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Endocannabinoids potently protect the newborn brain against AMPA-kainate receptor-mediated excitotoxic damage

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1 Brain lesions induced in newborn mice or rats by the glutamatergic agonists ibotenate (acting on NMDA and metabotropic receptors) or *S*-bromowillardiine (acting on AMPA-kainate receptors) mimic some aspects of white matter cysts and transcortical necrosis observed in human perinatal brain damage associated with cerebral palsy. Exogenous and endogenous cannabinoids have received increasing attention as potential neuroprotective agents in a number of neurodegenerative disorders of the adult. One recent study showed neuroprotection by the cannabinoid agonist WIN-55212 in a newborn rat model of acute severe asphyxia.

2 The present study was designed to assess the neuroprotective effects of the endogenous cannabinoid anandamide using a well-defined rodent model of neonatal excitotoxic brain lesions.

3 In this model, anandamide provided dose-dependent and long-lasting protection of developing white matter and cortical plate reducing the size of lesions induced by *S*-bromowillardiine. Anandamide had only marginal neuroprotective effect against ibotenate-induced cortical grey matter lesions. Anandamide-induced neuroprotection against AMPA-kainate receptor-mediated brain lesions were blocked by a CB1 antagonist but not by a CB2 antagonist. Furthermore, anandamide effects were mimicked by a CB1 agonist but not by a CB2 agonist. Real-time PCR confirmed the expression of CB1 receptors, but not CB2 receptors, in the untreated newborn neocortex. Finally, neuroprotective effects of anandamide in white matter involved increased survival of preoligodendrocytes and better preservation of myelination.

4 The present study provides experimental support for the role of endocannabinoids as a candidate therapy for excitotoxic perinatal brain lesions.
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Abbreviations: ACPA, arachidonylcyclopropylamide; CP, cerebral palsy

Introduction

The major brain lesions associated with cerebral palsy (CP) are periventricular leukomalacia (periventricular white matter lesion) in preterm infants and cortico-subcortical lesions in term infants (Volpe, 2001). Several preconception, prenatal and perinatal factors implicated in the pathophysiology of brain lesions associated with CP include hypoxia-ischemia, endocrine imbalances, genetic factors, growth factor deficiency, abnormal competition for growth factors, excess free reactive oxygen species production, maternal infection yielding excess cytokines, and other proinflammatory agents (Willoughby and Nelson, 2002; Mesples *et al*., 2005).

Excess release of glutamate could represent a molecular mechanism common to some of these risk factors. Accordingly, injection of glutamate agonists into the striatum, the neocortex or the periventricular white matter of newborn rodents or rabbits produces histological lesions that mimic CP-associated brain damage characterized by cystic periventricular leukomalacia and hypoxic-ischemic or ischemic-like cortical and striatal lesions (McDonald *et al*., 1988; Barks and Silverstein, 1992; Marret *et al*., 1995; Acarin *et al*., 1999; Follett *et al*., 2000; Sfaello *et al*., 2005a) . The use of specific agonists for the different classes of glutamatergic receptors has demonstrated that oligodendrocyte precursor cell death is a key event in periventricular white matter lesions induced by agonists acting on or alpha-3-amino-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate receptors (Follett *et al*., 2000), while microglial activation plays a major role in periventricular white matter lesions induced by agonists acting on *N*-methyl-D-aspartate (NMDA) receptors (Tahraoui *et al*., 2001).

Despite major improvements in neonatal care, there is still no specific treatment for perinatal brain lesions (Gressens

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& Spedding, 2004). The endocannabinoid system is an emerging target for drug discovery, because it is involved in the regulation of many cellular and physiological functions (Fernandez-Ruiz *et al*., 2000; Fride, 2002). The endocannabinoid system constitutes the endogenous lipids anandamide, 2-arachidonoylglycerol and noladin ether, and the cannabinoid CB1 and CB2 receptors, as well as the proteins involved in the synthesis and inactivation of the endocannabinoids (van der Stelt *et al*., 2002).

Endogenous cannabinoids are clearly a major site for drug action – and do not share the deleterious effects of cannabis which swamps the endogenous system by stimulating all the receptors. The distribution of cannabinoid receptors in the brain shows how important they are in modulating the brain schemes shown above with differential distribution in hippocampus and amygdala (Freund *et al*., 2003). The massive endocannabinoid distribution in the basolateral amygdala is responsible for the anxiolytic effects of cannabis.

There is a clear link between the cannabinoid system and the GABAergic system. For example, endogenous anandamide inhibits CA1 neurones *via* interneurones and this effect is mediated by CB1 receptor stimulation. The inhibition is very local feedback system, involving only a few interneurones. Depolarization-induced suppression of inhibition (DSI) is a calcium-dependent, retrograde-signalling process mediated by endocannabinoids by a reduction of GABA release via presynaptic cannabinoid receptor (CB1) activation. DSI lasts around one minute while synaptic long term depression (LTD – usually loss of AMPA receptors from the synapse) is long lasting.

However, in early life the GABAergic system is depolarizing rather than hyperpolarizing (up to ~P8–P10 in mice) and this obviously has major effects on the drugs acting on the GABA system, particularly as the time for the GABA hyperpolarization/depolarization switch is not known in man, and may vary with brain areas. Bernard *et al*. (2005) showed that retrograde signaling of the cannabinoid system can substitute for the GABA system in early development, controlling synaptic transmission and preventing epileptic discharges. As there is a continuing problem of CP in the newborn, and the use of benzodiazepine activators of the GABAergic system is used for controlling epileptic activity in these children (perhaps inappropriately if the GABA hyperpolarization/depolarization switch has not taken place in all brain areas), it seems essential to determine if endogenous cannabinoid agonists can have neuroprotective effects in newborn animals. The use of synthetic agonists may be inappropriate as there will be no local inactivation of these substances and they may, therefore, swamp the local control systems, resulting in impaired developmental synaptic plasticity (Bernard *et al*., 2005).

