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Research Article

A Cannabinoid Receptor Type 1 (CB1R) Agonist Enhances the Developmental Neurotoxicity of Acetaminophen (Paracetamol)

Gaëtan Philippot,[*,1] Stefan Hallgren,[*] Torsten Gordh,[†] Anders Fredriksson,[‡] Robert Fredriksson,[§] and Henrik Viberg[*]

[*]Department of Environmental Toxicology, Evolutionary Biology Centre; [†]Department of Surgical Sciences and [‡]Department of Neuroscience, Uppsala University, 752 36 Uppsala, Sweden; and [§]Department of Molecular Neuropharmacology, Uppsala Biomedical Centre, Husargatan 3, 751 24 UPPSALA, Sweden

[1]To whom correspondence should be addressed at Department of Environmental Toxicology, Evolutionary Biology Centre, Uppsala University, Norbyv. 18A, 752 36 Uppsala, Sweden. Fax: 018-471 6425; E-mail: gaetan.philippot@ebc.uu.se.

ABSTRACT

Acetaminophen (AAP; also known as paracetamol) is the most used and only recommended analgesic and antipyretic among pregnant women and young children. However, recent findings in both humans and rodents suggest a link between developmental exposure to AAP and adverse neurobehavioral effects later in life. We hypothesized that the cannabinoid receptor type 1 (CB1R) may be involved in the developmental neurotoxicity of AAP, owing to its interaction with the endocannabinoid system. Here we test if CB1R agonist WIN 55 212-2 (WIN) and AAP can interact when exposure occurs during a neurodevelopmental stage known for increased growth rate and for its vulnerability to AAP exposure. We exposed male NMRI mice on postnatal day 10 to different combinations of AAP and WIN. Adult mice, neonatally co-exposed to AAP and WIN, displayed a significant lack of habituation in the spontaneous behavior test, when compared with controls and single agent exposed mice. These adult adverse effects may at least in part be explained by a reduction of transcript levels of hippocampal synaptophysin (*Syp*) and tropomyosin receptor kinase B (*Trkb*), and cerebral cortical fatty acid amide hydroxylase (*Faah*), 24 h after exposure. These findings are consistent with our hypothesis that AAP and WIN can interact when exposure occurs during early postnatal brain development in mice. Assuming our results are relevant for humans, they raise concerns on AAP safety because it is the only recommended analgesic and antipyretic during pregnancy and early life.

Key words: developmental toxicity; acetaminophen (paracetamol); CB1R; spontaneous behavior; habituation.

Acetaminophen (AAP; also known as paracetamol) is the most widely used analgesic and antipyretic worldwide and is in most countries available over-the-counter (OTC). In Northern and Western Europe, the estimated prevalence of using AAP during pregnancy is 51–61% and in Northern America 49% (Lupattelli *et al.*, 2014). It has also been estimated that most neonates and toddlers have been medicated with AAP (Hawkins and Golding, 1995; Walsh *et al.*, 2007). AAP reaches the fetal/neonatal brain after passing the placenta (Levy *et al.*, 1975) and the blood brain barrier (Kumpulainen *et al.*, 2007) and may therefore affect brain development. Maternal intake of AAP has over the past years been linked to attention-deficit/hyperactivity disorder (ADHD) symptoms, hyperkinetic disorder, and other adverse behavioral outcomes later in life (Avella-Garcia *et al.*, 2016; Brandlistuen *et al.*, 2013; Liew *et al.*, 2014; Stergiakouli *et al.*, 2016; Thompson *et al.*, 2014). Experimental investigations in rodents have also shown that developmental exposure to AAP is linked to reduced spatial learning, changed spontaneous behavior, decreased habituation to a novel home cage, reduced anxiolytic, and analgesic response to AAP and effects on various neurotransmitters

(e.g., serotonergic, noradrenergic, dopaminergic, and neurotrophic) in adults (Blecharz-Klin *et al.* 2015, 2016, 2017; Philippot *et al.*, 2017; Viberg *et al.*, 2014). The safety using AAP is currently being discussed, and the European Medicines Agency's (EMA) Pharmacovigilance Risk Assessment Committee (PRAC) still agrees with the PRAC Rapporteur's view from 2015 (de Fays *et al.*, 2015) that a causal relationship between AAP exposure during pregnancy and neurodevelopmental outcomes could not be established (EMA, 2017).

The mammalian brain undergoes a rapid growth period, called the brain growth spurt (BGS), which is recognized to be highly vulnerable to toxic insults (Davison and Dobbing, 1968; Eriksson, 1997). In humans, the BGS begins during the third trimester of pregnancy, reaches a peak around birth, and continues up to 2 years of age (Davison and Dobbing, 1968; Dobbing and Sands, 1979). Since AAP exposure is common throughout pregnancy and in early life, AAP exposure encompasses this period of increased vulnerability. In rodents, the BGS is completely postnatal—in mice, it starts a few days after birth and continues for the next 3–4 weeks postnatally, peaking around postnatal day (PND) 10 (Davison and Dobbing, 1968). Comparing this period between humans and rodents the timescales and timing relative to birth are different; however, the chronology of these developmental events are remarkably similar (Semple *et al.*, 2013).

A fraction of AAP metabolizes to *N*-arachidonoyl 4-aminophenol (AM404), an activator of the endocannabinoid system through both direct and indirect activation of cannabinoid receptor type 1 (CB1R) (Bertolini *et al.*, 2006; Mallet *et al.*, 2008). The endocannabinoid system is already present during early brain development and is important in progenitor cell proliferation (Aguado *et al.*, 2005), neuronal migration (Berghuis *et al.*, 2005), and correct axonal and neurite outgrowth (Berghuis *et al.*, 2007). Developmental interference with this system has been shown to cause long-lasting effects on locomotor activity and cognitive function, emotional disturbances and increased sensitivity to other drugs (Campolongo *et al.*, 2011).

