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Title: High accumulation of methadone compared to buprenorphine in fetal rat brain after maternal exposure

Mette Kongstorp, Inger Lise Bogen, Tom Stiris and Jannike Mørch Andersen

*Section for Drug Abuse Research, Department of Forensic Sciences, Oslo University Hospital, Oslo,
Norway (M.K., I.L.B. and J.M.A.)*

Institute of Clinical Medicine, Faculty of Medicine, University of Oslo, Oslo, Norway (M.K. and T.S.)

*Department of Pharmacy, Faculty of Mathematics and Natural Sciences, University of Oslo, Oslo,
Norway (J.M.A.)*

Institute of Basic Medical Sciences, Faculty of Medicine, University of Oslo, Oslo, Norway (I.L.B.)

Department of Neonatal Intensive Care, Oslo University Hospital, Oslo, Norway (T.S.)

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Corresponding author:

Mette Kongstorp

Address: PO Box 4950 Nydalen, N-0424 Oslo, Norway

Phone: 0047 40476700

Email: mette.kongstorp@ous-hf.no

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Abbreviations: GD, gestation day; NAS, neonatal abstinence syndrome; OMT, opioid maintenance treatment; PND, postnatal day; UHPLC-MS/MS, ultra-performance liquid chromatography-tandem mass spectrometry; USVs, ultrasonic vocalizations.

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Abstract:

Experimental animal studies are valuable to reveal a causal relationship between prenatal exposure to opioid maintenance treatment (OMT) and subsequent effects. However, previous animal studies of OMT during pregnancy have been criticized for their lack of clinical relevance due to the use of high drug doses and the absence of pharmacokinetic data. Hence, the aim of this study was to determine blood and brain concentrations in rat dams, fetuses and offspring after continuous maternal exposure to methadone or buprenorphine during gestation and to examine the offspring for neonatal outcomes and withdrawal symptoms. Female rats were implanted with a 28-day osmotic minipump delivering methadone (10 mg/kg/day), buprenorphine (1 mg/kg/day) or vehicle 5 days prior to mating. Continuous exposure to methadone or buprenorphine induced stable blood concentrations in the dams of 0.25 ± 0.02 μM and 5.65 ± 0.16 nM, respectively. The fetal brain concentration of methadone (1.89 ± 0.35 nmol/g) was twice as high as that in the maternal brain, while the fetal brain concentration of buprenorphine (20.02 ± 4.97 pmol/g) was one third of the maternal brain concentration. The opioids remained in the offspring brain several days after the exposure ceased. Offspring prenatally exposed to methadone but not buprenorphine displayed reduced body weight and length and increased corticosterone levels. No significant changes in ultrasonic vocalizations were revealed. Our data in rat fetus and neonates indicate that OMT with buprenorphine may be a better choice than methadone during pregnancy.

Significance Statement:

Concern has been raised about the use of opioid maintenance treatment during pregnancy due to the important role of the endogenous opioid system in brain development. Here, we show that the methadone concentration in the fetal rat brain was twice as high as that in the maternal brain, while the buprenorphine concentration was one third. Furthermore, buprenorphine allowed more favorable birth outcomes, suggesting that buprenorphine may be a better choice during pregnancy.

Introduction

The recommended therapy for opioid-dependent pregnant women is opioid maintenance treatment (OMT) with methadone or buprenorphine (WHO, 2014). OMT patients generally take better care of their health and are less likely to relapse to illicit drug use compared to those not receiving such treatment (Fischer et al., 1998; Minozzi et al., 2013). However, OMT during pregnancy may not be without risk to the unborn child. The endogenous opioid system plays a central role in neurobiological development (Sargeant et al., 2008), and opioid receptors are expressed early in embryonic life (Kim et al., 2006). In addition, because of the immaturity of the blood-brain barrier (Wolburg and Lippoldt, 2002), the fetal brain may be particularly vulnerable to exogenous opioid exposure.

Of all infants born to mothers in OMT, 40-90% show signs of neonatal abstinence syndrome (NAS) (Jones et al., 2010; Welle-Strand et al., 2013; Wurst et al., 2016). Prenatal exposure to methadone or buprenorphine has been linked to an increased risk of preterm birth (Fajemirokun-Oduyei et al., 2006; Cleary et al., 2012; Lemon et al., 2018), lower birth weight (Sarfi et al., 2009; Cleary et al., 2012; Norgaard et al., 2015) and smaller head circumference (Welle-Strand et al., 2013; Mactier et al., 2014; Bier et al., 2015). Increasing evidence also indicates a relationship between prenatal OMT exposure and long-lasting negative effects on different outcomes, such as impaired cognitive functioning and attention, and behavioral, psychomotor and visual problems (de Cubas and Field, 1993; Sundelin Wahlsten and Sarman, 2013; Nygaard et al., 2015; Nygaard et al., 2017; Monnelly et al., 2018). However, since OMT is associated with multiple confounding factors, e.g. poly-substance use and genetic and socioeconomic differences (Konijnenberg and Melinder, 2011), it is difficult to draw conclusions about prenatal opioid exposure and its subsequent effects. To confirm a causal relationship, controlled experimental studies with methadone and

buprenorphine are needed, but due to ethical concerns, such studies cannot be performed in pregnant women.

Previous animal studies of OMT during pregnancy have been criticized for their lack of clinical relevance due to the use of high drug doses (Farid et al., 2008; Ross et al., 2015). Since pharmacokinetic data of methadone and buprenorphine in rodents are missing, we do not know if the doses that have usually been administered result in blood concentrations comparable to those reported in humans (Gordon et al., 2010; Concheiro et al., 2011; Bartu et al., 2012). Moreover, there is a lack of knowledge concerning distribution of methadone and buprenorphine to the fetus. The aim of this study was therefore to investigate the blood and brain opioid concentrations in rat fetuses and offspring continually exposed to methadone or buprenorphine through exposure of the pregnant dams. The fetal opioid concentrations were compared with the blood and brain opioid concentrations in the dams. Moreover, the pups were examined for neonatal outcomes and symptoms of withdrawal the first two weeks after birth.