Exogenously administered endocannabinoids have been shown to exert neuroprotection in a variety of *in vitro* and *in vivo* models of adult neuronal injury (Baker *et al*., 2003; Di Marzo *et al*., 2004) including 6-hydroxydopamine toxicity (Lastres-Becker *et al*., 2005), beta-amyloid-induced toxicity (Ramirez *et al*., 2005), and neurodegeneration in models of multiple sclerosis (Ortega-Gutierrez *et al*., 2005) or excitotoxicity (Marsicano *et al*., 2003; Veldhuis *et al*., 2003). The proposed mechanisms include, among others, blockade of microglial activation (Ramirez *et al*., 2005), increase in brain-derived neurotrophic factor (Khaspekov *et al*., 2004), reduction of calcium influx (Nadler *et al*., 1993), and antioxidant activity (El-Remessy *et al*., 2003). Despite the rapid enzymatic degradation of endocannabinoids, neuroprotection was observed even if exogenous endocannabinoids were administered systemically in the absence of inhibitor of the fatty acid amid hydrolase (FAAH) which is involved in the hydrolysis of endogenous cannabinoids such as anandamide (Lastres-Becker *et al*., 2005).

To our knowledge, only a few studies have addressed the potential neuroprotective effects of cannabinoids for the neonatal brain. In one study (Hansen *et al*., 2001), it was shown that anandamide accumulates in neonatal rat models characterized by widespread neurodegeneration as a consequence of altered glutamatergic neurotransmission. In another study (Martinez-Orgado *et al*., 2003), the authors showed neuroprotection by the cannabinoid agonist WIN-55212 in an *in vivo* newborn rat model of acute severe asphyxia. This neuroprotective effect was blocked by a specific CB1 receptor antagonist. Conversely, it was shown that a specific CB1 receptor antagonist was neuroprotective against NMDA-induced neuronal cell death in newborn rats (Hansen *et al*., 2002).

The present study was designed to assess the neuroprotective effects of the endocannabinoid anandamide using in a well-defined mouse model of neonatal excitotoxic brain lesions (Marret *et al*., 1995; Tahraoui *et al*., 2001; Husson *et al*., 2002; Husson *et al*., 2005; Sfaello *et al*., 2005b), which mimics several aspects of brain damage associated with human CP.

Methods

Animals and drugs

Swiss mice and Sprague Dawley rats of both sexes were used for this study. Experimental protocols were approved by the institutional review committee, meet the INSERM guidelines, and were carried out in accordance with the Guide for the Care and use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. All drugs were purchased from Tocris (Bristol, U.K.) except for nabilone which was obtained from Servier. Ibotenate was diluted in phosphate-buffer saline (PBS) containing 0.01% acetic acid. *S*-bromowillardiine, MK-801, and NBQX were diluted in PBS. Anandamide, arachidonylcyclopropylamide (ACPA), AM251, AM630, JWH133, nabilone, and URB597 were diluted in Tocrisolve™.

Ibotenate activates NMDA and metabotropic glutamatergic receptors while *S*-bromowillardiine activates both AMPA and kainate receptors. MK801 is a NMDA receptor antagonist and NBQX is an AMPA/kainate receptor antagonist. Anandamide is an endogenous cannabinoid activating both CB1 and CB2 receptors, ACPA is a selective CB1 agonist, JWH133 is a selective CB2 agonist, nabilone is a CB1 and CB2 agonist, AM251 is a selective CB1 antagonist, and AM630 is a selective CB2 antagonist (Howlett *et al*., 2002). URB597 is selective inhibitor of the FAAH (Fegley *et al*., 2005).

Excitotoxic brain lesions

We induced excitotoxic brain lesions by injecting ibotenate (10 μg) or *S*-bromowillardiine (15 μg) into developing mouse and rat brains, as previously described (Marret *et al*., 1995;

Gressens *et al*., 1997; Tahraoui *et al*., 2001; Husson *et al*., 2002). Controls received intracerebral ibotenate (10 μg) + MK-801 (1 μg) or *S*-bromowillardiine (15 μg) + NBQX (20 μg). Briefly, on postnatal day (P) 5, pups anesthetized with isoflurane were kept under a warming lamp to maintain body temperature. They were injected intracerebrally (into the neopallial parenchyma) with ibotenate or *S*-bromowillardiine on the fifth postnatal day (P). Intracerebral injections were performed with a 25-gauge needle on a 50-μl Hamilton syringe mounted on a calibrated microdispenser. The needle was inserted 2 mm below the skin's external surface. The needle tip was placed in the frontoparietal area of the right hemisphere, 2 (mice) or 2.5 mm (rats) from the midline in the lateral-medial plane, and three (mice) or 4 mm (rats) from the bregma in the rostro-caudal plane. Histopathological observation confirmed that the needle tip always reached the periventricular white matter. Two 1-μl boluses of ibotenate or *S*-bromowillardiine were injected at 20-s intervals. The needle was left in place for an additional 20 s.

Experimental groups

Pups from at least two different litters were used in each experimental group, and data were obtained from two or more successive experiments.

In the first set of experiments, P5 mouse pups were intracerebrally injected with 10 μg ibotenate, 10 μg ibotenate + 20 μg MK801, 15 μg *S*-bromowillardiine or 15 μg *S*-bromowillardiine + 20 μg NBQX.

In the second set of experiments, 10 μg ibotenate or 15 μg *S*-bromowillardiine were intracerebrally injected into P5 mouse pups and anandamide (0.01, 0.03, 0.1, 0.3, 1, 3, or 10 mg kg^{-1}), ACPA (1, 3, or 10 mg kg^{-1}), nabilone (1 or 10 mg kg^{-1}), JWH133 (1 or 10 mg kg^{-1}), anandamide (10 mg kg^{-1}) + AM251 (20 mg kg^{-1}), anandamide (10 mg kg^{-1}) + AM630 (20 mg kg^{-1}), AM251 (20 mg kg^{-1}), AM630 (20 mg kg^{-1}), anandamide (1 mg kg^{-1}) + URB597 (0.3 mg kg^{-1}), or vehicle (controls) were administered intraperitoneally immediately following the excitotoxin injection.

