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TITLE PAGE

Endocannabinoid Enhancement Protects Against Kainic Acid-Induced Seizures and Associated Brain Damage

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Abbreviations:

AM374 = palmitylsulfonyl fluoride; AM251 = N-(piperidin-1-yl)-5-(4-iodophonyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; FAAH = fatty acid amide hydrolase; KA = kainic acid; MAPK = mitogen-activated protein kinase; ERK = extracellular signal regulated-kinase; AEA = anandamide; CB1 = cannabinoid CB1 receptor; BDP = spectrin breakdown product; SEM = standard error of the mean

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ABSTRACT

Endocannabinoids are released in response to pathogenic insults, and inhibitors of endocannabinoid inactivation enhance such on-demand responses that promote cellular protection. Here, palmitylsulfonyl fluoride (AM374), an irreversible inhibitor of fatty acid amide hydrolase (FAAH), was injected IP into rats to test for systemic endocannabinoid enhancement. AM374 caused a prolonged elevation of anandamide levels in several brain regions including the hippocampus, and resulted in rapid activation of the extracellular signal regulated-kinase / mitogen-activated protein kinase (ERK/MAPK) pathway that has been linked to survival. To evaluate the neuroprotective nature of the FAAH inhibitor, we tested AM374 in a seizure model involving rats insulted with kainic acid (KA). AM374 was injected immediately following KA administration, and seizure scores were significantly reduced throughout a 4-h observation period. The KA-induced seizures were associated with calpain-mediated cytoskeletal breakdown, reductions in synaptic markers, and loss of CA1 hippocampal neurons. FAAH inhibition protected against the excitotoxic damage and neuronal loss assessed 48 h post-insult. AM374 also preserved pre- and postsynaptic markers to levels comparable to those found in non-insulted animals, and the synaptic marker preservation strongly correlated with reduced seizure scores. Regarding behavioral deficits in the excitotoxic rats, AM374 produced nearly complete functional protection, significantly improving balance and coordination across different behavioral paradigms. These data indicate that AM374 crosses the blood brain barrier, enhances endocannabinoid responses in key neuronal circuitries, and protects the brain against excitotoxic damage.

INTRODUCTION

Increased understanding of the endogenous cannabinoid (endocannabinoid) system in recent years has led to a marked expansion in the medicinal indications for cannabinoid drugs (for reviews see Grundy, 2002; Karanian and Bahr, 2006). The endocannabinoid system has been identified as a potential target for the treatment of several disorders of the central nervous system (Lastres-Becker et al., 2002; Maccarrone et al., 2003; Ramirez et al., 2005; Marsicano et al., 2003), including epilepsy and excitotoxicity (Marsicano et al., 2003; Karanian et al., 2005b; Monory et al., 2006). Endocannabinoid responses indeed have been linked to protection against a variety of neuropathogenic insults.

The neuroprotective effects elicited by the endocannabinoid system may involve modulation of ion channels (Deadwyler et al., 1993; Shen and Thayer, 1996, 1998; Mu et al., 1999) as well as signaling pathways elicited by cannabinoid CB1 receptors. The CB1 receptor, which recognizes the endocannabinoids anandamide (AEA) and 2-arachidonoyl glycerol, is linked to several pro-survival signaling cascades including phosphatidylinositol-3 kinase (PI3K), protein kinase B/Akt, brain-derived neurotrophic factor (BDNF), focal adhesion kinase (FAK), mitogen-activated protein kinase (MAPK) and its subtype extracellular signal regulated-kinase (ERK) (Bonni et al., 1999; Galve-Roperh et al., 2002; Derkinderen et al., 2001; 2003; Gomez del Pulgar et al., 2002; Molina-Holgado et al., 2002; Derkinderen et al., 2003; Khaspekov et al., 2004; Karanian et al., 2005a/b). Disruption of CB1 receptor signaling has been shown to increase excitotoxic vulnerability and seizure susceptibility, as well as attenuate neuronal maintenance (Wallace et al., 2002, 2003; Marsicano et al., 2003; Clement et al., 2003; Khaspekov et al., 2004; Karanian et al., 2005a). These studies indicate the importance of endocannabinoid responses in

cell survival and the viability of neuronal networks.

The endocannabinoid system is comprised of at least two types of cannabinoid receptors, an endocannabinoid transport system, and endocannabinoid-degrading enzymes. Cannabinergic inactivation involves internalization of endocannabinoids by the highly selective carrier-mediated transport system (Di Marzo et al., 1994; Beltramo et al., 1997; Piomelli et al., 1999; Fegley et al., 2004) as well as enzymatic degradation. Fatty acid amide hydrolase (FAAH), which is distributed throughout the brain, is thought to be the primary mediator for the hydrolysis of released endocannabinoids (Cravatt et al., 1996, 2001; Egertova et al., 2003). Enhancement of endocannabinoid responses can be achieved with disruption of FAAH activity (Cravatt et al., 2001; Kathuria et al., 2003), as well as through inhibition of the transporter (Giuffrida et al., 2000; Fegley et al., 2004; Karanian et al., 2005b). Thus, cannabinergic tone can be enhanced by blocking the inactivation of endogenous ligands.

