International Journal of Neuropsychopharmacology (2011), **14**, 505–517. © CINP 2011
doi:10.1017/S1461145710001562

ARTICLE

THEMATIC SECTION
Consequences of Developmental Exposure to Drugs, Hormones or Altered Environment

CINP

Cannabinoid exposure in pubertal rats increases spontaneous ethanol consumption and NMDA receptor associated protein levels

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Abstract

Recent evidence suggests an involvement of the endocannabinoid system in the regulation of emotional behaviour and ethanol intake. Here we investigated age-specific acute behavioural effects of the cannabinoid receptor agonist WIN 55,212-2 (WIN) on anxiety-related behaviour and voluntary ethanol consumption in rats. Animals were treated with WIN (1.2 mg/kg)/vehicle at puberty onset on postnatal day (PD) 40, or at adulthood (PD 100). Animals were tested in the elevated plus-maze (EPM) and the light/dark emergence test (EMT) and for the initial response to alcohol in a free-choice ethanol consumption paradigm. Acute WIN treatment increased anxiety-related behaviours, and this effect was found to be partially more pronounced in pubertal than adult rats. Additionally, increased intake of higher ethanol solutions after cannabinoid treatment was only observed in pubertal rats. These drug-induced behavioural changes during puberty are paralleled by induction of the NR1 subunit of the NMDA receptor in the medial prefrontal cortex and the striatum. Moreover, pubertal but not adult WIN administration increased the levels of the scaffold protein Homer in these brain regions. Enhanced CB_1 receptor levels in the reinforcement system were also observed in pubertal compared to adult rats. These data support the notion that puberty is a highly vulnerable period for the aversive effects of cannabinoid exposure. In particular, augmented ethanol intake in pubertal cannabinoid-exposed animals might be related to some extent to increased emotional behaviour and in particular to enhanced NMDA and CB_1 receptor signalling.

Received 14 June 2010; Reviewed 22 July 2010; Revised 21 October 2010; Accepted 30 November 2010; First published online 7 January 2011

Key words: Anxiety, cannabinoid, ethanol, NMDA, puberty.

Introduction

Products of the hemp plant *Cannabis sativa*, are mainly used for their euphoric and relaxing effects. However, cannabis can also produce dysphoric reactions and states of anxiety (Hall & Solowij, 1998; Patton *et al*. 2002). This apparent paradox is also seen in several animal models where cannabinoids can induce both anxiogenic- and anxiolytic-like responses, depending upon dosage, behavioural paradigms, context (familiar/novel), species and the genetic strain (Valverde, 2005; Viveros *et al*. 2005*b*). However, only few studies have so far investigated if age or developmental status might also influence cannabinoid effects on anxiety-related behaviours.

It has been shown that age of onset of cannabis consumption is an important factor for acute and later consequences of cannabinoid exposure. Consumption/administration of cannabinoids during pubertal development induces lasting cognitive, motivational, social and morphological alterations in humans and rats which are not seen after an identical cannabis use/treatment in adulthood (Schneider, 2008). Although most of the literature about acute cannabinoid effects in laboratory animals comes from adult rodents, there

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are also some results obtained from juvenile and pubertal rats, showing both anxiogenic (McGregor *et al*. 1996; Romero *et al*. 2002; Schneider *et al*. 2008; Schramm-Sapyta *et al*. 2007) and anxiolytic (McGregor *et al*. 1996) effects of acute cannabinoid treatment. Hence, there is only limited data on cannabinoid effects in the context of emotional behaviour during development (Viveros *et al*. 2005*a*).

Recent evidence suggests that the endocannabinoid (ECB) system plays a key role in determining the reinforcing effects of alcohol, since both drugs activate the reward system (for review see Hungund & Basavarajappa, 2004; Mechoulam & Parker, 2003; Spanagel, 2009). The CB_1 receptor antagonist rimonabant (SR141716) has been shown to reduce ethanol intake in rats (Arnone *et al*. 1997; Colombo *et al*. 1998; Gallate & McGregor, 1999; Gallate *et al*. 2004) and cannabinoid agonists increase the motivation for ethanol consumption (Gallate & McGregor, 1999). Furthermore, in CB_1 knockout mice reduced ethanol self-administration (Naassila *et al*. 2004; Thanos *et al*. 2005) and ethanol-induced place preference (Houchi *et al*. 2005; Thanos *et al*. 2005) has been demonstrated. However, little is known about age-specific actions of cannabinoids on ethanol reward and ethanol intake. So far it has only been shown that an age-dependent decline in ethanol preference observed in aged mice compared to younger mice may be linked to a parallel decline in CB_1 receptor signalling (Wang *et al*. 2003). In general, puberty has been identified as a period associated with higher rates of substance use and abuse (Patton *et al*. 2004), and since we observed in our previous work stronger acute behavioural cannabinoid effects in pubertal compared to adult rats (Schneider *et al*. 2008), it might be possible that the modulatory action of the ECB system on ethanol intake is altered as well during pubertal development.

In the present study we therefore investigated the effects of acute administration of the synthetic cannabinoid receptor agonist WIN 55,212-2 (WIN) on anxiety-related behaviour, using the elevated plus-maze (EPM) and the light/dark emergence test (EMT), and on spontaneous ethanol consumption in a free-choice intake paradigm in pubertal [postnatal day (PD) 40] and adult (PD 100) rats.

To correlate the WIN-induced age-dependent behavioural changes with alterations at the molecular level we employed immunoblots to investigate whether acute CB_1 receptor activation would have an impact on the abundance of *N*-methyl-D-aspartate (NMDA) receptor-associated proteins in the reward system [medial prefrontal cortex (mPFC) and the ventral striatum]. The NMDA receptor complex is one of the primary targets of the action of ethanol (Spanagel, 2009; Vengeliene *et al*. 2008) and the composition of post-synaptic scaffolding complexes (e.g. Homer protein expression) in the mesolimbic neurocircuitry plays a crucial role in ethanol-induced neuroplasticity (Obara *et al*. 2009; Szumlinski *et al*. 2005, 2008). To correlate the age-dependent WIN-induced behavioural changes, we assessed protein levels of different NMDA receptor subunits and Homer in pubertal and adult rats following WIN exposure and also quantified the developmental expression profile of the CB_1 receptor.