AAP intake during pregnancy or neonatal life affects the endocannabinoid system; therefore, we hypothesized that the effects seen after developmental exposure to AAP may be due to AAP-induced activation of the CB1R. Because the chronology of key neurodevelopmental events is similar between human and mice, the neonatal mouse is an excellent model for studying potential neurodevelopmental effects of AAP in humans and for evaluating our hypothesis that developmental exposure to AAP in combination with the CB1R agonist Win 55 212-2 (WIN) may have more severe effects on brain development than AAP alone. To clarify the effects of AAP on neurodevelopment, studies to verify the various proposed hypotheses regarding the underlying mechanism(s) are warranted. Here we aim to elucidate the potential role of the CB1R in the AAP-induced developmental neurotoxicity.

MATERIALS AND METHODS

Experiments were conducted in accordance with the Directive of European Parliament and of the Council of 22 September 2010 (2010/63/EU), after approval from the local ethical committee (Uppsala University and Agricultural Research Council).

Animals and Chemicals

Pregnant Naval Medical Research Institute (NMRI) mice (from Charles River Laboratory) were purchased from Scanbur (Sollentuna, Sweden) and maintained individually in macrolon cages in a temperature-controlled (22°C) and light-controlled (12 h light/dark cycle) room with a relative humidity in the range 45–65%. All experimental animals had free access to standardized pellet food (Lactamin, Stockholm, Sweden) and tap water. The pregnant NMRI mice were checked for birth once daily (18.00) and day of birth was designated PND 0. Litter sizes were adjusted within 48 h after birth to 10–12 pups (containing both sexes) by euthanizing excess pups.

Acetaminophen (Paracetamol Fresenius Kabi, 10 mg ml^{-1}; Fresenius Kabi AB, Sweden; CAS no. 103-90-2) was purchased from Apoteksbolaget, Uppsala, Sweden, and a stock solution containing 6 mg AAP ml^{-1} saline (0.9% sodium chloride in water) was made. The CB1R agonist WIN 55 212-2 ((R)-(+)-[2, 3-Dihydro-5-methyl-3[(4-morpholinyl)methyl]pyrrolo[1, 2, 3-de]-1, 4-benzoxazinyl]-(1-naphthalenyl) methanone mesylate salt; CAS no. 131543-23-2) was purchased from Sigma Aldrich and a stock solution was made containing 0.2 mg WIN ml^{-1} saline.

Treatment, Sampling, and Exposure Groups

In this study two separate experiments were conducted: one experiment for the spontaneous behavior recordings that contained 6 exposure groups and another experiment for the gene expression and protein level analyses containing a total of 4 exposure groups. All exposures were made by subcutaneous injection in the neck on PND 10 in a volume of 5 ml kg^{-1}. Control mice from both experiments were injected with saline vehicle (0.9% NaCl). In this study only male mice were used.

Exposure groups in the spontaneous behavior experiment. For the recordings of spontaneous behavior, a total of 6 exposure groups were used. Male mice were exposed on PND 10 to either saline vehicle, AAP (30 mg kg^{-1}, single dose), AAP (30 + 30 mg AAP kg^{-1}, 4 h apart), WIN (1 mg WIN kg^{-1}, single dose), a combination of AAP/WIN (30 mg AAP/1 mg WIN kg^{-1}, single dose) or a combination of AAP/WIN (30 mg AAP + 30 mg AAP/1 mg WIN kg^{-1}, 4 h apart) where WIN was administered with AAP only at the second administration (Table 1). At the age of around 4 weeks, after weaning, male offspring were separated from their female siblings, which were euthanized, and were kept with their male siblings from each treatment group. Litters contained 4–7 animals. When the animals reached 2 months of age they were subjected to spontaneous behavior testing.

Exposure groups for biochemical analyses. For the gene expression and protein level analyses 4 exposure groups were used. Male pups, randomly selected from different litters, were administered with either AAP (30 + 30 mg AAP kg^{-1}, 4 h apart), WIN (1 + 1 mg WIN kg^{-1}, 4 h apart) or a combination of AAP and WIN (30 mg AAP/1 mg WIN + 30 mg AAP/1 mg WIN kg^{-1}, 4 h apart) on PND 10 (Table 1). Pups were killed by decapitation 24 h after exposure and brains were dissected on an ice-cold glass plate where frontal cortex, parietal cortex and hippocampus were collected and individually snap frozen in liquid nitrogen and then stored at −80°C until assayed.

Behavioral Tests

Spontaneous behavior in a novel home environment measures the integration of sensory input into motor output and tests the animals' ability to integrate new information with information previously attained, and hence the mice ability to habituate. Habituation is a nonassociative form of learning and is considered cognitive function (Daenen *et al.*, 2001; Groves and Thompson, 1970; Wright *et al.*, 2004). Habituation capability is here defined as a decrease in registered counts for the

Table 1. Exposure Groups, Dosage, and Abbreviations for Exposure Groups in *Experiment 1* (Spontaneous Behavior Recordings) and in *Experiment 2* (Biochemical Analyses)

