Cellular/Molecular

Dual Modulation of Endocannabinoid Transport and Fatty Acid Amide Hydrolase Protects against Excitotoxicity

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The endocannabinoid system has been suggested to elicit signals that defend against several disease states including excitotoxic brain damage. Besides direct activation with CB_1 receptor agonists, cannabinergic signaling can be modulated through inhibition of endocannabinoid transport and fatty acid amide hydrolase (FAAH), two mechanisms of endocannabinoid inactivation. To test whether the transporter and FAAH can be targeted pharmacologically to modulate survival/repair responses, the transport inhibitor *N*-(4-hydroxyphenyl)-arachidonamide (AM404) and the FAAH inhibitor palmitylsulfonyl fluoride (AM374) were assessed for protection against excitotoxicity *in vitro* and *in vivo*. AM374 and AM404 both enhanced mitogen-activated protein kinase (MAPK) activation in cultured hippocampal slices. Interestingly, combining the distinct inhibitors produced additive effects on CB_1 signaling and associated neuroprotection. After an excitotoxic insult in the slices, infusing the AM374/AM404 combination protected against cytoskeletal damage and synaptic decline, and the protection was similar to that produced by the stable CB_1 agonist AM356 (*R*-methanandamide). AM374/AM404 and the agonist also elicited cytoskeletal and synaptic protection *in vivo* when coinjected with excitotoxin into the dorsal hippocampus. Correspondingly, potentiating endocannabinoid responses with the AM374/AM404 combination prevented behavioral alterations and memory impairment that are characteristic of excitotoxic damage. The protective effects mediated by AM374/AM404 were (1) evident 7 d after insult, (2) correlated with the preservation of CB_1-linked MAPK signaling, and (3) were blocked by a selective CB_1 antagonist. These results indicate that dual modulation of the endocannabinoid system with AM374/AM404 elicits neuroprotection through the CB_1 receptor. The transporter and FAAH are modulatory sites that may be exploited to enhance cannabinergic signaling for therapeutic purposes.

Key words: AM374; AM404; cannabinoid CB_1 receptor; endocannabinoid system; hippocampus; neuroprotection

Introduction

Medicinal indications for cannabinoid drugs have expanded markedly in recent years. The growing list includes potential treatments for chemotherapy complications (Sharma et al., 2005), tumor growth (Bifulco et al., 2004), pain (Calignano et al., 1998), Parkinson's disease (Maccarrone et al., 2003; Fernandez-Espejo et al., 2004), Huntington's disease (Lastres-Becker et al., 2002), Alzheimer's disease (Ramirez et al., 2005), multiple sclerosis (Mestre et al., 2005), amyotrophic lateral sclerosis (Raman et al., 2004), glaucoma (El-Remessy et al., 2003), as well as traumatic brain injury (Panikashvili et al., 2001), cerebral ischemia (Nagayama et al., 1999), and other excitotoxic insults (Shen and Thayer, 1998; Marsicano et al., 2003). The protective effects may involve signal transduction pathways linked to cannabinoid CB_1 receptors that recognize the endocannabinoids anandamide and 2-arachidonylglycerol (Bouaboula et al., 1995; Derkinderen et al., 1998, 2003; Galve-Roperh et al., 2002; Karanian et al., 2005). Blocking CB_1 receptors pharmacologically was found to disrupt neuronal maintenance and increase excitotoxic vulnerability (Khaspekov et al., 2004; Karanian et al., 2005), and mice that lack the receptors were more susceptible to ischemia (Parmentier-Batteur et al., 2002) and seizure induction (Marsicano et al., 2003). The findings indicate that CB_1 signaling is important for neuronal survival.

In the brain, CB_1 receptors are joined by a specific transporter and a fatty acid amide hydrolase (FAAH) to make up the endocannabinoid system. The transporter and FAAH are part of a two-step process for cannabinergic inactivation. First, internalization of endocannabinoids is facilitated by the highly selective carrier-mediated transport system (Di Marzo et al., 1994; Beltramo et al., 1997; Hillard et al., 1997; Piomelli et al., 1999; Fegley et al., 2004). Inhibitors of the transport system have been shown to enhance the effects of exogenous cannabinoid ligands (Giuffrida et al., 2000; Fegley et al., 2004; Hájos et al., 2004). FAAH activity is the second step of endocannabinoid inactivation. The hydrolase is distributed throughout the brain and is thought to be the primary mediator for the hydrolysis of released endocannabinoids (Schmid et al., 1985; Cravatt et al., 1996, 2001; Tsou et al.,

1999; Egertova et al., 2003; Morozov et al., 2004). As with transport inhibition, disrupting FAAH activity through genetic and pharmacological means also enhances endocannabinoid signaling (Cravatt et al., 2001; Kathuria et al., 2003). Together, these studies indicate that cannabinoid signals can be enhanced by increasing the level of endogenous ligands with inhibitors of the endocannabinoid transporter and FAAH.

Here, we tested whether blocking endocannabinoid inactivation leads to a level of CB_1 signaling that is sufficient to protect against excitotoxicity *in vitro* and *in vivo*. By blocking inactivation processes with inhibitors of the transporter and FAAH, endogenous CB_1 signaling was enhanced. The selective inhibitors were combined to produce additive modulation of endocannabinoid tone. The efficient dual modulation of the endocannabinoid system was evaluated for neuroprotection against (1) molecular indicators of pathology in the excitotoxic hippocampal slice model, (2) pathogenic indicators *in vivo* after intrahippocampal injection of excitotoxin, and (3) functional deficits induced in the hippocampal lesion model.