In the third set of experiments, 15 μg *S*-bromowillardiine were intracerebrally injected into P5 mouse pups and intraperitoneal (i.p.) anandamide (10 mg kg^{-1}) was administered at immediately, 4, 8, 12, or 24 h after the excitotoxin injection.

In the last set of experiments, 15 μg *S*-bromowillardiine were intracerebrally injected into P5 rat pups and i.p. anandamide (10 mg kg^{-1}) was administered immediately after the excitotoxin injection.

Determination of lesion size

Mouse and rat pups were kill by decapitation 5 (P10) or 25 (P30) days after the excitotoxic challenge. Brains were fixed immediately in 4% formalin and remained in this solution for 5 days. Following paraffin embedding, 16-μm-thick coronal sections were cut. Every third section was stained with cresyl-violet. The size of neocortical and white matter lesions can be defined by the length on three orthogonal axes: the lateral-medial axis (in a coronal plane), the radial axis (also in a coronal plane, from the pial surface to the lateral ventricle), and the fronto-occipital axis (in a sagittal plane). In previous studies (Marret *et al*., 1995; Gressens *et al*., 1997; Husson *et al*., 2002), we found an excellent correlation among the measurements from the three axes of the excitotoxic lesions. Based on these findings, we cut serial sections of the entire brain in the coronal plane for this study. This permitted an accurate and reproducible determination of the sagittal fronto-occipital diameter (which is equal to the number of sections where the lesion was present multiplied by 16 μm). This measure was used as an index of the lesion volume.

Immunohistochemistry for oligodendrocytes and myelin

To study the neuroprotective effect of TPM on oligodendrocytes and myelin, P5 rat pups were injected with 15 μg *S*-bromowillardiine and 10 mg kg^{-1} i.p. anandamide, or 15 μg *S*-bromowillardiine and i.p. PBS. Animals were killed at 1 (P6) or 9 (P14) days after injections. Myelin basic protein (MBP, a marker of myelin; 1/1000, Chemicon, Temecula, CA, U.S.A.) immunostaining was performed on formalin-fixed, 15-μm thick, paraffin sections. O4 (a marker of preoligodendrocytes; 1/500, generous gift from Dr PA Rosenberg, Boston, MA, U.S.A.) immunofluorescence staining was performed on 4% paraformaldehyde-fixed, 10-μm thick, frozen sections. For 04 staining, detection of labeled antigens was performed with secondary antibody conjugated to Texas red (Vector) and nuclei were counter-stained with *bis*-benzimide.

Five animals were included in each group. For each animal, several sections were immunoreacted in successive experiments, and two different investigators blinded to the experimental groups independently performed the analyses. MBP immunostaining was evaluated in a qualitative manner in the white matter at the site of the lesion. O4 labeled cells were quantified at the level of neocortical lesion and/or underlying white matter cystic lesion. For each animal, counts were performed in 0.0625 mm^2 area of the most affected section.

Real time RT–PCR for CB1 and CB2 receptors mRNA

Mouse pups ($n = 5$ per group) were sacrificed on P5 immediately before or 3 h after intracerebral injection with ibotenate, *S*-bromowillardiine or PBS. Brains were removed and tissues immediately adjacent to the site of excitotoxin injection (see above) were collected from the different animals for RNA extraction. Total RNA was extracted as previously described (Lelievre *et al*., 2002), followed by DNaseI treatment (TURBO DNA-*free*™, Ambion, Austin, TX, U.S.A.). For each sample, 600 ng were used in reverse transcription (iScript kit from BioRad, Hercules, CA, U.S.A.). The following oligonucleotides (Oligo6 software, Molecular Biology Insights, Cascade, CO, U.S.A.) 5′- TAATTGCTGTGTTGCCTCTC C-3′ and 5′-TCCGATCCAGAACATCAGGTA-3′, 5′- GGCA GTGTGACCATGACCTT-3′ and 5′-GGTAGGCGGGTAA CACAGACA-3′ were used as sense and antisense primers, for CB1 and CB2 receptors, respectively. PCR amplification resulted in the generation of a single band at 107 and 90 bp corresponding to the region 1288-1395 and 514–604 of the previously published sequences (NCBI access numbers NM007726 and NM009924) of mouse CB1 and CB2 receptors, respectively. A primer set (5′- TGGTGAAAAGGACCTCTC GAA-3′ and 5′-TCAAGGGCATATCCAACAACA-3′, as sense and antisense, respectively) for the mouse hypoxanthine guanine phosphoribosyl transferase (HPRT) gene was used to standardize the experiments. These primers amplified an 90 bp

region encoding the nucleotides 578–668 of the published sequence (access number NM013556) of the mouse mRNA. Real-time PCR was set up using sybergreen-containing supermix from BioRad, for 45 cycles (20 s denaturation at 96°C, 20 s annealing at 62°C, and 20 s extension at 72°C). Amplification specificity was assessed by melting curve and sequencing analyses. Quantification of each PCR samples was made using standard curves made from serial dilutions of control samples. Relative expression levels of genes of interest were calculated as the difference between the specific ratios (CB receptor/HPRT) and were further adjusted on the basis of their differences in C_T values (number of cycles to reach threshold). Experiments were independently run three times. In each experiment, samples were performed in quadruplicates.

Statistical analysis

All variables were found normally distributed based on Skewness and Kurtosis analyses. Data were analyzed with a Student's *t*-test or a univariate ANOVA (GraphPad Prism version 3.03 for Windows, GraphPad Software, San Diego, CA, U.S.A.). When ANOVA showed significant differences among multiple experimental groups, multiple comparisons of treated vs control groups were performed using Dunnett's *post hoc* test.