Exposure (Dosage)	Abbreviation
Experiment 1 (spontaneous behavior recordings)	
Control	C
AAP (30 mg kg^{-1}, single dose)	AAP
AAP (30 + 30 mg AAP kg^{-1}, 4 h apart)	AAP + AAP
WIN (1 mg WIN kg^{-1}, single dose)	WIN
AAP/WIN (30 mg AAP/1 mg WIN kg^{-1}, single dose)	AAP/WIN
AAP/WIN (30 mg AAP + 30 mg AAP/1 mg WIN kg^{-1}, 4 apart, where WIN was administered with AAP only the second administration)	AAP + AAP/WIN
Experiment 2 (biochemical analyses)	
Control	C
AAP (30 + 30 mg AAP kg^{-1}, 4 h apart)	AAP + AAP
WIN (1 + 1 mg WIN kg^{-1}, 4 h apart)	WIN + WIN
AAP/WIN (30 mg AAP/1 mg WIN + 30 mg AAP/1 mg WIN kg^{-1}, 4 h apart)	AAP/WIN + AAP/WIN

measured variables: locomotion, rearing, and total activity, over time.

Locomotion: counting took place when the mouse move horizontally through the low-level grid of infra-red beams.

Rearing: registered when the mouse moved vertically, thereby interrupting a single high-level beam, at a rate of counts per second hence the number of counts obtained was proportional to the time spent rearing.

Total activity: all types of vibrations within the test cage, ie, those caused by animal movements (e.g. locomotion and rearing), shaking (tremors), and grooming were registered by a pickup (needle mounted on a lever with a counterweight), connected to the brim of the test cage.

In this study, we assess habituation capability to a novel home cage in mice. We defined habituation as a decrease in registered counts over a 60-min period for the measured variables: locomotion, rearing, and total activity. Nine mice (2 months of age) from each exposure group were tested for spontaneous behavior (Rat-O-Matic, ADEA Elektronik AB, Uppsala, Sweden) as described previously (Fredriksson, 1994).

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Relative expression levels of mRNA, from 8 animals per exposure group (24 h after exposure), were measured as described previously in Hallgren and Viberg (2016), using quantitative real-time PCR. RNA extraction and reverse transcription to cDNA were done with Aurum Total RNA extraction columns (Bio-Rad, Stockholm, Sweden) and iSCRIPT (BioRad, Stockholm, Sweden), respectively. Gene transcription of *brain-derived neurotrophic factor* (*Bdnf*), *tropomyosin receptor kinase B* (*Trkb*; encoded by *neurotrophic receptor tyrosine kinase 2* [*Ntrk2*]), *synaptophysin* (*Syp*), *postsynaptic density protein 95* (*Psd-95*; encoded by *large homolog 4* [*Drosophila*] [*Dlg4*]), *cannabinoid receptor type 1* (*Cb1r*; encoded by *cannabinoid receptor 1* [*Cnr1*]) and *fatty acid amide hydroxylase* (*Faah*) was normalized against transcription of housekeeping genes *Pkg-1* and *Gapdh* for each sample. The gene-specific PCR primers are listed in Table 2. The efficiency of each primer pair was determined from a standard curve with pooled cDNA. Annealing temperature was 61°C. Gene transcription analyses of the genes of interest were analyzed with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Each sample was run as a triplicate. To ensure the amplification of a single product, a melt curve for each qPCR reaction was performed (melt curve ranging from 55°C to 95°C).

Slot Blot Analysis

Target proteins, from 4 to 6 animals per exposure group (24 h after exposure) were assessed with the semi-quantitative Slot Blot technique as described earlier (Lee *et al.*, 2015; Viberg *et al.*, 2008a). The specificity of antibodies of synaptophysin (Calbiochem, 573822) and PSD-95 (Millipore, MABN68) had previously been evaluated by Viberg *et al.* with Western blot procedure, which showed that the antibodies were specific for the protein intended.

Data Analysis and Statistics

Normality of residuals and homogeneity of variances were tested for behavior, gene transcript, protein, and body weight data using Kolmogorov–Smirnov test and Bartlett's test, respectively. We used standard parametric statistics whenever the assumptions of such models were fulfilled (only adult weight data and *Syp* gene transcript levels in the hippocampus had to be normalized by log-transformation). Data from the spontaneous behavior observations (treatment, time, and treatment × time, between subjects, within subjects, and interaction factors, respectively) were evaluated with an ANOVA, using a split-plot design with pairwise testing using Tukey's HSD test. Differences in body weights, mean relative transcript levels and relative protein levels were evaluated with a one-way ANOVA followed by Tukey's HSD test. Graphical illustrations, normality testing, homogeneity of variances and impact of exposure on body weights, gene transcripts, and protein levels were made in GraphPad Prism version 5.01 (GraphPad software Inc., California) and analyses of spontaneous behavior were made in SAS 9.1 software.

RESULTS

There were no signs of toxic symptoms in any of the mice during the experiments. Body weights were measured on PND 10 and at sacrifice in the mice killed 24 h after exposures. There were no significant effects of exposures on body weights in these neonates ($p > .05$). In the mice raised until 2 months of age, body weights were measured on PND 10 and at sacrifice. In these mice there were no significant effects of exposures on body weight gain at sacrifice ($p > 0.05$).

Spontaneous Behavior

The results from the spontaneous behavior variables locomotion, rearing, and total activity in 2-month-old male mice after

Table 2. Gene-Specific Primer Sequences Used for qPCR.

