

Paracetamol (Acetaminophen) Administration During Neonatal Brain Development Affects Cognitive Function and Alters Its Analgesic and Anxiolytic Response in Adult Male Mice

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Paracetamol (acetaminophen) is one of the most commonly used drugs for the treatment of pain and fever in children, both at home and in the clinic, and is now also found in the environment. Paracetamol is known to act on the endocannabinoid system, involved in normal development of the brain. We examined if neonatal paracetamol exposure could affect the development of the brain, manifested as adult behavior and cognitive deficits, as well as changes in the response to paracetamol. Ten-day-old mice were administered a single dose of paracetamol (30 mg/kg body weight) or repeated doses of paracetamol (30 + 30 mg/kg body weight, 4 h apart). Concentrations of paracetamol and brain-derived neurotrophic factor (BDNF) were measured in the neonatal brain, and behavioral testing was done when animals reached adulthood. This study shows that acute neonatal exposure to paracetamol (2 × 30 mg) results in altered locomotor activity on exposure to a novel home cage arena and a failure to acquire spatial learning in adulthood, without affecting thermal nociceptive responding or anxiety-related behavior. However, mice neonatally exposed to paracetamol (2 × 30 mg) fail to exhibit paracetamol-induced antinociceptive and anxiogenic-like behavior in adulthood. Behavioral alterations in adulthood may, in part, be due to paracetamol-induced changes in BDNF levels in key brain regions at a critical time during development. This indicates that exposure to and presence of paracetamol during a critical period of brain development can induce long-lasting effects on cognitive function and alter the adult response to paracetamol in mice.

Key Words: behavior; developmental neurotoxicity; neonatal; paracetamol (acetaminophen).

Paracetamol (acetaminophen, CAS number 103-90-2) is one of the most commonly used drugs for the treatment of pain and fever in children, available without prescription, and one of the most prescribed drugs in hospitals (Läkemedelsverket, 2009a). Studies have shown that the majority of all toddlers have been medicated with paracetamol (Hawkins and Golding, 1995;

Walsh *et al.*, 2007). In addition, paracetamol also may constitute an environmental pollutant as it is readily detected in the sewage effluent water and rivers in Europe and in U.S. natural waters (Kolpin *et al.*, 2002; Roberts and Thomas, 2006; Wu *et al.*, 2012).

Paracetamol acts on the endocannabinoid system for its analgesic effect. The analgesic activity of both paracetamol and tetrahydrocannabinol (THC, delta-9-tetra-hydro-cannabinol) is prevented by the blockade of cannabinoid CB₁ receptors (Bertolini *et al.*, 2006; Dani *et al.*, 2007; Mallet *et al.*, 2008; Ottani *et al.*, 2006; Ruggieri *et al.*, 2008), which has also been seen with the anandamide transport inhibitor AM404, a known metabolite of paracetamol (Anderson, 2008; Fride, 2005). The endocannabinoid system is involved in the development of the brain, and the CB₁ receptor is required for normal axonal growth and fasciculation. Embryonic CB₁-receptor signaling may participate in the correct establishment of neuronal connectivity, which may be compromised by maternal cannabis consumption (Vitalis *et al.*, 2008; Watson *et al.*, 2008). Epidemiological studies indicate that prenatal cannabinoid exposure can result in behavioral problems in children (Day *et al.*, 1994; Goldschmidt *et al.*, 2000).

Investigations show that paracetamol is rapidly distributed to the rat brain and that the concentration becomes significantly higher in the brain than in other compartments, eg, serum (Ara and Ahmad, 1980; Kumpulainen *et al.*, 2007). High doses of paracetamol (200–250 mg/kg body weight [bw]) can cause apoptosis in cortical neurons in the rat brain, supporting recent *in vitro* results claiming paracetamol to be potentially neurotoxic (Posadas *et al.*, 2010). The recommended dose of paracetamol in newborns and toddlers is 7.5–15 mg/kg up to 4 times a day, and it is also used in preterm neonates (maximum dose 60 mg/kg bw per day) for up to 10 days (Anderson and Allegaert, 2009).

During fetal and neonatal life, a series of rapid fundamental developmental changes occur, including maturation of dendritic and axonal outgrowth, the establishment of neural connections, and the synaptogenesis and proliferation of glia cells,

with accompanying myelination and acquisition of many new motor and sensory abilities (Bolles and Woods, 1964; Davison and Dobbing, 1968; Dobbing and Sands, 1979; Kolb and Whishaw, 1989), leading to the maturation of the brain into its highly developed adult functions, referred to as the brain growth spurt (BGS) (Davison and Dobbing, 1968; Dobbing and Sands, 1979). In human, the BGS begins during the third trimester of pregnancy and continues throughout the first 2 years of life, whereas in rodents the BGS is neonatal, spanning the first 3–4 weeks of life, peaking around postnatal day 10. Brain-derived neurotrophic factor (BDNF), a neurotrophin, widely expressed in the brain, with a distinct ontogeny pattern during the BGS, promotes neuronal survival but also regulates cell migration, axonal and dendritic outgrowth, and formation and function of synapses (Cui, 2006; Huang and Reichardt, 2001). Several studies support the idea that BDNF is both interacting with the endocannabinoid system and can be affected by cannabinoid use (De Chiara *et al.*, 2010; D'Souza *et al.*, 2009; Fishbein *et al.*, 2012), which could have implications during development, which implicates that other substances acting in a similar manner maybe also can affect the levels of BDNF.