Here, we tested whether efficient blockage of endocannabinoid inactivation with the irreversible FAAH inhibitor AM374 elevates AEA in the brain and leads to a level of CB1 signaling that is sufficient to protect against excitotoxic damage. Modulation of the endocannabinoid system with AM374 was evaluated for neuroprotection against i) kainate-induced seizures, ii) molecular and cellular indicators of excitotoxic pathology, and iii) excitotoxic behavioral impairments. The results indicate that enhancement of endocannabinoid responses through FAAH inhibition reduces seizure activity and the seizure-induced brain damage.

METHODS

Animals

Sprague-Dawley rats (Charles River Labatories; Wilmington, Massachusetts) were housed following guidelines from National Institutes of Health. The animals were handled daily for 3-5 days in order to facilitate acclimation prior to behavioral testing. Experiments were initiated at postnatal day 21-24.

Chemicals and antibodies

The FAAH inhibitor palmitylsulfonyl fluoride (AM374) and CB1 antagonist N-(piperidin-1-yl)-5-(4-iodophonyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251) was synthesized through an established route (Gatley et al., 1996; Deutsch et al., 1997). Kainic acid (KA) was obtained from Tocris (Ellisville, Missouri). Affinity-purified antibodies to GluR2/3 (Bahr et al., 1996) and to the calpain-mediated spectrin fragment BDP were used as previously described (Karanian et al., 2005b). Other antibodies utilized include those to the active form of ERK and total ERK (Cell Signaling, Beverly, Massachusetts), synapsin II (Cal-Biochem, San Diego, California), and actin (Sigma, St. Louis, Missouri).

Endocannabinoid Detection

After systemic injection of AM374 or vehicle consisting of phosphate-buffered saline (PBS) with DMSO, brain regions were rapidly dissected, dropped into liquid nitrogen, and subjected to a methanol / chloroform extraction procedure. Chromatographic separation was achieved using an Agilent Zorbax SB-CN column (2.1x50 mm) on a Finnigan TSQ Quantum Ultra triple quad mass spectrometer (Thermo Electron, San Jose, California) with an Agilent

1100 HPLC on the front end (Agilent Technologies, Wilmington, Delaware). The mobile phase consists of 10 mM ammonium acetate, pH 7.3 and methanol in the following gradient: initial conditions are held at 10% for 1 min, increased linearly from 60% to 75% from 1.5 to 10 min, then to 95% in 0.5 min (flow rate = 0.5 ml/min); the autosampler was kept at 4º C to prevent analyte degradation. Eluted peaks were ionized via atmospheric pressure chemical ionization in SRM mode. Deuterated AEA from 0-500 pg/ul were used for the standard curve, and levels in brain per g tissue were determined (correlation coefficient; r = 0.99).

Activation of extracellular signal regulated-kinase (ERK)

Sprague-Dawley rats were administered an IP injection of vehicle or 8 mg/kg AM374. The animals were sacrificed 15-90 min post-injection and brain tissue was rapidly dissected. The hippocampus, frontal cortex and neocortex samples were homogenized in lysis buffer consisting of 15 mM HEPES (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA and the protease inhibitor cocktail. Protein content was determined and equal protein aliquots assessed by immunoblot for active pERK2 with antibodies specific for MEK-dependent phosphorylation sites in the catalytic core of ERK2 as well as total ERK as described (Bahr et al., 2002; Karanian et al., 2005a).

KA-induced seizures and subsequent drug administration

Sprague-Dawley rats were injected first with PBS (n=17) or 10 mg/kg KA (n=21). Immediately following the KA injection, the rats were administered a subsequent IP injection with either vehicle or 1-8 mg/kg AM374. Following the injections, animals were monitored for seizure activity, 24-48 h post-injection animals were assessed for behavioral changes, at 48 h post-injections animals were sacrificed for immunoblot and histological analysis as described. A

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subset of animals assessed at 48 h post-injection for molecular and cellular changes were administered 3 mg/kg AM251 30-min prior to receiving the excitotoxin and AM374.

Seizure Scoring

Animals were observed for 4 h following injections. Seizures were counted and scored for each hour post-injection. Briefly, blinded raters continuously observed the animals throughout the 4-h period. The seizure counts and score was recorded every 15 min, representative of the seizure expression during that period. The seizure rating scale consisted of the following six stages: stage 0, normal behavior; stage 1, freezing, staring, mouth or facial movements; stage 2, rigid posture, head nodding or isolated twitches; stage 3, tail extension, unilateral-bilateral forelimb clonus, or repetitive scratching; stage 4, rearing with one or both forepaws extended; stage 5, clonic seizures with loss of posture, jumping, and falling; stage 6, severe tonic seizures.

After 48 h post-injection, brains were rapidly removed with ice-cold homogenization 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 cocktail containing 4-(2-aminoethyl) benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, and aprotinin. The hippocampal tissue was homogenized in lysis buffer as previously described (Bahr et al., 2002), protein content determined, and assessed for cytoskeletal, and synaptic markers.