Results

Overall mortality was low in the present study (<3% of the animals injected with ibotenate or *S*-bromowillardiine). No significant difference was observed in a test of contingency (exact Fisher test) when the different treatment groups were compared to the animals injected with ibotenate plus vehicle or with *S*-bromowillardiine plus vehicle. Epileptic manifestations including clonic or tonic seizures and apneas were observed in all ibotenate-treated animals. However, treatment with anandamide, ACPA, nabilone, AM251 or AM630 did not induce any detectable difference in severity and frequency of seizures (frequency of seizures was quantitatively assessed during a 10-min period, once every hour during the first 6 h following excitotoxin injection; severity of seizures was qualitatively assessed according to the same schedule) when compared to controls (data non shown).

Mouse pups injected on P5 with intracerebral ibotenate or *S*-bromowillardiine and i.p. vehicle developed cortical lesions and periventricular white matter cysts (Figure 1a). The cortical lesion was typically characterized by neuronal loss in all neocortical layers and almost complete disappearance of neuronal cell bodies along the axis of excitotoxin injection. Ibotenate-induced lesions were totally abolished by cointracerebral injection of MK-801, a specific NMDA receptor antagonist (Figure 2). *S*-bromowillardiine-induced lesions were totally abolished by co-intracerebral injection of NBQX, a specific AMPA-kainate receptor antagonist (Figure 3a). P5 rats injected with intracerebral *S*-bromowillardiine and i.p. PBS displayed cortical and white matter lesions similar to those observed in P5 mice (data not shown).

In P5 mice, i.p. anandamide induced a moderate neuroprotection of the cortical plate against ibotenate-induced lesions but had no detectable effect on ibotenate-induced white matter lesions when observed on P10 (Figure 2). This moderate

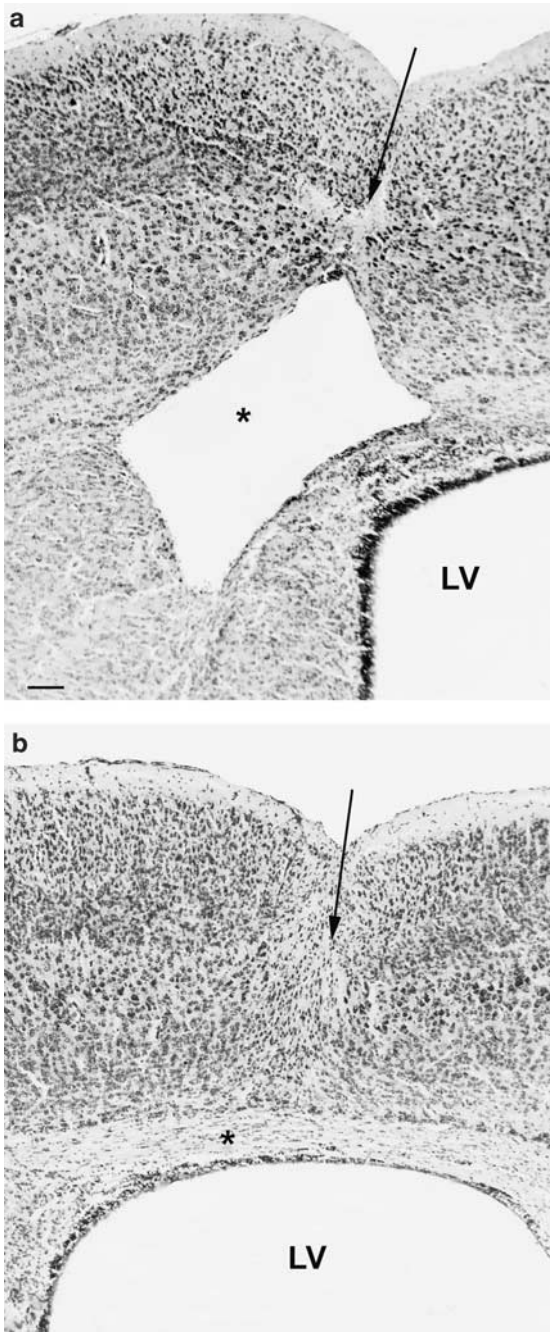


Figure 1 TPM protects against *S*-bromowillardiine-induced brain lesions. Cresyl violet-stained sections showing brain lesions induced by *S*-bromowillardiine injected at P5 and studied at the age of P10. (a) Brain from a pup cotreated with intracerebral *S*-bromowillardiine and i.p. vehicle, showing the typical neuronal loss in layers II–VI (arrow) and the white matter cystic lesion (*). (b) Brain from pup cotreated with intracerebral *S*-bromowillardiine and i.p. anandamide (10 mg kg^{-1}). LV, lateral ventricle. Bar = 40 μm.

protective effect was independent of the dose used. The addition of URB597 (0.3 mg kg^{-1}), an inhibitor of FAAH, to anandamide (1 mg kg^{-1}) did not enhance anandamide-induced neuroprotection (Figure 2).