Materials and Methods

Animals

The present study was approved by the Norwegian Animal Research Authority (Norwegian Food Safety Authority, Oslo, Norway) and performed in accordance with the laws and regulations controlling experiments and procedures on research animals in Norway. Female (350.4 ± 5.9 g, $N = 75$) and male (12 weeks, $N = 38$) Sprague-Dawley rats were obtained from Taconic (Ejby, Denmark). Upon arrival, all animals were allowed one week of habituation. The rats were housed two to three per cage with free access to food and water under an artificial 12-hour light/dark cycle, $50 \pm 10\%$ humidity and $21 \pm 2^\circ\text{C}$. The study was performed in two separate experiments. In the first experiment, the blood and brain opioid concentrations

in dams and fetuses were examined during gestation. In the second experiment, the disturbance of the dams was reduced to a minimum to avoid the possible influence of maternal stress, and offspring effects were examined. A total of 320 offspring were included in the study.

Drugs

Methadone-HCl (MW 345.91; Sigma, Oslo, Norway) and buprenorphine-HCl (MW 504.11; Chiron AS, Trondheim, Norway) were dissolved in sterile water (Fresenius Kabi, Hadeland, Norway). Sterile water was used as vehicle due to the low solubility of buprenorphine in saline.

Therapeutic doses of methadone and buprenorphine have been reported to be 20-260 mg/day and 0.8-32 mg/day, respectively, in pregnant women (Gordon et al., 2010; Concheiro et al., 2011; Bartu et al., 2012; Welle-Strand et al., 2013), corresponding to 0.3-3.7 mg/kg/day methadone and 0.01-0.45 mg/kg/day buprenorphine. In humans, methadone and buprenorphine have half-lives of 20-30 hours (Saxon et al., 2013), however, methadone and buprenorphine are eliminated much faster in rodents ($t_{1/2} = 2-2.5$ hours) (Ohtani et al., 1994; Andersen et al., 2011). To compensate for the rapid metabolism, the female rats were exposed to 10 mg/kg/day methadone or 1 mg/kg/day buprenorphine throughout gestation. The drug doses are the same as previously used (Hutchings et al., 1992; Robinson and Wallace, 2001; Hung et al., 2013; Chen et al., 2015), and are based on the salt form.

Pump implantation

An osmotic minipump was used to maintain stable and continuous exposure of the dams to opioids. Five days prior to mating (one female to one drug-naïve male), the female rat was implanted with a 28-day osmotic minipump with a 2.5 μ l/hour delivery rate (2ML4; Alzet, Cupertino, CA) under isoflurane anesthesia (Baxter, Deerfield, IC). The pumps were filled

with methadone-HCl (initial dose 10 mg/kg/day, $N = 26$), buprenorphine-HCl (initial dose 1 mg/kg/day, $N = 33$) or vehicle (sterile water, $N = 16$) under sterile conditions. For an overview of the experimental set up, see Fig. 1. A small lateral incision was made in the dorsal area behind the neck, the pump was inserted subcutaneously (s.c.) with the opening facing the anterior direction, and the incision site was closed with non-absorbable stitches. The animals were allowed to recover on heating pads under observation before they were placed in individual cages. Metacam (0.3 mg/kg, s.c.) was given as an analgesic during and 5-6 hours after the surgery.

Blood and brain sampling from dams and fetuses

Blood samples were collected from the dams one day after the implantation of the pump (gestation day (GD) -4), on the day of mating (GD 0) and on GD 7, 10, 14, 17 and 21 (Fig. 1). After 5-10 minutes on a heating pad, the animal was restrained, the hind leg was shaved, Vaseline was applied and the *lateral saphenous* vein was punctured with a cannula (25 G). Approximately 250 μ l of blood was collected in a tube containing 30 μ l heparin (100 IU/ml; LEO Pharma AS, Lysaker, Norway) to inhibit clotting. In duplicate, 100 μ l of each blood sample was transferred to a 5 ml tube and immediately frozen in liquid N₂.

On GD 22, blood and brain tissue were collected from dams and fetuses. The dams were anesthetized by isoflurane, and blood was collected by cardiac puncture with a syringe (21 G cannula) prefilled with 80 μ l heparin. In duplicate, 100 μ l of each blood sample was transferred to a 5 ml tube and immediately frozen in liquid N₂. An abdominal incision was made, the embryonic sacs containing the fetuses were opened and the fetuses were immediately decapitated. Trunk blood was collected from each fetus and pooled (2 to 5 offspring) to obtain a volume of 100 μ l in a tube containing 10 μ l heparin. The brains were collected from 4 separate fetuses from each litter and immediately frozen in N₂. After the

embryonic sacs were removed, the dam was decapitated, and the cerebrum was removed, rinsed with 0.9% NaCl and quickly frozen in liquid N₂. After blood and brain harvesting, the osmotic pump was released, and the remaining solution was extracted.

Blood and brain sampling from offspring

The day of birth was defined as postnatal day (PND) 0. On PND 1, 7 and 14, one or two offspring from each litter were randomly chosen for blood and brain harvesting. The pups were anesthetized by isoflurane, and a blood sample was collected by cardiac puncture using a syringe (25 G cannula) prefilled with 80 µl heparin. In duplicate, 100 µl of each blood sample was transferred to a 5 ml plastic tube and immediately frozen in liquid N₂. After blood sampling, the pups were decapitated, and the brains were harvested, weighed and frozen immediately in liquid N₂. All blood samples and brain tissue were stored at -80°C for further analysis.

Determination of methadone, buprenorphine and corticosterone concentrations in blood and brain tissue

The cerebrum was homogenized in ice-cold 0.32 M sucrose (100 mg brain/ml) containing cOmplete protease inhibitor cocktail (Roche, Basel, Switzerland), batched and stored at -80°C for analysis.