Target Name	Accession No.	Forward Primer (5′–3′)	Reverse Primer (5′–3′)
Gapdh	NM_008084.3	GGGCTCCCTAGGCCCCTCCTCTTAT	CACCCCAGCAAGGACACTGAGCAAG
Pgk-1	NM_008828.3	CTCCGCTTTCATGTAGAGGAAG	GACATCTCCTAGTTTGGACAGTG
Bdnf	NM_001285416.1	GAAGAGCTGCTGGATGAGGAC	TTCAGTTGGCCTTTGGATACC
Trkb	NM_001025074.2	TGGACCACGCCAACTGACATT	GAATGTCTCGCCAACTTGAG
Syp	NM_009305.2	ACAGCAGTGTTCGCTTTCA	GGGTCCCTCAGTTCCTTG
Psd-95	NM_007864.3	TCTGTGCGAGAGGTAGCAGA	AAGCACTCCGTGAACTCCTG
Cb1r	NM_007726.4	TGAAGTCGATCTTAGACGGCC	GTGGTGATGGTACGGAAGGTA
Faah	NM_010173.4	GCCACACGCTGGTCCCCTTC	AGAGCAGCCACCATCACTGAACAG

exposure to AAP, WIN, or different combinations of both (all exposure groups are shown in Table 1) are shown in Figure 1. There was significant treatment × time interactions ($F_{10.96}$ = 90.81, $p < .0001$; $F_{10.96} = 110.72$, $p < .0001$; $F_{10.96} = 115.34$, $p < .0001$) for locomotion, rearing and total activity variables, respectively.

Control mice displayed normal habituation, ie, they displayed a distinct decrease in activity over the 60 min of spontaneous behavior recording for all three variables, in accordance with previous studies (Fredriksson, 1994; Philippot *et al.*, 2016, 2017; Viberg *et al.*, 2014).

Pairwise testing, using Tukey's *post-hoc* test, showed that between control animals and mice neonatally exposed to a single dose of AAP there was no significant difference in any of the three 20 min periods in any of the three variables. However, mice exposed to 2 doses of AAP, 4 h apart (AAP + AAP), displayed activity levels that were significantly different from the controls. More specifically, locomotor, rearing and total activity levels these mice were reduced during the first 20 min of testing, and increased during the second and third 20 min periods of testing compared with controls. Mice that had been exposed to a single dose of WIN displayed no significant alterations in activity compared to control animals for any of the three variables for any of the 3 time periods. Activity levels of mice neonatally exposed to a combination of AAP and WIN (AAP/WIN) as one single dose were significantly different from the activity levels of control animals, those exposed to a single dose of AAP and those exposed to WIN. These animals had a decreased locomotor, rearing and total activity compared to controls, AAP-exposed and WIN-exposed animals during the first 20 min period of testing. During the second and third 20 min of testing the same animals displayed increased locomotor, rearing and total activity compared with control animals, AAP-exposed and WIN-exposed animals. Mice that neonatally received repeated AAP doses together with a single WIN dose (AAP + AAP/WIN) displayed altered spontaneous behavior compared with all other exposure groups, including AAP/WIN-exposed animals. The activity levels were significantly decreased compared with control animals (among other groups) during the first 20 min of testing and increased compared with all other exposure groups during the second and third 20 min of testing for locomotion, rearing and total activity. All significances are shown in Figure 1.

Effects on Gene Transcript Levels

Twenty-four hour after subcutaneous injection of either AAP (AAP + AAP), WIN (WIN + WIN) or AAP, and WIN (AAP/WIN + AAP/WIN), gene transcript levels of *Bdnf*, *Trkb*, *Cbr1*, *Faah*, *Syp*, and *Psd-95* were measured in the frontal cortex, parietal cortex, and hippocampus (Figure 2).

In the hippocampus, there was a statistically significant effect of exposure on *Trkb* transcript levels as determined by one-way ANOVA ($F_{3.28} = 5.26$, $p = .0053$). Tukey's *post-hoc* test revealed a significant reduction of transcript levels of *Trkb* following neonatal exposure to both AAP + AAP ($p < .05$) and WIN + WIN ($p < .05$) alone and to AAP/WIN + AAP/WIN ($p < .01$) compared with controls. Hippocampal transcript levels of *Syp* was also affected by exposure ($F_{3.28} = 3.83$, $p = .022$). Here, a decrease of *Syp* transcript levels were observed in mice neonatally exposed to WIN + WIN ($p < .05$) and AAP/WIN + AAP/WIN ($p < .05$). In the frontal cortex, one-way ANOVA did not reveal any significant effect of exposure on gene transcript levels ($F_{3.28} = 2.66$, $p = .068$); however, Tukey's *post-hoc* test revealed a significant decrease in transcript levels of *Faah* following AAP/WIN + AAP/WIN ($p < .05$) but not to AAP + AAP or WIN + WIN.

Synaptic Density

Exposures to AAP (AAP + AAP), WIN (WIN + WIN) or AAP, and WIN (AAP/WIN + AAP/WIN) had no significant effect on SYP or PSD-95 in the neonatal frontal cortex, parietal cortex or in hippocampus 24 h after exposure ($p > .05$) (Figure 3).

DISCUSSION

To investigate the influence of CB1R activation on AAP-induced neurotoxicity at the peak of the growth rate of the developing mouse brain, we exposed 10-day-old NMRI mice to either AAP, the CB1R agonist WIN or different combinations of both substances. This study shows that neonatal co-exposure to WIN and clinically relevant doses of AAP interact to alter spontaneous behavior and reduce habituation capability in the adult mouse, which may, at least in part, be explained by reduced neonatal transcript levels of *Syp*, *Faah*, and *Trkb*.