Materials and Methods

Chemicals and antibodies. The cannabinoid compounds *R*-methanandamide (AM356), *N*-(4-hydroxyphenyl)-arachidonamide (AM404), palmitylsulfonyl fluoride (AM374), *N*-(morpholin-4-yl)-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM281), and *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM251) were synthesized as described previously (Abadji et al., 1994; Beltramo et al., 1997; Deutsch et al., 1997; Lan et al., 1999). Glutamatergic ligands and antagonists were obtained from Tocris (Ellisville, MO). The monoclonal antibody against synaptophysin was obtained from Chemicon (Temecula, CA). Affinity-purified antibodies to glutamate receptor 1 (GluR1) and to the calpain-mediated spectrin fragment BDP_N were also used (Bahr et al., 1995, 2002). Other antibodies used include those to the active form of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) (Cell Signaling, Beverly, MA), active focal adhesion kinase (FAK) (Upstate Biotechnology, Lake Placid, NY), synapsin II (Calbiochem, San Diego, CA), and actin (Sigma, St. Louis, MO).

Hippocampal slice cultures. Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) were housed following guidelines from the National Institutes of Health. As described previously (Bahr et al., 1995; Karanian et al., 2005), the brains were rapidly removed at 11–12 d postnatal, and 400 μm hippocampal slices were positioned on Millicell-CM inserts (Millipore, Bedford, MA). The cultures were supplied periodically with fresh media consisting of basal medium Eagle's (50%), Earle's balanced salts (25%), horse serum (25%), and defined supplements (Bahr et al., 1995). Slices were maintained in culture for a 15–20 d maturation period before experiments were initiated.

Activation of ERK and FAK. Hippocampal slices were incubated for 30 min at 37°C with 10–50 μM AM356 in the absence or presence of the CB_1 antagonist AM281 (10 μM). Other slices were treated with 100–500 nM AM374, 10–50 μM AM404, or a combination of the two in the absence or presence of either AM281 or 1, 4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126) (Calbiochem). The slices were then harvested in ice-cold buffer consisting of 0.32 M sucrose, 5 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.6 μM okadaic acid, 50 nM calyculin A, and a protease inhibitor mixture containing 4-(2-aminoethyl)-benzenesulfonyl fluoride, pepstatin A, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), bestatin, leupeptin, and aprotinin. Samples were homogenized in lysis buffer consisting of (in mM) 15 HEPES, pH 7.4, 0.5 EDTA, 0.5 EGTA, and the protease inhibitor mixture. Protein content was determined, and equal protein aliquots were assessed by immunoblot for the phosphorylated active form of FAK (pFAK) with antibodies specific for its Tyr397 phosphorylation site, and for the active ERK2 isoform (pERK2), with antibodies specific for MAPK kinase (MEK)-dependent phosphorylation sites in the catalytic core of ERK2, as described previously (Bahr et al., 2002; Karanian et al., 2005). Blots were stained routinely for a

protein load control (e.g., actin). Anti-IgG–alkaline phosphatase conjugates were used for secondary antibody incubation, and development of immunoreactive species was terminated before maximum intensity to avoid saturation. Integrated optical density of the bands was determined at high resolution with BIOQUANT software (R & M Biometrics, Nashville, TN).

In vitro excitotoxicity. Cultured hippocampal slices were treated with 100 μM AMPA for 20 min. Immediately after the insult, AMPA was removed and the excitotoxic stimulation was rapidly quenched with two 5 min washes containing glutamate receptor antagonists CNQX and MK801 (5-methyl-10,11-dihydro-5*H*-dibenzo[a,d]-cyclohepten-5,10-imine), as described previously (Bahr et al., 2002). The antagonists block any further activity of NMDA- and AMPA-type glutamate receptors, allowing for a controlled, reproducible excitotoxic insult. The slices were then incubated for 24 h with either 100 μM AM356 or the drug combination of AM374/AM404 (500 nM and 50 μM, respectively). At such time, slices were rapidly harvested, homogenized in lysis buffer, and analyzed by immunoblot for BDP_N, GluR1, and protein load control.

In vivo excitotoxicity. Adult Sprague Dawley rats (175–225 g) were anesthetized with a solution of ketamine (10 mg/kg, i.p.) and xylazine HCl (0.2 mg/kg, i.p.). Using stereotaxic coordinates (−5.3 mm from bregma, −2.5 mm lateral), a 2.5 μl injection was administered to the right dorsal hippocampus (−2.9 mm from skull surface). Vehicle consisted of 50% DMSO in a PBS solution. The insult included 63 nmol of AMPA excitotoxin in the absence or presence of AM356 (250 nmol) or the drug combination of AM374 (0.75 nmol) and AM404 (75 nmol). After the injections, wounds were sutured and the animals were placed back in their home cage for recovery. After 4–7 d, the brains were rapidly removed and either fixed in 4% paraformaldehyde or the dorsal hippocampal tissue was dissected and snap frozen in dry ice. The hippocampal tissue was homogenized in lysis buffer, and protein content was determined and assessed for cytoskeletal, synaptic, and signaling markers.

Histology. Brains fixed in paraformaldehyde were cryoprotected in 20% sucrose for 24 h. They were sectioned at 35 μm thickness using an American Optical (Buffalo, NY) AO860 precision sliding microtome and mounted on Superfrost slides (Fisher Scientific, Pittsburgh, PA). Tissue was Nissl stained, dehydrated through ethanol solutions, and coverslipped.

Behavioral testing. Animals that received intrahippocampal injections were assessed for motor changes 4–7 d after injection. The animals were placed in the center of a locomotor box, during which time the rodents' gross motor movements were monitored using a photobeam activity system as described previously (Kosten et al., 2005). Total move time was assessed as the mean number of seconds in which the animal exhibits motion during three 10 min sessions. A separate study evaluated memory function using a fear-conditioning paradigm slightly modified from one described previously (Kosten et al., 2005). Briefly, the rodents were placed in a chamber for 3 min and presented with seven pairings, each 1 min apart, of a 10 s tone (2.9 kHz, 82 dB) that coterminated with a 1 s footshock (1 mA). After training, animals received intrahippocampal injections of vehicle, AMPA, or AMPA with AM374/AM404. After 4–7 d, the rodents were placed back in the chamber and baseline movement assessed for 3 min. Freezing behavior (inactivity for ≥3 s) was then monitored as tone was delivered seven times, each 1 min apart.