The concentrations of methadone and buprenorphine in the blood and brain tissue and the blood concentration of corticosterone were measured using an ultra-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method established in our laboratory. Working solutions for 5 calibrators and 4 quality controls were prepared separately in 5 mM ammonium formate buffer (pH 3.1). The calibrators (1-2000 nM for methadone, 0.1-200 nM for buprenorphine and 0.35-1430 nM for corticosterone) and quality controls (1.2-1200 nM for methadone, 0.12-120 nM for buprenorphine and 0.4-800 nM for

corticosterone) were prepared by diluting 50 μ l of the working solutions with 100 μ l blood from unexposed rats. Fifty microliters of an internal standard (methadone- d_9 , buprenorphine- d_6 , and corticosterone- d_4) and 100 μ l ammonium bicarbonate (0.2 M, pH 9.3) were added to each sample before vortexing. Next, 1.2 ml ethyl acetate:heptane (1:4) was added, and the samples were mixed and centrifuged at 4°C at 4750 rpm (3600 x g) for 4 minutes. The organic phase was then transferred to a 5 ml glass tube, evaporated to dryness under a stream of N₂ at 50°C, reconstituted with 100 μ l 90:10 ammonium formate buffer (10 mM, pH 3.1):MeOH, and transferred to auto sampler vials.

The samples were analyzed using an Acquity UPLC system coupled to an Xevo-TQS triple quadrupole mass spectrometer with an electrospray ionization interface (Waters, Milford, MA). Methadone, buprenorphine and corticosterone were separated on a BEH C₁₈ column (2.1 x 50 mm, 1.7 μ m; Waters) using gradient elution with a mobile phase consisting of 5 mM ammonium formate buffer, pH 10.2 (solvent A) and MeOH (solvent B), a flow rate of 0.4 ml/minute and a column temperature of 60°C. The gradient had a total run time of 5 minutes with the following profile: 0-0.1 minutes; 5% B, 0.1-0.18 minutes; 5-30% B, 0.18-1.6 minutes; 30-50% B, 1.6-2.3 minutes; 50-90% B, 2.3-2.7 minutes; 90% B, 2.7-2.76 minutes; 90-98% B, 2.76-3.5 minutes; 98% B, 3.5-3.6 minutes; 98-5% B, 3.6-5 minutes; 5% B. The injection volume was 5 μ l. MS/MS analysis was performed with positive ionization using multiple reaction monitoring (MRM) mode. A capillary voltage of 3 kV, source temperature of 150°C, desolvation gas temperature of 500°C, cone gas flow of 300 l/hour and desolvation gas flow of 1000 l/hour were used. The MRM transitions for the compounds had the following values: methadone: m/z 310.22 > 223.10 (cone voltage (cv) 30 V, collision energy (ce) 25 eV) and m/z 310.22 > 265.16 (cv 30 V, ce 18 eV), buprenorphine: m/z 468.31 > 396.22 (cv 60 V, ce 40 eV) and m/z 468.31 > 414.26 (cv 60 V, ce 40 eV), corticosterone: m/z 347.20 > 121.10 (cv 25 V, ce 25 eV) and m/z 347.20 > 329.10 (cv 25 V, ce 15 eV).

Acquisition and processing were performed using Masslynx software (version 4.1, Waters). The limits of quantification were 1 nM for methadone, 0.1 nM for buprenorphine, and 0.7 nM for corticosterone. The retention times for methadone, buprenorphine and corticosterone were 2.9, 3.2 and 2.4 minutes, respectively. The correlation coefficient was over 0.99, and the deviation of the quality controls was lower than $\pm 20\%$.

Neonatal outcomes

On PND 1, all litters were examined for the number of offspring and the number of stillborn, and all pups were examined for sex, body weight and body length.

Ultrasonic vocalization

Ultrasonic vocalizations (USVs) were examined in 2 randomly selected pups from each litter on PND 1, 3, 7 and 14. Each pup was isolated from its dam and littermates and injected (i.p.) with naltrexone (1 mg/kg) or an equal volume of 0.9% NaCl 15 minutes prior to recording. USVs were recorded for 6 minutes using the UltraVox 3.0 Ultrasound Microphone (Noldus Information Technology, Wageningen, The Netherlands). During recording, the pup was placed in a plastic cage with regulated heating, and the microphone was placed approximately 15 cm above the animal. After recording, the pup was returned to its home cage. The recordings were analyzed by a researcher blinded to the treatment of the pups using the UltraVox TX 3.0 software (Noldus Information Technology). All calls with a frequency of 20-60 kHz were counted (Hofer et al., 2002).

Data and statistical analysis

All data are presented as the mean \pm S.E.M., unless stated otherwise. For the analysis of treatment over time, repeated-measures analysis of variance (ANOVA) was used followed by Tukey's post hoc test (Fig. 2). For comparisons between two groups, a two-tailed Student's *t*

test was used (Fig. 3). One-way ANOVA followed by Tukey's post hoc test was used to compare the three treatment groups (Table 1 and Fig. 4). *P*-values below 0.05 were considered statistically significant. The statistical analyses were conducted by SPSS (version 25, Chicago, IL).

Results

A total of 75 female rats were implanted with an osmotic minipump. Seven animals were euthanized during pregnancy due to health problems or complications with the pump. Six vehicle-exposed, 5 methadone-exposed, and 13 buprenorphine-exposed females did not mate successfully.

Maternal blood concentrations of methadone and buprenorphine

The maternal blood concentrations were stable at 0.25 ± 0.02 μM for methadone and 5.65 ± 0.16 nM for buprenorphine throughout gestation (GD 7-21; Fig. 2A and B). No significant differences were observed in body weight between the vehicle-, methadone- or buprenorphine-exposed dams (Fig. 2C). By GD 21, the mean weight gain was 72.0 ± 6.5 g. Due to the weight gain, the initial doses of 10 mg/kg/day methadone and 1 mg/kg/day buprenorphine were reduced to approximately 8 mg/kg/day and 0.8 mg/kg/day, respectively. On GD 22, the drug concentration of the solutions remaining in the pump was $99 \pm 4\%$ of the initial concentration (data not shown).