Methods

Subjects

Male and female Wistar rats were imported from Harlan-Winkelmann (Germany) and housed together in pairs under standard conditions on a 12-h light/dark cycle (lights on 07:00 hours). They received free access to tap water and were fed *ad libitum.* After 3 wk male rats were removed from the breeding cages. The litters were culled to eight pups directly after birth (all male if possible). In order to avoid litter effects we attempted to assign equal proportions of rats of each litter to the different treatment groups (Zorilla, 1997). A total of 92 first-generation offspring male Wistar rats from our own breeding colony were used for the study.

After weaning on PD 21, male pups were housed in a different room in groups of six (Macrolon cage type IV) under standard conditions on a 12 h light/dark cycle (lights on 06:00 hours). They received free access to tap water and were fed *ad libitum.*

The experiments were performed in accordance with the NIH ethical guidelines and the European Communities Council Directive 86/609/EEC for the care and use of laboratory animals for experiments, and were approved by the local animal care committee (Cologne, Germany).

Drugs

WIN 55,212-2 (WIN) (Sigma-Aldrich, Germany) was dissolved in 0.1% Tween-80 and diluted in saline (0.9%). The drug was administered intraperitoneally at a dose of 1.2 mg/kg. That specific dose was chosen since we have found previously that this concentration of WIN has an effect on behavioural performance (e.g. memory performance and attentional processing) without affecting basic locomotor activity (Drews *et al*. 2005; Schneider & Koch, 2002).

Injection volumes were 1 ml/kg. The experimenter was not aware of the drug treatment of the animals.

Experimental design

Behavioural testing and biochemical analysis was assessed in pubertal (PD 40) and adult (PD 100) rats. Puberty onset in male rats occurs around PD 40 and sexual maturity is reached around PD 60. However, afterwards the animals are still considered to be young adults (late-adolescence) and therefore rats at PD 100 were taken as adult controls (for detailed review see Schneider, 2008). One group of rats was tested for emotional behaviour, starting 30 min after WIN treatment. Therefore, rats were first tested in the light/dark EMT followed 30 min later by the EPM test. Preliminary experiments in our laboratory proved that the sequence of anxiety tests used in the present study had no influence on behaviour during later testing and additionally the effects of WIN on behaviour did not differ if tested 30 or 60 min after injection. Behavioural performance for EPM and EMT was videotaped (digital handycam, Sony, USA) and evaluated offline by a trained experimenter blind to group assignment.

A second cohort of animals was used for investigating the effects of acute WIN treatment on spontaneous alcohol consumption. Alcohol and water were provided 1 h after the cannabinoid injection. Constant background noise was provided by a radio during the light phase of the light/dark cycle in the holding room and for behavioural testing.

For Western blot analysis a third naive group of age-matched animals (PD 40 and PD 100) was used.

Behavioural testing

Light/dark EMT

The EMT took place in a plastic box ($25 \times 80 \times 33$ cm) which consisted of two different compartments ($25 \times 40 \times 33$ cm), separated by a dividing wall with a hole in the centre that allowed the animals free access to both sides. The first compartment, with black walls could be closed by a lid and was used as the start box. The second compartment had white walls and was brightly illuminated.

Rats were initially placed in the dark, closed compartment and their behaviour was recorded for 5 min after the start box was opened. Subsequent video analysis scored the latency of rats to emerge from the dark compartment into the light compartment, the emergence frequency, the duration of time spent in the light compartment, the amount of rearings and risk assessment behaviour (only head or forepaws are placed in the open compartment without concomitant movement of the hindlimbs, even if the rat subsequently entered the area). The apparatus was thoroughly cleaned with 70% ethanol between sessions.

EPM

The EPM consisted of a plastic plus-shaped apparatus with two open (50×10) and two enclosed arms ($50 \times 10 \times 40$ cm), elevated 70 cm above the floor. All arms extended from a central square (10×10 cm).

At the beginning of each trial, rats were placed on the central square of the plus maze, facing a closed arm. Each rat was tested for 5 min and the following parameters were analysed from videotapes: number of entries into open or closed arms (an entry was defined if all four paws were placed on that arm), time spent in open and closed arms, head dips (the whole head is lowered beneath the edge of an open arm) and risk assessment (only head or forepaws are placed in an open arm without concomitant movement of the hindlimbs, even if the rat subsequently entered the area). Percentage of open arm entries

$$\frac{\text{open arm entries}}{\text{open} + \text{closed arm entries}} \times 100$$

and percentage of time spent in open arms

$$\frac{\text{open arm time}}{\text{open} + \text{closed arm time}} \times 100$$

were calculated as well. The total number of closed arm entries was used as an index of general activity. The apparatus was thoroughly cleaned with 70% ethanol between sessions.