Effects on the developing nervous system, in contrast to effects on the mature systems where regulatory mechanisms generally are more calibrated, are more likely to be permanent (Davison and Dobbing, 1968; Rice and Barone, 2000). During the BGS many new motor and sensory abilities are acquired (Bolles and Woods, 1964). The long-lasting effect of xenobiotic interference during the BGS has been shown in many previous studies where both commonly used anesthetics and analgesics, such as AAP (Philippot *et al.*, 2017; Viberg *et al.*, 2014), Δ^9-tetrahydrocannabinol (THC) (Philippot *et al.*, 2016), ketamine (Viberg *et al.*, 2008b), and propofol (Pontén *et al.*, 2011) have shown to be neurotoxic, manifested as changes in home-cage spontaneous behavior and reduced habituation capability. Habituation is a non-associative form of learning where sensory input transforms into motor output and alterations in habituation rates can be due to disturbances in sensory, motor or cognitive function (Daenen *et al.*, 2001; Groves and Thompson, 1970; Wright *et al.*, 2004). Measurement of spontaneous home-cage behavior recordings is a highly sensitive method, capable of detecting very small behavioral disturbances. Since developmental effects in general may appear subtle in contrast to, eg, acute effects of a

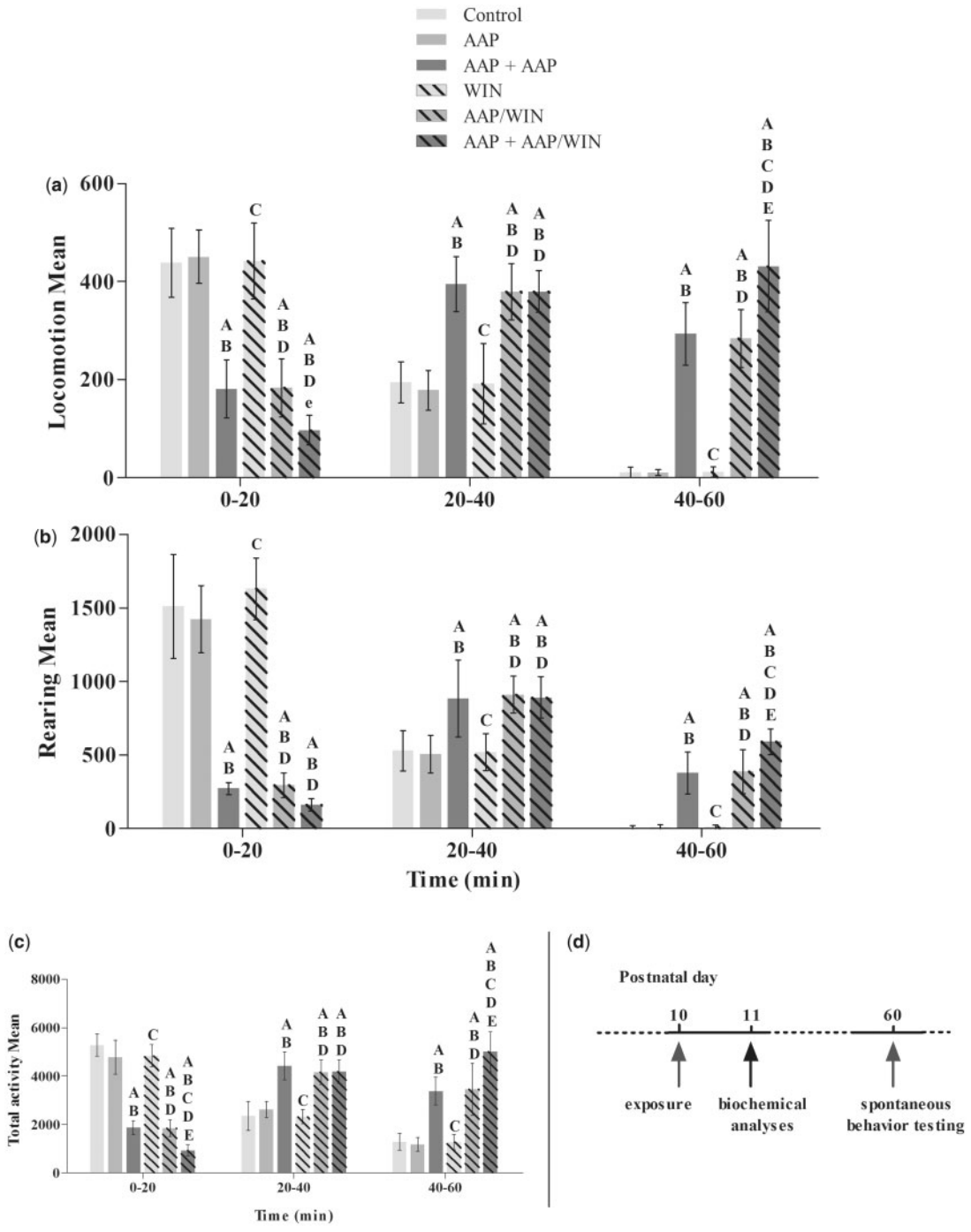

Figure 1. Spontaneous behavior in 2-month-old male NMRI mice following PND 10 exposure to either saline vehicle (control), AAP (30 mg AAP kg^{-1}, single dose), AAP + AAP (30 + 30 mg AAP kg^{-1}, 4 h apart), WIN (1 mg WIN kg^{-1}, single dose), AAP/WIN (30 mg AAP/1 mg WIN kg^{-1}, single dose), or AAP + AAP/WIN (30 mg AAP + 30/1 mg AAP/WIN kg^{-1}, 4 h apart). Split-plot ANOVA was used to assess the variables (a) locomotion, (b) rearing, and (c) total activity, over time. Exposures, biochemical analyses, and behavioral testing was done on PND 10, 11 and at 2 months of age, respectively, according to the (d) experimental outline. Pairwise testing between exposure groups was performed using Tukey's HSD test and statistical differences within each 20-min spell are indicated as: (A) significantly different versus control, $p \leq .01$; (B) significantly different versus AAP, $p \leq .01$; (C) significantly different versus AAP + AAP, $p \leq .01$; (D) significantly different vs. WIN, $p \leq .01$; (E) significantly different versus AAP/WIN, $p \leq .01$; (e) significantly different versus AAP/WIN, $p \leq .05$. The height of the bars represent the mean ± SD of 9 animals.