In P5 mice, i.p. anandamide induced a significant and dose-dependant neuroprotection against *S*-bromowillardiine-induced cortical plate and white matter lesions when observed

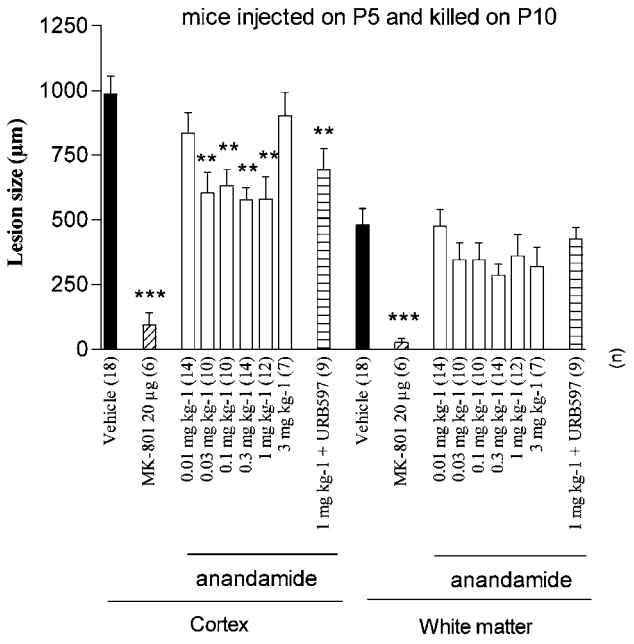


Figure 2 Anandamide has marginal effects on the size of ibotenate-induced lesions. Mouse pups were injected with intracerebral ibotenate on P5 and sacrificed on P10. Pups were injected with a single i.p. injection of vehicle, anandamide or anandamide + URB597 (0.3 mg kg^{-1}) immediately after ibotenate injection or with intracerebral MK-801 concomitantly with ibotenate. Bar represents mean length of the brain lesions at P10 + s.e.m. Asterisks indicate statistically significant difference from black bars; $^{**}P<0.01$; $^{***}P<0.001$ in ANOVA with Bonferroni's multiple comparison tests.

on P10 (Figures 1b and 3a) or P30 (Figure 3b). Similar effects were observed in rat pups injected on P5 and killed on P10 (Figure 3c). In mice, these neuroprotective effects of anandamide were mimicked by i.p. ACPA, a CB1 agonist but not by i.p. nabilone, a CB1 and CB2 agonist, and not by i.p. JWH133, a CB2 agonist (Figure 3d). In mice, anandamide neuroprotective effects against *S*-bromowillardiine were abolished by AM251, a CB1 antagonist, but not by AM630, a CB2 antagonist (Figure 3e).

When i.p. injection of anandamide followed the excitotoxic challenge, neuroprotection was a function of time. Protection was observed in mice receiving anandamide within the first 4 h after *S*-bromowillardiine administration (Figure 3f).

In rats, i.p. administration of anandamide (10 mg kg^{-1}, i.p.) largely prevented *S*-bromowillardiine-induced reduction of MBP staining when analyzed 9 days after the insult (Figure 4a and b). Similarly, when compared to a control PBS injection, i.p. injection of anandamide (10 mg kg^{-1}, i.p.) significantly increased the density of cells labelled with O4, a marker of preoligodendrocytes, when examined between 24 h after *S*-bromowillardiine administration (Figure 4c and e).

Quantitative real time PCR showed high levels of CB1 receptor mRNA while CB2 receptor mRNA was barely detectable in untreated P5 neocortex (Figure 5a). CB1 mRNA expression was not modified by intracerebral injection with ibotenate or *S*-bromowillardiine (Figure 5b). In contrast, CB2 mRNA expression was significantly enhanced 3 h after the injection of ibotenate of *S*-bromowillardiine (Figure 5c). However, the levels of CB2 mRNA were still much lower than the levels of CB1 mRNA.

Discussion

The present study provides experimental support for the consideration of endocannabinoids as a candidate therapy for reducing the risk of excitotoxic perinatal brain lesions. In this mouse model, endocannabinoids protected the developing white matter and cortical plate in a dose-dependent and long-lasting manner against an AMPA/kainate receptor-mediated challenge. Endocannabinoids had only a marginal effect on NMDA receptor-mediated cortical brain lesions. Endocannabinoid-induced neuroprotection of white matter involved increased survival of preoligodendrocytes and increased preservation of myelination.

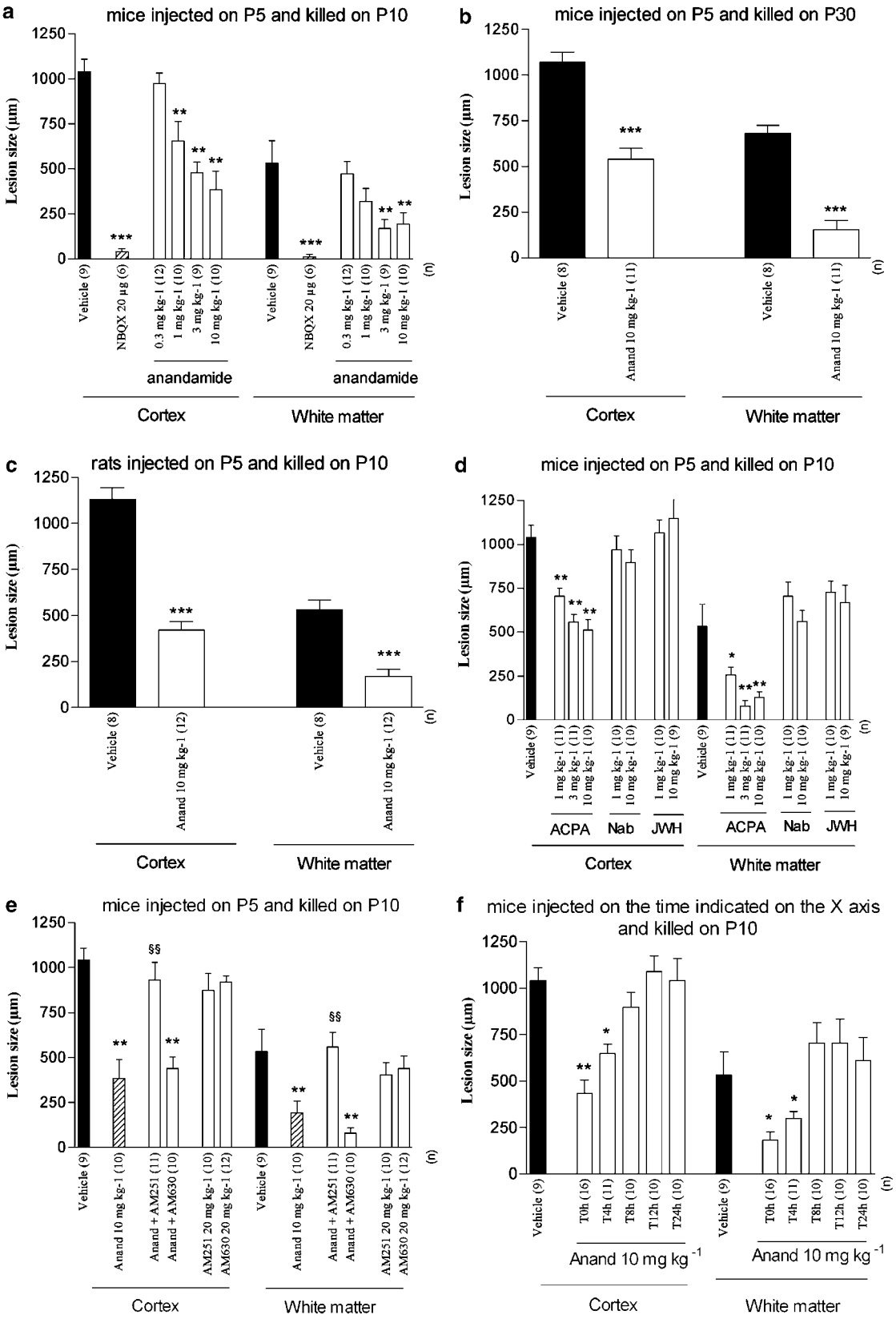
Neuroprotection conferred by endocannabinoids on excitotoxic injury in the developing brain: comparison of our findings with those of others