Recently we have shown that neonatal exposure to certain pharmaceutical drugs, such as ketamine and propofol (Ponten *et al.*, 2011; Viberg *et al.*, 2008a) and environmental pollutants, such as DDT, PCBs, PBDEs, and PFCs (Eriksson, 1998; Eriksson *et al.*, 1992; Johansson *et al.*, 2008; Viberg *et al.*, 2003) can cause similar persistent disturbances in behavior, including learning and memory deficits in adult animals. Some of these compounds also affect the levels of BDNF (Ponten *et al.*, 2011; Viberg *et al.*, 2008b). We have also reported that neonatal exposure to the anesthetic agent propofol decreases the anxiolytic effect of adult diazepam treatment in mice (Ponten *et al.*, 2011).

This study was undertaken to investigate whether neonatal exposure to paracetamol affects the levels of BDNF in the neonatal brain and the adult cognitive function and/or alters its analgesic and anxiolytic effect.

MATERIALS AND METHODS

Chemicals and Animals

Pregnant NMRI mice (from Scanbur, Sollentuna, Sweden) were housed individually in plastic cages and supplied standardized pellet food (Lactamin, Stockholm, Sweden) and tap water *ad libitum* in a room with a temperature of 22°C and a 12/12-h cycle of light and dark. At the age of 4–5 weeks, litters were separated (females sacrificed) and males were kept in their litters (in treatment groups) with their siblings, and placed and raised in groups of 4–7, in a room for male mice only. Only male mice were used in this study. Experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC), after approval from the local ethical committees (Uppsala University and Agricultural Research Council) and the Swedish Committee for Ethical Experiments on Laboratory Animals, approval number C185/9.

Paracetamol (Perfalgan, 10 mg/ml Bristol-Meyers Squibb, Princeton, New Jersey) was purchased (Apoteksbolaget, Uppsala, Sweden), and the purity of the product was not checked because it is a licensed medical drug approved by the Swedish Medical Products Agency. On postnatal day 10, both male

and female pups were administered 30 (single) or 30 + 30 mg (repeated) paracetamol/kg bw (4 h apart), or vehicle (0.9% NaCl) in a volume of 5 ml/kg by SC injection in the neck. The recommended dose for suppositories containing paracetamol is 12.5 mg/kg bw in newborns and toddlers (Fass, 2012). The recommended dose for Perfalgan injection fluid is 7.5 mg paracetamol/kg bw, where the maximum daily dose should not exceed 30 mg/kg bw (Fass, 2012; FDA, 2012). Our doses are higher than the recommended doses, but in the same range, and the exposure occurs on 1 day only, in contrast to what is often common in daily life.

HPLC-EC Assay of Paracetamol Concentration

Six control male animals were sacrificed 1 h after injection with saline; 6 male animals injected with 30 mg paracetamol/kg bw were sacrificed at each time point, 1, 2, or 4 h after the injection; and 6 male animals injected with 30 + 30 mg paracetamol were sacrificed 1 h after the second injection. The frozen tissue was homogenized in 0.1 M perchloric acid, 5 ml/g tissue. The homogenate was centrifuged (16 700 × g, 4°C, 15 min) and filtered (0.45 μ filter). Assay of paracetamol was performed using high-performance liquid chromatography with electrochemical detection (HPLC-EC). In order to fit the standard curve, a suitable aliquot of the supernatant was diluted with a mobile phase up to 300 μl, of which 20 μl was injected into the HPLC-EC system. This procedure was needed due to the wide concentration range of paracetamol in brain tissue (μg/mg brain tissue).

The HPLC system consisted of a Biscoff pump model 2250, an autosampler/autoinjector fitted with tray cooling kept at 5°C (Midas, Spark Holland), an analytical column (Reprosil-Pur, C18-AQ, 250 × 4 mm, 5 μm) fitted with a guard column (A. Maisch, Deutschland) kept at 30°C, and a Coulochem II ESA multi-electrode detector fitted with a Model 5011-A dual analytical cell (ESA Analytical, Chelmsford, Massachusetts) operating at an oxidation potential of +350 mV. The mobile phase, pH 3.0 ± 0.1, consisted of 100 mM NaH₂PO₄, 0.5 mM 1-octanesulfonic acid, 1 mM EDTA, and methanol 14%. The flow rate was 0.7 ml/min.

ELISA Assay to Measure the BDNF Concentration

Nine male mice from the control group and 9 male mice from the repeated treatment group (30 + 30 mg paracetamol/kg bw), randomly selected from 9 different litters, were sacrificed 24 h after exposure, and frontal cortex, parietal cortex, and hippocampus were dissected out and sonicated in 20 volumes (wt/vol) of ice-cold lysis buffer (137 mM NaCl; 20 mM Tris-HCl, pH 8.0; 1 mM phenyl-methyl-sulfonyl fluoride; 0.5 mM sodium vanadate; 1% NP40; 10% glycerol; 10 μg/ml aprotinin; 1 μg/ml leupeptin). These brain regions were used in order to allow comparison with previous studies on ketamine and propofol (Ponten *et al.*, 2011; Viberg *et al.*, 2008a). The homogenate was centrifuged for 20 min at 20 000 × g at 4°C, and the supernatant was acidified (pH < 3) with HCl and neutralized back to pH 7.6 with NaOH. The Promega E_{max} ImmunoAssay System (Promega 7699) was used to determine the amount of BDNF according to the technical bulletin. In brief, BDNF was captured with a monoclonal antibody against BDNF, then bound to a specific, polyclonal antibody (pAb) against BDNF, and detected by using a specific anti-IgY antibody conjugated to horseradish peroxidase (HRP). Unbound conjugate was removed, and the color change was measured in a microplate reader at 450 nm. The amount of BDNF was proportional to the color change and compared to a standard curve. In these experiments, 9 hippocampi and frontal and parietal cortices from each treatment group were used. The cross-reactivity to other neurotrophic factors is less than 3%, and the purity of the anti-BDNF antibodies was greater than 95%. In these experiments, 8 hippocampi and frontal and parietal cortices from each treatment group were used.