Statistical analyses. Mean integrated densities for antigens and separate groups of behavioral data were evaluated using ANOVA and Tukey's *post hoc* tests.

Results

AM374 and AM404 promote CB_1 signaling

To test whether inhibitors of endocannabinoid transport and FAAH enhance CB_1 receptor responses, we used hippocampal slice cultures prepared from rats at postnatal days 12 and 13. The cultured slices express compensatory responses to injury and survival signaling pathways that are similar to those found in the adult brain (Bahr et al., 2002; Khaspekov et al., 2004; Karanian et al., 2005). As shown in Figure 1, *A* and *B*, stimulation of CB_1 receptors with the stable agonist AM356 results in the activation of ERK as well as FAK, a signaling event upstream of the ERK/
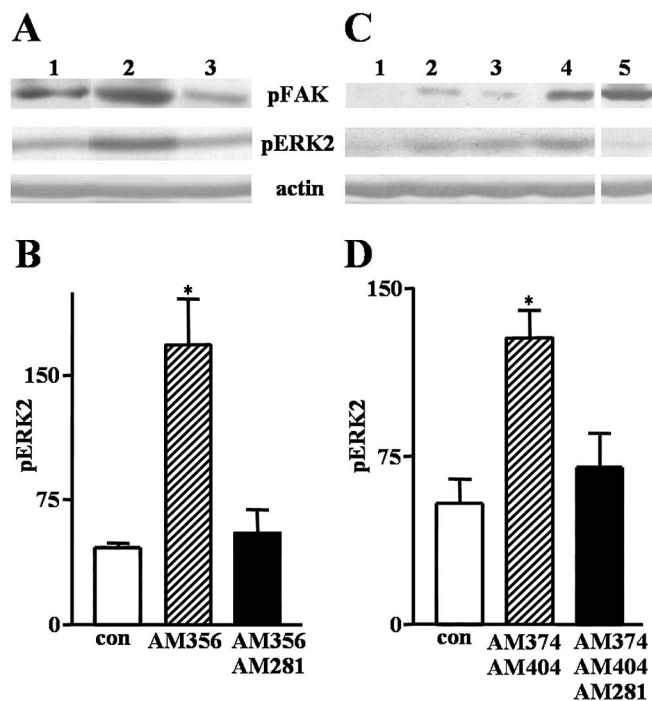
Figure 1. CB_1 signaling by direct receptor activation (**A**, **B**) and disruption of endocannabinoid inactivation (**C**, **D**). Hippocampal slice cultures were treated with selective agents for 30 min in groups of six to eight slices each and rapidly homogenized in the presence of phosphatase inhibitors for parallel assessment of pFAK, pERK2, and actin on single immunoblots. **A**, Untreated slices were used to determine basal antigen levels (lane 1), whereas other slices were exposed to the CB_1 agonist AM356 in the absence (lane 2) or presence (lane 3) of the antagonist AM281. **B**, Integrated optical densities for pERK2 (mean ± SEM) were determined by image analysis (ANOVA; $p = 0.014$). **C**, Treatment groups include control slices (lane 1) and slices treated with the FAAH inhibitor AM374 (lane 2), the transporter inhibitor AM404 (lane 3), the AM374/AM404 combination (lane 4), or AM374/AM404 with 20 μM U0126 (lane 5). **D**, Image analysis data for pERK2 are shown (ANOVA; $p = 0.015$). The asterisks represent significant *post hoc* tests compared with control and AM281-treated slices. con, Control.

Figure 2. Excitotoxic protection through direct CB_1 activation (**A**, **B**) and disruption of endocannabinoid inactivation (**C**, **D**) *in vitro*. Hippocampal slice cultures were infused with an excitotoxic level of AMPA for 20 min, followed by a 10 min washout period. A subset of cultures then received either the CB_1 agonist AM356 (10 μM) or the combination of FAAH inhibitor AM374 (0.5 μM) and transport inhibitor AM404 (50 μM). The slices were harvested 24 h after insult in groups of six to eight each, along with untreated sister cultures, and assessed by immunoblotting for calpain-mediated spectrin breakdown product BDP_N (**A**, **C**) and the postsynaptic marker GluR1 (**B**, **D**). Representative antigen staining is shown with a protein load control (con) from the same blots. The bar graphs contain integrated optical densities (mean ± SEM) as determined by image analysis.

MAPK pathway (Derkinderen et al., 1998; Karanian et al., 2005). The active pFAK and pERK2 were increased over basal levels expressed by control slices.