As mentioned above, only a few studies have addressed the potential neuroprotective effects of cannabinoids for the neonatal brain. In one study (Hansen *et al*., 2001), it was shown that anandamide accumulates in neonatal rat models of neurodegeneration. In another study (Martinez-Orgado *et al*., 2003), the authors showed neuroprotection by the cannabinoid agonist WIN-55212 in a newborn rat model of acute asphyxia. As it has been previously shown that, in this neonatal rat model, hypoxic-ischemic brain lesions are largely mediated by excess glutamate release (Ikonomidou *et al*., 1989; Hagberg *et al*., 1994), limitation of excitotoxic damage as demonstrated in the present study might underlie some of the neuroprotective effects afforded by WIN-55212 against hypoxic-ischemic insult. In addition, exogenously administered endocannabinoids have been shown to exert neuroprotection against neuronal excitotoxicity in cell culture and in adult rodents (Marsicano *et al*., 2003; Veldhuis *et al*., 2003; Chen *et al*., 2005).

Figure 3 Anandamide significantly mitigated *S*-bromowillardiine-induced lesions. Mouse or rat pups were injected with intracerebral *S*-bromowillardiine on P5. (a) Mouse pups were injected with a single i.p. injection of vehicle or anandamide immediately after *S*-bromowillardiine injection, or with intracerebral NBQX concomitantly with *S*-bromowillardiine. Pups were sacrificed on P10. (b) Mouse pups were injected with a single i.p. injection of vehicle or anandamide (Anand) immediately after *S*-bromowillardiine injection. Pups were killed on P30. (c) Rat pups were injected with a single i.p. injection of vehicle or anandamide (Anand) immediately after *S*-bromowillardiine injection. Pups were killed on P10. (d) Mouse pups were injected with a single i.p. injection of vehicle, ACPA, nabilone (Nab), or JWH133 (JWH) immediately after *S*-bromowillardiine injection. Pups were sacrificed on P10. (e) Mouse pups were injected with a single i.p. injection of drug of combination of drugs indicated on the X-axis immediately after *S*-bromowillardiine injection. Pups were killed on P10. (f) Mouse pups were injected with a single i.p. injection of anandamide administered at immediately, 4, 8, 12, or 24 h after *S*-bromowillardiine injection. Pups were sacrificed on P10. Bar represents mean length of the brain lesions at P10 or P30 + s.e.m. Asterisks indicate statistically significant difference from black bars; $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ in ANOVA with Bonferroni's multiple comparison tests.

Mechanistic basis for the neuroprotection conferred by endocannabinoids on excitotoxic brain lesions

Endocannabinoids have been shown to be neuroprotective through numerous mechanisms involving blockade of microglial activation (Ramirez *et al*., 2005), increase in brain-derived neurotrophic factor (Khaspekov *et al*., 2004), reduction of calcium influx (Nadler *et al*., 1993), and antioxidant activity (El-Remessy *et al*., 2003). Although all these mechanisms are potentially neuroprotective against neonatal excitotoxicity