Behavior Tests

In all behavior tests, the same person performed the tests, and the tester was blinded to the different treatments given to the animals. The animals were randomly selected from the different litters in each treatment group, meaning that certain mice may have been part of more than one behavioral tests. The order of the tests was the same as presented here, starting with the spontaneous behavior test at 2 months of age, but with a period of 1 week in between the different tests.

Spontaneous Behavior

At 2 months of age, 9 male mice, randomly taken from 9–11 different litters in each treatment group, were subjected to spontaneous behavior testing in a novel home environment, between 0800 and 1200h, under the same light and temperature conditions as detailed above. The reason for testing the animals at 2 months of age is that the animals are young adults where most of the brain development is finished and also to allow for comparison with previous studies on ketamine and propofol (Ponten *et al.*, 2011; Viberg *et al.*, 2008a). Motor activity was measured for 60 min, divided into 3×20 min periods, in an automated device consisting of cages (40×25×15 cm, same size as the regular home cage) placed within 2 series of infrared beams (low and high level) (Rat-O-Matic, ADEA Elektronik AB, Uppsala, Sweden). Three variables were measured:

Locomotion. Registered horizontal movement through the low-level grid of infrared beams, aimed 10 mm above the bedded floor.

Rearing. Registered vertical movement, at a rate of 4 counts per second, when a single high-level beam was interrupted, ie, the number of counts was proportional to time spent rearing. Infrared beams were aimed 80 mm above the bedded floor.

Total activity. All types of vibration within the cage, ie, those caused by animal movements, shaking (tremors), and grooming, were registered by a pickup (mounted on a lever with a counterweight), connected to the brim of the test cage.

Radial Arm Maze

The radial eight-arm maze testing, sensitive to impairments in spatial learning (Olton and Werz, 1978; Olton *et al.*, 1978), started 1 week after the end of the spontaneous behavior testing. The radial arm maze was constructed from plywood and the 8 arms (each 36 cm long, 7 cm wide, and 2 cm high enclosing walls) of the maze extended radially from a central hub (16×16 cm²) fitted on a tripod 70 cm above the floor in the center of the test room. The animals were tested 3 consecutive days, 1 trial per day. The mice had free access to water, but in order to increase the motivation, the mice were deprived of food for 12 h before the trial on the first day and 12 h before the trial on days 2 and 3. For learning trials, a food pellet (approximately 10 mg) was placed at the extremity of each arm, hidden behind a small shield (1 cm wide and 2 cm high) to prevent mice from seeing the food pellet. Eight male mice from each of the 3 treatment groups were tested in the radial arm maze. The mouse was placed in the central hub and then monitored for its instrumental learning performance, ie, the latency until all 8 pellets were collected (maximum time 10 min) and the number of arms visited in collecting all 8 pellets subtracted by 8 provided the number of errors per animal. Errors were defined as re-entries to arms already visited.

Hot Plate

The hot plate test, traditionally used when testing the effectiveness of analgesics (Eddy and Leimbach, 1953), used a clear Plexiglas box, heated to 52±0.1°C. Eighteen male mice from each of the 3 neonatally exposed treatment groups were tested in the hot plate test 1 week after the completion of the radial arm maze test. Nine of the animals in each treatment group (vehicle, 30 mg paracetamol/kg bw, or 30 + 30 mg paracetamol/kg bw) were pretreated with a SC injection of vehicle (0.9% NaCl), and the other 9 animals were pretreated with 30 mg paracetamol/kg bw, 30 min before testing. The mice were placed on the hot plate and observed for the occurrence of licking or lifting of the hind paw or jumping. The reaction latency was recorded as soon as one of these symptoms occurred, and the animal was quickly taken of the hot plate. If no reaction occurred within 40 s, the animal was taken from the hot plate and given the latency of 40 s (De Vry *et al.*, 2004). The reason for the pretreatment was to evaluate if neonatal paracetamol treatment affected the adult pain sensation and/or if neonatal exposure to paracetamol could affect the adult response to reexposure to paracetamol by altering the analgesic/antinociceptive effect.

Elevated Plus Maze

The test procedure, giving information about anxiety-like behavior, is adopted from Lister (1987). In the plus-maze apparatus, 2 diametrically placed

open arms (white floor with no wall, 30×6 cm) faced 2 closed arms (black floor with walls, 30×6×30 cm) mounted 50 cm above the floor. The mouse was placed on the central platform (white floor, 6×6 cm) of the apparatus, facing “north” toward the closed arms. The number of entries into open and closed arms and the total time spent in each of the arms were measured with video monitoring equipment for 5 min. Arm entry was defined as all 4 paws in the arm. One week after the completion of the hot plate test, at the age of 3 months, 18 male mice from each of the 3 neonatally exposed treatment groups were tested in the elevated plus maze. Nine of the animals in each treatment group (vehicle, 30 mg paracetamol/kg bw, or 30 + 30 mg paracetamol/kg bw) were pretreated with a SC injection of vehicle (0.9% NaCl), and 9 animals were pretreated with 30 mg paracetamol/kg bw, 30 min before testing. The reason for the pretreatment was to evaluate if the neonatal paracetamol treatment affected the adult anxiety-related behavior and/or if neonatal exposure to paracetamol could change the adult response to reexposure to paracetamol, altering the antianxiolytic effect of paracetamol.

Statistical Analysis

Data obtained from the spontaneous behavior and radial arm maze tests were statistically evaluated with an ANOVA using a split-plot design, with pairwise testing using Tukey’s HSD test (honestly significant differences) (Kirk, 1968).

Data obtained from the concentration analysis, elevated plus maze, and hot plate tests were statistically evaluated with a 2-way ANOVA with Tukey’s HSD *post hoc* test.

