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Blocking cannabinoid activation of FAK and ERK1/2 compromises synaptic integrity in hippocampus☆

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Abstract

The cannabinoid CB_1 receptor allows endocannabinoids to act as intercellular and retrograde messengers in the central nervous system. Endocannabinoid actions have been implicated in both synaptic plasticity and neuroprotection. Here, cannabinergic activation of extracellular signal regulated-kinase (ERK) and focal adhesion kinase (FAK) occurred correspondingly in long-term hippocampal slice cultures. The stable endocannabinoid analogue R-methanandamide activated ERK1/ERK2 subtypes of mitogen-activated protein kinase (MAPK) through the upstream activator MAPK kinase (MEK). R-methanandamide also promoted FAK signaling, but in a MEK-independent manner. Both events of ERK and FAK activation were selectively blocked by *N*-(morpholin-4-yl)-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM281), a cannabinoid CB_1 receptor antagonist, and the blockage was associated with a gradual decline in synaptic markers. Interestingly, the integrin antagonist Gly–Arg–Gly–Asp–Ser–Pro also caused the disruption of R-methanandamide-mediated ERK and FAK responses and upset the integrity of excitatory synapses. These results suggest that the endocannabinoid system supports synaptic maintenance through linkages with MAPK pathways and integrin-related FAK signaling.

Keywords: Hippocampus; Excitotoxicity; FAK; MAPK; Survival signaling

1. Introduction

The endocannabinoid system, involving at least two receptor types, is known to have several influences on the central nervous system. The CB_1 class of cannabinoid receptors is found abundantly in several brain regions including the hippocampus (Herkenham et al., 1990; Marsicano and Lutz, 1999; Tsou et al., 1999), and has been implicated in memory encoding and neuroprotection (see review by Guzman et al., 2001). A second class of cannabinoid receptors, CB_2, is found primarily in the periphery (Griffin et al., 1999). The cannabinoid system transmits signals mediated by endogenous cannabinoids, a family of lipids including anandamide and 2-arachidonylglycerol (see Mechoulam et al., 1998). Cannabinoid signaling can be induced with selective agonists or, alternatively, by increasing circulating anandamide levels with inhibitors of endocannabinoid transport and anandamide amidase (also known as fatty acid amide hydrolase) (Beltramo et al., 1997; Deutsch et al., 1997; Gifford et al., 1999; Fegley et al., 2004). The resultant activation of the cannabinoid CB_1 receptor triggers signal transduction events that can influence synaptic mechanisms and compensatory responses.

Cellular responses that elicit synaptic plasticity and neuroprotection may involve cannabinoid CB_1 receptors and their link to a variety of signaling elements including G proteins, mitogen-activated protein kinase (MAPK), and focal adhesion kinase (FAK). Cannabinoid agents activate

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signal transduction pathways regulated by the Gi/Go family of G-proteins (Howlett and Mukhopadhyay, 2000; Breivogel et al., 2001), including those linked to MAPK kinase (MEK) and its substrate extracellular signal regulated-kinase (ERK) (Bouaboula et al., 1995; Kobayashi et al., 2001; Galve-Roperh et al., 2002; Derkinderen et al., 2003). The ERK pathway promotes synaptic maintenance and cell survival (Bonni et al., 1999; Bahr et al., 2002; Marsicano et al., 2003), thus, may be related to the neuroprotective nature of endocannabinoid signaling. Cannabinergic compounds have been shown to protect neurons from several types of pathogenic insults including ischemia (Nagayama et al., 1999; Sinor et al., 2000), traumatic brain injury (Panikashvili et al., 2001), seizures (Wallace et al., 2002), and excitotoxin exposure (Shen and Thayer, 1998; Marsicano et al., 2003).

Neuroprotective cannabinoid signaling may also involve integrin-type adhesion responses including activation of the tyrosine kinase FAK (Derkinderen et al., 1996, 2001b). Integrins activate multiple signaling events including the ERK/MAPK pathway, and they maintain synaptic structure during brain development (Miranti and Brugge, 2002; Milner and Campbell, 2002). As is the case with ERK, FAK activity is linked to cell survival (Frisch et al., 1996; Liu et al., 2003), and both ERK and FAK signaling have been implicated in the action of cannabinoid agents. Thus, the cannabinoid system may stimulate complex pathways that promote cellular viability.