Spontaneous ethanol consumption

For the evaluation of ethanol consumption, the animals were singly housed on testing days. Ethanol intake was assessed over a period of 24 h (from 16:00 to 16:00 hours) during which the rats had free access to four different ethanol concentrations (0.5%, 1%, 5%, 10% ethanol) and water. The animals had no previous experience with ethanol, since we were interested in the behaviour upon the first contact with this drug. The volume of ethanol intake was calculated as grams of ethanol/kg of body weight (g/kg) per animal separately for all four different ethanol concentrations and also as total ethanol intake (g/kg) for all concentrations taken together. Additionally, the mean ethanol and water intake during the 24-h period was assessed and ethanol preference was calculated as percentage dependent upon the total fluid intake

$$\frac{100}{\text{total fluid intake (ml)}.} \times \text{water or ethanol intake (ml)}$$

Table 1. Light/dark emergence test (EMT) performance after acute WIN treatment in pubertal and adult rats. A general cannabinoid treatment effect was observed for emergence latency, time spent in the lit compartment and rearing behaviour for pubertal and adult rats, respectively

	Pubertal treatment		Adult treatment	
Light/dark EMT	Vehicle	WIN	Vehicle	WIN
Emergence frequency	6.5 ± 0.7	4.5 ± 0.8	4.9 ± 1.1	3.3 ± 1.1
Emergence latency (s)	11.7 ± 3.4	77.3 ± 30.9	10.0 ± 2.1	74.4 ± 42.6
Emergence duration (s)	85.4 ± 10.9	46.4 ± 9.1	113.8 ± 24.9	81.0 ± 24.6
Risk assessment	13.1 ± 1.1	11.0 ± 1.1	8.3 ± 0.8	6.0 ± 1.3
Rearing	11.3 ± 1.6	5.1 ± 1.1	11.3 ± 2.3	4.9 ± 1.6

Data are expressed as mean ± S.E.M.

Western blot analysis

Brain lysate preparation

One hour following administration of WIN or vehicle to adult or pubertal rats, animals were sacrificed and the brains were taken. The ventral striatum and mPFC were dissected and homogenized in 1 ml lysis buffer (10 mM Tris–HCl, 2 mM EDTA; pH 8.0) containing a cocktail of protease inhibitors (Invitrogen, Germany) on crushed ice with a glass homogenizer. In preparation for immunoblotting, aliquots were mixed with an equal volume of 2 × Laemmli reducing sample buffer and heated to 95 °C for 5 min.

Immunoblotting

Using 12% gels, protein samples (20 μg) were separated by SDS–PAGE, transferred onto PVDF membranes, and probed with the following antibodies: rat anti-EVH1 (panHomer) serum (Klugmann *et al*. 2005) at 1:1000 dilution, mouse anti-NR1 (1:1000, Millipore, Germany), rabbit anti-NR2b (1:1000, Millipore, Germany), mouse anti-α-tubulin (1:400 000, Sigma-Aldrich, Germany), and rabbit anti-β-actin (1:2000; 13E5, Cell Signalling, Germany). For CB_1 detection, brain lysates were separated on NuPAGE® Novex bis-tris mini gels 4–12% (Invitrogen, Germany). After transfer, membranes were incubated with the rabbit anti CB_1 receptor antibody (1:500; Cayman Chemical, Estonia). Bound primary antibodies were detected by the appropriate HRP-conjugated secondary antibodies (Dianova, Germany) followed by a chemiluminescent substrate, and the signals captured on film. Densitometric measurement of immunoblot signals was performed using ImageJ software. Signals were standardized to β-actin (CB_1) or tubulin.

Statistical analysis

Treatment effects of the cannabinoid agonist WIN or vehicle on behavioural performance (EPM, EMT, total ethanol intake and preference) and Western blot results in pubertal and adult rats were evaluated using two-way ANOVA, followed by *post-hoc* Tukey *t* tests for pairwise comparisons. Ethanol intake (g/kg) for the different ethanol concentrations was analysed by a two-way ANOVA for repeated measure followed by *post-hoc* Tukey *t* tests for pairwise comparisons. The descriptive statistics was based on means + S.E.M. and a value of $p < 0.05$ was considered to represent a significant effect.

Results

Light/dark EMT

Statistical analysis revealed a significant WIN treatment effect for emergence latency ($F_{1,38} = 4.96$, $p < 0.05$), time spent in the open compartment ($F_{1,38} = 4.36$, $p < 0.05$) and rearing behaviour ($F_{1,38} = 14.08$, $p < 0.05$) compared to vehicle-treated controls. No treatment effects were observed for risk assessment ($F_{1,38} = 3.23$, $p > 0.05$) and emergence frequency ($F_{1,38} = 3.71$, $p < 0.05$) (Table 1) (pubertal, WIN group: $n = 13$; vehicle: $n = 12$; adult, WIN group: $n = 9$; vehicle: $n = 8$).

EPM

Two-way ANOVA revealed a general significant WIN treatment in the EPM effect for time spent in open arms ($F_{1,38} = 9.25$, $p < 0.05$), time spent in closed arms ($F_{1,38} = 22.01$, $p < 0.05$), percentage open arm time ($F_{1,38} = 10.27$, $p < 0.05$) and percentage open arm entries ($F_{1,38} = 4.47$, $p < 0.05$). A significant interaction effect for

Table 2. Effects of acute WIN treatment on Elevated Plus-Maze performance in pubertal and adult rats. A general treatment effect was observed for time spent in open arms, time spent in closed arms, percentage time spent in open arms and percentage open arm entries. Additionally, an age effect was observed for time spent in open and closed arms and percentage open arm time. Significant interaction effects were detected for open arm entries, head dips and risk assessment, with WIN injection inducing only a significant effect in pubertal treated rats ($p<0.05$ for age-dependent WIN effects is indicated by asterisks)

	Pubertal treatment		Adult treatment	
EPM	Vehicle	WIN	Vehicle	WIN
Time spent in open arms (s)	13.4±4.3	2.84±1.1	45.8±9.9	20.3±6.2
Time spent in closed arms (s)	255.3±6.3	280.3±3.4	218.4±11.1	263.1±6.8
Time spent in open arms (%)	5.1±1.6	1.1±0.4	17.4±4.7	7.1±2.4
Open arm entries	2.0±0.5	0.5±0.2*	2.8±0.6	2.1±0.6
Closed arm entries	7.3±1.2	6.1±1.3	8.8±1.4	8.7±1.6
Open arm entries (%)	21.2±4.6	10.5±4.3	24.4±4.0	17.1±3.7
Head dips	6.3±0.9	2.9±0.5*	5.5±0.7	4.3±0.5
Risk assessment	11.9±1.1	8.2±1.1*	10.5±0.8	9.9±1.2

Data are expressed as mean±S.E.M.