Data obtained from the BDNF analysis were statistically evaluated with an unpaired, 2-tailed Student’s *t* test.

RESULTS

There were no visual signs of toxicity in the control or paracetamol-exposed mice at any given time during the experimental period nor were there any significant differences in body weight between the different groups (data not shown).

Concentration of Paracetamol in Neonatal Mouse Brain

Results from the measurement of the paracetamol concentration in the brain at different time points after SC exposure to 30 mg (single) or 30 + 30 mg (repeated) paracetamol/kg bw at an age of 10 days are shown in Table 1.

The paracetamol concentration in the neonatal brain declined significantly ($p \leq .01$) from 4.95 µg/g, 1 h after exposure to 30 mg paracetamol/kg bw, to 0.17 µg/g 2 h after exposure. This means that 96.5% of the paracetamol is eliminated or metabolized during this 60-min period. In neonatal mice given 2 SC injections of paracetamol (30 + 30 mg/kg bw, 4 h apart), the concentration of paracetamol 1 h after the second injection was 4.86 µg/g, not significantly different from the concentration 1 h after the single injection of 30 mg paracetamol/kg bw (4.95 µg/g).

Concentration of BDNF in neonatal mouse brain

Results from the measurement of the BDNF concentration in frontal cortex, parietal cortex, and hippocampus 24 h after SC exposure to 30 + 30 mg (repeated) paracetamol/kg bw at an age of 10 days are shown in Table 2.

Student’s *t* test revealed that neonatal exposure to paracetamol changes the levels of BDNF in different brain regions. Twenty-four hours after SC exposure to 30 + 30 mg (repeated)

TABLE 1
Concentration of Paracetamol ($\mu\text{g/g}$ Brain, Fresh Weight) in the Brain at Different Time Points After Exposure to Paracetamol on Postnatal Day 10^a

Time After Exposure (h)	Exposure Group		
	Control ($\mu\text{g/g}$)	30 mg/kg bw ($\mu\text{g/g}$)	30+30 mg/kg bw ($\mu\text{g/g}$)
1	0	4.95 ± 0.83 [#]	4.86 ± 1.44 [#]
2	n.a.	0.17 ± 0.08 ^{**}	n.a.
4	n.a.	0.01 ± 0.01 ^{**}	n.a.

Note. ^aTen-day-old male NMRI mice were exposed to a SC injection of 30 mg paracetamol/kg bw, 30+30 mg paracetamol/kg (4 h apart), or 0.9% saline vehicle in the same manner. Six animals were killed 1, 2, or 4 h after treatment, and the concentration of paracetamol was measured in the whole brain. The statistical evaluation was made using 1-way ANOVA and pairwise testing using Tukey's HSD *post hoc* test. The statistical differences are indicated by [#] $p < .01$, compared with the concentration in the control group; ^{**} $p \leq .01$, compared with the concentration 1 h after exposure to 30 mg paracetamol/kg bw. The number of observations in each group at each time point was 6, and n.a. means were not analyzed.

TABLE 2
Concentration of BDNF (ng/g Brain, Fresh Weight) in Different Brain Regions 24-h Exposure to Paracetamol on Postnatal Day 10^a

Brain Region	Exposure Groups	
	Control (ng/g)	30+30 mg/kg bw (ng/g)
Frontal cortex	1.16 ± 0.34	3.28 ± 2.32 ^{**}
Parietal cortex	10.18 ± 1.49	7.56 ± 1.67 ^{***}
Hippocampus	2.77 ± 0.61	3.05 ± 1.20

Note. ^aTen-day-old male NMRI mice were exposed to SC injections of 30+30 mg paracetamol/kg (4 h apart) or 0.9% saline vehicle in the same manner. Nine control animals and 9 paracetamol-exposed animals were killed 24 h after treatment, and the concentration of BDNF was measured in frontal cortex, parietal cortex, and hippocampus. The statistical evaluation was made using a 2-sided, paired *t* test. The statistical difference is indicated by ^{**} $p \leq .01$ and ^{***} $p \leq .001$ compared with the control group. The number of observations in each group was 8–9.

paracetamol/kg body, the BDNF concentration had increased significantly ($p \leq .01$) with 183% in frontal cortex, compared with the control animals, and in parietal cortex, a significant decrease ($p \leq .001$) of 26% was seen. In hippocampus, there were no difference seen in BDNF concentration between paracetamol-exposed animals and control animals.

Spontaneous Behavior in 2-Month-Old Male Mice

Results from the 3 spontaneous behavioral variables locomotion, rearing, and total activity in 2-month-old NMRI mice, after neonatal SC exposure to 30 mg or 30 + 30 mg paracetamol/kg bw, are shown in Figure 1.

Neonatal exposure to paracetamol showed that there were significant treatment \times time interactions 2 months after exposure