In the present report, we studied cannabinoid signaling in organotypic slice cultures prepared from hippocampus, a brain region that is both essential for certain memory systems and targeted by a variety of neurological disorders. The hippocampus is well studied for its important synaptic connections, related plasticity mechanisms, and distinct vulnerability to neurodegeneration. Hippocampal slice cultures, in the absence of systemic variables, provide a sensitive model system that exhibits cellular and genetic responses comparable to those found in vivo (e.g., see Bahr and Bendiske, 2002; Bahr et al., 2002; Caba and Bahr, 2004). Their three-dimensional nature maintains features characteristic of the adult hippocampus, such as the circuitry, morphological integrity, and organization of neuronal subfields (Stoppini et al., 1991; Bahr, 1995). Specific pharmacological tools were utilized including (i) R-methanandamide, a high affinity, metabolically stable analogue of anandamide (Abadji et al., 1994), (ii) the competitive antagonist selective for cannabinoid CB_1 receptors *N*-(morpholin-4-yl)-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM281) (Gifford et al., 1997), and (iii) the Gly–Arg–Gly–Asp–Ser–Pro peptide (GRGDSP) that blocks integrin-type matrix recognition and disrupts structural and functional plasticity in hippocampal synapses (Bahr et al., 1997; Bahr, 2000). The described results indicate that the cannabinoid system is linked to MEK/ERK and FAK compensatory signaling in the hippocampus, possibly as part of a synaptic maintenance pathway.

2. Materials and methods

2.1. Organotypic hippocampal slice cultures

Sprague–Dawley rat pups (Charles River Laboratories; Wilmington, Massachusetts) were housed with their mother following guidelines from National Institutes of Health. The animals were allowed 4–5 days of acclimatization prior to sacrifice. The litter was sacrificed at the age of 11–12 days postnatal. Transverse hippocampal slices (400 μm) were rapidly prepared and placed in groups of 6–10 slices per Millicell-CM insert (Millipore; Bedford, Massachusetts). Media consisting of 50% basal medium Eagle, 25% Earle's balanced salts, 25% horse serum, and defined supplements (Bahr et al., 1995, 2002) were changed every other day. Slice cultures typically had a 20-day maturation period before experiments were initiated.

2.2. ERK and FAK activation

The agonist R-methanandamide (compound AM356) and other cannabinoid agents were synthesized as previously described (Abadji et al., 1994; Gifford et al., 1997). Slice cultures were treated with 0.01–100 μM R-methanandamide for 10–60 min at 37 °C in the presence or absence of the cannabinoid CB_1 receptor antagonist AM281 (10 μM). Selectivity experiments induced kinase activation with the glutamatergic agonist α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA). Other slices received R-methanandamide for 1 h in the presence or absence of either the MEK inhibitor 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126; 20 μM) or the integrin antagonist GRGDSP (100 μM). To terminate the experiment, the culture inserts were rapidly flooded 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 (Sigma; St. Louis, Missouri) containing 4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, and aprotinin. Each experimental condition was represented by homogenate samples prepared from 6–8 hippocampal slices each. Selective antibodies were used to determine the degree of enzyme activation by immunoblot.

2.3. Disruption of signaling and induction of neuropathology

In order to disrupt cannabinoid signaling, slices were exposed to the antagonist AM281 (10 μM) for 0–5 days. Freshly prepared antagonist was applied each day for the 5-day exposure period. The hippocampal slices were then analyzed by immunoblot for calpain-mediated spectrin breakdown and the synaptic markers synapsin II, synaptophysin, and the AMPA-type glutamate receptor subunit GluR1. Disruption of integrin-type signaling was achieved

by exposing slices to 100 μM of the integrin antagonist GRGDSP for 4 days. The tissue was infused with GRGDSP each day for the 4-day exposure period.

For induction of excitotoxic pathology, slices were pretreated with or without 100 μM GRGDSP for 24 h and then exposed to 100 μM AMPA in the absence or presence of GRGDSP for 20 min. Immediately following the insult, slices were rapidly quenched for 10 min with the glutamate receptor antagonists CNQX and MK801 (Tocris; Ellisville, Missouri) as previously described (Bahr et al., 2002). Slices were assessed 24 h post-insult for spectrin breakdown, GluR1, synapsin II, and actin.

2.4. Immunoblot analysis

Slices were harvested by gently removing them from the culture membrane with a soft brush. Slices were sonicated in groups of 6–8 in lysis buffer containing protease inhibitors as previously described (Bahr et al., 2002). Protein content was determined with a bovine serum albumin standard, and equal aliquots of the slice samples were denatured in SDS at 100 °C. 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 (Cell Signaling; Beverly, Massachusetts), active FAK and total FAK (Upstate Biotechnology; Lake Placid, New York), calpain-mediated spectrin breakdown product (Bahr et al., 1995), synaptophysin (Chemicon; Temecula, California), synapsin II (Cal-Biochem; San Diego, California), actin (Sigma), and to the AMPA receptor subunit GluR1 (Bahr et al., 1996). 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).

2.5. Histology

Slices were fixed for 24 h in cold 0.1 M phosphate buffer plus 4% paraformaldehyde, and cryoprotected in a 20% sucrose solution. Slices were then rinsed with phosphate buffer and gently removed from the insert. Slices were mounted directly onto coated slides, or sections were cut at 35-μm thickness then mounted. The tissue was Nissl stained, dehydrated through ethanol solutions, and cover slipped in permount.