treatment and age was detected for open arm entries ($F_{1,38}=4.21$, $p<0.05$), head dips ($F_{1,38}=4.07$, $p<0.05$) as well as risk assessment ($F_{1,38}=4.14$, $p<0.05$). *Post-hoc* analysis revealed that only pubertal rats showed a significant decrease for open arm entries ($p=0.002$), head dips ($p<0.001$) and risk assessment ($p=0.002$) compared to vehicle-treated pubertal controls, whereas no significant difference was observed in the adult treatment groups ($p>0.05$). Finally, an age effect was found for time spent in open arms ($F_{1,38}=17.70$, $p<0.05$), time spent in closed arms ($F_{1,38}=13.17$, $p<0.05$) and percentage open arm time ($F_{1,38}=16.60$, $p<0.05$). No treatment effects were observed on closed arm entries ($F_{1,38}=0.26$, $p>0.05$) (Table 2) (pubertal, WIN group: $n=13$; vehicle: $n=12$; adult, WIN group: $n=9$; vehicle: $n=8$).

Spontaneous ethanol consumption

Acute WIN treatment significantly altered ethanol intake (Fig. 1). Statistical analysis revealed a significant interaction effect of treatment and age for the total ethanol intake (g/kg) ($F_{1,28}=4.74$, $p<0.05$) (Fig. 1*a*). *Post-hoc* analysis indicated that WIN administration significantly increased ethanol intake specifically in pubertal rats compared to vehicle-treated pubertal ($p<0.001$) and adult ($p<0.001$) controls. No such WIN effect was observed in the adult treatment group ($p>0.05$). Furthermore, within vehicle-treated animals, pubertal rats consumed more ethanol than adult controls ($p=0.003$). A significant interaction effect was also observed for percentage ethanol preference ($F_{1,28}=7.56$, $p<0.05$) (Fig. 1*b*). *Post-hoc* analysis revealed that ethanol preference was significantly increased only in pubertal WIN-treated rats compared to vehicle-treated pubertal controls ($p=0.004$) and WIN-treated adult rats ($p<0.001$). No treatment effects were observed in the adult treatment group ($p>0.05$). Finally, when analysing the intake of the different ethanol concentrations (g/kg), a significant interaction effect was observed in pubertal rats (ANOVA: $F_{3,48}=2.83$; $p<0.05$) (Fig. 1*c*) with the intake of the 5% ($p=0.001$) and 10% ($p=0.035$) ethanol solutions being significantly increased in cannabinoid-treated animals compared to controls.

No such effects were found in adult rats (ANOVA: $F_{1,30}=0.13$, $p>0.05$) (Fig. 1*d*) (pubertal, WIN group: $n=9$; vehicle: $n=9$; adult, WIN group: $n=7$; vehicle: $n=7$).

Biochemical analysis

Western blot for NMDA receptor subunits (NR1 and NR2b) and Homer

Statistical analysis revealed a significant interaction effect between age and cannabinoid treatment for NR1 in the striatum (ANOVA: $F_{1,16}=4.75$, $p<0.05$) and the mPFC (ANOVA: $F_{1,16}=12.89$, $p<0.05$) (Fig. 2*a*, *b*). *Post-hoc* analysis showed that acute WIN administration significantly increased NR1 expression compared to vehicle-treated controls only in pubertal rats (striatum: $p=0.001$; mPFC: $p=0.021$). Furthermore, pubertal WIN-treated rats had significantly higher NR1 levels in both regions compared to adult WIN-treated animals (striatum: $p=0.001$; mPFC: $p=0.002$). In addition, in the mPFC adult WIN treatment

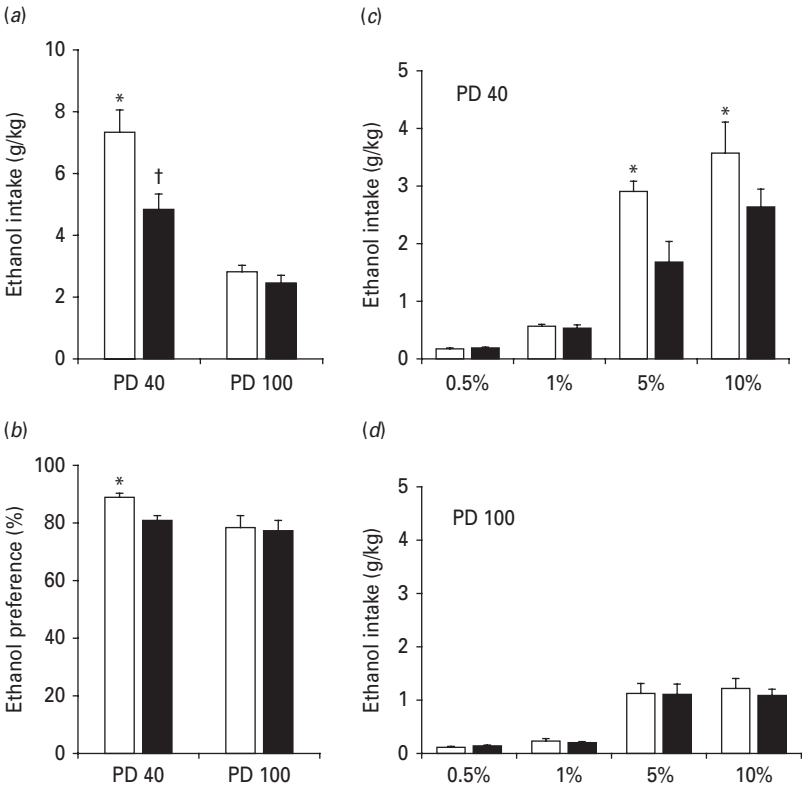

Fig. 1. Acute WIN effects on spontaneous ethanol intake in pubertal and adult rats. (*a*) WIN significantly increased the total ethanol intake (g/kg) in pubertal rats compared to vehicle-treated controls and WIN-treated adult animals. Additionally, vehicle-treated pubertal rats were found to have a higher ethanol intake than adult controls. (*b*) Percentage ethanol preference was also specifically increased in pubertal WIN-treated rats only compared to vehicle-treated pubertal controls and WIN-treated adult animals. (*c*) Furthermore, when analysing the ethanol intake of the different ethanol concentrations provided, a significant increase was observed specifically for 5% and 10% ethanol in pubertal rats. (*d*) No such increase was observed in adult animals. Data are expressed as mean + S.E.M., $p < 0.05$ detected after *post-hoc* analysis are indicated for treatment effects by asterisks (*) and for age effects by daggers (†). ■, WIN; □, vehicle.