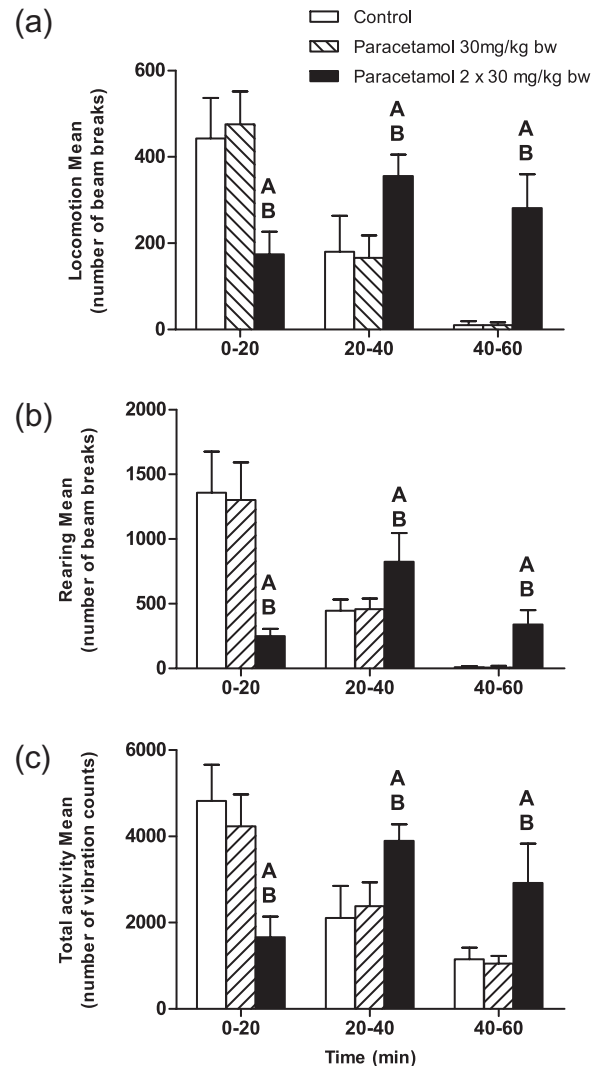


FIG. 1. Spontaneous behavior in 2-month-old NMRI male mice exposed to 0.9% saline vehicle, 30 mg paracetamol/kg bw, or 30 + 30 mg paracetamol/kg bw (4 h apart) on postnatal day 10. The data were subjected to an ANOVA with split-plot design, and there were significant group \times period interactions ($F_{4,48} = 62.56$; $F_{4,48} = 65.94$; $F_{4,48} = 49.95$) for the variables locomotion (a), rearing (b), and total activity (c), respectively. Pairwise testing between paracetamol-exposed and control animals was performed using Tukey's HSD tests. The statistical differences are indicated as (A) significantly different vs. controls, $p \leq .01$. The height of the bars represents the mean value and SD of 9 animals; (B) significantly different vs. paracetamol 30 mg/kg bw, $p \leq .01$. The height of the bars represents the mean value and SD of 9 animals.

($F_{4,48} = 62.56$; $F_{4,48} = 65.94$; $F_{4,48} = 49.95$) ($p < .001$; $p < .001$; $p < .001$) for the locomotion, rearing, and total activity variables, respectively (Fig. 1). Pairwise testing showed that in control mice and mice exposed to a single paracetamol dose, there was a distinct decrease in activity in all 3 behavioral variables over the 60-min period, which is normal for control animals and shows a normal habituation to a novel home environment. Male mice receiving the repeated doses of paracetamol showed a difference in activity for locomotion, rearing, and total activity, ie, significantly decreased

activity ($p \leq .01$) during the first 20 min (0–20 min) period and a significantly increased activity ($p \leq .01$) during the last 20-min period (40–60 min) compared with the control animals and the animals exposed to the single dose of paracetamol.

Radial Arm Maze Learning in 2-Month-Old Male Mice

Results from the 2 radial arm maze variables, total time and numbers of errors, in 2-month-old NMRI mice after neonatal SC exposure to 30 mg (single) or 30 + 30 mg (repeated) paracetamol/kg bw, are shown in Figure 2.

Neonatal exposure to paracetamol showed that there were significant treatment \times day interactions 2 months after exposure ($F_{4,48} = 12.99$; $F_{4,48} = 7.56$) ($p < .001$; $p < .001$) for both the total time variable and number of errors variable (Fig. 2). Pairwise testing showed that in control mice and mice exposed to a single paracetamol dose, there was a distinct decrease in time and number of errors over the 3 consecutive days, which is normal for control animals and shows the ability to improve spatial learning. Male mice receiving the repeated doses of paracetamol showed a significantly longer total time ($p \leq .01$) finding all 8 food pellets and significantly more errors ($p \leq .01$) on

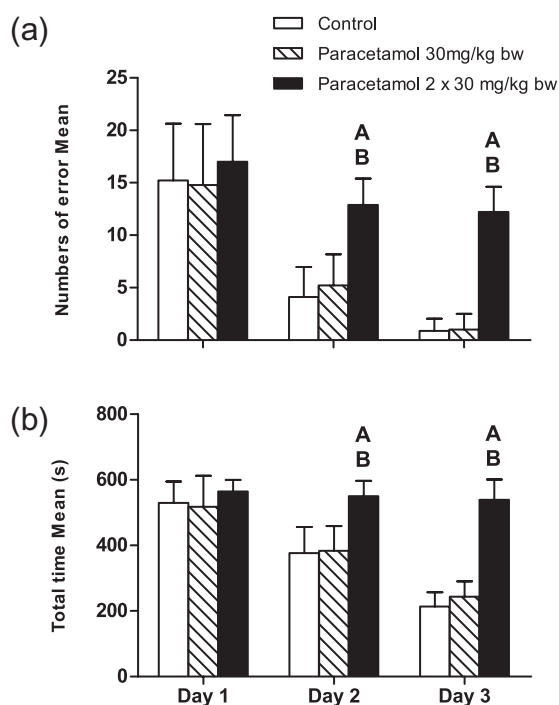


FIG. 2. Radial arm maze acquisition performance in 2-month-old NMRI male mice exposed to 0.9% saline vehicle, 30 mg paracetamol/kg bw, or 30 + 30 mg paracetamol/kg bw (4 h apart) on postnatal day 10. Number of errors and total time (seconds) were measured for 3 consecutive days. The data were subjected to an ANOVA with split-plot design, and there were significant group \times period interactions ($F_{4,48} = 12.95$; $F_{4,48} = 7.56$) for the variables errors (a) and total time (b), respectively. Pairwise testing between paracetamol-exposed and control animals was performed using Tukey's HSD tests. The statistical differences are indicated as (A) significantly different vs. controls, $p \leq .01$; (B) significantly different vs. paracetamol 30 mg/kg bw, $p \leq .01$. The height of the bars represents the mean value and SD of 9 animals.

test days 2 and 3 compared with the control animals and the animals exposed to the single paracetamol dose.