Immunoblot analysis

Protein content was determined in the homogenized hippocampal samples with a bovine serum albumin standard, and equal aliquots of the samples were denatured in SDS at 100 ºC. The

samples were then separated by SDS-polyacrylamide gel electrophoresis and blotted to nitrocellulose. Immunodetection was achieved by incubating blots overnight at 4°C with separate antibodies to the active form of ERK and total ERK, calpain-mediated spectrin breakdown product, synapsin II, and the AMPA receptor subunit GluR2/3. Blots were routinely stained for a protein load control (e.g., actin). Anti-IgG-alkaline phosphatase conjugates were used for secondary antibody incubation. Development of immunoreactive species used the 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium substrate system and was terminated prior to maximum intensity in order to avoid saturation. Integrated optical density of the bands was determined at high resolution with BIOQUANT software (R & M Biometrics; Nashville, Tennessee).

Histology

Brain tissue was fixed in paraformaldehyde for 24 h and then paraffin embedded. The paraffin blocks were sectioned at 5-μm thickness and mounted on slides. The embedded tissue was heated at 60°F for 30 min, dehydrated through ethanol solutions, and stained with Hematoxylin and Eosin.

Behavioral Testing

Animals were assessed for excitotoxic-induced behavioral changes 24-48 h post-injection. In the first behavioral test animals were placed with front paws over a bar and hind paws touching surface in order to measure time before movement. Time to front paw movement was recorded for a maximum of 30 sec. The second behavioral test involved balance on a suspended beam as modified from previously described (Hamm et al., 1994). The animals were

placed on a beam (1.2 cm diameter) suspended 15 cm above a padded surface and assessed for time to fall with a maximum time of 30 sec. A rota-rod paradigm was used for the third behavioral test to assess motor coordination. As modified from previously described (Hamm et al., 1994; Kabova et al., 1999), animals received 10 consecutive training sessions on the rotating rod (10 rpm). Following the final training the animals were allowed to rest and then tested for ability to maintain balance and coordination on the rota-rod for a maximum of 30 sec. A subset of animals was monitored for locomotor activity in a novel environment. The rats were assessed for distance of exploration in an open field (106 cm x 54 cm) that was divided into eight segments. The animals were placed in the center of the open field and monitored for the total number of segments crossed during the five min session.

RESULTS

AM374 Elevates Anandamide levels and Promotes CB1 Signaling. The irreversible FAAH inhibitor AM374 or vehicle was systemically administered to 20 rats to test for elevation of endocannabinoid levels in the brain and enhancement of CB1 responses. Rats were given an IP injection of vehicle (control) or 8 mg/kg AM374, and brains were removed 45-180 min later. Brain regions were rapidly dissected, frozen in liquid nitrogen, and assessed for endocannabinoid levels by mass spectrometry that recognizes the signature of AEA as it elutes from the reverse-phase column. Levels of hippocampal AEA were significantly elevated 2.5 ± 0.37 fold following systemic administration of AM374 (see Fig. 1). Significant increases in AEA were found in other brain regions including a 4.8 ± 1.2 fold elevation in frontal cortical tissue.

The AM374-induced increases in AEA led to enhanced endocannabinoid signals. As shown in Fig. 2, systemic administration of the FAAH inhibitor results in the activation of MAPK/ERK signaling which has been linked to the endocannabinoid system (Derkinderen et al., 2003; Karanian et al., 2005a/b). The representative blots in Fig. 2A show an increased level of the phosphorylated active form of the ERK2 isoform (pERK2) within 15 min following AM374 administration. The dug resulted in the activation of ERK2 in several brain regions including the hippocampus, frontal cortex and neocortex (Fig. 2B). The most robust pERK2 activation was evident at 90 min post-injection for each brain region. AM374 increased pERK2 activation above basal levels by 400% in the hippocampus, 650% in the frontal cortex, and 284% in the neocortex. As a control, total ERK levels were found not to change with drug treatment across the different time points. Taken together, these results indicate that AM374 crosses the blood brain barrier and enhances AEA levels to promote cannabinergic signaling.

FAAH Inhibition Reduces Seizure Severity. Enhancement of endocannabinoid responses with the irreversible FAAH inhibitor AM374 was tested for protective features in an excitotoxicity model. In order to initiate excitotoxic seizures, rats were injected with 10 mg/kg KA. Immediately following KA administration, animals were injected with either vehicle or 1-8 mg/kg AM374 and seizure activity was scored for 4 h. The FAAH inhibitor reduced seizure severity in a dose-dependent fashion as compared to animals that only received vehicle after the excitotoxin injection (Fig. 3A, ANOVA: $p<0.0001$). Additional non-parametric analyses were conducted on the data resulting in a Kruskal-Wallis statistic of 33 ($p<0.0001$), and the Mann-Whitney test between the KA rats and KA plus 5-8 mg/kg AM374 was also significant ($p = 0.0002$).

Figure 3B shows KA-induced seizure scores across the post-insult rating period, data for each hour being normalized to the KA only animals. AM374-induced protection was significant in the first hour of seizure induction, and the protection progressively improved in subsequent hours. Compared to KA rats that did not receive drug, AM374 reduced seizure severity by 47% in hour 1, by 44% in hour 2, by 70% in hour 3, and by 86% in hour 4. AM374 not only reduced the overall seizure severity, but also appears to accelerate the rate of seizure decay. Thus, disruption of FAAH activity with the irreversible FAAH inhibitor effectively reduces excitotoxin-induced seizure events.