Blood and brain concentrations of methadone in dams, fetuses and offspring

Following the continuous exposure to methadone throughout gestation, the blood methadone concentration in the dams on GD 22 was 0.30 ± 0.04 μM , while the brain concentration was 2.8 times higher (0.84 ± 0.10 nmol/g; *P* < 0.001; Fig. 3A). The fetal blood concentration on GD 22 was 0.48 ± 0.08 μM , while the fetal brain concentration was 1.89 ± 0.35 nmol/g,

which was 4 times higher than that in the blood ($P < 0.01$; Fig. 3A). Moreover, the methadone concentration in the fetal blood was 1.6 times higher than that in the maternal blood ($P < 0.01$), and the fetal brain concentration was 2.3 times higher than the maternal brain concentration ($P < 0.01$). On PND 1, the offspring blood and brain concentrations were $0.02 \pm 0.01 \mu\text{M}$ and $0.13 \pm 0.05 \text{ nmol/g}$, respectively (Fig. 3C). On PND 7, no methadone was detected in the offspring blood, while the offspring brain concentration was $0.01 \pm 0.01 \text{ nmol/g}$, which was significantly lower than on PND 1 ($P < 0.01$; Fig. 3C).

Blood and brain concentrations of buprenorphine in dams, fetuses and offspring

After the continuous exposure to buprenorphine throughout gestation, the blood buprenorphine concentration in the dams on GD 22 was $5.69 \pm 1.44 \text{ nM}$, while the brain concentration was 10 times higher ($60.22 \pm 8.21 \text{ pmol/g}$; $P < 0.001$; Fig. 3B). The fetal blood concentration on GD 22 was $4.56 \pm 0.93 \text{ nM}$, while the brain concentration was $20.02 \pm 4.97 \text{ pmol/g}$, which was 4 times higher than that in the fetal blood ($P < 0.05$; Fig. 3B). While the fetal and maternal blood concentrations were within the same range, the fetal brain concentration was one third of the maternal brain concentration ($P < 0.001$; Fig. 3B). On PND 1, the offspring blood and brain concentrations were $0.36 \pm 0.11 \text{ nM}$ and $4.53 \pm 0.92 \text{ pmol/g}$, respectively (Fig. 3D). On PND 7, no buprenorphine was detected in the offspring blood, while the brain concentration was $0.80 \pm 0.27 \text{ pmol/g}$, which was significantly lower than on PND 1 ($P < 0.01$; Fig. 3D).

Neonatal outcomes after prenatal exposure to methadone or buprenorphine

Maternal exposure to methadone or buprenorphine did not affect litter size, the number of stillborn pups or the sex ratio (Table 1). Compared with the vehicle- and buprenorphine-exposed offspring, the pups prenatally exposed to methadone showed significantly reduced body weight ($P < 0.01$) and body length ($P < 0.001$) on PND 1. However, when the litters (N

= 10) instead of the individual pups ($N = 99-119$) were compared, no significant differences were observed in body weight or length. No differences were observed in the offspring brain weight (Table 1).

Blood corticosterone concentrations and USVs in offspring prenatally exposed to methadone or buprenorphine

On PND 1, pups prenatally exposed to methadone exhibited significantly higher blood levels of the stress hormone corticosterone compared both the vehicle- and buprenorphine-exposed offspring ($P < 0.05$; Fig. 4A). No differences were observed on PND 7 or 14. The USV recordings did not reveal any significant differences between the three groups on PND 1, 3, 7 or 14 (Fig. 4B, left panel). Moreover, compared to saline pretreatment, pretreatment with naltrexone (1 mg/kg) 15 minutes prior to recording did not affect vocalization (data not shown), which is consistent with the low opioid concentrations detected in the offspring (Fig. 3C and D). However, the number of calls varied considerably between the individual pups from all groups (Fig. 4B, right panel). On PND 7, approximately 50% of the opioid-exposed offspring displayed more calls than the pup exhibiting most calls in the vehicle group. Similar findings were observed on PND 3 for the methadone-exposed offspring (Fig. 4B, right panel).

Discussion

In the present study, we show that continuous exposure to methadone or buprenorphine, at doses of 10 and 1 mg/kg/day, respectively, provided stable blood concentrations in pregnant rats that were comparable to those reported in pregnant women in OMT. The concentration of methadone in the fetal brain was twice as high as that in the maternal brain, while the fetal brain concentration of buprenorphine was one third of the maternal brain concentration. Moreover, offspring prenatally exposed to methadone, but not buprenorphine, displayed reduced body weight and length and increased blood corticosterone level the day after birth.

Previous animal studies of prenatal exposure to methadone or buprenorphine have been criticized for their lack of clinical relevance due to the use of high drug doses and the absence of pharmacokinetic data (Farid et al., 2008; Ross et al., 2015). To our knowledge, only one previous study has reported methadone concentrations in pregnant rats after repeated daily administrations (Hutchings et al., 1976), however, blood sampling was performed at one single undefined time point. The present data are therefore the first to demonstrate that the continuous exposure to high doses of methadone (10 mg/kg/day) or buprenorphine (1 mg/kg/day), used previously (Hutchings et al., 1992; Robinson and Wallace, 2001), induces stable blood concentrations of $0.25 \pm 0.02 \mu\text{M}$ and $5.65 \pm 0.16 \text{ nM}$, respectively, throughout gestation. These concentrations are in the same range as plasma concentrations of pregnant women in OMT, which have been reported to be 0.08 – 1.7 μM for methadone and 0.3 – 16.0 nM for buprenorphine (Doberczak et al., 1993; Johnson et al., 2001; Drozdick et al., 2002; Jansson et al., 2007; Concheiro et al., 2010; Gordon et al., 2010; Concheiro et al., 2011; Bartu et al., 2012). In agreement with previous studies that have reported accumulation of methadone and buprenorphine in the rat brain after acute exposure (Gabrielsson et al., 1985; Pontani et al., 1985), we also found higher opioid concentrations in the brain than in the blood of pregnant rats.