Hot Plate Test in 3-Month-Old Male Mice

Results from the hot plate test, after pretreatment with saline or 30 mg paracetamol/kg bw in 3-month-old NMRI mice, after neonatal SC exposure to 30 mg (single) or 30 + 30 mg (repeated) paracetamol/kg bw, are shown in Figure 3.

There were significant treatment effects regarding the time spent on the hot plate before and after the adult challenge to paracetamol (2-way ANOVA, $F_{5,48} = 50.17$) ($p < .0001$), and pairwise testing showed significant differences between controls and paracetamol-treated mice.

Pretreatment with saline 30 min before testing. There were no significant differences in the time on the hot plate in animals neonatally exposed to paracetamol compared with control animals, indicating that neonatal paracetamol exposure does not affect the pain sensitivity in the adult.

Pretreatment with 30 mg paracetamol/kg bw, 30 min before testing. Control animals spent a significantly longer time on the hot plate ($p \leq .01$) than control animals pretreated with saline, showing an expected significant analgesic/antinociceptive effect of the adult paracetamol treatment. In contrast, mice exposed neonatally to single or repeated doses of paracetamol and pretreated with paracetamol spent a significantly shorter time ($p \leq .01$) on the hot plate than the control animals.

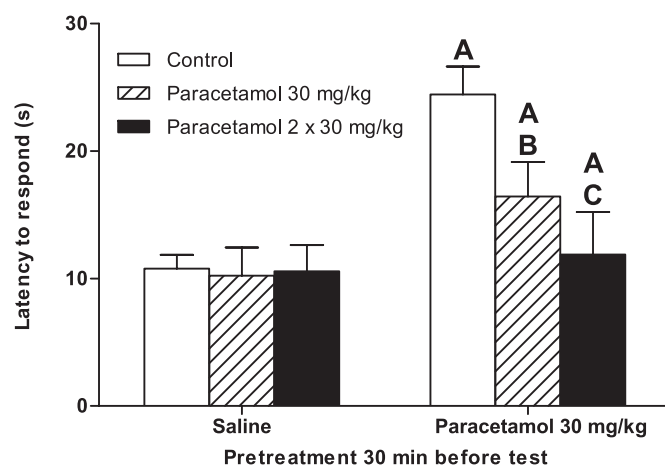


FIG. 3. Hot plate performance, 30 min after a SC injection of 0.9% saline vehicle or 30 mg paracetamol/kg bw, in 3-month-old NMRI male mice exposed to 0.9% saline vehicle, 30 mg paracetamol/kg bw, or 30 + 30 mg paracetamol/kg bw (4 h apart) on postnatal day 10. The time spent on the hot plate before signs of pain was recorded. The data were subjected to an ANOVA with split-plot design, and there were significant treatment group effects ($F_{5,48} = 50.17$) for the variable time spent on hot plate. Pairwise testing between paracetamol-exposed and control animals was performed using Tukey's HSD tests. The statistical differences are indicated as (A) significantly different vs. its own treatment group pretreated with saline, $p \leq .01$; (B) significantly different vs. controls pretreated with paracetamol, $p \leq .01$; (C) significantly different vs. single-dose paracetamol, pretreated with paracetamol, $p \leq .01$. The height of the bars represents the mean value and SD of 9 animals.

Furthermore, there was also a significant difference between the 2 paracetamol-exposed groups, where the animals exposed neonatally to the repeated doses spent a shorter time on the hot plate than the animals exposed to the single dose ($p \leq .01$), indicating a dose response–related decrease in the adult analgesic/antinociceptive effect of paracetamol.

Elevated Plus Maze Behavior in 3-Month-Old Male Mice

Results from the elevated plus maze variables, percentage of total time spent in the open arms, and percentage of entries into the open arms, after pretreatment with saline or 30 mg paracetamol/kg bw in 3-month-old NMRI mice, after neonatal SC exposure to 30 mg (single) or 30 + 30 mg (repeated) paracetamol/kg bw, are shown in Figure 4.

There were significant treatment effects regarding the variables percent of total time spent in the open arms and percent of entries into the open arms, before and after the adult challenge to paracetamol (2-way ANOVA, $F_{5,48} = 13.98$; $F_{5,48} = 14.99$) ($p < .0001$), and pairwise testing showed significant differences between controls and paracetamol-treated mice.

Pretreatment with saline, 30 min before testing. There were no significant differences in animals neonatally exposed to paracetamol compared with control animals, indicating that neonatal paracetamol exposure does not affect anxiety-like behavior.

Pretreatment with 30 mg paracetamol/kg bw, 30 min before testing. In control animals, the percent of total time spent in the open arms and percent of entries into the open arms increased significantly ($p \leq .01$) compared with the animals pretreated with saline, showing the anxiolytic effect of paracetamol. In contrast, pairwise testing between paracetamol-exposed and control groups showed a significant change in both test variables for the groups exposed neonatally to the repeated dose of paracetamol. These animals spent a significantly shorter time and made fewer entries ($p \leq .01$) into the open arms than the control animals. Furthermore, after the pretreatment with 30 paracetamol/kg bw, the animals exposed neonatally to the single dose of paracetamol also spent significantly less time in the open arms ($p \leq .05$) compared with the animals neonatally exposed to the vehicle. These results indicate that neonatal exposure to paracetamol changes the adult anxiolytic response to paracetamol.