3. Results

To study cannabinoid signaling and its influence on synapses, hippocampal slice cultures were prepared and assessed for stability by Nissl staining and consistent levels of synaptic markers. Cannabinoid receptors were rapidly activated in cultured slices using the selective agonist R-methanandamide. Fig. 1A shows that the activated forms of ERK1/2 MAPK (pERK1/2) and FAK (pFAK) displayed distinct increases in cultures treated with R-methanandamide. The phosphorylated pERK2 species reached a level 288±26% of that found in control slices ($P<0.001$, one-sample t-test; $n=7$), and the corresponding change in pFAK was 315±61% of control ($P=0.02$; $n=5$). The potent agonist produced threshold activation of ERK and FAK in the 10–100 nM range (Fig. 1B), and induction of the active kinases was clearly evident at the earliest time point of 10 min (Fig. 1C). The active pERK1 and pERK2 were assessed with antibodies that selectively recognize the MEK-dependent phosphorylation sites in the catalytic core of the isoforms. The increase in the 125-kDa pFAK was found using antibodies specific for its Tyr-397 phosphorylation site. The immunostaining also revealed a 3-fold increase in a larger species of 130–136 kDa (pFAK133; see asterisk in Fig. 1A and B). As controls, actin and total FAK levels (Fig. 1A) as well as total ERK1/2 (not shown) were found to be unchanged in the R-methanandamide-treated samples.

Both R-methanandamide-mediated ERK and FAK activation were blocked by the cannabinoid CB_1 receptor antagonist AM281. In Fig. 2A, the activated levels of ERK and FAK are shown in slice samples that were treated with R-methanandamide for 60 min (left lanes). In the right lanes, AM281 prevented the activation effects of R-methanandamide and, as a result, pERK2 and pFAK remained at levels similar to those found in control slices (also see Table 1 and Fig. 1C). Selectivity of the AM281 blockage was confirmed by showing that the cannabinoid CB_1 receptor antagonist had no influence on kinase activation events mediated through AMPA-type glutamate receptors (Fig. 3 and Table 1).

The MEK inhibitor U0126 also was used to help define the cannabinoid signaling pathway. While AM281 disrupted both ERK and FAK activation, U0126 produced differential effects on the two types of kinases (Fig. 2B). When U0126 was co-administered with R-methanandamide, the level of pERK remained at or below control levels while pFAK exhibited little change (also see Table 1). Thus, U0126 completely blocks the cannabinoid receptor-mediated ERK response without affecting the corresponding FAK activation. The lack of a U0126 effect on pFAK was accompanied by little to no effect on pFAK133 (90–103% of levels found in slices treated with R-methanandamide alone). Parallel immunoblot samples shown in Fig. 2B confirm that there was no change in either total FAK or total ERK in the experiments. The results indicate that FAK is activated in a MEK-independent manner.

Since disrupting MAPK signaling events with U0126 has been previously shown to compromise hippocampal synapses (Bahr et al., 2002), we tested whether disruption of endogenous cannabinoid signals affects synaptic integrity.