Neuroprotection. The KA and KA plus AM374 groups were also assessed for molecular and cellular indicators of excitotoxicity. At 48 h post-injection, brains were rapidly dissected under ice-cold conditions and hippocampal tissue assessed by immunoblot with specific antibodies. The hippocampus from KA-exposed animals exhibited a pronounced level of calpain-mediated cytoskeletal breakdown (BDP) as well as loss of synaptic markers synapsin II and GluR2/3 (Fig. 4A). Note that cytoskeletal BDP is often associated with a loss of synaptic and dendritic integrity (Neumar et al., 2001; Bahr et al., 2002; Karanian et al., 2005b), and we have previously reported that enhancement of endocannabinoid responses protects against such pathogenic manifestations (Karanian et al., 2005b). When AM374 was injected immediately following the KA insult, spectrin BDP measured 48 h later was reduced by 89% (Fig. 4B, ANOVA: $p<0.01$). Thus, the FAAH inhibitor provides nearly complete cytoskeletal protection from KA-induced excitotoxicity.

In addition to the protective effects on cytoskeletal integrity, AM374 reduced the

synaptic decline resulting from excitotoxin exposure. KA reduced the postsynaptic marker GluR2/3 along with the presynaptic marker synapsin II by 60-70% (see Fig. 4A). Interestingly, the synaptic decline was strongly correlated with seizure severity (Fig. 5; $r = -0.84$, $p<0.0001$), evident by decreased GluR2/3 immunoreactivity in animals with increased seizure scores. Excitotoxin-exposed animals treated with AM374 exhibited synaptic protection with greater than 78% preservation of GluR2/3 (Fig. 4C; ANOVA: $p<0.0001$). AM374 also completely protected the presynaptic marker synapsin II (Fig. 4D; ANOVA: $p<0.01$). AM374's neuroprotective effects were blocked by the selective CB1 antagonist N-(piperidin-1-yl)-5-(4-iodophonyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251). In Fig. 6A, a subset of animals was systemically injected with 3 mg/kg AM251 30-min prior to receiving the excitotoxin and AM374. The immunoblots, assessed 48 h post-injection, show that AM374's cytoskeletal and synaptic protection is attenuated by pretreatment with AM251. These results indicate that inhibition of FAAH provides protection through CB1 receptors in the excitotoxic rat.

In order to assess cellular changes, brains from the different treatment groups were rapidly dissected 48 h post-insult, then fixed and sectioned for staining with hematoxylin and eosion. As shown in photomicrographs of CA1 of hippocampus, exposure to the excitotoxin resulted in neuronal compromise. Compared to control tissue (Fig. 6B), the KA insult dramatically reduced viable neurons and induced pyknotic changes in the pyramidal subfield (Fig. 6C). AM374 promoted cell survival as confirmed by the absence of neuronal loss and pyknotic effects in tissue from an excitotoxic animal treated with the FAAH inhibitor (Fig. 6D). As was the case for cytoskeletal and synaptic protection, pretreatment with AM251 prevented

AM374's effect on the cellular protection (Fig. 6E). FAAH inhibition, thus, protects against excitotoxin-induced neuronal compromise, by acting through the CB1 receptor.

Functional Protection. Three behavioral paradigms were employed to test the irreversible FAAH inhibitor for functional protection. The first behavioral test assessed onset of front paw movement when placed on a raised bar as a measure of the animal's coordination to negotiate the raised position. As shown in Figure 7A, KA-induced neurodegeneration was associated with a marked delay in initial movement assessed 24 h post-insult, over the basal movement exhibited by vehicle-injected control animals. Initiation of activity was returned to near control levels when endocannabinoid inactivation was inhibited by injection of AM374 immediately following the excitotoxin exposure (Fig. 7A, ANOVA: $p<0.001$; post-hoc test compared to insult only data: $p<0.01$). Note that the KA alteration was not due to gross impairment in motor ability since there was no change across treatment groups in explorative distance when placed in a novel open field (Fig. 7B).

The animals were also assessed for changes in balance and coordination 24-48 h post-insult. In Figure 8A, the balance beam test resulted in a significant decrease in the time to fall for KA rats as compared to control animals. AM374 administered immediately after KA prevented the excitotoxic-induced disruption of balance (ANOVA: $p<0.001$; post-hoc test compared to insult only data: $p<0.01$). A rota-rod paradigm was used as our third test since it is a task sensitive to excitotoxic brain injury and can be used to assess coordination separate from the balance beam (Kabova et al., 1999; Hamm et al., 1994). At 48 h post-injections, rats were trained on the rota-rod and subsequently tested for their ability to maintain themselves for a continuous

period on the 10-rpm rotating-rod. The excitotoxic damage induced by KA exposure resulted in a marked reduction in rota-rod performance compared to control animals (Fig. 8B). AM374 protected against the excitotoxin-induced impairment as determined by analysis of variance ($p<0.01$), thus indicating improved motor coordination. The assessment of selective motor disturbances further supports that the irreversible FAAH inhibitor reduces excitotoxic brain defects. Taken together, these results demonstrate that enhancement of endocannabinoid responses protects against excitotoxic seizures and seizure-induced neuronal damage.