drug, analysis of home-cage behavior has become an excellent method when assessing long-term behavioral consequences following developmental exposures (Eriksson, 1997; Philippot *et al.*, 2016, 2017). One of the strength, associated with home-cage behavioral recordings, is the elimination of confounding effects induced by environmental novelty (Tecott and Nestler, 2004). The level of stress has an impact on exploratory behavior where high levels of stress suppress motor activity—however, stress up to moderate levels can be expected to be required to motivate exploration in the first place (Lever *et al.*, 2006). In the present study, we replicate previous findings that exposure of PND 10 mice to 30 + 30 mg AAP $kg^{-1,}$ 4 h apart, but not 30 mg

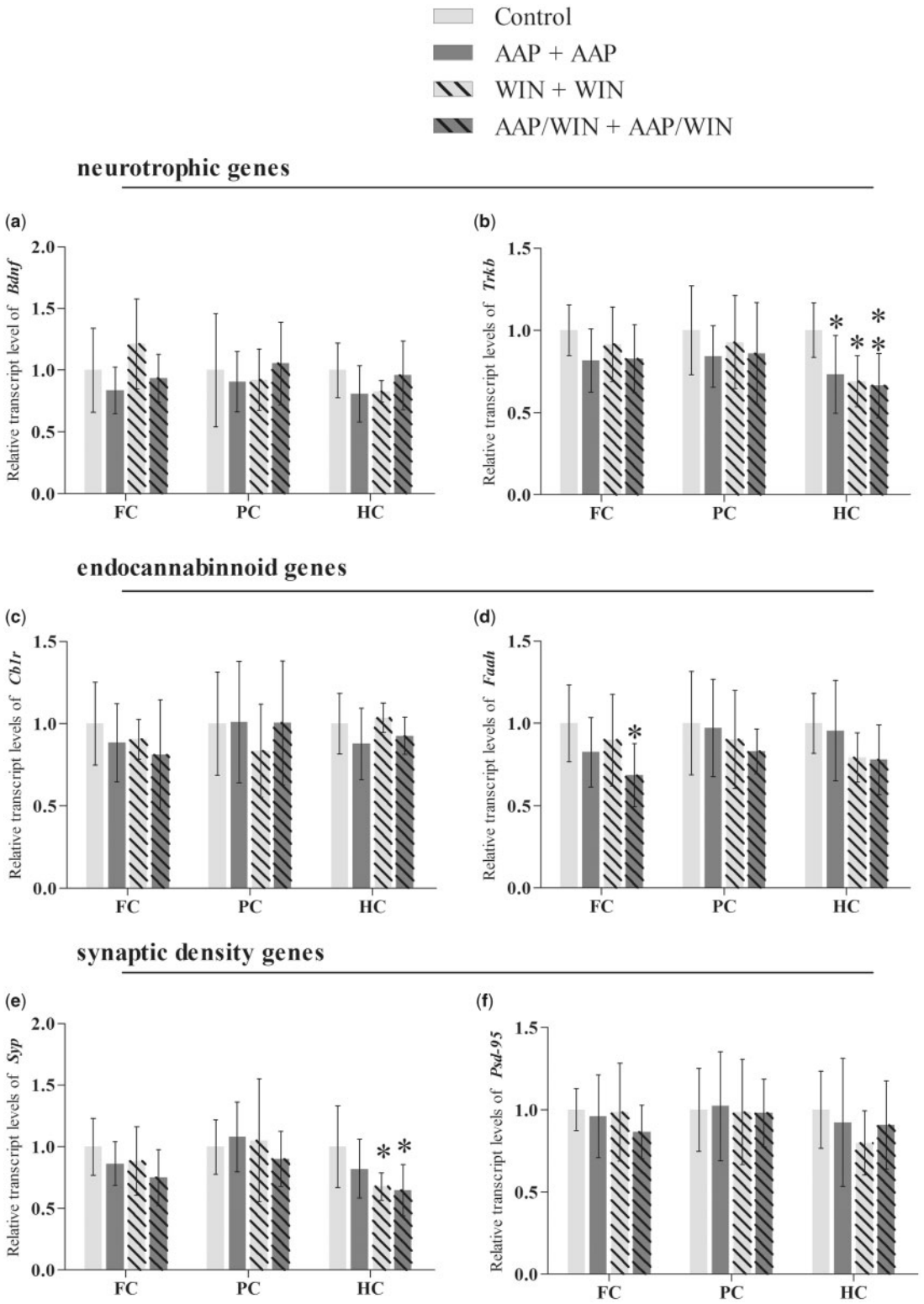

Figure 2. Relative gene transcription of (a) *Bdnf*, (b) *Trkb*, (c) *Cb1r*, (d) *Faah*, (e) *Syp*, and (f) *Psd-95* in male NMRI mice 24 h after exposure to either saline vehicle (control), AAP + AAP (30 + 30 mg AAP kg^{-1}, 4 h apart), WIN + WIN (1 + 1 mg WIN kg^{-1}, 4 h apart), or AAP/WIN + AAP/WIN (30 mg AAP/1 mg WIN + 30 mg AAP/1 mg WIN kg^{-1}, 4 h apart) on PND 10 in frontal cortex (FC), parietal cortex (PC) and hippocampus (HC). Data were subjected to one-way ANOVA followed by Tukey's HSD test. Statistical differences from post-hoc test are indicated as: (**) significantly different versus control, $p \leq .01$; (*) significantly different versus control, $p \leq .05$. Bars represent mean fold change ± SD of control animal transcription of 8 animals.