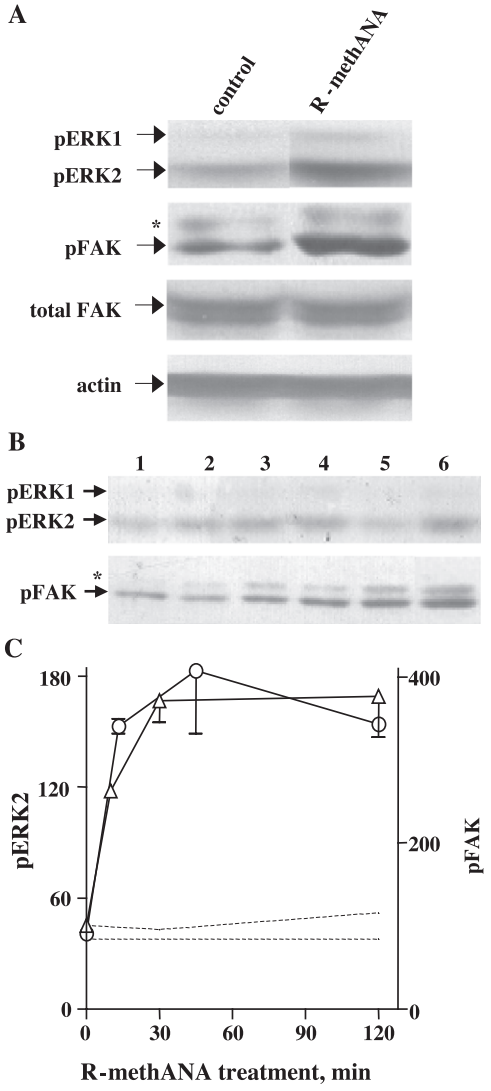

Fig. 1. The cannabinoid CB_1 receptor is linked to the activation of ERK1/2 and FAK in hippocampal slice cultures. Untreated control slices and slices exposed to R-methanandamide (R-methANA) were assessed for active pERK1 and pERK2, which were immunolabeled with antibodies specific for MEK-dependent phosphorylation sites. The active form of FAK (pFAK) and pFAK133 (*) was labeled with antibodies specific for FAK's Tyr-397 phosphorylation site. (A) Slices were treated with 100 μM R-methananda-mide for 30 min, harvested in the presence of phosphatase inhibitors, and assessed for the activated isoforms in groups of 6–8 slices each. Actin was measured on the same blots as a loading control. Identical samples were run on parallel immunoblots for staining of total FAK. (B) Dose-dependent activation events are shown in lanes 1–6 representing slices treated with 0, 0.01, 0.1, 1, 10, and 50 μM R-methanandamide, respectively. (C) Time-dependent increases in pERK2 (triangles) and pFAK (circles) were determined across 11–12 treated samples using image analysis (mean integrated optical density ±S.E.M.). Baseline levels of pERK2 (upper dotted line) and pFAK (lower line) were determined in the presence of the cannabinoid CB_1 receptor antagonist AM281 (10 μM).

Indeed, application of AM281 over a period of 0–5 days resulted in a gradual loss of three synaptic markers in the hippocampal slice cultures (Fig. 4A). Compromised synaptic integrity is indicated by decreased immunostaining of the presynaptic markers synaptophysin and synapsin II, in

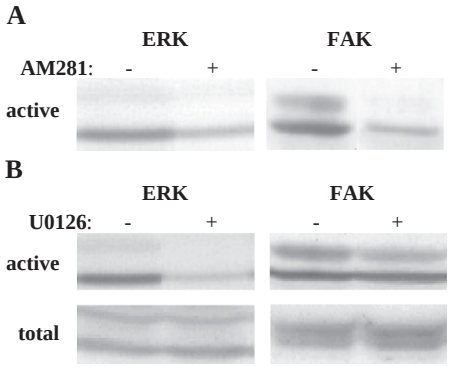

Fig. 2. Cannabinoid CB_1 receptor blockade inhibits R-methanandamide-mediated activation of both ERK and FAK, while U0126 only blocks ERK activation. Hippocampal slices were exposed to R-methanandamide for 1 h in the absence or presence of 10 μM AM281 (A) or 20 μM U0126 (B). Slices were assessed for the phosphorylated active forms of ERK1/2 and FAK with specific antibodies. Effects were verified across 5–7 separate groups of slices. The same samples were run on parallel immunoblots for staining of total ERK and total FAK.

addition to reduced staining of the postsynaptic glutamate receptor subunit GluR1. No reductions were evident after only 1 day of treatment, but between 1 and 5 days, highly significant correlations were observed between AM281 exposure time and the synaptic decline (Fig. 4B). The synaptic markers were reduced by 33–45% after 3 days of AM281 treatment, and by 62–73% after 5 days. There was no change in actin levels across the 5-day treatment period, nor was there any evident production of calpain-mediated spectrin breakdown product (Fig. 4A), a marker of excitotoxicity and other types of toxic events (Vanderklish and Bahr, 2000). Thus, AM281 has a selective effect on hippocampal tissue, resulting in reduced expression of synaptic proteins.

Blocking endocannabinoid signaling also causes synaptic decline before obvious signs of cellular atrophy. Histological analysis determined that the major neuronal subfields appear intact and display typical neuronal densities in both the non-treated control (Fig. 5A) and the 5-day AM281-treated tissue (Fig. 5B). Under closer examination of the

Table 1
Effects of AM281 and MEK inhibitor on R-methanandamide-mediated activation of ERK and FAK

Treatment group	pERK2	pFAK
Control	100	100
R-methanandamide	288±26	315±61
R-methanandamide+AM281	111±12**	128±14*
R-methanandamide+U0126	47±8**	276±43
AMPA	170±38	235±5
AMPA+AM281	169±49	207±90

Hippocampal slice cultures were treated as in Figs. 2 and 3, and levels of pERK2 and pFAK were determined by immunoblot and image analysis. Means±S.E.M. were determined from 3–7 slice groups, then normalized to control levels (the latter set to 100%). Unpaired *t*-test compared to R-methanandamide treatment alone.

* $P<0.03$.

** $P<0.01$.

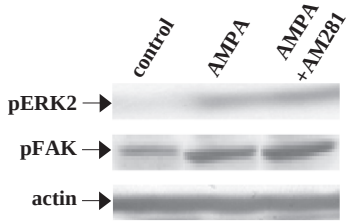

Fig. 3. The cannabinoid CB_1 receptor antagonist AM281 does not alter AMPA-induced activation of ERK and FAK. Cultured hippocampal slices were treated with 100 μM of the glutamate receptor agonist AMPA for 2 h in the absence or presence of 50 μM AM281. Untreated control slices and AMPA-exposed slices were assessed for the phosphorylated active forms of ERK and FAK with specific antibodies. Image analysis was used to determine that there was no change in AMPA-induced ERK or FAK activation in the presence of AM281 (see Table 1).