induced a significant reduction in NR1 expression compared to vehicle-treated adult controls ($p = 0.023$). No significant differences in NR1 expression were detected for both regions between vehicle-treated pubertal (Pub) and adult rats ($p > 0.05$) (WIN-Pub, Veh-Pub, WIN-Adult, Veh-Adult: $n = 5$).

Acute WIN treatment did not affect protein levels of NR2b in the striatum (ANOVA: $F_{1,16} = 0.12$, $p > 0.05$) but we observed a slight but significant ($p = 0.023$) reduction of NR2B after WIN treatment in the mPCF of adult animals compared to vehicle-treated adult controls ($F_{1,16} = 4.79$, $p < 0.05$) (Fig. 2*c*, *d*). Furthermore, we also detected an age effect, with vehicle-treated pubertal rats expressing higher NR2B levels than adult animals ($p = 0.039$) (WIN-Pub, Veh-Pub, WIN-Adult, Veh-Adult: $n = 5$).

Similar WIN effects as for NR1 could be detected for levels of the NMDA receptor adaptor protein Homer (Fig. 2*e*, *f*). A significant interaction effect of age and WIN treatment was found for the striatum (ANOVA: $F_{1,16} = 4.70$, $p < 0.05$) and the mPFC (ANOVA: $F_{1,16} = 8.16$, $p < 0.05$). *Post-hoc* analysis revealed that WIN administration significantly increased Homer protein levels exclusively in pubertal rats (striatum: $p = 0.009$; mPFC: $p = 0.01$). In addition, vehicle-treated pubertal rats differed significantly in Homer expression in the striatum from adult controls ($p = 0.001$). In the mPFC cannabinoid-treated pubertal rats showed higher Homer protein levels compared to cannabinoid-treated adults ($p = 0.001$) (WIN-Pub, Veh-Pub, WIN-Adult, Veh-Adult: $n = 5$).

Western blot for CB_1

Statistical analysis detected a significant age effect of CB_1 receptor protein levels between pubertal and adult rats (Fig. 3), independent of WIN treatment, with pubertal rats showing higher CB_1 receptor expression

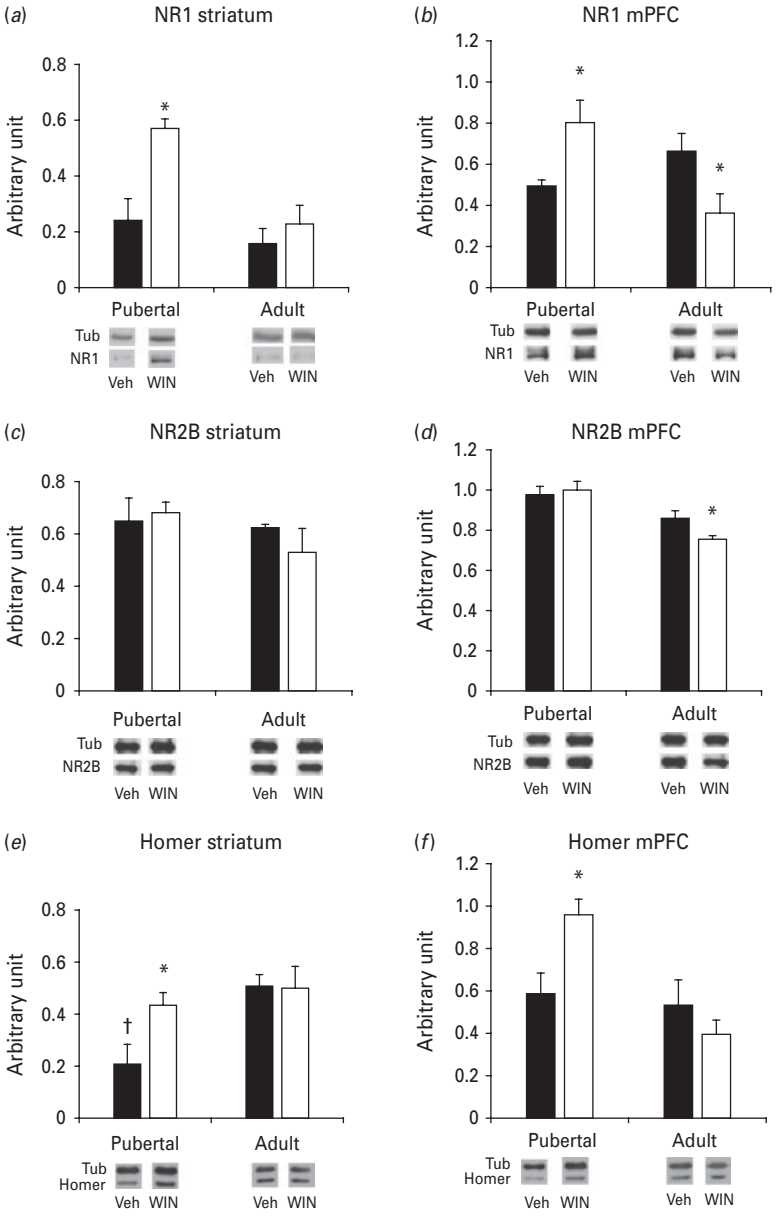

Fig. 2. Acute effects of WIN in pubertal and adult rats on protein levels of (*a*, *b*) NR1, (*c*, *d*) NR2B and (*e*, *f*) Homer in the striatum and mPFC, respectively, detected by Western blot analysis. Pubertal WIN treatment significantly increased NR1 and Homer levels in the striatum and mPFC. In adult rats a decrease in NR1 and NR2B was specifically detected for the mPFC. Additionally, lower Homer expression levels were found in the striatum of pubertal vehicle-treated rats compared to adult controls. Data are expressed as mean + S.E.M., $p < 0.05$ after *post-hoc* analysis for treatment effects is indicated by asterisks (*) and for age effects by daggers (†). ■, WIN; □, vehicle.