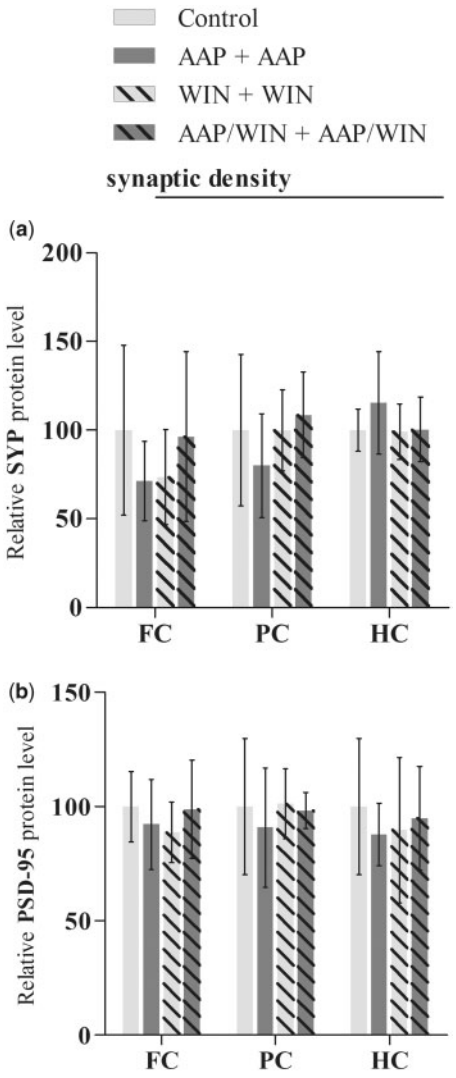

Figure 3. Relative protein levels of (a) SYP and (b) PSD-95 were measured in male mice 24 h after exposure to either saline vehicle (control), AAP + AAP (30 + 30 mg AAP kg^{-1}, 4 h apart), WIN + WIN (1 + 1 mg WIN kg^{-1}, 4 h apart), or AAP/WIN + AAP/WIN (30 mg AAP/1 mg WIN + 30 mg AAP/1 mg WIN kg^{-1}, 4 h apart) on PND 10 in frontal cortex (FC), parietal cortex (PC) and hippocampus (HC). Data were subjected to one-way ANOVA followed by Tukey's HSD test. Bars represent mean relative change ± SD of control animal protein levels of 6 animals (except for FC in panel "a" where n = 4 for the AAP/WIN + AAP/WIN exposure group).

AAP kg^{-1}, affected adult spontaneous behavior and habituation capability in a novel home cage (Viberg *et al.*, 2014). Neonatal exposure to a single dose of WIN (1 mg/kg) did not affect adult behavior, in turn, showing that this particular dose is not sufficient to cause developmental neurotoxicity. Interestingly, the present study demonstrates that neonatal co-exposure to AAP and WIN can interact to induce adverse effects on adult spontaneous behavior, as WIN exposure enhanced the adverse neurobehavioral effects of AAP. Disruption of habituation was noted for both locomotion and rearing, and measuring disruption of both these exploratory behaviors gives a more robust indication of the habituation disruption than analysis of only one variable.

AAP affects the endocannabinoid system through the metabolite AM404. This metabolite has both a direct and indirect effect on the CB1R (Bertolini *et al.*, 2006). The endocannabinoid system is an important signaling system during brain development and is important in both structural and functional aspects of neural development (Aguado *et al.*, 2005; Berghuis *et al.*, 2005, 2007). Here, we show that WIN and AAP exposure in a combination decreased cerebral cortical transcript levels of endocannabinoid-associated enzyme *Faah*, which is an enzyme essential in endocannabinoid signaling (Cravatt *et al.*, 2001); however, AAP and WIN alone had no effect of *Faah* transcript levels compared to the transcript levels in controls. Furthermore, the developmental neurotoxicity of CB1R agonism has previously been shown in our animal model as THC exposure on PND 10 gave adult behavioral alterations similar to those observed after AAP exposure (Philippot *et al.*, 2016). AAP also has effect on central cyclooxygenase (COX) activity (Flower and Vane, 1972) and a COX-mediated developmental neurotoxic mechanism is therefore possible. However, it seem less likely that the neurotoxic effects of AAP are due to an interaction with COX, because neonatal exposure to ibuprofen, with a known effect on central and peripheral COX activity, did not affect adult spontaneous behavior and habituation capability (Philippot *et al.*, 2016). Effects were also observed on gene transcript levels of *Trkb* following exposure to both AAP, WIN, and a combination of both. TRKB, and its associated activator BDNF, are highly important in neuronal function as they are required for both neuronal survival and differentiation during brain development and in synaptic and behavioral plasticity in the mature neurons, including hippocampal-dependent memory (Barnes and Thomas, 2008; Heldt *et al.*, 2007; Huang and Reichardt, 2003). Interestingly, the endocannabinoid system interact with BDNF-TRKB signaling, especially during brain development (Berghuis *et al.*, 2005; D'Souza *et al.*, 2009), and therefore (in line with our suggested hypothesis), the developmental effects of AAP may, at least in part, be caused by changes in BDNF and TRKB signaling. However, noteworthy on a hypothesis of such is the lack of effect on hippocampal *Bdnf* transcript levels following exposures. This may depend on either post-transcriptional changes of this protein or that the potential effects on *Bdnf* transcription occurs earlier that 24 h after exposure, and that the effect of this potential alteration manifests later as changes in transcript levels of *Trkb*. It is known that TRKB has an impact on learning and memory as TRKB conditional knockout animals show impaired spatial learning (Minichiello *et al.*, 1999), in turn consistent with our previous observation that neonatal mice exposed to AAP displayed reduced learning in radial arm maze in adulthood (Viberg *et al.*, 2014). Other potential neurotoxic mechanistic pathways following early exposure to AAP has been reviewed by Bauer *et al.* (2018).