Activation of ERK and FAK also occurred when 100 nM of the FAAH inhibitor AM374 and 10 μM of the transport inhibitor AM404 were applied individually to the cultures (Fig. 1C). Lower concentrations of the inhibitors were less effective at activating the signaling pathways. As a control, actin was found not to change with drug treatment, and total FAK and total ERK levels were shown previously to be unaffected. Interestingly, blocking both mechanisms of endocannabinoid inactivation with the AM374/AM404 combination resulted in an additive effect, producing a comparable level of cannabinergic signaling as that produced by the agonist AM356. The drug combination increased pFAK by 140–190% and pERK2 by 60–100% over those levels activated by the individual drugs. As also shown in Figure 1C (lane 5), inhibiting the upstream activator of MAPK, MEK, caused selective blockage of AM374/AM404-mediated ERK activation, as reported previously for agonist-induced ERK activation (Karanian et al., 2005). The effects of agonist treatment (Fig. 1B) as well as endocannabinoid potentiation by AM374/AM404 (Fig. 1D) were prevented by the CB_1 antagonist AM281. The data indicate that indirect pharmacological modulation of the endocannabinoid system is an efficient strategy to promote signaling through CB_1 receptors.
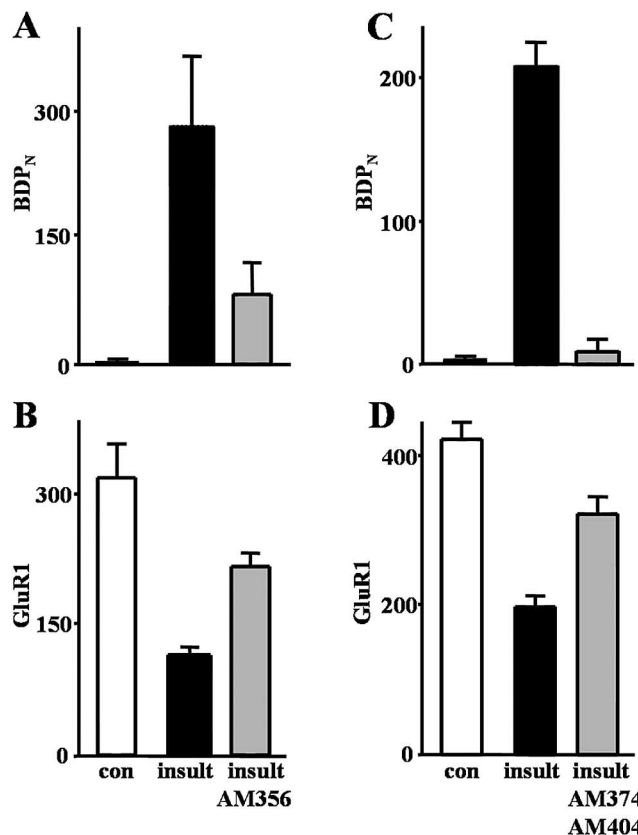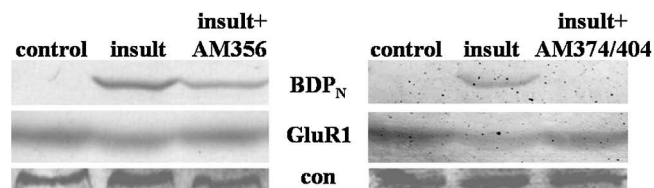
Neuroprotection *in vitro*

Indirect endocannabinoid potentiation with AM374/AM404 was tested for protective features in the excitotoxic hippocampal slice model and compared with the actions of a CB_1 agonist. The slice cultures were subjected to excitotoxic stimulation of AMPA-type glutamate receptors for 20 min, resulting in persistent cytoskeletal damage indicated by the calpain-mediated spectrin breakdown product BDP_N evident 24 h later. Post-insult activation of cannabinoid responses with the agonist AM356 reduced the cytoskeletal damage by 72% (Fig. 2A). Note that we also found, as shown by others (Shen and Thayer, 1998), that the cannabinergic system is effective against the overactivation of NMDA receptors. Here, NMDA-induced BDP_N levels [231 ± 39 (mean ± SEM); $n = 8$] were significantly reduced by activating CB_1 receptors (89 ± 19; $n = 8$; $p < 0.01$).

In the AMPA-treated slice cultures, the cytoskeletal break-

Table 1. Improved cytoskeletal and synaptic protection with dual blockage of endocannabinoid inactivation

Drug treatment	Reduction in BDP_N	Recovery of GluR1
AM374	$36 \pm 12\%$	$25 \pm 6\%$
AM404	$22 \pm 7\%$	$20 \pm 6\%$
AM374/AM404	$96 \pm 8\%*$	$56 \pm 5\%*$

Slice cultures were subjected to an AMPA insult as in Figure 2 and received AM374 (0.5 μM), AM404 (50 μM), or a combination of the two drugs. Slices harvested 24 hr, after insult were assessed for BDP_N and GluR1, and percentage changes from insult-alone slices were determined (ANOVAs; $p = 0.001$ and 0.003, respectively). The mean percentages $\pm$ SEM are listed ($n = 5$–8). $*p < 0.01$, *post hoc* tests compared with individual drug data.

down marker was also reduced by the AM374/AM404 combination, in this case by 96% (Fig. 2C). Note that excitotoxic calpain activation is commonly associated with reductions in synaptic markers (Riederer et al., 1992; Vanderklish and Bahr, 2000; Bahr et al., 2002; Munirathinam et al., 2002; Karanian et al., 2005), and across slice samples, AM356 (Fig. 2B) and AM374/AM404 (Fig. 2D) both attenuated the loss of the postsynaptic marker GluR1 by 50–70% (ANOVAs; $p < 0.01$). Thus, dual blockage of endocannabinoid inactivation with AM374 and AM404 produced the same level of excitotoxic protection *in vitro* as that produced by the direct activation of CB_1 receptors. When applied separately, AM374 and AM404 were less effective with regard to cytoskeletal and synaptic protection (Table 1).

Neuroprotection *in vivo*

The AM374/AM404 drug combination and the AM356 agonist were tested in an *in vivo* model of excitotoxic brain damage. To initiate excitotoxicity in adult rats, 63 nmol of AMPA were injected unilaterally into the dorsal hippocampus. The dorsal half of the hippocampus was dissected 4–7 d later, and the ipsilateral tissue exhibited a pronounced level of cytoskeletal breakdown as well as reductions in presynaptic and postsynaptic markers (Fig. 3A). As in the slice model, the excitotoxic cytoskeletal breakdown *in vivo* correlated with synaptic decline ($r = -0.74$; $p < 0.0001$), and cannabinoid responses were protective against the pathogenic manifestations. When AM356 was coinjected with the AMPA insult, spectrin BDP_N evident 4–7 d after insult was reduced by 78% (Fig. 3B). A similar reduction in cytoskeletal damage was found when endocannabinoid inactivation was blocked with AM374/AM404 during the excitotoxic insult (Fig. 4C). In fact, the drug combination provided what appeared to be complete cytoskeletal protection in 9 of the 12 animals examined.