A major concern of OMT during pregnancy is that methadone and buprenorphine cross the placenta and are distributed in fetal tissue (Sarman, 2000; Nanovskaya et al., 2002). We found the methadone concentration to be 1.6 times higher in fetal blood than in maternal blood, while the fetal blood concentration of buprenorphine was within the same range as the blood concentration in the dams. Similar ratios have been reported following a single injection of methadone in rats (Gabrielsson et al., 1985) and buprenorphine in mice (Coles et al., 2009). This somewhat lower transfer of buprenorphine than of methadone is also consistent with *in vitro* studies of the human placenta (Nekhayeva et al., 2005; Gordon et al., 2010).

The 4 times higher concentration of methadone and buprenorphine in the fetal brain than in the blood demonstrates that, as methadone or buprenorphine enters the fetal circulation, both drugs accumulate in neuronal tissue. This is concerning because the endogenous opioid system plays a key role in neurobiological development (Sargeant et al., 2008). Previous animal studies have shown that exposure to opioids during fetal or early life affects the migration and survival of neurons, leading to an overall inhibition of brain growth and development (Zagon and McLaughlin, 1977; Wu et al., 2001; Bajic et al., 2013), which may cause acute effects (Hutchings et al., 1979; Hein et al., 1988; Kunko et al., 1996) and long-lasting neurobiological changes. Indeed, exposure to methadone or buprenorphine during development has been shown to cause impaired learning and memory (Zagon et al., 1979; Van Wagoner et al., 1980; Chen et al., 2015), anxiety and depression (Daly et al., 2012; Hung et al., 2013; Chen et al., 2015), and altered pain thresholds in adolescent and adult rats (Zagon and McLaughlin, 1982; Chiang et al., 2015). There are also indications of negative long-lasting effects in children born to mothers in OMT (Konijnenberg and Melinder, 2011; Monnelly et al., 2019).

The methadone concentration in the fetal brain was twice as high as that in the maternal brain. Similar findings have been reported previously after a single injection (Peters et al., 1972). This is alarming as it suggests that methadone treatment during pregnancy leads to a larger burden for the fetal brain than for the maternal brain. In contrast to methadone, the buprenorphine concentration in the fetal brain was one third of the maternal brain concentration. Moreover, even though the buprenorphine dose used in the current study was one-tenth the methadone dose, the fetal brain concentration of buprenorphine was 95 times lower than that of methadone. It is important to note that methadone and buprenorphine are different with respect to being full vs. partial agonists, their affinity and dissociation rate for the μ -opioid receptor, and their action on NMDA receptors (Volpe et al., 2011; Drewes et al.,

2013). Thus, the effect of a drug on the developing brain is not only dependent on the drug concentration, but also its pharmacological properties.

The concentrations of methadone and buprenorphine in the 1-day-old pups were lower than the concentrations in the fetuses. Similar to fetuses, the opioid concentrations in neonates were considerably higher in the brain than in the blood. Moreover, methadone and buprenorphine were still present in the offspring brains on PND 7. We cannot completely exclude a minor drug exposure through lactation the first days after birth. However, since the pumps were empty around the time of delivery, our findings indicate slow drug elimination from the neonatal brain.

The pups prenatally exposed to methadone were born with significantly lower body weights and body lengths compared with those of the vehicle- and buprenorphine-exposed offspring. However, no significant differences were observed in these measures when comparing the litters instead of the individual pups. Similarly, some previous animal studies have reported reduced birth weights after prenatal methadone exposure (Hutchings et al., 1979; Robinson et al., 1991; Kunko et al., 1996; Robinson and Wallace, 2001), while others have reported no differences (Chiang et al., 2010; Chiang et al., 2015). In agreement with our results, rat pups prenatally exposed to buprenorphine seem to have more favorable birth outcomes compared with those of methadone-exposed pups (Hutchings et al., 1996; Chiang et al., 2010).

Additionally, human studies have reported a reduced incidence of preterm birth, higher birth weights and larger head circumferences after maternal treatment with buprenorphine compared with methadone (Welle-Strand et al., 2013; Zedler et al., 2016).

Offspring prenatally exposed to methadone exhibited increased blood levels of the stress hormone corticosterone compared with those in both the vehicle- and buprenorphine-exposed offspring. This may be explained by the more rapid elimination of methadone and may

therefore be a sign of opioid withdrawal (Hamilton et al., 2013). However, the increased level of corticosterone may also be due to methadone exposure itself (Bartolome and Kuhn, 1983). USVs, which are used as a measure of withdrawal in rodents (Barr et al., 1998), were not significantly different between the groups. However, the number of calls varied considerably between the individual pups, especially in the opioid-treated groups, where approximately 50% of the methadone- and buprenorphine-exposed offspring exhibited increased vocalizations on PND 7. Large variations in abstinences have also been reported in newborns (Jones et al., 2010; Welle-Strand et al., 2013; Wurst et al., 2016). In line with our observations, maternal treatment with methadone is associated with more severe NAS when compared with buprenorphine, shown by longer hospital stay and an increased need for treatment (Jones et al., 2010; Meyer et al., 2015).

In conclusion, our pharmacokinetic data show that the exposure regimen used in the present study and in previous animal studies of OMT, with continuous exposure to high doses of methadone (10 mg/kg/day) or buprenorphine (1 mg/kg/day), results in blood concentrations in pregnant rats comparable to those reported in pregnant women in OMT. Furthermore, we show that maternal treatment with methadone leads to a significantly higher brain concentration in the fetus compared to the dam. This suggests that methadone treatment during pregnancy entails a larger burden for the fetus than for the mother. In contrast, exposure to buprenorphine resulted in a significantly lower concentration in the fetal brain than in the maternal brain. Buprenorphine also allowed more favorable birth outcomes compared to methadone. Taken together, our data on short-term effects indicate that OMT with buprenorphine may be a better choice than methadone during pregnancy. However, prenatal exposure to OMT may have long-lasting consequences not revealed the first weeks after birth.