in the striatum (ANOVA: $F_{1,16} = 5.15$, $p < 0.05$) and mPFC ($F_{1,16} = 10.46$, $p < 0.05$) (WIN-Pub, Veh-Pub, WIN-Adult, Veh-Adult: $n = 5$).

Discussion

In the present study we showed that acute cannabinoid administration in pubertal rats increases anxiety-related behaviours and the initial consumption and preference of ethanol. Although emotional behaviour was also enhanced after acute WIN treatment in adult rats, no acute effects on ethanol consumption were detected in those animals. These drug-induced behavioural changes observed in pubertal rats were paralleled by induction of the NR1 subunit of the NMDA receptor in the mPFC and striatum. Moreover,

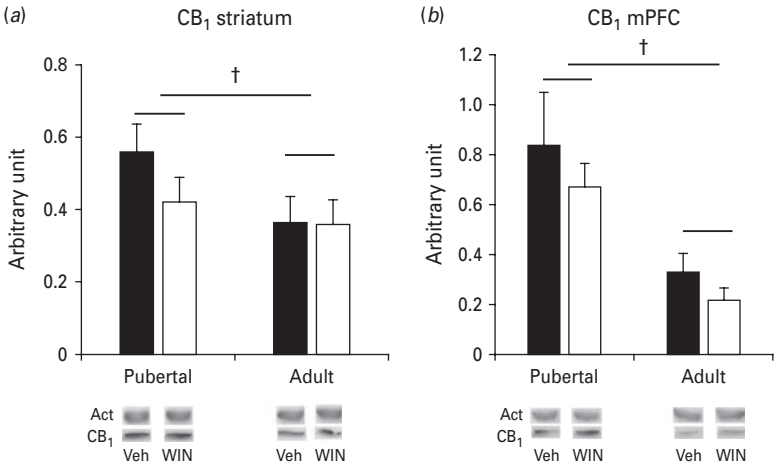

Fig. 3. No acute treatment effects of WIN on CB_1 receptor protein levels were detected in either pubertal or adult rats in the striatum and mPFC. However, an age effect was observed, indicating that the expression of CB_1 receptor levels was significantly higher in all pubertal rats compared to adult groups for both regions. Data are expressed as mean + S.E.M., $p < 0.05$ for the general age effect is indicated by daggers (†). ■, WIN; □, vehicle.

pubertal but not adult WIN treatment increased the levels of the scaffold protein Homer in these brain regions. These findings are consistent with earlier studies showing that cannabinoids induce stronger effects during pubertal development compared to adult animals (Schneider & Koch, 2003; Schneider, 2008; Schneider *et al*. 2008). Enhanced NMDA receptor complex mediated signalling as well as augmented CB_1 receptor availability in pubertal *vs*. adult animals may contribute to increased vulnerability for the adverse consequences of cannabinoid exposure during puberty.

Emotional behaviour

Acute WIN treatment increased anxiety-like behaviour in pubertal (PD 40) and adult (PD 100) rats in the EPM and the dark/light EMT. However, the anxiogenic effects of WIN were partially more pronounced in pubertal than adult animals. Increasing evidence implicates the involvement of the ECB system in the regulation of anxiety-related behaviour (Moreira & Lutz, 2008; Valverde, 2005; Viveros *et al*. 2005*b*; Witkin *et al*. 2005). Studies investigating age-dependent cannabinoid effects on emotional behaviour are rare and show discrepant findings. In rat pups at PD 12 the cannabinoid agonist CP 55,940 caused a dose-dependent anxiolytic-like reduction of ultrasonic vocalization that was reversed by the CB_1 receptor antagonist rimonabant (McGregor *et al*. 1996). However, in the same study and also reported by others in pubertal (PD 40) rats (Romero *et al*. 2002), high doses of CP 55,940 induced hyperreactivity and the rats emitted audible vocalizations when picked up by the experimenter, which might be interpreted as an aversive or anxiogenic-like response. Additionally, it was shown that administration of Δ^9-tetrahydrocannabinol (THC) in juvenile (PD 28) and late-pubertal (PD 65) male rats increased emotional responses and elevated stress hormone levels for a prolonged period after THC treatment at both ages (Schramm-Sapyta *et al*. 2007). Unfortunately, no adult controls or pubertal rats were used in this study, which would have indicated the age specifity of these findings. Taken together, these findings indicate that the effects of cannabinoids on emotional behaviour depend to a great extent upon the age of an animal and the developmental state (juvenile, prepubertal, pubertal or early adult).

In humans several reports have shown that early cannabis exposure is significantly associated with later anxiety and depression (Degenhardt *et al*. 2003; Goldschmidt *et al*. 2004; Gray *et al*. 2005; Leech *et al*. 2006; Patton *et al*. 2002; Poulin *et al*. 2005; Rey *et al*. 2002). These studies therefore lend support to our present findings that cannabinoids induce alterations in emotional behaviour in particular during specific vulnerable phases of neuronal development.

Notably, we also observed some age differences between pubertal and adult rats in the EPM, where pubertal rats spent less time in the open arms compared to adult rats. However, since we did not observe any age effects in the light/dark EMT and on other behavioural variables in the EPM such as frequency and percentage of open arm entries and head dips, this

difference in open arm time appears not be related primarily to an altered anxiety state in pubertal rats. It is well known that the stress responsiveness is increased during adolescent development (McCormick *et al*. 2010; Romeo, 2010), therefore, it might be possible that the vehicle injection *per se* acted as a stronger stressor in pubertal rats, which might also contribute to the slightly more pronounced WIN effects in pubertal than adult rats.