In addition to the protective effects on cytoskeletal integrity, AM356 reduced the postsynaptic GluR1 decline evident in the excitotoxic animals by an average of 58% (Fig. 3C). The presynaptic marker synapsin II was protected by a similar level (Fig. 3A). As with cytoskeletal protection, blocking endocannabinoid inactivation provided a similar, if not higher, degree of synaptic protection, as did the CB_1 receptor agonist. The AM374/AM404 combination protected GluR1 levels by an average of 74% (Fig. 4D). When individual animals were assessed, 8 of the 12 that received AM374/AM404 exhibited >90% preservation of the postsynaptic marker. Also, the presynaptic markers synapsin II and synaptophysin were almost completely protected by the drug combination (Fig. 4A). The effects of AM374/AM404 on cytoskeletal (Fig. 4C) and synaptic protection (Fig. 4D) were blocked by the CB_1 antagonist AM251, indicating that the effects were mediated through CB_1 receptors. These results indicate that direct and indirect activation of CB_1 signaling provides similar protection *in vivo*.

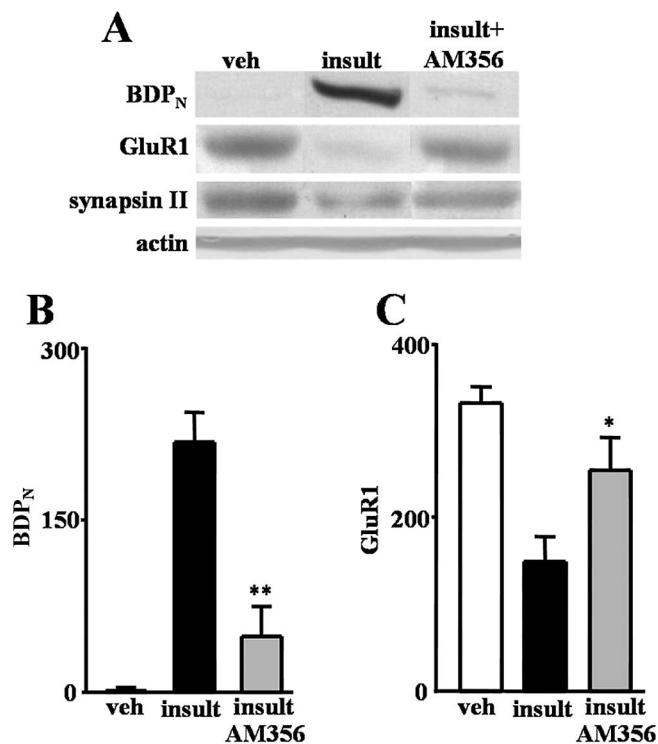Interestingly, blocking endocannabinoid transport and hy-

Figure 3. Direct CB_1 activation protects against hippocampal excitotoxicity *in vivo*. Groups of adult rats received a 2.5 μl unilateral injection into the dorsal hippocampus, containing vehicle only (veh; $n = 10$), 63 nmol of AMPA (insult; $n = 19$), or AMPA coadministered with 250 nmol of the CB_1 agonist AM356 ($n = 12$). At 4–7 d after injection, the brains were rapidly removed under ice-cold conditions containing protease inhibitors, and the ipsilateral dorsal hippocampus was dissected for immunoblotting. Spectrin breakdown product BDP_N, postsynaptic marker GluR1, presynaptic marker synapsin II, and actin were assessed on single immunoblots (**A**). Mean integrated optical densities $\pm$ SEM are shown for BDP_N (**B**; ANOVA; $p < 0.0001$) and GluR1 (**C**; $p < 0.001$). $*p < 0.05$, $**p < 0.001$, *post hoc* tests compared with insult-only data.

drolysis not only produced cytoskeletal and synaptic protection against the AMPA insult but also maintained important signaling pathways. Basal levels of activated pFAK and pERK were maintained by AM374/AM404 at levels found in vehicle-treated animals (Fig. 4B, compare lanes 1, 3). In addition, the AM251 antagonist correspondingly abolished the effects of AM374/AM404 on cytoskeletal protection, synaptic protection, and the maintenance of the FAK and ERK/MAPK pathways (Fig. 4A, B, lane 4). Pathogenic changes remained similar to those found with insult alone despite the coinjection of AM374/AM404; thus, the antagonist eliminated any indication of protection and did so without worsening the excitotoxic damage. None of the antigens tested were altered when the single AM251 injection was administered alone (Fig. 4A, B, lane 5), and the lack of cytoskeletal damage or synaptic decline confirmed that no toxicity is involved. Together, the results strongly suggest that the neuroprotection by AM374/AM404 is mediated through CB_1 receptor responses.