DISCUSSION

FAAH is a key regulatory site of cannabinergic signaling and pharmacological modulation of FAAH activity results in the enhancement of endocannabinoid responses. The selective and irreversible FAAH inhibitor AM374 was found to dramatically increase AEA levels in the brain and promote CB1 signaling. Fatty acid sulfonyl fluorides are two-to-three-orders of magnitude more selective for blocking FAAH than for displacing CB1 agonist binding (Deutsch et al., 1997), while their effect on endogenous AEA concentrations indirectly promotes cannabinergic responses. AM374 has a similar selectivity for FAAH as compared to monoglyceride lipase that hydrolyzes 2-arachidonoyl glycerol (Dinh et al., 2002). Disrupting endocannabinoid responses alters synaptic integrity, increases excitotoxic vulnerability, and attenuates survival responses (Parmentier-Batteur et al., 2002; Marsicano et al., 2003; Khaspekov et al., 2004; Karanian et al., 2005a/b). Conversely, the current study demonstrates that enhancing endocannabinoid levels through the selective inhibition of FAAH protects against KA-induced seizures and excitotoxic neurodegeneration. Moreover, modulating the

endocannabinoid system in this manner also ameliorated excitotoxic brain defects evident in behavioral tests.

AM374-mediated enhancement of endocannabinoid responses in our study was associated with reduced seizures in the KA rat. The KA excitotoxin is well known to induce seizures and pathology characteristic of epilepsy. We showed that FAAH inhibition provides a dose-dependent as well as accelerated rate of seizure recovery. The protection against seizures was evident throughout the seizure scoring period, suggesting that the drug has an extended effect on reducing excitotoxic progression. Note that while FAAH knockout mice were found to exhibit susceptibility to spastic and proconvulsant activity (Cravatt et al., 2001; Clement et al., 2003), dosing with the potent AM374 compound facilitated a controlled modulation of FAAH to promote protective cannabinergic signals. Corresponding with the present results, inhibiting endocannabinoid transport has been shown to reduce motor hyperactivity and seizure severity (Lastres-Becker et al., 2002; Marsicano et al., 2003), and several types of neuroprotection have been described with agonists that activate CB1 responses (see Bahr et al., 2006). When endocannabinoid signaling is disrupted through genetic or pharmacological means, the resultant proconvulsant conditions can lead to neuronal compromise (Wallace et al., 2002, 2003; Marsicano et al., 2003; Monory et al., 2006).

In the KA model, we also tested for protection against molecular and cellular consequences ascribed to epileptiform-type excitotoxic events. AM374 prevented KA-induced cytoskeletal damage and synaptic decline in the hippocampus. The cytoskeletal damage was assessed by measuring calpain-mediated BDP, a sensitive precursor to synaptic compromise and

subsequent neuronal pathology in *in vitro* and *in vivo* models (see Bahr et al., 2002; Karanian et al., 2005b). As in such studies, pharmacological reduction of excitotoxic cytoskeletal damage was associated with enhanced survival of important neuronal fields. Both indications of protection mediated by AM374 were prevented by the CB1 antagonist AM251. Corresponding with the cytoskeletal protection, disruption of endocannabinoid inactivation also provided nearly complete protection of pre- and postsynaptic protein expression. As in the case of the cytoskeletal and cellular protection, CB1 antagonism also prevented the synaptic preservation indicating that AM374's protective effects are mediated by CB1 receptors. It should be pointed out that KA-induced seizure severity correlates with synaptic marker decline, likely leading to the loss of synapse function. The reduction of the excitotoxic cascade through governed excitability and preserved integrity of synapses by AM374 would perhaps safeguard those synaptic signals that are important for neuronal maintenance (see Hayashi et al., 1999; McKinney et al., 1999; Bahr et al., 2002). The data support the idea that FAAH inhibition provides cellular protection against epileptiform excitotoxicity.

In addition to the cytoskeletal, synaptic, and cellular protection, AM374 effectively ameliorated behavioral indications of excitotoxic brain damage. The KA insult caused marked impairment of balance and coordination as indicated by reduced performance on three paradigms. The behavioral alterations were significantly improved when the FAAH inhibitor was administered immediately following the KA injection. The data strongly suggest preservation of brain function since the level of KA used did not affect gross motor ability as indicated by normal explorative motor behavior. Interestingly, the KA plus AM374 rats also did not exhibit deficits in explorative movement, suggesting that enhancement or maintenance of KA-induced

AEA levels does not produce adverse motor effects. Others have shown that AEA transport inhibitor LY2183240 elevates the endocannabinoid 5-fold in the brain, resulting in behaviorally relevant cannabimimetic responses in the absence of adverse effects on motor function (Moore et al., 2005). Our functional protection results correspond with previous work that showed inhibition of endocannabinoid inactivation attenuates excitotoxin-induced behavioral defects (Karanian et al., 2005b). FAAH inhibition has also been reported to reduce muscle spasticity in a multiple sclerosis model (Baker et. al., 2001). Together, the findings indicate that endocannabinoid modulation via FAAH inhibition protects against excitotoxicity and preserves brain function.