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Author contributions

Participated in research design: Kongstorp, Bogen, Stiris and Andersen

Conducted the experiments: Kongstorp, Andersen

Performed data analysis: Kongstorp

Wrote or contributed to the writing of the manuscript: Kongstorp, Bogen, Stiris and Andersen

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Figure legends

Fig. 1. Schematic overview of the study. Female rats were implanted with a 28-day osmotic minipump delivering vehicle (sterile water), methadone (10 mg/kg/day) or buprenorphine (1 mg/kg/day) 5 days prior to mating (gestation day (GD) -5). The maternal blood concentration of the administered opioid was measured the day after implantation (GD -4), on the day of mating (GD 0) and on GD 7, 10, 14, 17 and 21. On GD 22, the blood and brain concentrations of the administered opioid were measured in the dams and fetuses. The offspring were examined for neonatal outcomes on postnatal day (PND) 1, for opioid and corticosterone concentrations on PND 1 and 7, and for ultrasonic vocalizations on PND 1, 3, 7 and 14.

Fig. 2. The blood concentrations of A) methadone (μM) and B) buprenorphine (nM) in the female rats implanted with an osmotic minipump delivering methadone (10 mg/kg/day) or buprenorphine (1 mg/kg/day), respectively, 5 days prior to mating (gestation day (GD) -5). The blood concentrations were measured the day after implantation (GD -4), on the day of mating (GD 0) and on GD 7, 10, 14, 17 and 21, $N = 7$. C) The body weights of the dams exposed to vehicle, methadone or buprenorphine prior to and during gestation, $N = 5-12$. The data are presented as the mean \pm SEM, no significant differences were revealed, repeated measures ANOVA.

Fig. 3. The blood and brain concentrations of A) methadone (blood, μM ; brain, nmol/g) and B) buprenorphine (blood, nM; brain, pmol/g) in the dam and fetus on gestation day (GD) 22. The female rats were implanted with a 28-day osmotic minipump delivering methadone (10 mg/kg/day) or buprenorphine (1 mg/kg/day) 5 days prior to mating (GD -5). The blood and brain concentrations of C) methadone and D) buprenorphine in the offspring on postnatal days 1 and 7. The maternal blood and brain, $N = 7$; the fetal blood, $N = 5-7$ (pooled from 2-5 fetuses); the fetal brain, $N = 25-26$ (from 7 litters); and the offspring blood and brain, $N = 10-16$ (from 7-8 litters). The data are presented as the mean \pm SEM, * $P < 0.05$, **/ $\dagger\dagger$ $P < 0.01$,

***/ $\dagger\dagger\dagger$ $P < 0.001$, * compared to blood, \dagger compared to the respective maternal concentration, two-tailed paired student's t test, $^{##}$ $P < 0.01$ compared with PND1, two-tailed unpaired student's t test.

Fig. 4. Stress hormone levels and vocalizations in the offspring prenatally exposed to vehicle, methadone (10 mg/kg/day) or buprenorphine (1 mg/kg/day). A) The blood concentration (μ M) of corticosterone in the offspring on postnatal day (PND) 1, 7 and 14, $N = 9-16$ (from 7-9 litters). B) Ultrasonic vocalizations (USVs), measured as the number of calls, on PND 1, 3, 7 and 14, $N = 6-10$. The left panel displays the mean number of calls and the right panel the variations in the number of calls in each treatment group. The data are presented as the mean \pm SEM or as single data points, * $P < 0.05$ compared to vehicle, $\dagger P < 0.05$ compared to buprenorphine, one-way ANOVA followed by Tukey's post hoc test.

Table

Table 1. Litter size, number of stillborn, sex ratio, and pup body weight, body length and brain weight on postnatal day 1.

	Vehicle	Methadone	Buprenorphine
Litter size ^a	11.9 ± 0.8	11.1 ± 1.1	9.9 ± 0.8
Stillborn (number of dead/total) ^a	2/119	5/111	2/99
Sex ratio (male/female) ^a	1.1	1.2	1.4
Body weight (g) ^b	7.08 ± 0.13	6.59 ± 0.13 **††	7.17 ± 0.08
Body length (cm) ^b	5.24 ± 0.05	4.92 ± 0.05 ***†††	5.31 ± 0.05
Brain weight (g) ^c	0.26 ± 0.01	0.25 ± 0.01	0.25 ± 0.01

^a*N* = 10 litters; ^b*N* = 99-119 (from 10 litters); ^c*N* = 11-16 (from 10 litters). **/†† *P* < 0.01,

***/††† *P* < 0.001, * compared to vehicle, † compared to buprenorphine, one-way ANOVA,

Tukey's post hoc test. Not significant when comparing mean litter weight or length.

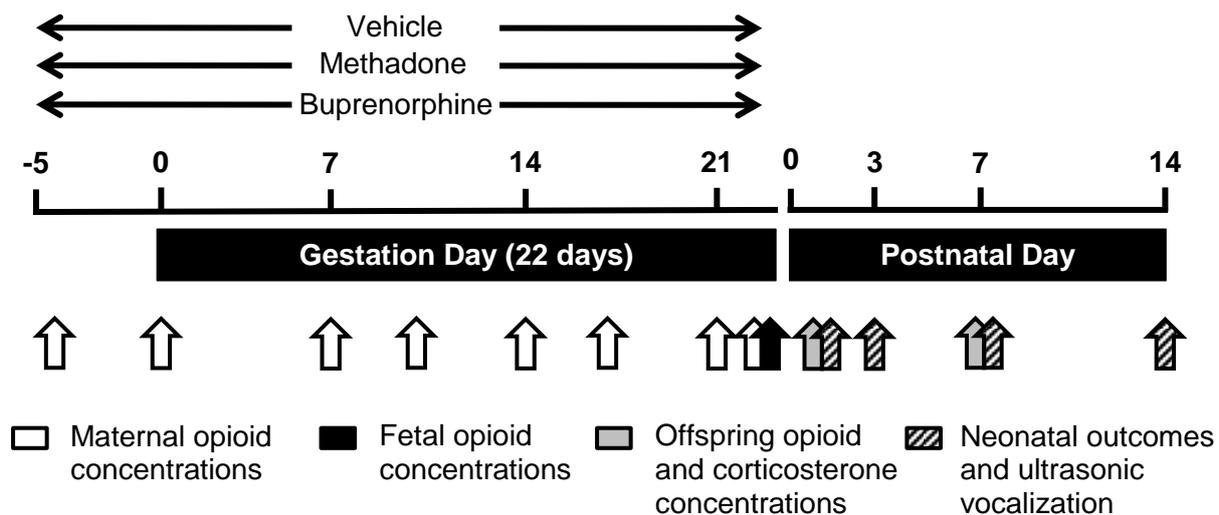


Fig. 1.