To confirm cellular protection by the AM374/AM404 combination, brains from the different treatment groups were rapidly dissected at 7 d after insult, fixed, and sectioned for staining by Nissl. Coronal sections confirmed the unilateral damage at the excitotoxin injection site in the dorsal hippocampus (Fig. 5A). In addition, the contralateral hippocampus had no evidence of spectrin BDP_N or associated synaptic decline as found in the ipsilateral tissue (Fig. 5B). The AMPA insult also caused a pronounced decrease in density of CA1 pyramidal neurons in the ipsilateral hippocampus, as well as an increase in pyknotic nuclei (Fig. 5D)
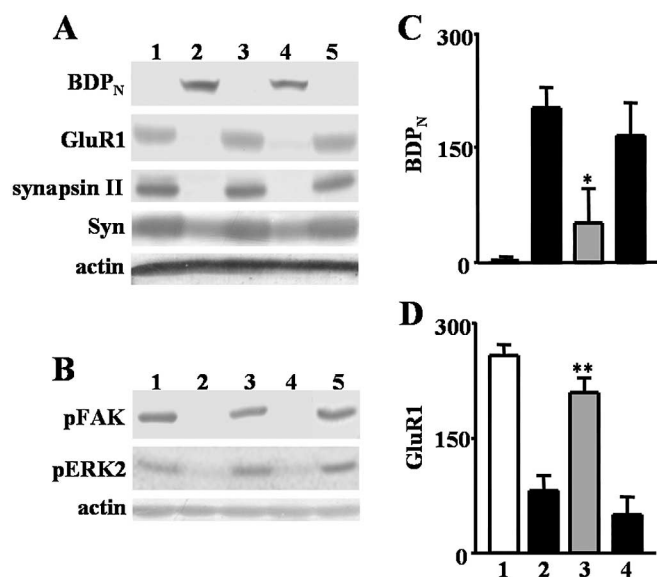
Figure 4. Disruption of endocannabinoid inactivation protects against hippocampal excitotoxicity *in vivo*. Adult rats received a 2.5 μl unilateral injection into the dorsal hippocampus, containing vehicle only (group 1; $n = 13$), a 63 nmol AMPA insult (group 2; $n = 20$), the AMPA insult coadministered with 0.75 nmol of AM374 and 75 nmol of AM404 (group 3; $n = 12$), AMPA coadministered with both the AM374/AM404 combination and 43 nmol of the CB_1 antagonist AM251 (group 4; $n = 5$), or AM251 alone (lane 5; $n = 3$). At 4–7 d after injection, ipsilateral dorsal hippocampal tissue was rapidly dissected using ice-cold buffers with protease inhibitors. *A, B*, Protection by the endocannabinoid transport and FAAH inhibitors is evident in blot samples from the five treatment groups (lanes 1–5, respectively), and the protection was blocked by AM251. AM251 alone had no effect on the six antigens stained. Syn, Synaptophysin. Mean integrated optical densities $\pm$ SEM are shown for BDP_N (*C*; ANOVA; $p < 0.0001$) and GluR1 (*D*; $p < 0.0001$). *$p < 0.05$, **$p < 0.001$, *post hoc* tests compared with insult-only data.

compared with tissue from control rats (Fig. 5C). Previous studies have reported a correspondence between calpain-mediated spectrin breakdown and subsequent cell death in the hippocampal CA1 subfield (Lee et al., 1991; Roberts-Lewis et al., 1994; Bahr et al., 2002). Accordingly, the AM374/AM404 drug combination
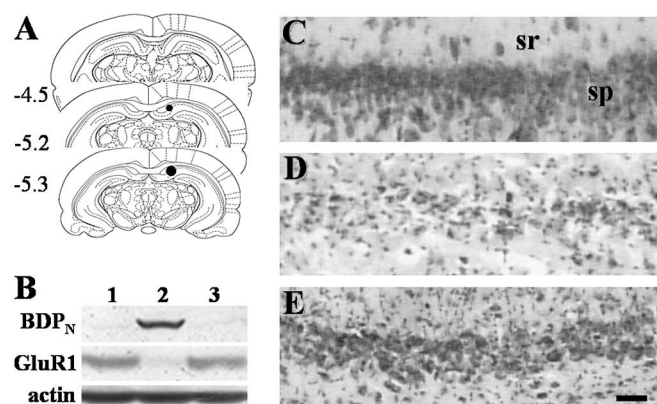

Figure 5. Disruption of endocannabinoid inactivation promotes cell survival. Rats subjected to dorsal hippocampal injections as in Figure 4 were killed 7 d after insult, and the brains were rapidly fixed for histology or dissected for immunoblot analyses. *A*, Nissl-stained coronal sections from nine animals confirmed lesion size and location as depicted by the black circles (millimeter values are posterior to bregma). *B*, The ipsilateral dorsal hippocampus from rats injected with vehicle only (lane 1) or the 63 nmol AMPA insult (lane 2) were assessed for BDP_N and GluR1 along side the contralateral sample from the AMPA-treated animal (lane 3). Photomicrographs of the ipsilateral CA1 field are shown for animals injected with vehicle (*C*), AMPA (*D*), or AMPA coadministered with 0.75 nmol of AM374 and 75 nmol of AM404 (*E*). Scale bar, 30 μm. sp, Stratum pyramidale; sr, stratum radiatum.
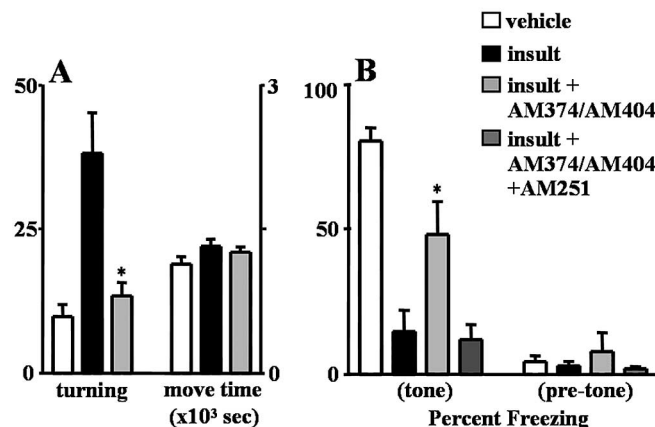

Figure 6. Disruption of endocannabinoid inactivation provides functional protection in the excitotoxic rat. *A*, In five to eight rats per treatment group, a 2.5 μl unilateral injection was administered into the dorsal hippocampus, containing vehicle only, a 63 nmol AMPA insult, or the AMPA insult coadministered with 0.75 nmol of AM374 and 75 nmol of AM404. At 4–7 d after injection, the animals were tested for turning behavior. Administration of AM374/AM404 during the insult reduced AMPA-induced turning (left bars; ANOVA; $p < 0.01$), whereas the mean move time remained unchanged (right bars). *B*, Using a fear-conditioning paradigm, different animals ($n = 13$–14) were conditioned to seven pairings of a tone that coterminated with footshock and were subjected to the intrahippocampal injections. At 4–7 d after injection, the animals were presented with tone in the absence of shock and assessed for freezing behavior. The AMPA insult alone caused memory impairment (left bars). Inhibiting endocannabinoid hydrolysis and transport with AM374/AM404, applied during the insult, reduced the memory impairment measured 4–7 d after injection (ANOVA; $p < 0.0001$). The baseline activity measured before the onset of the tone was unchanged across treatment groups (right bars). *$p < 0.05$, *post hoc* tests compared with insult alone.

that reduced excitotoxic cytoskeletal breakdown was found to prevent neuronal death and pyknotic changes (Fig. 5E).