The present findings further support that the endocannabinoid system provides an on-demand response to excitotoxic insults as previously implicated (see Marsicano et al., 2003; Karanian et al., 2005b). Excitotoxins including KA have been reported to selectively increase AEA levels in the brain by 3- to 13-fold, prompting the idea that CB1 responses facilitate endogenous protection (Hansen et al., 2001; Marsicano et al., 2003). The increase in the diffusible messenger after systemic KA administration in mice was transient, peaking after 20 min and returning to normal levels after 60 min. Controlled modulation of FAAH may prolong such transient responses since AM374 was still effective 3 h post-KA, increasing AEA 3-6-fold. Enhancement of the endocannabinoid system reduced KA-induced seizure severity, perhaps by reducing intracellular calcium via cannabinergic actions on voltage-gated calcium channels and modulation of potassium channels through inhibition of adenylyl cyclase (Deadwyler et al., 1993; Shen and Thayer, 1996, 1998; Mu et al., 1999), as well as by eliciting nonspecific and synapse-specific depression of excitatory circuits (Shen et al., 1996; Kim and Thayer, 2000;

Gerdeman and Lovinger, 2001; Singla et al., 2007). Induced calcium regulation is supported by the fact that FAAH inhibition markedly decreased calcium-dependent activation events of calpain that produce cytoskeletal fragments. The reduction in calcium would govern neuronal excitability, slow excitotoxic progression that expands out from zones expressing seizure activity, and attenuate calpain and other forms of pathogenesis that lead to cellular and functional compromise. Indeed, AM374 protected against KA-induced behavior defects, and previous work reported that endocannabinoid enhancement produces a neuroprotective correlation between reduced cytoskeletal breakdown mediated by calpain and improved performance in a fear-conditioning paradigm (Karanian et al., 2005b). In addition to calcium regulatory pathways, enhanced cannabinergic responses during the post-insult recovery period may also entail MAPK and other pro-survival signal transducers. AM374 appears to have more than a transient effect on the activation of the ERK/MAPK pathway, a pathway found to be through the upstream activator MEK and mediated by CB1 receptors (Derkinderen et al., 2003; Karanian et al., 2005a/b). The additional CB1 events that may be involved cover a wide range of pro-survival signal transduction elements including PI3K/Akt, BDNF, and FAK (see Hayashi et al., 1999; McKinney et al., 1999; Nagayama et al., 1999; Panikashvili et al., 2001; Gomez del Pulgar et al., 2002; Molina-Holgado et al., 2002; Maccarrone et al., 2003; Marsicano et al., 2003; Khaspekov et al., 2004; Karanian et al., 2005a), possibly explaining the broad protective effects of the endocannabinoid system across diverse disease states.

Endocannabinoids are elevated following seizure induction and are possibly synthesized and released on demand to prevent excitotoxic progression (Wallace et al., 2002, 2003; Marsicano et al., 2003). Accordingly, pharmacological modulation of endocannabinoid tone has become of great interest and several reports indicate the neuroprotective effects of

inhibiting FAAH activity (see Bahr et al., 2006 for a recent review). The present report is consistent with such results suggesting that compensatory signaling can be positively modulated through the inhibition of endocannabinoid hydrolysis with AM374. The endocannabinoid system is likely involved in a negative feedback loop to inhibit neuronal over-excitation as well as a positive feedback loop of cell maintenance signaling. To summarize, enhancement of endocannabinoid responses through inhibition of FAAH is sufficient to produce molecular, cellular, and functional protection in a model of epileptiform excitotoxicity. AM374 appears to cross the blood-brain barrier and effectively enhance endocannabinoid signaling. Such indirect modulation of endocannabinoid responses promotes recovery from seizure events and improves brain function. Thus, controlled FAAH inhibition poses a therapeutic avenue for epileptiform excitotoxicity.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. FAAH inhibition increases AEA levels in the brain. The irreversible FAAH inhibitor AM374 (8 mg/kg) or vehicle was systemically administered to rats. Across post-injection times, brain regions were rapidly dissected, flash-frozen in liquid nitrogen, and assessed for AEA levels by mass spectrometry. Time of elution (min) from the reverse phase column is noted for reference peaks. Arrows depict the AEA peaks generated from hippocampal tissue. (y-scale: 0-15%).

Figure 2. AM374 activates the ERK/MAPK pathway. Rats were given an IP injection of 8 mg/kg AM374 or vehicle. At 15-90 min following the injections, brain tissue was harvested and rapidly homogenized in the presence of phosphatase inhibitors. Brain tissue was assessed by parallel immunoblots for pERK2, and total ERK. **A:** The representative blots show tissue from a vehicle animal used to determine basal antigen levels, along with AM374-induced changes across time. **B:** Mean integrated optical densities for pERK2 were determined by image analysis for hippocampus (Hip), frontal cortex (FC), and neocortex (Neo).

Figure 3. AM374 reduces KA-induced seizure severity throughout the 4-h rating period. Seizures were initiated in rats by injection with 10 mg/kg of KA (insult). Following administration of KA (n=21), animals were immediately injected with either vehicle or 1-8 mg/kg AM374. Vehicle treated rats (con, n=17) did not receive KA or AM374. Seizures were monitored and scored by blinded raters for 4-h following injections. **A:** Mean seizure scores ± SEM are shown for the 4-h rating period (ANOVA $p<0.0001$; post-hoc test compared to insult only data: *** $p<0.001$). **B:** Seizure scores were normalized to KA (insult) and shown as mean ± SEM for each hour post-injection (Post-hoc tests of AM374's effect: $p<0.01$ - 0.0001).