DISCUSSION

This study shows that acute neonatal exposure to paracetamol (2×30 mg) results in altered locomotor activity on exposure to a novel home cage arena and a failure to acquire spatial learning in adulthood, without affecting thermal nociceptive responding or anxiety-related behavior. However, mice neonatally exposed to paracetamol (2×30 mg) fail to exhibit paracetamol-induced antinociceptive and anxiogenic-like behavior in adulthood. Behavioral alterations in adulthood may, in part, be

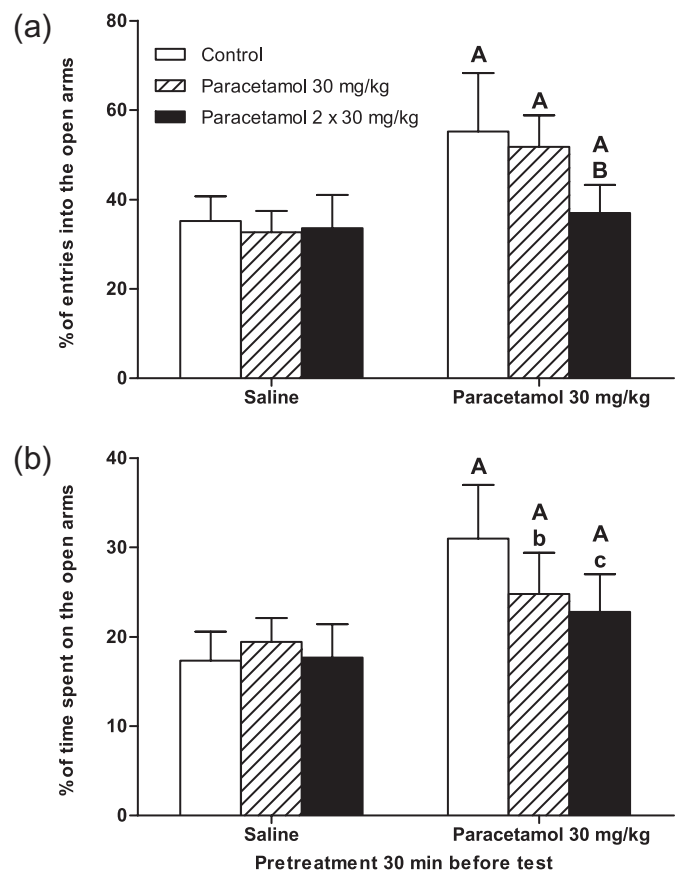


FIG. 4. Elevated plus maze performance 30 min after a SC injection of 0.9% saline vehicle or 30 mg paracetamol/kg bw in 3-month-old NMRI male mice exposed to 0.9% saline vehicle, 30 mg paracetamol/kg bw, or 30 + 30 mg paracetamol/kg bw (4 h apart) on postnatal day 10. The number of entries into open arms and time spent on open arms, expressed in percent of total, were measured for 5 min. The data were subjected to an ANOVA with split-plot design, and there were significant treatment group effects ($F_{5,48} = 13.83$; $F_{5,48} = 14.42$) for the variable entries into open arms (a) and time spent in open arms (b), respectively. Pairwise testing between paracetamol-exposed and control animals was performed using Tukey's HSD tests. The statistical differences are indicated as (A) significantly different vs. its own treatment group pretreated with saline, $p \leq .01$; (B) significantly different vs. controls pretreated with paracetamol, $p \leq .01$; (b) significantly different vs. controls pretreated with paracetamol, $p \leq .05$; (C) significantly different vs. single-dose paracetamol, pretreated with paracetamol, $p \leq .01$; (c) significantly different vs. single-dose paracetamol, pretreated with paracetamol, $p \leq .05$. The height of the bars represents the mean value and SD of 9 animals.

due to paracetamol-induced changes in BDNF levels in key brain regions at a critical time during development. BDNF is involved in several important processes of brain development, and the levels can be affected by compounds acting on the cannabinoid system, like THC (D'Souza *et al.*, 2009), and paracetamol has been shown to have similar mechanisms (Ottani *et al.*, 2006). The changes seen here may therefore be critical for the adult functional effects. Differential effects in different parts of the brain can have several explanations, eg, different amounts of the compound reaching the specific brain regions and/or retention of the compound, due to the blood flow to that

specific part. Furthermore, different brain regions can be in different stages of development and have differential metabolic pathways, affecting the mode of action of the compound. In this specific case, we do not yet know the reason for the different effects on BDNF levels in different brain regions.

Analysis of spontaneous behavior to a novel home environment (Fredriksson, 1994) showed a normal habituation in control animals and in the mice neonatally exposed to the single dose of paracetamol, whereas repeated doses of paracetamol reduced habituation and caused hyperactivity in the adult mice. This type of behavioral disturbance and effect on habituation is considered to be a disturbance in cognitive function (Daenen *et al.*, 2001; Groves and Thompson, 1970; Wright *et al.*, 2004). Earlier studies have also shown an altered spontaneous behavior in adult animals, neonatally exposed to the anesthetic agents ketamine and propofol (Ponten *et al.*, 2011, Viberg *et al.*, 2008a). Also here changes in neonatal concentrations of BDNF was observed (Ponten *et al.*, 2011), and these changes were also different in different brain regions just like in this study. Effects on cognitive function was further supported in the radial arm maze test, where these mice displayed a reduced spatial working memory. Several recent studies indicate that exposure to the mechanistically related compound THC can affect the spatial working memory in rodents (Fadda *et al.*, 2004; O'Shea and Mallet, 2005; Silva de Melo *et al.*, 2005; Varvel *et al.*, 2001). In addition, the endocannabinoid anandamide is known to induce deficits in short-term memory (Iversen, 2003; Juszczak and Swiergiel, 2009). Furthermore, epidemiological studies have shown that prenatal exposure to THC can induce increased hyperactivity, impulsivity, and inattention symptoms in children (Goldschmidt, Day and Richardson, 2000).

