0.09] vs 0.11 [0.09–0.14]; $p = 0.8$) (Supplementary Fig. S3).

## DISCUSSION

This is the first study reporting effects of caffeine treatment on development of PT animals from birth to young adulthood. Of note, unlike rodents, the rabbit is a perinatal "brain developer,"
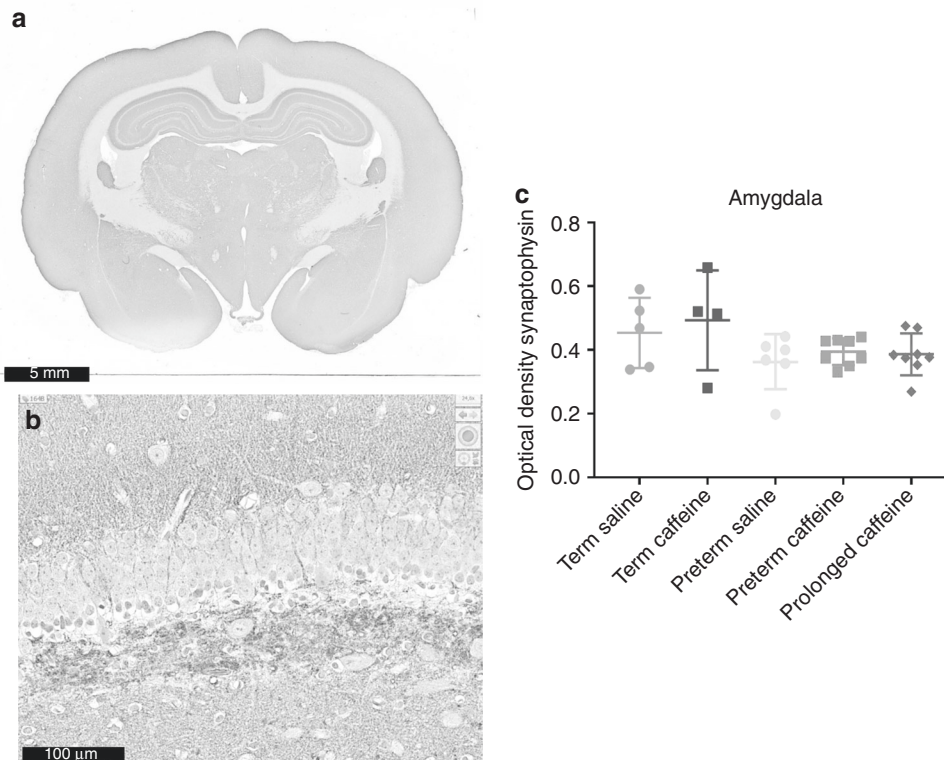
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**Fig. 5  Synaptophysin stain with DAB and hematoxylin background.** Overview of the brain (**a**) with magnification of the hippocampus (**b**) where synaptophysin expression is mainly seen above and below the dentate gyrus. On the right, optical density of synaptophysin expression in the amygdala (**c**).

similar to humans, thus representing an excellent model for assessment of neuro-cognitive development. In addition, the effects of caffeine were longitudinally assessed in both PT and term rabbits, including both functional (neurobehavioral testing) and histological (immunohistochemistry) outcomes.

We administered caffeine at a clinical equivalent dose and route of administration, as this was previously demonstrated to lead to effective caffeine blood levels in the PT rabbit model.[31] We treated animals until postconceptional day 36, which corresponds with human week 37 in terms of brain development, the time period when caffeine treatment is usually ceased.

As expected, PT rabbits were born with lower body weights. During the first weeks of life, they became comparable in size but by young adulthood age saline-treated rabbits displayed an altered neurobehavior, i.e., less exploration and more anxiety, and when treated with saline, pups had lower survival rates. On histologic examination, we observed a lower neuron density in the hippocampus.

Neonatal caffeine treatment in PT infants has been shown to be beneficial to prevent bronchopulmonary dysplasia and at 18 months to improve survival without morbidity.[7,10] At school age, caffeine-treated children have less developmental coordination disorder; however, when children grow older, the beneficial effect seems to fade out.[8] In animal studies, conflicting results are found when assessing the effect of caffeine on brain development.[32] Furthermore, to our knowledge, we are the first to evaluate the long-term effects of neonatal caffeine treatment following PT birth. Pups were delivered at day 29, which is the beginning of the brain growth spurt in a rabbit,[33] which corresponds to 24–28 weeks in the human in terms of brain development.

PT pups were much smaller than term rabbits. In the early neonatal period, PT pups showed delayed growth but afterwards they started to catch up with term pups so that, at 3 months of age, body weights were comparable. This mimics the human situation where PT infants catch up in weight during childhood.[34,35] In humans, it was seen that caffeine temporarily decreased weight gain in the first weeks of life.[7] Although in our study no difference was seen between caffeine- and saline-treated PT pups, it did take three times as long for caffeine pups to catch up with controls compared to saline pups. In the term group, we could see no difference between the caffeine- and saline-treated pups. This is contradictory to earlier findings where it has been demonstrated that caffeine-treated rat pups had a lower weight gain than control pups; however, the use of caffeine was associated with exposure to either chronic unpredictable stress or hyperoxia, and different dose regimens were used.[36,37]

Survival was lower in the saline-treated PT pups compared to controls unlike caffeine-treated PT pups. In other animal studies, exposure to caffeine has not yet been shown to improve survival. In humans, the CAP trial did not show an improved survival at 18–21 months; however, there was a higher rate of survival without neurologic morbidity.[10] In rabbits, this might be translated to crude mortality since severe neurologic disability in rabbits results in death as rabbit does will stop caring for weak pups resulting in survival of the healthiest.