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Figure 4. FAAH inhibition reduces KA-induced cytoskeletal damage and synaptic decline. Rats received KA insult (10 mg/kg, IP) immediately followed by administration of either vehicle or 5-8 mg/kg AM374 (n=4-8). At 48 h post-injection, hippocampal tissue for immunoblotting was rapidly dissected from these animals and from non-insulted control rats (con). Spectrin breakdown product BDP, postsynaptic marker GluR2/3, presynaptic marker synapsin II, and actin were assessed on single immunoblots (**A**). Mean integrated optical densities ± SEM are shown for BDP (**B**; ANOVA: $p<0.01$), GluR2/3 (**C**; ANOVA: $p<0.0001$) and synapsin II (**D;** ANOVA: $p<0.01$). Post-hoc tests compared with insult only data: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Figure 5. Synaptic decline correlates with seizure severity. Treatment groups from Figure 3A were used so that each animal was assessed and designated a seizure score to correspond with the synaptic marker. The seizure activity was scored for 4 h post-injection by blinded raters. At 48 h post-injection, hippocampal tissue was rapidly dissected under ice-cold conditions and evaluated by immunoblot for the postsynaptic marker GluR2/3. The data set was subjected to regression analysis in order to determine the correlation coefficient ($r = -0.84$) for the apparent linear relationship between seizure severity and synaptic compromise ($p<0.0001$).

Figure 6. The neuroprotection promoted by inactivation of FAAH is CB1 receptor mediated. Rats were given an injection of KA followed by administration of either vehicle or 5-8 mg/kg AM374. A subset of animals was administered 3 mg/kg AM251 30 min prior to KA and AM374. The animals were sacrificed at 48 h post-injection, and brain tissue was assessed by

immunoblot or fixed for histology. **A:** Immunoblots show that pretreatment with the specific CB1 antagonist attenuates AM374's cytoskeletal (BDP) and synaptic protection (GluR1, synapsin II). Note that actin remained unchanged across treatment groups. Fixed hippocampal tissue was paraffin embedded, sectioned, and stained with hematoxylin and eosin. Photomicrographs of the CA1 field are shown for animals injected with vehicle (**B**, con), KA (**C**, insult), KA and AM374 (**D**), or AM251 followed by KA and AM374 (**E**). Exposure to the excitotoxin resulted in neuronal loss and pyknotic changes that were ameliorated by AM374. The cellular protection elicited by AM374 was prevented by pretreatment with the specific CB1 antagonist. sp, stratum pyramidale; sr, stratum radiatum. Size bar: 30 μm.

Figure 7. Disruption of endocannabinoid inactivation provides functional protection in the excitotoxic rat. Rats received an injection of vehicle (con, n=5-11) or KA (insult, n=6) immediately followed by administration of either vehicle, or 5-8 mg/kg AM374 (n=7-9). At 24-48 h post-injection, the animals were evaluated for alterations in movement. **A:** Mean time (sec) to onset of movement ± SEM is shown for the different treatment groups. The KA insult alone delayed front paw movement and treatment with AM374 protected against the excitotoxic-induced behavioral impairment (ANOVA: $p<0.001$; post-hoc test compared with insult only data: $^{**}p<0.01$). **B:** A subset of rats was monitored for locomotor activity in a novel open field. The distance of exploration was unchanged across treatment groups as measured by total number of segments crossed.

Figure 8. FAAH inhibition protects against excitotoxic-induced disruption of balance and coordination. The animals that received injections of vehicle or KA (insult) immediately

followed by vehicle, or 5-8 mg/kg AM374 in Figure 7, were also assessed for balance and motor coordination. **A:** The KA insult disrupted balance as presented by mean time to fall (sec) ± SEM off the elevated beam. Treatment with AM374 protected against the excitotoxic-induced behavioral impairment (ANOVA: $p<0.001$). **B:** A rota-rod paradigm was used to assess coordination 48 h post-injection. Animals exposed to the excitotoxin alone had a marked impairment in coordination as determined by time on the rotating-rod (10 rpm). The insult-exposed animals that were immediately treated with AM374 had improved rota-rod performance compared to the excitotoxin alone (ANOVA: $p<0.01$). Post-hoc tests compared with insult only data: $*p<0.05$, $**p<0.01$.