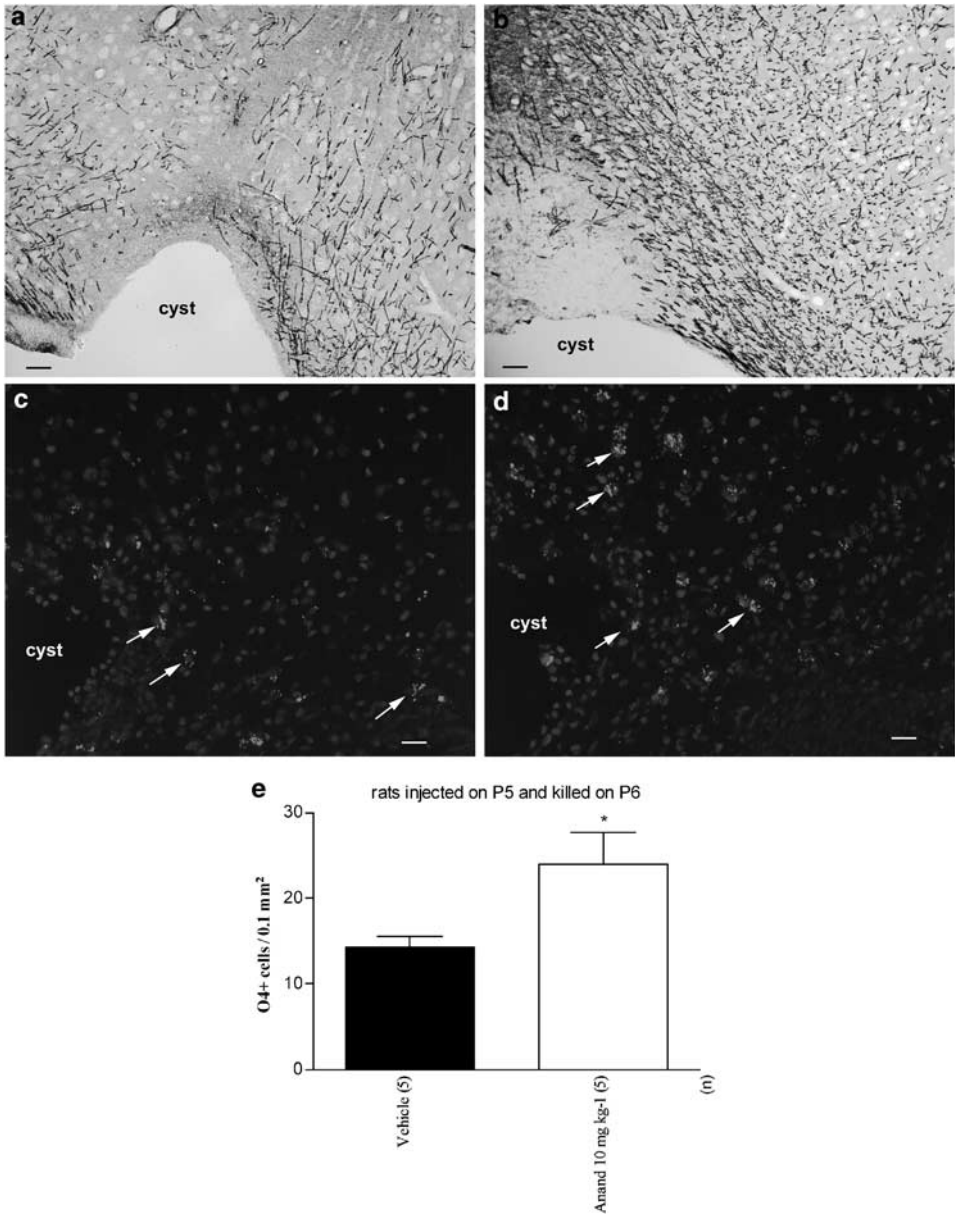


Figure 4 Effects of a single i.p. injection of anandamide (10 mg kg^{-1}) on *S*-bromowillardiine-induced changes in white matter myelin and pre-oligodendrocytes. (a, b) MBP immunostaining 9 days after *S*-bromowillardiine-vehicle (a) or *S*-bromowillardiine-anandamide (b) injection on P5. Bar = 40 μm. (c, d) O4 immunostaining 24 h after *S*-bromowillardiine-vehicle (c) or *S*-bromowillardiine-anandamide (d) injection on P5. Arrows point to examples of O4-positive cells. Bar = 20 μm. (e) Quantitative analysis of O4-stained cells in the periventricular white matter at the lesion site. Bars represent means ± s.e.m. Asterisk indicates statistically significant difference from vehicle group in a Student's *t*-test; $*P<0.05$.

(Gressens & Spedding, 2004), the precise mechanism(s) by which endocannabinoids are acting in the present study remain(s) to be determined.

Glutamate receptor-subtype specificity When comparing different glutamate receptors agonists, endocannabinoid-induced neuroprotection was much greater against an AMPA/kainate receptor-mediated insult when compared to NMDA receptor-mediated injury. These differences in neuroprotective effects of endocannabinoids probably reflect differences in mechanisms of injury when mediated by the different classes of glutamate receptor (Tahraoui *et al*., 2001).

The lack of dose-dependency of anandamide effects in the ibotenate model could be due to the rapid degradation of anandamide by the FAAH. However, two results argue against this hypothesis: (i) the addition of URB597, an inhibitor of the FAAH, did not enhance anandamide-induced neuroprotection; (ii) anandamide-mediated neuroprotection against *S*-bromowillardiine-induced lesions exhibits a classical dose–response curve.

CB receptors involved in the neuroprotective effects of endocannabinoids Several evidence support a key role of CB1 receptors in this endocannabinoid-mediated neuroprotection: (i) Endocannabinoid-induced neuroprotection was