Functional protection

Two behavioral correlates of neuronal damage were used to test the AM374/AM404 combination for functional protection. The first involves perseverative turning shown to be induced by AMPA injections into the brain and by selective hippocampal damage (Mickley et al., 1989; Smith et al., 1996). As shown in the left data set of Figure 6A, intrahippocampal AMPA injections were found associated with steady, slow bouts of turning assessed 4–7 d after insult. The excitotoxic brain damage caused a fourfold to sixfold increase over the basal turning exhibited by vehicle-injected control animals. The increased turning was reduced to near control levels when endocannabinoid inactivation was disrupted by coinjecting AM374 and AM404 with the excitotoxin (ANOVA; $p = 0.0017$). The reduction in turning was more pronounced than that produced by the agonist AM356 (data not shown). Although enhancing cannabinoid responses would be expected to reduce motor behavior, this is not likely the case here because animals were assessed for exploratory movement several days after drugs were administered. In addition, total move time in the locomotor box per session was not changed in drug-treated animals or by the insult itself (Fig. 6A, right data set). Thus, assessment of a selective behavioral disturbance further supports that the AM374/AM404 combination protects against excitotoxic brain damage.

The second behavioral correlate of brain damage measured was memory impairment. A fear-conditioning paradigm was used because this learning task is sensitive to electrolytic and excitotoxic lesions in the dorsal hippocampus (Anagnostaras et al., 1999; Zou et al., 1999). Rats were trained to fear an innocuous

stimulus, in this case consisting of both the context of an operant chamber and a tone. Seven pairings of a 10 s tone with an aversive footshock were given 2–2.5 h before the unilateral AMPA injection into the dorsal hippocampus. When re-exposed to the conditional environment/tone 4–7 d later, control rats exhibited the adaptive fear response of freezing, whereas AMPA-injected rats did not (Fig. 6B, left data set). Thus, the excitotoxic damage decreased fear conditioning likely by impairing consolidation processes of the hippocampus. As evident in Figure 6B, endocannabinoid inactivation with the AM374/AM404 combination protected such hippocampal processes and, as a result, significantly reduced the memory impairment causing improved fear responses (ANOVA; $p < 0.0001$). The rats demonstrated storage of the conditioning chamber and tone, and these are the same animals that exhibited pronounced reduction in spectrin breakdown product and recovery of synaptic markers. Moreover, reduced BDP_N levels exhibited a significant correlation with improved performance in the fear-conditioning task ($r = -0.68$; $p < 0.01$), as did increased levels of the postsynaptic protein GluR1 ($r = 0.73$; $p < 0.01$). The CB_1 antagonist AM251 blocked the AM374/AM404-mediated functional protection (Fig. 6B), corresponding with its blockage effects on cytoskeletal and synaptic protection. As a control, baseline locomotor activity was determined in a subset of animals during a time period immediately before testing for memory storage. Baseline freezing was characteristically low and did not differ across the treatment groups (Fig. 6B, right data set). Together, these results demonstrate that indirect endocannabinoid potentiation protects against excitotoxic hippocampal damage that disrupts memory consolidation and/or recall.

Discussion

The data presented indicate that the endocannabinoid transporter and FAAH are sites of modulation that allow pharmacological enhancement of protective endocannabinergic signals. Selective inhibitors of the transporter and FAAH caused additive augmentation of endogenous signaling events mediated by the cannabinoid CB_1 receptor. Disruption of such signals has been shown to prevent neuronal maintenance processes and increase vulnerability to brain damage (Parmentier-Batteur et al., 2002; Marsicano et al., 2003; Khaspekov et al., 2004; Karanian et al., 2005). Here, blocking endocannabinoid inactivation enhanced cannabinergic activity and ameliorated cellular disturbances associated with excitotoxicity. Modulating the endocannabinoid system in this way also prevented excitotoxic behavioral abnormalities including memory impairment. Collectively, these results indicate that increasing endocannabinoid responses leads to molecular, cellular, and functional protection against excitotoxic insults such as stroke and traumatic brain injury.

The dual modulation of the endocannabinoid system used a combination of the transport inhibitor AM404 and the FAAH inhibitor AM374. AM404 has been shown to enhance the action of anandamide *in vivo* (Fegley et al., 2004) and in hippocampal slices (Hájos et al., 2004). AM374 also has been shown to increase the levels of the endocannabinoid in neuroblastoma cells (Deutsch et al., 1997) and to increase the modulatory action of low-level exogenous anandamide in the hippocampus (Gifford et al., 1999). Separately, the two drugs were found to trigger cannabinergic activation of the FAK and ERK/MAPK pathways in the cultured hippocampal slice model. In combination they caused pronounced FAK and ERK responses similar to those triggered by a CB_1 agonist, and the ERK activation was selectively blocked by a MEK inhibitor. The ERK pathway is of particular interest because

it promotes synaptic maintenance and cell survival (Bahr et al., 2002; Marsicano et al., 2003) and thus may be related to the excitotoxic protection elicited by endocannabinoids. Activation events elicited by AM374/AM404 were also blocked by the CB_1 antagonist AM281. These data indicate that disruption of two distinct mechanisms of endocannabinoid inactivation, transport and hydrolysis, causes potentiation of endocannabinoid tone.