On neurobehavioral testing at 3 months of age, saline-treated pups covered less distance and were more likely to remain in the peripheral zone, in contact with the side wall. This behavior is indicative of an increased anxiety, as the center is seen as a dangerous area (for predators) and the wall provides security on one side. Anxiety could not be seen in the caffeine-treated PT pups; they expressed behavior comparable to term pups. This suggests that anxiety induced by PT birth is restored or prevented by neonatal caffeine treatment. In literature, it has already been described that caffeine treatment might decrease anxiety although that was found when treating term born neonatal rats[38] and in a model of sepsis in adult rats.[39] Different brain regions are

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involved in fear and anxiety regulation including the prefrontal cortex, hypothalamus, hippocampus, and amygdala. In our histologic findings, we could only find differences between the PT and term groups in neuron density in the hippocampus and a trend for decreased synaptic density in the amygdala. This corresponds with earlier findings in prematurity in rabbits where van der Merwe et al. described a severe impact in the hippocampus at the first day of life.[40] Our findings, however, demonstrate a less severe impact than that previously described. This could be explained by survival of the healthiest. As we only examine our rabbits at 12 weeks, it could be that the rabbits with the most severe brain damage died during the neonatal period. Another explanation might be that rabbits have some kind of "recovery mechanism." In earlier work, we demonstrated that rabbits who suffer from an anesthetic insult during fetal life have severe brain damage at birth, which became unrecognizable at 7 weeks of age.[41] A similar mechanism might be present here. Lastly, it might be that our analysis of neuronal and synaptic density is not sensitive enough to detect subtle changes. Neural plasticity may lead to similar structural characteristics in PT and term brains at 3 months of age, whereas more subtle differences in neurotransmitters may account for differences in functionality. Caffeine exerts its neuronal effects through binding to different subtypes of the AR. Both subtypes A1 and A2A are known to be involved in anxiety-related behavior; mice lacking A1 or A2A receptors have high anxiety levels.[42,43] Possibly these receptors or their sensitivity and functionality are altered in early neonatal life and these changes become undetectable in later life with our measurements.

PT birth in the absence of inflammation or hypoxia has been shown to disrupt the maturation of pre-oligodendrocytes and alter the functionality of oligodendrocytes in rabbits.[40] However, in the present study we could not detect any differences in myelination between groups. This again might be explained by the plasticity these neonatal brains possess. In addition, survival of the healthiest might also play a role here introducing an attrition bias in the study population.

Lastly, we could see no difference in cognitive abilities in the ORT. In contrast to our previous findings,[29] rabbits could recognize the novel object after a 24-h interval. The memory of the rabbit was furthermore visible when rabbits were introduced to the OF at the last testing days when no signs of anxiety were visible due to recognition of the testing environment.

This study has some limitations. First, we have not included neuroimaging analyses such as brain magnetic resonance imaging, which is commonly used in clinical studies. Furthermore, we did not weigh the brains, therefore we do not have information about total brain weight or volume. Second, owing to survival of the healthiest, PT pups with severe impairment might have died during the neonatal period. As a consequence, the overall long-term outcome of PT pups might be improved, whereas in humans these infants would survive due to intensive neonatal care. Third, we did not test our rabbit for neurologic abilities at birth, so we have no comparison whether PT rabbits improved/deteriorated and whether indeed the "better" rabbits survived. Lastly, we did not evaluate distribution or density of the AR. Future studies exploring subtle differences in the AR may help explain behavioral differences.

One of the main strengths of this study consists of the choice of the animal model, the rabbit, which is a suitable model for neonatal brain development. Rabbits are perinatal brain developers, i.e., they have their brain growth spurt at the end of pregnancy and the beginning of neonatal life, comparable to humans unlike rodents (postnatal) or sheep and non-human primates (prenatal).[33] Furthermore, the rabbit has frequently been used to examine neurobehavioral consequences of prematurity and other perinatal insults (intraventricular hemorrhage,[44] maternal surgery in pregnancy,[41] hypoxia,[45] intrauterine growth restriction,[46] and infection[47]). Through a foster care model, rabbits are excellent animals for long-term follow-up of PT birth enabling assessment of both functional and structural aspects of brain development. Finally, our pups were raised to young adulthood allowing them sufficient time to recover from neonatal insults.

## CONCLUSION
This is the first study reporting long-term effects of caffeine in an animal model of PT birth. At a clinically relevant dose and duration, caffeine appeared to be safe without affecting the density of neurons, astrocytes, synapses, or myelin fibers at young adult age. PT rabbits had more anxious behavior than term rabbits; however, this was not observed in PT pups exposed to caffeine. Lower neuron density in the PT rabbits was not influenced by caffeine exposure. Future studies might explore the effects of caffeine in PT pups with acquired brain damage.

## AUTHOR CONTRIBUTIONS
L.v.d.V., S.G., M.B., D.L., and J.D. designed the experiments. L.v.d.V., S.G., and M.B. performed data acquisition. L.v.d.V., E.G., and B.H. analyzed the data. L.v.d.V., M.B., and D.L. drafted the article. All authors revised, adapted, and approved final version of the manuscript.

## ADDITIONAL INFORMATION
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