Initial ethanol intake

In order to assess the initial reaction to ethanol, the intake of different ethanol solutions was measured in alcohol-naive animals. Acute WIN treatment increased ethanol consumption in pubertal rats, in particular the intake of the higher ethanol concentrations (5% and 10%). No such effects were seen in adult animals. Additionally, acute WIN administration increased percentage ethanol preference and the total ethanol intake specifically in pubertal rats.

It has been suggested that the ECB system plays a major role in determining the reinforcing effects of alcohol (Hungund & Basavarajappa, 2004; Mechoulam & Parker, 2003; Spanagel, 2009). The human CB_1 receptor gene (CNR1) may play a role in the development of alcoholism (Benyamina *et al*. 2011; Zuo *et al*. 2007). SR141716 reduces ethanol intake in rats (Arnone *et al*. 1997; Colombo *et al*. 1998; Gallate & McGregor, 1999; Gallate *et al*. 2004) and cannabinoid agonists increase the motivation for ethanol consumption (Gallate & McGregor, 1999). Electrophysiological data revealed that the effects of ethanol in the mesolimbic reward pathway relay upon stimulation of CB_1 receptors, suggesting the necessity of the ECB system for full development of an appetitive ethanol response (Perra *et al*. 2005). Furthermore, CB_1 knockout mice show reduced ethanol self-administration (Naassila *et al*. 2004; Thanos *et al*. 2005), reduced ethanol-induced place preference (Houchi *et al*. 2005; Thanos *et al*. 2005), and ethanol withdrawal symptoms are completely absent in these animals (Racz *et al*. 2003). In line with these observations we show in the present study that pubertal rats have higher ethanol intake and preference than adult animals, which is paralleled by higher CB_1 receptor protein levels in pubertal rats compared to adults.

Apart from the reinforcing effects of alcohol, several studies indicate that anxiety-related behaviour might be a modulating factor in initial alcohol consumption. It has been suggested that some individuals may be predisposed to alcohol drinking because of high innate anxiety levels (Novak *et al*. 2003; Schuckit & Hesselbrock, 1994) and anxiety disorders in adolescents are significant predictors of the subsequent onset and persistence of alcohol use and alcoholism (Zimmermann *et al*. 2003). In animal studies it has been shown that elevated anxiety measures in rats correlate with high alcohol consumption during the initiation of alcohol drinking behaviour (Spanagel *et al*. 1995). However, conflicting findings have been reported from rat lines specifically selected and bred for high alcohol intake (Spanagel, 2009). Altogether animal research and epidemiological studies demonstrate the existence of a complex relationship between anxiety-related behaviour and alcohol consumption.

Besides the ethanol intake stimulating effects of WIN in pubertal rats, differences were also observed between pubertal and adult vehicle-treated controls. Total ethanol intake was significantly increased in pubertal compared to adult animals. This finding is consistent with previous studies showing different rewarding effects of ethanol in pubertal than juvenile or adult rats (Philpot *et al*. 2003) and higher beer intake and binge-like drinking patterns during puberty (Hargreaves *et al*. 2009). Additionally, adolescent rats are less sensitive to ethanol's aversive properties than adults, measured by conditioned taste aversion (Vetter-O'Hagen *et al*. 2009). Our finding of enhanced ethanol intake in non-treated pubertal animals, and also the increased stimulatory effects of WIN on pubertal ethanol intake, might be related to some extent to the higher activity of the ECB system at puberty onset (for review see Schneider, 2008) and the higher CB_1 receptor expression observed in the present study.

Biochemical analysis

CB_1 receptors are abundantly expressed in the mPFC and striatum, brain areas known to be involved in emotional behavioural outputs and rewarding effects of drugs such as marijuana or alcohol (Lutz, 2007). In our present study we detected various differences in these brain regions between pubertal and adult rats regarding expression of basal CB_1 receptor protein levels and also reponsiveness towards the acute effects of WIN for NMDA receptor-associated proteins.

Acute WIN treatment did not affect CB_1 receptor expression in both pubertal and adult rats. However, a general age effect was observed with pubertal rats expressing higher protein levels of CB_1 than adults. These results are consistent with an earlier study showing alterations in CB_1 receptor radioligand binding in rats in the limbic forebrain, ventral mesencephalon,

and striatum. Binding increased gradually starting from PD 10, and reached maximum values around PD 40 in male animals (Rodriguez de Fonseca *et al*. 1993). Afterwards CB_1 binding was found to be decreased when measured in adult rats at PD 70. These CB_1 receptor dynamics were most pronounced in the ventral mesencephalon and striatum. Therefore, our present results indicate that the heightened receptor sensitivity at the beginning of puberty at PD 40, might be a function of higher innate CB_1 protein levels compared to adulthood. These findings also support our behavioural data where we observed stronger WIN effects in pubertal than in adult rats.