The hippocampus is abundant in cannabinoid CB_1 receptors, which are found in many brain regions (Herkenham et al., 1990; Tsou et al., 1999). A second type of cannabinoid receptor, the CB_2 class, is only present in the periphery (Griffin et al., 1999; Palmer et al., 2002). The relationship between CB_1 receptors and prosurvival FAK and ERK signaling seems to support the maintenance of hippocampal neurons (Derkinderen et al., 1998; Karanian et al., 2005) and may explain the protective properties linked to the CB_1 receptors (Nagayama et al., 1999; Panikashvili et al., 2001; Maccarrone et al., 2003; Marsicano et al., 2003). In addition, the CB_1 receptors are involved in other repair systems including brain-derived neurotrophic factor that is dependent on the ERK/MAPK pathway (Derkinderen et al., 2003; Khaspekov et al., 2004) and phosphatidylinositol 3-kinase (PI3K) that makes up a potential FAK/PI3K/ERK repair pathway (Galve-Roperh et al., 2002; Liu et al., 2003). It has also been suggested that activated CB_1 receptors lead to the inhibition of voltage-sensitive calcium channels (Mackie and Hille, 1992; Guo and Ikeda, 2004), perhaps contributing to reductions in excitotoxic progression.

Levels of endocannabinoids are elevated after neuronal injury (Panikashvili et al., 2001; Maccarrone et al., 2003; Marsicano et al., 2003), indicating a potential compensatory response comprising possibly several CB_1-linked signaling events involved in cellular repair. The present report suggests that such compensatory signaling can be positively modulated through the inhibition of endocannabinoid transport and hydrolysis with the AM374/AM404 drug combination. Previous reports have described various types of beneficial effects by individually inhibiting the transport activity (Lastres-Becker et al., 2002; Marsicano et al., 2003; Bifulco et al., 2004; Fernandez-Espejo et al., 2004; Mestre et al., 2005) or FAAH (Maccarrone et al., 2003; Bifulco et al., 2004). *In vitro* and *in vivo* models were used here to show that dual modulation of the endocannabinoid system protects against cellular and functional consequences ascribed to excitotoxic events, such as stroke and traumatic brain injury.

Cytoskeletal damage was assessed by measuring the calpain-mediated spectrin breakdown product BDP_N, a sensitive precursor to neuronal pathology in animal models of excitotoxic insults (Lee et al., 1991; Saido et al., 1993; Roberts-Lewis et al., 1994; Bahr et al., 1995, 2002; Saatman et al., 1996; Munirathinam et al., 2002; Zhu et al., 2005) and human brain injury (McCracken et al., 1999). AM374/AM404 reduced the excitotoxic damage induced in the hippocampus. The level of cytoskeletal protection produced by the drug combination was similar, if not more robust, than that elicited by the stable CB_1 agonist AM356. Correspondingly, the AM374/AM404 combination that promotes cytoskeletal protection also was found to promote cell survival.

Disrupting endocannabinoid inactivation with AM374/AM404 also provided synaptic protection. Synaptic and dendritic compromise is often found associated with calpain-induced cytoskeletal damage (Neumar et al., 2001; Bahr et al., 2002; Munirathinam et al., 2002). As shown here, AMPA-induced excitotoxic progression caused presynaptic and postsynaptic decline in the hippocampal slice model. *In vivo*, the pronounced level of synaptic decline evident in dorsal hippocampal tissue samples indicates global synaptopathogenesis produced by the excito-

toxic insult. As with cytoskeletal protection, indirect modulation of the endocannabinoid system with AM374/AM404 produced the same or better synaptic protection compared with treatment with the CB$_1$ agonist. The protection of synaptic integrity would provide an added beneficial effect because the activity of glutamatergic synapses in the hippocampus is vital for neuronal maintenance (Bambrick et al., 1995; Bahr et al., 2002). Thus, the neuroprotective effects may have extended beyond the site at which AM374/AM404 was injected. In addition, the modulators of the endocannabinoid system preserved basal levels of activated FAK and ERK, two kinases involved in synaptic maintenance signaling and the levels of which are dramatically reduced in the *in vivo* model of excitotoxicity.

In addition to the cytoskeletal, synaptic, and cellular protection, two behavioral correlates of excitotoxic brain damage were also reduced by the AM374/AM404 drug combination, implicating endocannabinoid modulation in the protection of normal brain function. The selective disturbance of perseverative turning in the unilateral excitotoxic rat model was prevented when AM374/AM404 was coadministered with the insult. The excitotoxic animals also exhibited memory impairment as indicated by the lack of recall of pre-insult fear conditioning. The memory dysfunction was significantly prevented by the dual blockage of endocannabinoid inactivation mechanisms. Of particular interest is the corresponding preservation of the glutamate receptor subunit GluR1, synaptic vesicle markers, and basal levels of active ERK. Note that GluR1 (Schmitt et al., 2004), the synaptic vesicle protein synaptotagmin IV (Ferguson et al., 2000), and the ERK/MAPK pathway within hippocampal neurons (Shalin et al., 2004) have been shown to play important roles in memory functions, including fear conditioning. The results described here show that indirect enhancement of endocannabinoid responses protects against excitotoxic hippocampal damage and preserves mechanisms necessary for memory encoding.

To summarize, blocking endocannabinoid inactivation with the drug combination of AM374 and AM404 protects against excitotoxicity both *in vitro* and *in vivo*. Such enhancement of cannabinergic responses may be a strategy with which to reduce potential problems stemming from chronic treatment with CB$_1$ agonists. The results support the hypothesis that transporter and FAAH inhibition indirectly enhance neuroprotective signaling, and this represents a unique approach for dual modulation of the endocannabinoid system for therapeutic purposes.

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