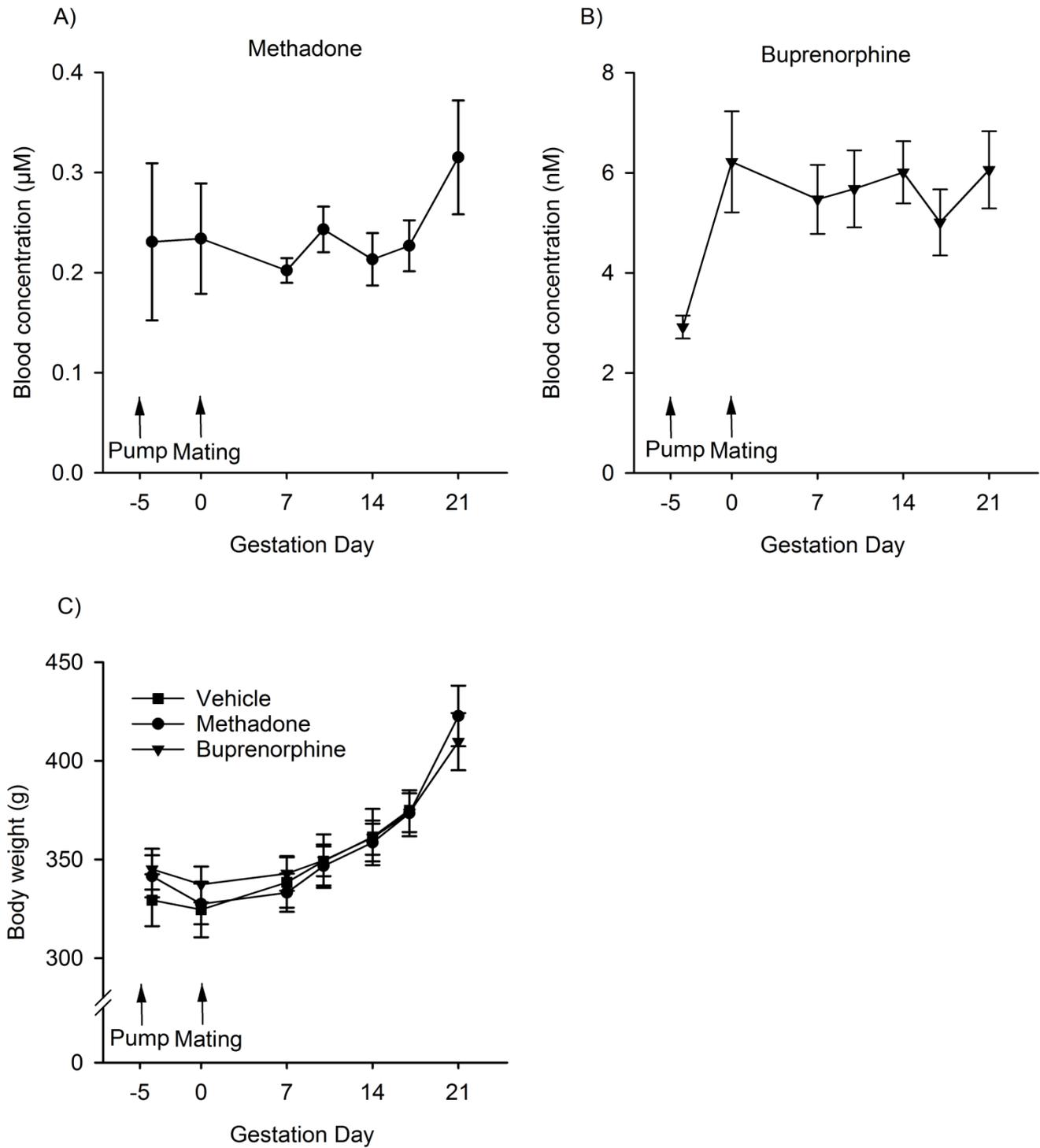


Fig. 2.