Additionally, we observed an increase in expression of NR1 in the striatum and mPFC after acute WIN treatment of pubertal but not adult rats, whereas NR2B was only altered region-specifically in adult WIN-treated animals. A WIN-induced decrease in NR1 and NR2B was detected in the mPFC of adult rats. It is not clear whether these regulations are caused by reduced receptor degradation or increased *de novo* synthesis. Functional NMDA receptors are assembled from heteromeric subunits including NR1 and at least one of NR2A-D or NR3 (Nakanishi *et al*. 1998). NR1 is the channel-forming subunit and the NR2 subunits have been proposed to have modulatory effects on channel activity (Monyer *et al*. 1992). Since the magnitude of WIN-induced decrease for NR2B in adults was less than for NR1, it appears possible that other NR2 subunits may have been co-regulated, thereby contributing to altered NMDA receptor physiology. However, in this study we focused on NR2B because there is compelling evidence that this subunit may constitute a genetic risk factor for stress-induced alcohol drinking and alcoholism (Sillaber *et al*. 2002). Composition of NMDA receptors containing NR1/NR2B subunits confers high sensitivity to the inhibitory effects of alcohol, as opposed to NR1/NR2C or NR1/NR2D receptors (Allgaier, 2002) and acute alcohol administration inhibits NMDA receptor function (Roberto *et al*. 2004). Moreover, different studies suggest a role for NR2B-containing rather than NR2A-containing NMDA receptors in modulating the intoxicating effects of ethanol (Boyce-Rustay & Holmes, 2005, 2006). An important role of NR2B in alcohol-induced behaviour is also indicated by the high relative abundance of this subunit in the cortex and striatum compared to other brain regions (Goebel & Poosch, 1999).

The up-regulation of NR1, the major NMDA receptor subunit, after acute CB_1 receptor activiation in pubertal animals suggests enhanced glutamatergic signalling paralleling the initiation of alcohol consumption. This result is supported by a report on WIN-induced acute activation of NMDA receptors and concomitant release of Ca^{2+} from intracellular stores in cultured cerebellar granule cells (Netzeband *et al*. 1999). Although inhibitory effects of cannabinoid function have been described to depress NMDA-elicited Ca^{2+} signals *in vitro* (Hampson & Deadwyler, 1998), it seems likely that cannabinoid-mediated enhancing effects on NMDA receptor function and intracellular Ca^{2+} might occur under moderate CB_1 receptor stimulation and/or short-term duration. Another explanation for the different effects of WIN in pubertal *vs.* adult animals might be differences in the molecular composition of constituents of the post-synaptic signalling complex, known as the post-synaptic density.

Homer proteins are critical for regulating the functional architecture of excitatory synapses and long Homer proteins expressing a coiled-coiled domain (CC-Homers) have been shown to recruit group I mGluRs, inositol-1,4,5-triphosphate receptors (IP3Rs) and NR2B to the post-synaptic density. By multimerization they cross-link mGluRs and NMDA receptors to the endoplasmic reticulum membrane and modulate intracellular Ca^{2+} dynamics (Klugmann & Szumlinski, 2008). This adapter function makes Homer an attractive candidate for enabling a functional interplay between the CB_1 and NMDA receptor signalling pathways. Indeed, direct involvement of Homer in ECB-mediated neuroplasticity has been indicated in recent studies. NMDA receptors are connected via PSD-95, GKAP, and SHANK to Homer that in turn binds to proteins involved in ECB signalling such as mGluRs and diacylglycerol lipase (DGL) (Klugmann & Szumlinski, 2008). Jung *et al*. (2007) have shown that Homer proteins enhance retrograde ECB signalling by promoting plasma membrane expression of DGL-α in a neuroblastoma cell line. Moreover, the cocaine-induced attenuation of retrograde ECB signalling known to underlie long-term depression has been correlated with enhanced expression levels of Homer proteins (Fourgeaud *et al*. 2004). Interestingly, the induction of ECB-mediated synaptic plasticity is also induced after stimulation of the immediate early gene isoform, Homer 1a. This isoform, known to be induced by neuronal activity (Brakeman *et al*. 1997), suppresses metabotropic-mediated but enhances depolarization-mediated endocannabinoid production (Roloff *et al*. 2010).

In the present study, we detected an age-specific induction of NR1 and CC-Homer after acute WIN treatment specifically in pubertal rats. Importantly, CC-Homer proteins in corticolimbic brain regions have

been shown to be critical for emotional behaviour (Klugmann & Szumlinski, 2008). Their regulation by various drugs of abuse is not only subregionally selective, but also drug-specific and isoform-specific. In the context of alcohol, Homer2a/b is up-regulated within the accumbens, but Homer1b/c is unchanged (Obara *et al*. 2009; Szumlinski *et al*. 2008). In the context of cocaine, both Homer1b/c and Homer2a/b are down-regulated within the accumbens, but Homer2a/b is up-regulated in the PFC (Ary & Szumlinski, 2007; Ben Shahar *et al*. 2009; Swanson *et al*. 2001). Moreover, these effects are observed 24 h (alcohol studies) or 3 wk (cocaine studies) following the last drug exposure. It is not known whether or not CC-Homers or their associated glutamate receptors change within the brain at shorter withdrawal time-points (i.e. at 1 h post-injection as observed in the present study). NR1 and long Homer isoforms are constitutively expressed, and it is not clear what cellular mechanisms might have accounted for short-term changes in the respective protein levels. It appears intuitive that NR1 levels may be increased by a reduced turnover or internalization while transcriptional or translational regulations can not be excluded. Our data suggest that relatively quick changes in NR1 and CC-Homer protein expression can occur following acute drug treatment that are likely to affect glutamate signalling of relevance to drug reward.

Interestingly, we observed lower CC-Homer protein expression in the striatum of vehicle-treated pubertal than adult rats. This result matches the reported developmental expression profile of Homer 1 and 2 in different brain regions, including the striatum (Shiraishi *et al*. 2004), where increasing protein levels were detected throughout the early postnatal developmental period in mice.

Conclusion

We were able to demonstrate with the present study that administration of a cannabinoid agonist induces stronger behavioural effects in pubertal rats compared to adults, by increasing anxiety-related behaviours and initial ethanol intake. These behavioural alterations were paralleled by differential inductions of NMDA receptor-associated proteins in pubertal and adult rats. The present data further demonstrate the susceptibility of the pubertal developmental period for the aversive behavioural consequences of cannabinoid exposure, which might be related to enhanced activity of the ECB system and concomitant alterations in glutamatergic signalling during this specific developmental period.

Acknowledgements

We thank Theresa Brand and Ruth Jelinek for excellent technical assistance. Miriam Schneider was supported by DFG (SCHN 958/3–1).

Statement of Interest

None.

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