CA1 subfield, pyramidal neurons in the non-treated control (Fig. 5C) and the AM281-treated slices (Fig. 5D) exhibited similar morphology and cellular organization.

We next used a pharmacological tool distinct from AM281 to test for the corresponding disruption of ERK and FAK activation. Adhesion responses involving ERK and FAK are mediated in large part by integrins, a family of matrix receptors that contain a binding site for the RGDS domain. Accordingly, we used the integrin antagonist GRGDSP to test for its effects on cannabinergic activation of ERK and FAK. Fig. 6A shows the activated levels of pERK and pFAK in R-methanandamide-treated slices, and the prevention of such activation by GRGDSP (right lanes). The integrin antagonist caused pERK1/2, pFAK, and pFAK133 to remain at levels similar to those found in control slices. Incubation with GRGDSP alone for an extended time period had no apparent effects on basal levels of pERK and pFAK (data not shown).

Similar to AM281 actions, blocking integrin-type signals with GRGDSP not only disrupted activation of ERK and FAK, but also altered the structural composition of hippocampal synapses. Hippocampal slice cultures were found to be vulnerable to synaptic decline when the integrin receptors were blocked for 4 days with GRGDSP peptide (Fig. 6B). Synaptic compromise is indicated by the 40–50% reductions in the postsynaptic marker GluR1 ($P<0.05$) and in the presynaptic markers synapsin II and synaptophysin ($P<0.03$). The significant reduction in immunoblot staining of synaptic markers occurred while actin levels remained unchanged. These results indicate that integrin signaling is involved in synaptic maintenance.

GRGDSP also made hippocampal tissue more vulnerable to the over-excitation of excitatory synapses. Slice cultures insulted with the excitotoxin AMPA for 20 min expressed a calpain response verified by the presence of spectrin breakdown 24 h post-insult (Fig. 6C, middle lane). A separate group of slices was pre-incubated with GRGDSP for 24 h, and then the integrin antagonist was co-administered with the AMPA insult. Disrupting integrin responses in this manner caused a 6-fold increase in excitotoxic calpain activation (right blot lane), indicating enhanced vulnerability to the AMPA exposure. The

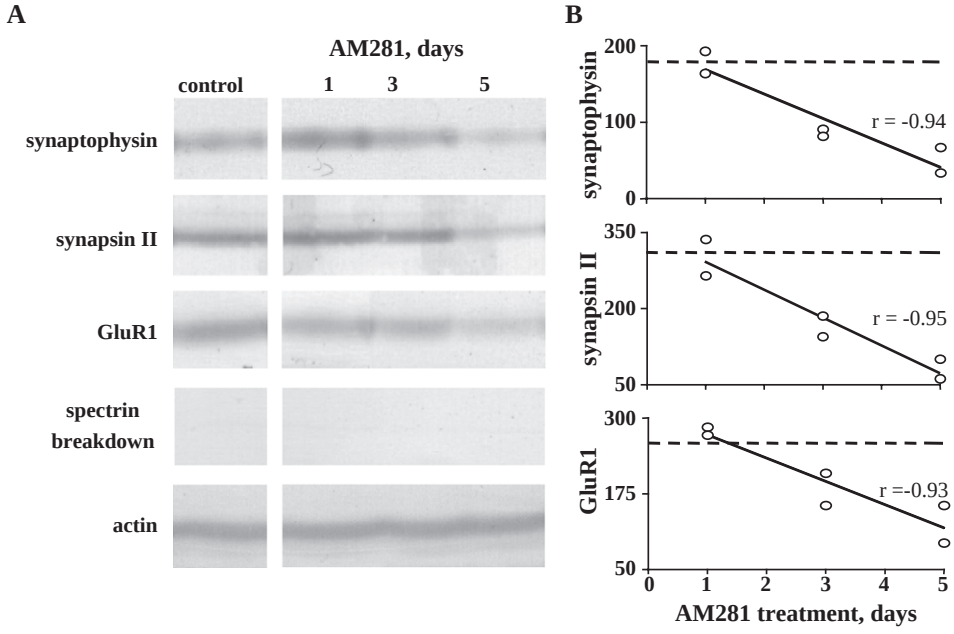

Fig. 4. The endogenous cannabinoid system is necessary for synaptic maintenance in hippocampal slice cultures. Slices were exposed to the cannabinoid CB_1 receptor antagonist AM281 for 0–5 days, and the harvested samples were examined by immunoblot (A). Image analysis was used to assess presynaptic markers synaptophysin and synapsin II, and the postsynaptic marker GluR1 (B). Basal levels of the markers had a typical variance of ±9% ($n=4$; see dotted lines). Each data set from 1–5 treatment days was subjected to regression analysis in order to determine the correlation coefficient (r) for the apparent linear relationships ($P<0.01$ for each marker). Note that the lack of immunoblot staining for calpain-mediated spectrin breakdown product confirmed that no overt toxicity is involved. The selective effect on synaptic decline was also evaluated by staining identical samples for actin.