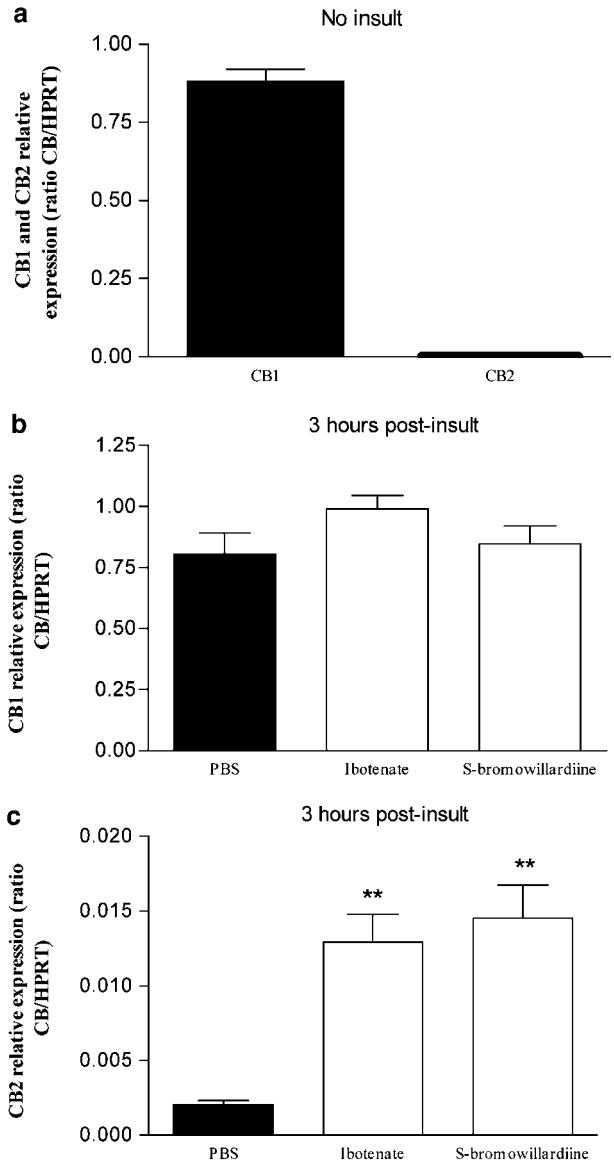


Figure 5 CB1 receptors are highly expressed in the P5 neocortex. (a) Real time PCR quantification of CB1 and CB2 receptor mRNAs in neocortices of untreated P5 mice. (b, c) Real time PCR quantification of CB1 (b) and CB2 (c) receptor mRNAs in neocortices of P5 mice intracerebrally injected 3 h earlier with PBS, ibotenate, or *S*-bromowillardiine. Data are presented as mean CB receptor/HPRT ratios + s.e.m. Asterisks indicate statistically significant difference from black bars; $^{**}P<0.01$ in ANOVA with Bonferroni's multiple comparison tests.

mimicked by a CB1 agonist (ACPA) and not by a CB2 agonist (JWH133). The lack of neuroprotective effect of nabilone, which acts on both CB1 and CB2 receptors, might be due to the fact that CB1 receptors activated by nabilone in the brain might be different from CB1 receptors activated by other cannabinoids (Diana *et al*., 2003). (ii) This neuroprotective effect was blocked by a CB1 antagonist (AM251) and not by a CB2 antagonist (AM630). (iii) Real-time PCR showed high levels of CB1 receptor mRNA while CB2 receptor mRNA was barely detectable in untreated P5 neocortex. We observed a similar profile of CB1 and CB2 mRNA expression in adult mouse brains (data not shown), in agreement with previous studies showing a predominance of CB1 receptors in the brain while CB2 receptors are largely expressed on the immune cells (Howlett *et al*., 2002).

In the present excitotoxic mouse model, a postinsult inflammatory response appears around 4 h following the glutamatergic agent, peaks around 24 h and last for several days after the insult (Tahraoui *et al*., 2001). This inflammatory response plays a deleterious role in the lesion and strategies targeting this immune response have been shown to be neuroprotective provided drugs are administered over a protracted period following the insult (Dommergues *et al*., 2003). In the present study, we observed, within a few hours after the excitotoxic insult, a significant increase in CB2 mRNA expression which could correspond to CB receptors expressed by inflammatory cells. In this context, CB2 agonists, which have been shown to limit inflammatory responses (Croxford and Yamamura, 2005), could also have a neuroprotective effect in the present model if they were given in a repeated manner following the excitotoxic insult.

Potential implications for the neuroprotection of human neonates

The mouse brain is lissencephalic, while its periventricular white matter thickness is much smaller and brain maturation is different than in humans, which limit the extrapolation of observations in rodents to humans. However, excitotoxic white matter lesions in the newborn mice mimic several key aspects of human periventricular leukomalacia, including the periventricular location, initial cystic appearance, secondary evolution towards a glial scar, early death of preoligodendrocytes, deleterious effect of inflammatory cytokines and the discrete ontogenetic window of the periventricular white matter's sensitivity to damage (Marret *et al*., 1995; Gressens *et al*., 1997; Dommergues *et al*., 2000; Follett *et al*., 2000; Tahraoui *et al*., 2001; Sfaello *et al*., 2005a). Similarly, excitotoxic cortical plate lesions mimic lesions observed in asphyxiated human term neonates (Ikonomidou *et al*., 1989; Hagberg *et al*., 1994; Marret *et al*., 1995).

The fact that endocannabinoids protected both periventricular white matter and cortical plate against an AMPA/kainate receptor-mediated challenge suggests that endocannabinoids could be a candidate therapy for preterm and term human neonates at risk of perinatal brain lesions. However, endogenous agents have specific inactivation systems and therefore may run less risk of interfering with ongoing developmental profiles than artificial ligands. This is an important issue, as endocannabinoids have major roles in embryonic implantation (associated with low anandamide levels), in neural development, in suckling (Fride, 2004a, b) and in developmental gene expression profiles (Fernandez-Ruiz *et al*., 2004). Furthermore, schizophrenia is a developmental disorder and cannabis use has been associated with its onset (Zammit *et al*., 2002; Fergusson *et al*., 2005). Prenatal exposure to the CB1 receptor agonist WIN 55,212-2 caused disruption of learning and decreased emotional reactivity of offspring and changes in NMDA function (Antonelli *et al*., 2005). Thus interventions targeted at the cannabinoid system need to be minimal during development (Bernard *et al*., 2005), and endogenous agonists may be less deleterious.

As previously shown (Follett *et al*., 2000; Volpe, 2001), white matter preoligodendrocytes express high levels of AMPA/kainate receptors and exhibit a selective vulnerability to overactivation of these receptors. This pathway seems to play a major role in the pathogenesis of white matter lesions observed in preterm infants. Therefore, modulating this pathway, as demonstrated in the present study with endocannabinoids, could represent an efficient neuroprotective strategy in this population at high risk to develop CP.

In conclusion, the present study demonstrates that endocannabinoids are neuroprotective in a mouse model of neonatal excitotoxic brain lesions mimicking brain damage observed in human neonates.

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