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Figure 1

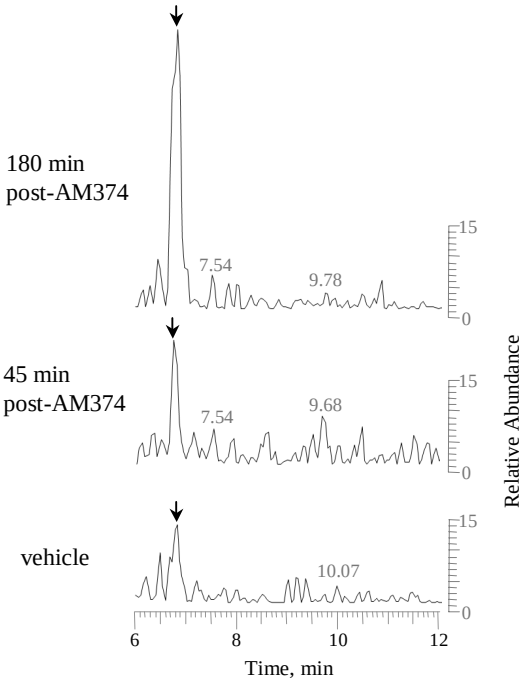

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Figure 2

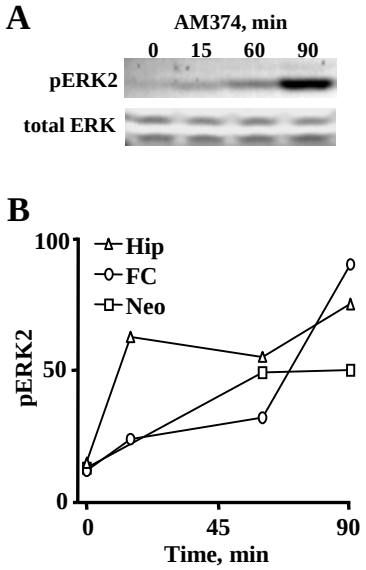

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Figure 3

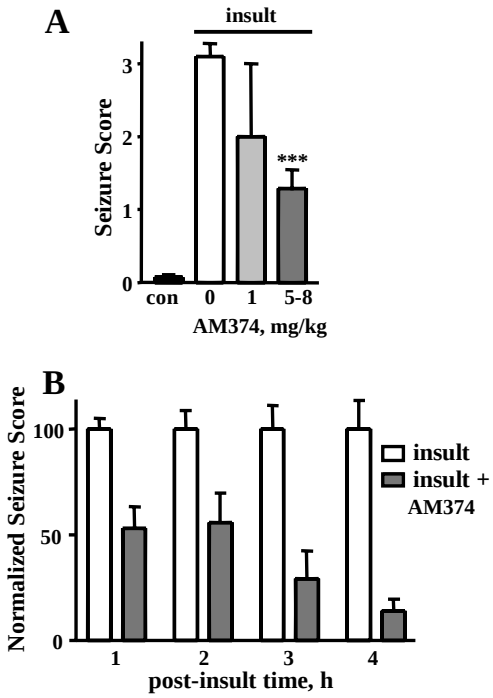

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Figure 4

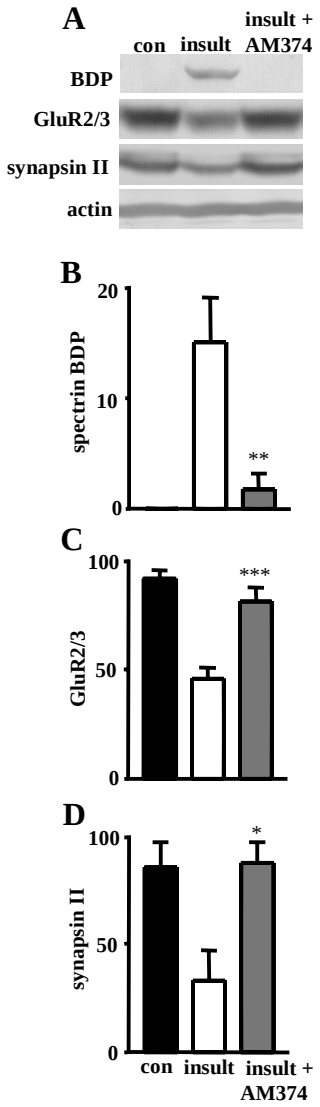

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Figure 5

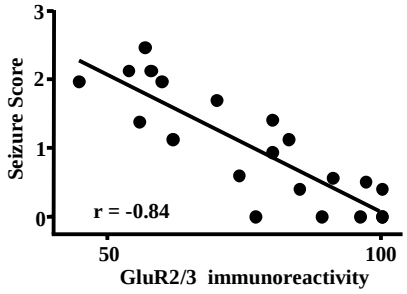

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Figure 6

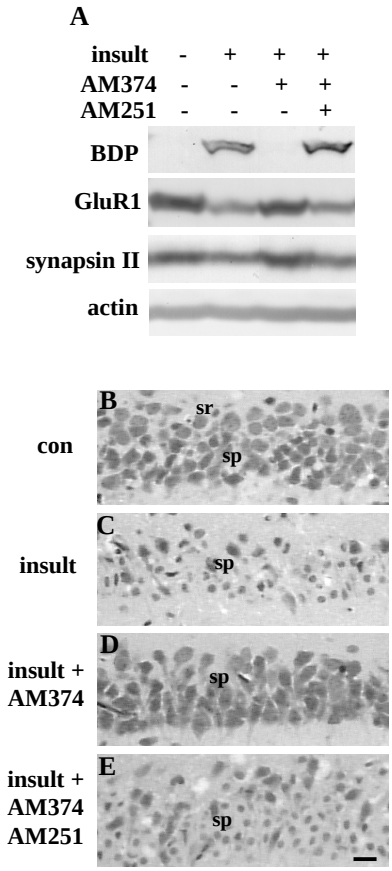

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Figure 7

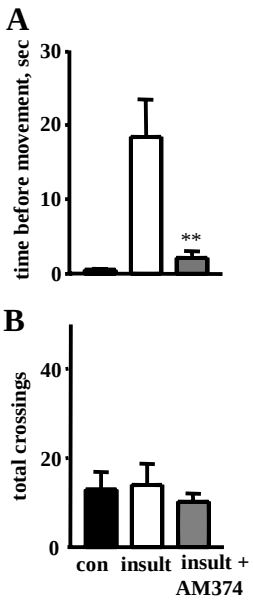

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Figure 8