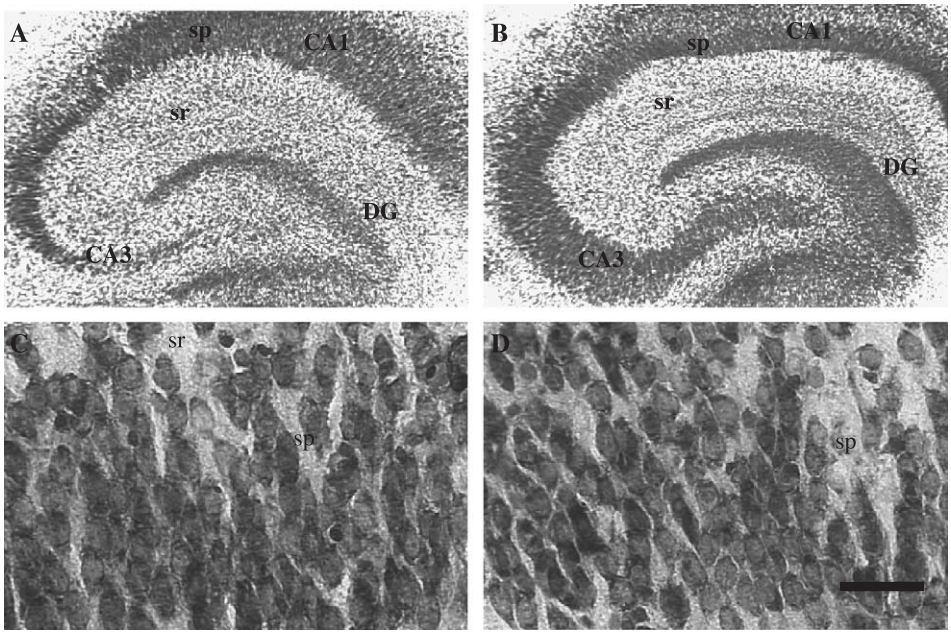

Fig. 5. Disruption of endogenous cannabinoid signaling does not alter tissue integrity in the hippocampal slice culture. Control slices (A, C) and slices treated with AM281 for 5 days (B, D) were fixed and Nissl stained. At low power (A, B), hippocampal subfields CA1, CA3, and dentate gyrus (DG) showed no AM281 effect. Note that regardless of treatment, variability was evident with regard to lamina width of CA3 and the stratum granulosum across different cultures. As shown at high power, AM281-treated slices exhibited similar density and morphology among CA1 pyramidal neurons (D) as found in the control slices (C). sp, stratum pyramidal; sr, stratum radiatum. Scale bar: A and B, 500 μm; C and D, 50 μm.

vulnerable tissue also exhibited a corresponding effect on the postsynaptic marker GluR1. The 35% reduction in GluR1 evident 24 h post-insult was enhanced to a 60% decline by the GRGDSP treatment (Fig. 6C). Incubation with GRGDSP alone for 24 h did not cause calpain-mediated spectrin breakdown, nor did it affect basal levels of synaptic markers (not shown). These findings suggest that integrin contacts with the extracellular matrix are necessary for synaptic maintenance, and perturbation of the resultant signaling renders hippocampal tissue more vulnerable to excitotoxicity.

4. Discussion

This report shows that cannabinergic activity through cannabinoid CB_1 receptors is linked to signaling pathways well known for their involvement in survival and cell maintenance. Such activity in hippocampus is disrupted by a cannabinoid CB_1 receptor antagonist and by an inhibitor of integrin-type adhesion responses, both resulting in gradual synaptic decline. The current results, thereby, further indicate the importance of cannabinoid receptors for stable synapses in the central nervous system. In addition, the cultured hippocampal slice model proved useful to study synaptic integrity and the effects of disrupting endocannabinoid signals involving MAPK and FAK.

Activation of hippocampal cannabinoid CB_1 receptors with R-methanandamide induced rapid MAPK signaling through the ERK1 and ERK2 isoforms. The effects of a selective cannabinoid receptor antagonist and a MEK inhibitor established that the ERK activation was a receptor-mediated event and occurred via an upstream MEK response. Cannabinoid CB_1 receptors have been previously implicated in different types of MAPK signaling pathways (Bouaboula et al., 1995; Derkinderen et al., 2001a; Galve-Roperh et al., 2002; Marsicano et al., 2003). The endogenous agonists anandamide and 2-arachidonylglycerol have both been linked to ERK through MEK (Kobayashi et al., 2001; Derkinderen et al., 2003). The cannabinoid CB_1 receptor-ERK connection was confirmed using conditional mutant mice that lack expression of the cannabinoid receptor in principal hippocampal neurons (Marsicano et al., 2003). Mechanisms that increase levels of the phosphorylated active forms of ERK could not be triggered in the mutant mice.