Synaptic density was assessed in the frontal cortex, parietal cortex, and in the hippocampus, of the neonatal mouse (on both mRNA and protein levels) by measuring two synaptic markers: SYP (presynaptic) and PSD-95 (postsynaptic). Lower levels of *Syp* mRNA transcripts were observed in the hippocampus 24 h after exposure to WIN and the AAP/WIN combination. Being a reliable marker for of synaptic abundance, lower *Syp* transcript levels gives an indication of loss of synapses following neonatal exposure to CB1R agonist exposure. In contrast, on a protein level no significant alterations in synaptic density were observed in mice 24 h after exposure to AAP, WIN, or a combination of both. Because gene expression and protein levels both were evaluated 24 h after exposure, there may be a latency on observable effects between transcript levels of a gene and the gene product itself, ie, the protein. Nonetheless, more research is needed to evaluate potential AAP-induced changes in neuronal micromorphology and/or density.

We have previously shown that AAP metabolism and/or elimination from the neonatal mouse brain is rapid as 96% of the parent compound had disappeared from 1 to 2 h after exposure (Viberg *et al.*, 2014). The same study showed that AAP concentrations in the brain was close to zero 4 h after the first dose. Furthermore, the concentration-time profile of AAP metabolite AM404 in the brain has been shown to be similar to that of AAP (Muramatsu *et al.*, 2016). The dose used in this study ($30 + 30$ mg kg^{-1}) roughly corresponds to a human equivalent dose of 4.9 mg kg^{-1} (Reagan-Shaw *et al.*, 2008), thereby making the dose lower than doses used in humans (recommended dose of AAP in newborns and toddlers is 7.5–15 mg kg^{-1} up to four times a day [Anderson and Allegaert, 2009]). Moreover, the time scales of brain development are notably different between mice and humans, however, many of the key processes are remarkably similar (Semple *et al.*, 2013). We have previously shown that AAP exposure when the brain growth rate is rapidly increasing (PND 3) or is at its peak (PND 10), altered adult spontaneous behavior and habituation capability in both male and female mice (Philippot *et al.*, 2017). These time points during neonatal brain development in mice are comparable with the beginning of the third trimester and the time around birth, respectively, in humans (Semple *et al.*, 2013). In contrast, AAP exposure on PND 19, a time when the mouse brain largely has attained adult size, did not affect above-mentioned adult spontaneous behavior (Philippot *et al.*, 2017). This illustrates the high vulnerability of the neonatal mouse brain during brain development and the presence of a critical period when the brain shows increased vulnerability to AAP exposure. As previously mentioned, PND 10 AAP exposure affects adult behavior in both male and female mice; however, the impact of WIN on AAP developmental neurotoxicity in female mice remains to be investigated. There is also an increasing body of epidemiological evidence suggesting a link between developmental intake of AAP and ADHD-like symptoms (Avella-Garcia *et al.*, 2016; Liew *et al.*, 2014; Stergiakouli *et al.*, 2016; Thompson *et al.*, 2014) and other adverse neurobehavioral outcomes (Brandlistuen *et al.*, 2013) later in life. However, EMA's AAP recommendations remain unchanged as there are no safe alternatives to AAP for treating pain or fever during the last trimester of pregnancy and neonatal life. From a public health perspective, the continuous evaluation of potential harm of developmental exposure to AAP is of essence, especially since AAP is highly available to the public as an OTC drug. Also, early childhood neurodevelopmental problems are associated with mental health problems in childhood, adolescence and adulthood (Beyer *et al.*, 2012; Lavigne *et al.*, 1998), in turn, imposing additional economic burdens for healthcare.

In conclusion, we have reported the neurodevelopmental effects of neonatal exposure to AAP in NMRI mice. Consistent with our hypothesis of the involvement of the CB1R, there was a more severe AAP-induced neurotoxic effect in the adult mouse following neonatal co-exposure to the CB1R agonist WIN. This extends our knowledge on (1) the delicate role of the CB1R during the BGS and (2) its potential role in the developmental neurotoxicity of AAP. In the evaluation of developmental neurotoxicity of AAP, mechanistic insights are highly needed. Based on our previous studies (where developmental exposures to THC, but not ibuprofen, affected adult spontaneous behavior and habituation capability), together with results presented herein, we propose a CB1R-mediated initiation in the adverse outcome pathway(s) of AAP. CB1R activation may in turn have effect on important factors/receptors during brain development such as BDNF and TRKB, which may ultimately lead to changed spontaneous behavior and altered habituation when introduced to a new home cage detected later in life. The present findings provide insights that may aid and direct future AAP research which in turn might lead to changed AAP recommendations and healthcare practice.

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