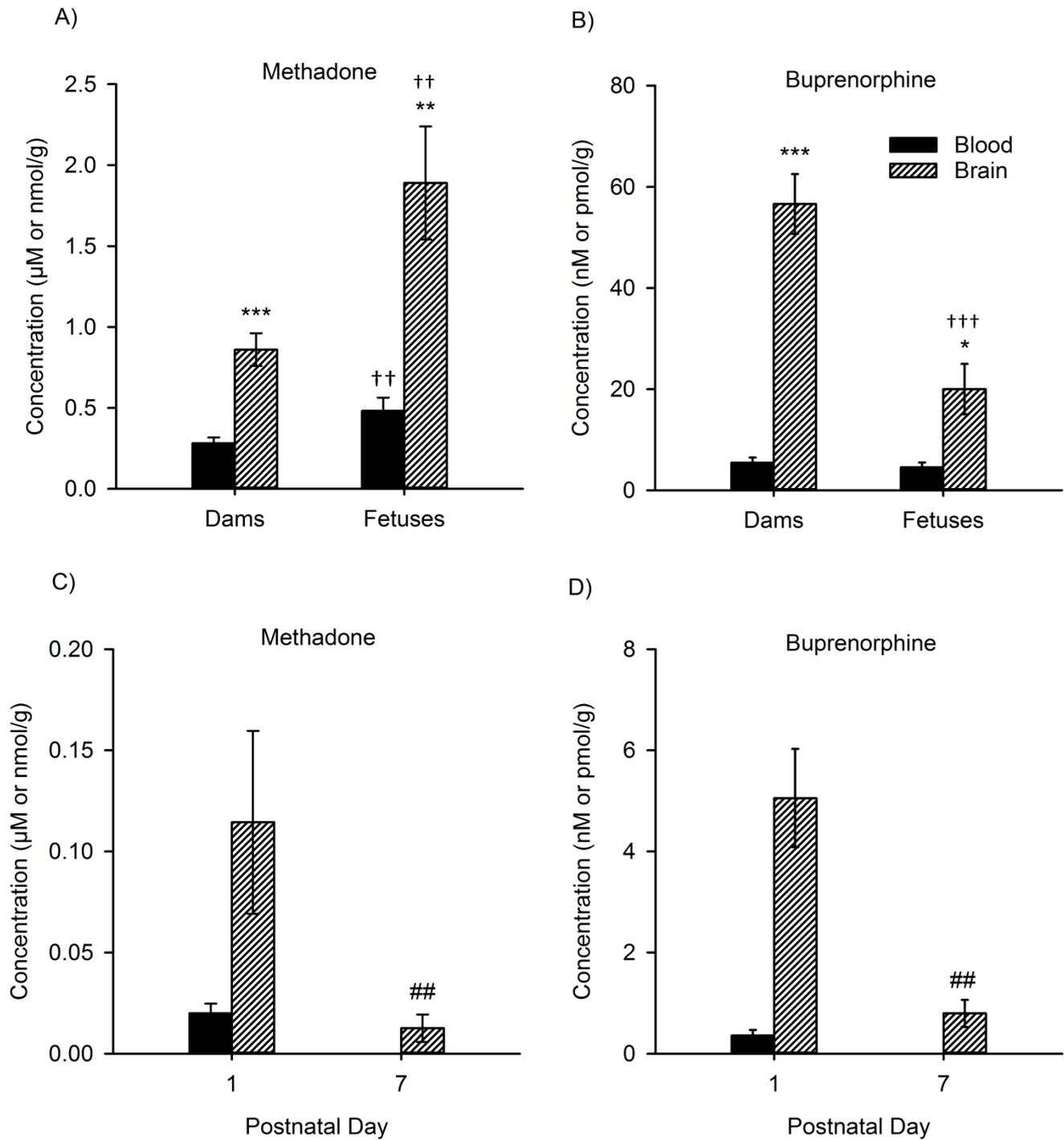


Fig. 3.

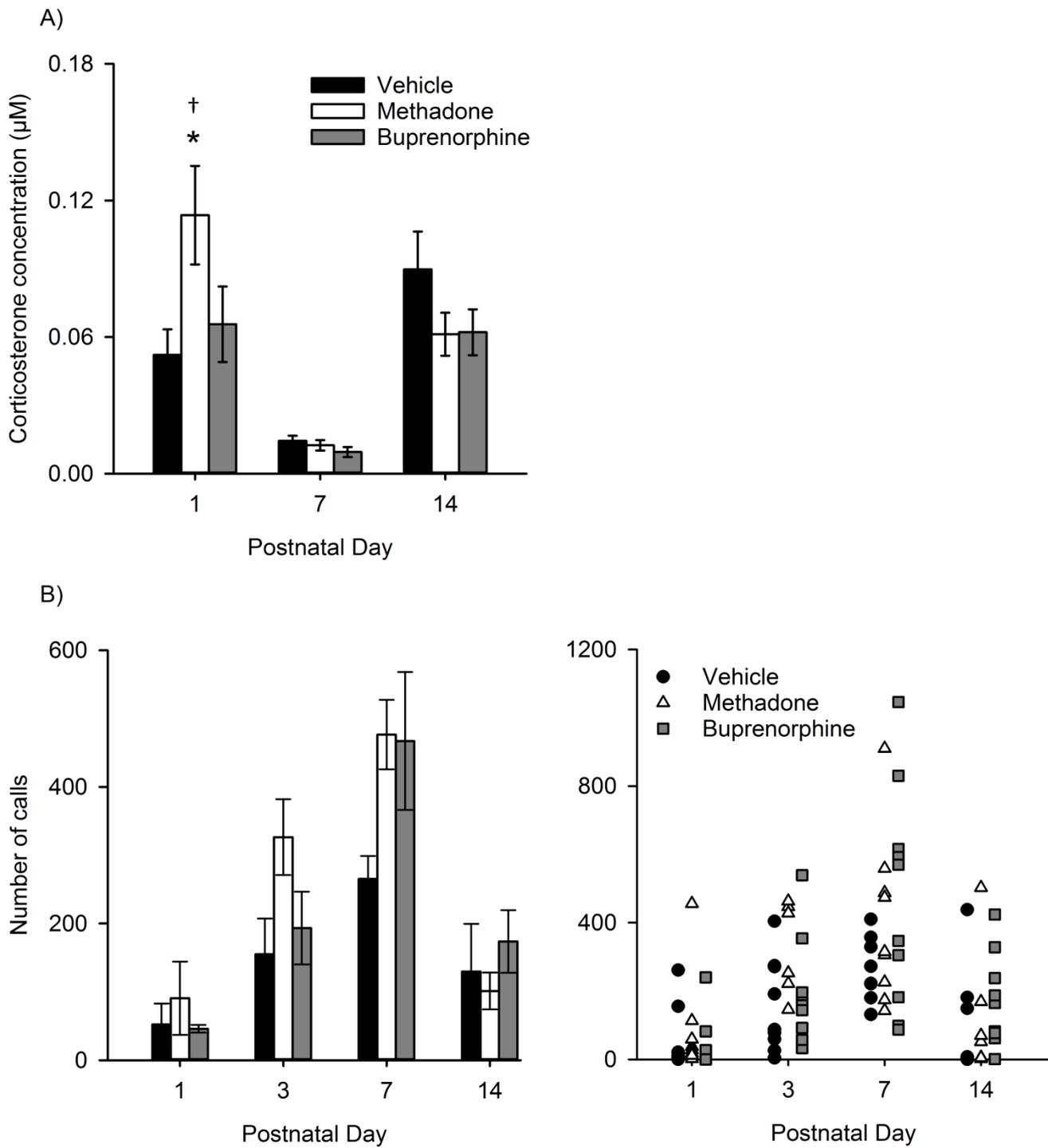


Fig. 4 .