Coinciding with the R-methanandamide-mediated ERK response in hippocampal tissue was increased levels of activated FAK. Corresponding activation of ERK and FAK was evident across both dosage and temporal profiles of the R-methanandamide effect. Thus, cannabinoid CB_1 receptors may be connected to cell maintenance by more than one signaling pathway. FAK is an integrin-dependent signaling element involved in neuronal survival (Frisch et al., 1996; Girault et al., 1999). Studies in acute hippocampal slices linked FAK to anandamide and 2-arachidonylglycerol responses, and provided strong evidence that cannabinoid activity regulates a neuronal splice isoform of FAK that is preferentially expressed in brain (Derkinderen et al., 1996, 2001b). This FAK isoform is shown here phosphorylated on

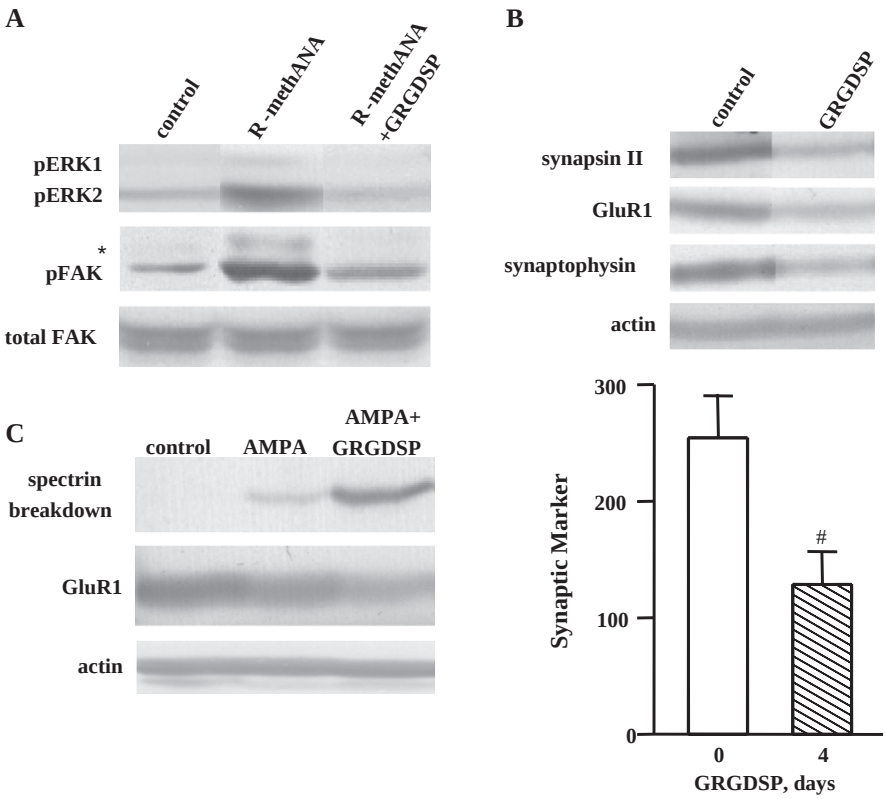

Fig. 6. Inhibition of integrin-type signaling blocks R-methanandamide-mediated ERK and FAK activation, leads to synaptic decline, and enhances excitotoxic vulnerability. Cultured hippocampal slices were exposed to R-methanandamide (R-methANA) for 1 h in the absence or presence of the integrin antagonist GRGDSP, then assessed for phosphorylated active forms of ERK and FAK, as well as for total FAK (A). Also immunolabeled was the pFAK133 species (*). In panel (B), untreated slices and slices exposed to 100 μM GRGDSP for 4 days were analyzed by immunoblot for the synaptic markers synapsin II, GluR1, and synaptophysin, as well as for actin protein levels. Mean integrated optical densities for the postsynaptic marker GluR1 are shown (±S.E.M., n=3–5; unpaired t-test: $^{\#}P<0.05$). In panel (C), slice cultures were subjected to excitotoxicity by exposure to AMPA for 20 min (middle lane). A second group of slices was pre-incubated with GRGDSP for 24 h before the insult was applied in the presence of the integrin antagonist (right lane). Slices were harvested 24 h post-insult and analyzed along side untreated control slices for calpain-mediated spectrin breakdown, GluR1, and actin.

Tyr-397 along with a slightly larger species, named pFAK133, also identified with the phosphorylation state-specific antibody. Related to pFAK133, evidence of endocannabinoids causing the activation of a similar species has been found with a monoclonal antiphosphotyrosine (Derkinderen et al., 1996) and with antibodies against FAK's phosphotyrosine-397 (Derkinderen et al., 2001b). Perhaps this larger species is a spliced variant expressed in the hippocampus.