In this study, we could also see that the analgesic effect of paracetamol, in the adult animal, was altered after neonatal exposure to paracetamol. When the adult animals were treated with a dose of paracetamol, the neonatally saline-injected animals stayed a significantly longer time on the hot plate than saline-treated controls. In contrast, this analgesic/antinociceptive effect was not seen in animals neonatally exposed to the single or repeated doses of paracetamol, but the animals exposed neonatally to the repeated dose of paracetamol stayed the shortest time on the hot plate, showing a clear dose-dependent alteration in the response to adult paracetamol treatment, when neonatally exposed to paracetamol.

Paracetamol is metabolized to the active metabolite AM404 (Hogestatt *et al.*, 2005), which is considered to have anxiolytic effects (Bortolato *et al.*, 2006; Patel and Hillard, 2006). In this study, neonatal exposure to paracetamol did not directly affect the adult anxiety-like behavior in the elevated plus maze, as no difference was seen between controls and the paracetamol-treated mice. However, the adult reaction to paracetamol was different and control animals treated as adults with paracetamol showed the antianxiolytic-like effect of paracetamol. The antianxiolytic effect of paracetamol was eliminated when animals neonatally exposed to the repeated doses of paracetamol

were treated with paracetamol as adults. This altered antianxiolytic effect was partly seen after the single neonatal dose of paracetamol, indicating a dose-dependent induction of this altered antianxiolytic response to paracetamol.

Altered adult response to pharmaceutical agents or chemicals, after neonatal exposure, has recently been observed by our research group, reporting that neonatal exposure to the anesthetic agent propofol abolishes the adult reaction to a diazepam injection. Furthermore, animals were injected with diazepam before the elevated plus maze test, and a clear antianxiolytic effect of the diazepam could be seen in the control animals, which could not be seen in the animals neonatally exposed to propofol. This altered response to the effects of diazepam was dependent on the neonatal propofol dosage (Ponten *et al.*, 2011). Altered susceptibility to agents in adult age has also been reported after neonatal exposure to nicotine, effects related to impaired development of the cholinergic nicotinic receptors. It was shown that neonatal exposure to nicotine inhibited/reduced the development of certain subtypes of nicotinic receptors (low-affinity nicotinic binding sites, corresponding to the $\alpha 7$ -subunit), and these animals also showed an opposite reaction in spontaneous behavior activity after an adult injection of nicotine (Ankarberg *et al.*, 2001; Eriksson *et al.*, 2000). Furthermore, animals neonatally exposed to low doses of nicotine were also more susceptible to adult exposure to an acetylcholine esterase inhibitor (the organophosphate paraoxon). In these animals, a persistent cognitive defect was also seen after adult exposure to paraoxon (Ankarberg *et al.*, 2004).

In this study, a possible explanation for the different effects on cognitive function and altered response to adult paracetamol exposure may be a changed pattern in the development of CB1 receptors during brain development because the timing of paracetamol coincides with the BGS, when the endocannabinoid system also undergoes several vital developmental changes (Fride, 2004, 2005; Vitalis *et al.*, 2008; Watson *et al.*, 2008), which is also true for BDNF that has a distinct ontogeny pattern during the BGS and is part of several important developmental processes, including interactions with the endocannabinoid system (Cui, 2006; Huang and Reichardt, 2001).

Measurements of paracetamol concentration in the brains of exposed mice showed that paracetamol is taken up and transported to the neonatal brain. This has previously been seen in rodents, along with the fact that the brain concentration is significantly higher than the concentration in serum (Ara and Ahmad, 1980; Kumpulainen *et al.*, 2007). The metabolism and/or elimination of paracetamol from the brain is rapid, shown by the significant decrease of paracetamol from 1 to 2 h after exposure, when 96% of the parent compound had disappeared. In studies of human newborns, it has been observed that the half-life of paracetamol in serum is relatively long (hours) (Allegaert *et al.*, 2004; van Lingen *et al.*, 1999), but no data are available concerning the newborn human brain. Four hours after the first paracetamol dose, the concentration is close to zero, but still measurable. Interestingly, the second dose of paracetamol does

not increase the brain concentration of paracetamol compared with the first dose. This indicates that the difference in neurotoxic effect seen between single and repeated exposure to paracetamol is dependent on the time that the compound is present in the brain. Unfortunately, we did not have the resources to make quantitative measurements of the bioactive metabolite AM404 or the endogenous cannabinoid anandamide, both believed to be involved in the analgesic pathway of paracetamol (Hogestatt *et al.*, 2005). Concerning the doses used in this study, 2 major remarks can be made. Firstly, the 2 different doses in this study are individually slightly higher than what is recommended for newborns and toddlers (Läkemedelsverket, 2009a,b). On the other hand, not even the repeated exposure group exceeded the highest recommended daily dosing, and when comparing the dosage in this study with the repeated dosage over several days, which is commonly used (Anderson and Allegaert, 2009), the doses administered in this study are therefore highly relevant. Secondly, with detected levels of paracetamol in the environment and medical use, concerns must be raised because there are similarities in the effects of paracetamol on BDNF and cognitive function with the effects earlier seen for several environmental toxicants, as an interactive effect between the compounds cannot be excluded.

In conclusion, this study shows that one of the most commonly used pharmaceutical drugs, paracetamol, can act as a developmental neurotoxic agent, affecting cognitive function and altering adult responsiveness to paracetamol and thereby its antianxiolytic and analgesic effect. These results are supported by several recent studies with other compounds, both pharmaceuticals and environmental pollutants, with similar mechanistic action and must therefore be taken seriously. Whether our findings are relevant for the possible effects in human remains to be studied, but our findings at least call for attention concerning the use of paracetamol in young children.

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