The MEK inhibitor U0126 blocked the R-methananda-mide-mediated ERK response without blocking the increased phosphorylation of FAK and pFAK133. The described results indicate that, although both ERK and FAK are activated by cannabinoid signaling, activation of FAK-related species is induced in a manner independent of MEK. Blocking integrin signaling, on the other hand, disrupted the increased phosphorylation of both FAK and ERK isoforms. These findings agree with previous reports that FAK activation is an upstream event leading to a MAPK/ERK pathway (Derkinderen et al., 1998; Yang et al., 2003).

The signaling protein phosphatidylinositol 3-kinase (PI3K) also has been shown to be necessary for cannabi-noid-induced ERK activation, and the pro-survival action of cannabinoid receptor agonist 3-(1,1-dimethylheptyl)-(-)11-hydroxy-Δ^8-tetrahydrocannabinol (HU-210) required both PI3K and ERK signaling (Galve-Roperh et al., 2002). Interestingly, an anti-apoptotic survival pathway in epithelial cells has been shown to involve FAK, PI3K, and ERK signaling (Liu et al., 2003). A compensatory response mediated by the cannabinoid system is also evident with the fact that (1) endocannabinoids are elevated after neuronal injury and (2) administering the cannabinoid agents results in neuroprotection (Panikashvili et al., 2001; Milton, 2002; Marsicano et al., 2003). It stands to reason, then, that disruption of endogenous cannabinoid signals would lead to neuronal destabilization as found in this report. Correspondingly, enhanced vulnerability to excitotoxic insults occurs in mice lacking expression of cannabinoid CB_1 receptors (Parmentier-Batteur et al., 2002; Marsicano et al., 2003). These results indicate that endocannabinoid signaling is necessary for maintaining neuronal health, although an earlier report found that blockade of cannabinoid receptor activity evokes neuroprotection against an excitotoxic insult (Hansen et al., 2002).

MEK/ERK signaling is important insofar as it is involved in neuronal maintenance and repair (Bonni et al., 1999; Bahr et al., 2002), possibly mediated by the induction of neurotrophic factors (Hayashi et al., 1999; Derkinderen et al., 2003). Together with the current results, the findings indicate that inhibiting either component of the cannabinoid receptor-MAPK pathway would disrupt neuronal viability. Experiments with AM281, an antagonist that blocks both anandamide and 2-arachidonylglycerol from binding the CB_1 class of cannabinoid receptors, indicate that the receptors are involved in the maintenance of synapses. AM281 prevents not only cannabinoid-mediated ERK activation, but also the activation of the integrin-related tyrosine kinase FAK. ERK may represent a convergent pathway for trophic factors and integrin signals (Giancotti and Ruoslahti, 1999; Liu et al., 2003; Reginato et al., 2003). As reported here, AM281 selectively compromised the expression of synaptic proteins in hippocampal tissue without altering actin levels. The synaptic decline was also distinct in that it occurred gradually without any overt signs of cellular toxicity or changes in neuronal morphology. The results indicate that blockade of endocannabinoid signaling causes selective disruption of synaptic maintenance.

Blocking integrin signaling with GRGDSP also disrupted R-methanandamide-mediated ERK and FAK activation in the hippocampal slice model, and, as in the case with AM281, caused a loss of synaptic integrity. Disruption of the integrity of excitatory synapses was also indicated by the observation that slices treated with GRGDSP exhibited increased vulnerability to an excitotoxic insult. Blocking MEK/ERK signaling alone caused a similar type of enhanced vulnerability (Bahr et al., 2002). The GRGDSP effect is rather selective in that basal synaptic responses are not altered by the integrin antagonist (Bahr et al., 1997), nor did the peptide have any acute effects on the expression of important synaptic proteins. The data suggest that cannabinoid CB_1 receptor signaling in hippocampal synapses is negatively influenced when select adhesion contacts are compromised. As cannabinoid signaling appears to be involved in the maintenance and modulation of central synapses, similar connections have been made between integrin-type chemistries and synaptic structure and plasticity (Bahr and Lynch, 1992; Bahr et al., 1997; Benson et al., 2000; Chan et al., 2003). Adhesion contacts between integrins and the extracellular matrix are known to maintain cell health and synaptic structure during development (Benson et al., 2000). Thus, cannabinoid signaling appears to converge with integrin-type pathways, and the resultant cascade involving FAK and ERK responses is necessary for stable synaptic function.

To summarize, we report that cannabinoid CB_1 receptors in hippocampus generate important signals for synapse maintenance. Cannabinoid signals activate pathways linked to survival, and inhibition of these signaling pathways compromises synaptic integrity. Together with previous work, the current results strongly suggest that increasing endocannabinoid tone would promote mechanisms for synaptic stability and neuroprotection. In addition, the results indicate that the cannabinoid responses represent an endogenous compensatory system involving MEK/ERK, FAK/MEK/ERK, and/or FAK/PI3K/ERK and possible other unidentified signaling pathways. Also note that while neuroprotection studies have focused primarily on direct activation of cannabinoid CB_1 receptors via agonists, indirect alternatives may have merit by amplifying the cannabinoid system through the modulation of its endogenous components.

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