Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 466 (2003) 73–83
www.elsevier.com/locate/ejphar

Chronic ethanol inhibits the anandamide transport and increases extracellular anandamide levels in cerebellar granule neurons

Balapal S. Basavarajappa[a,b], Mariko Saito[b], Thomas B. Cooper[a,b,c], Basalingappa L. Hungund[a,b,c,*]

[a] New York State Psychiatric Institute, New York, NY 10032, USA
[b] Nathan S. Kline Institute for Psychiatric Research, Bldg. #39, 140-Old Orangeburg Road, Orangeburg, NY 10962, USA
[c] Department of Psychiatry, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA

Received 21 December 2002; received in revised form 21 February 2003; accepted 28 February 2003

Abstract

Ethanol increases extracellular anandamide levels in neuronal cells. However, the molecular mechanisms by which this occurs are unknown. Chronic exposure of cerebellar granule neurons to ethanol increased the levels of anandamide accumulated in the cellular medium. Anandamide uptake was saturable and was inhibited (30% at 3 min) in response to chronic exposure to ethanol. Chronic ethanol treatment did not alter the K_m, but significantly decreased V_{max} of anandamide transport (33%) ($P<0.0001$). Fatty acid amide hydrolase activity was not affected by chronic ethanol treatment. Anandamide transport processes are independent of cannabinoid CB1 receptor, as cannabinoid CB1 receptor knockout mice exhibited time-dependent anandamide transport and cannabinoid CB1 receptor antagonists did not alter the effects of chronic ethanol on anandamide transport. Furthermore, anandamide transport was inhibited by acute ethanol in a time- and dose-dependent manner. Interestingly, acute ethanol-induced inhibition of anandamide transport was abolished in neurons exposed to chronic ethanol, suggesting that the anandamide transport processes may play a role in the development of long-term cellular tolerance to ethanol.

Keywords: Cerebellar granule neuron; Ethanol; Anandamide transport; Endocannabinoid; Cannabinoid CB1 receptor

1. Introduction

Chronic exposure to ethanol has been shown to modulate the endogenous cannabinoid (endocannabinoid) system in central nervous system (Basavarajappa and Hungund, 2002). Manipulation of endocannabinoid receptor function has been found to alter drinking behavior in rodents (Arnone et al., 1997; Colombo et al., 1998, 2002; Gallate and McGregor, 1999; Gallate et al., 1999; Rodriguez de Fonseca et al., 1999; Freedland et al., 2001), suggesting that endocannabinoid receptor system may be a potential mechanism for alcoholism. To date, two endogenous cannabinoid substances that mimic the pharmacological actions of Δ^9-tetrahydrocannabinol, the active ingredient of marijuana and other synthetic agonists (Mechoulam and Fride, 1995), have been isolated and characterized. These include anandamide and 2-arachidonylglycerol (Devane et al., 1992; Mechoulam et al., 1995; Stella et al., 1997). Both anandamide and 2-arachidonylglycerol have been shown to bind specifically to cannabinoid CB1 receptors in the brain (Devane et al., 1992; Mechoulam et al., 1995; Sugiura et al., 1995). Recent evidence indicates that the cannabinoid CB1 receptor signal transduction system plays a role in the pharmacological actions of ethanol, including voluntary ethanol consumption (Arnone et al., 1997; Basavarajappa et al., 1998; Colombo et al., 1998; Basavarajappa and Hungund, 1999a,b, 2002; Gallate and McGregor, 1999; Gallate et al., 1999; Rodriguez de Fonseca et al., 1999). However, the exact molecular mechanism by which cannabinoid CB1 receptors regulate ethanol consumption remains unknown. Recently, we demonstrated that chronic exposure to etha-

* Corresponding author. Nathan S. Kline Institute for Psychiatric Research, Bldg. #39, 140-Old Orangeburg Road, Orangeburg, NY 10962, USA. Tel.: +1-845-398-5452; fax: +1-845-359-7029.
E-mail address: Hungund@nki.rfmh.org (B.L. Hungund).

0014-2999/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0014-2999(03)01557-7

nol enhances the formation of the endogenous cannabinoid CB1 receptor agonists anandamide and 2-arachidonylglycerol in neuronal cells (Basavarajappa and Hungund, 1999a; Basavarajappa et al., 2000). In these studies, the majority of the synthesized anandamide and 2-arachidonylglycerol (85–90%) remained in the medium even after 72 h exposure to ethanol (Basavarajappa and Hungund, 1999a; Basavarajappa et al., 2000). Anandamide has a variety of regulatory functions, including mediating retrograde signals from depolarized postsynaptic neurons to presynaptic terminals, thereby reducing neurotransmitter release (Ohno-Shosaku et al., 2001).

In mammalian cells, termination of anandamide signaling at the cannabinoid CB1 receptors occurs through an uptake mechanism that transports anandamide into the cell where it subsequently undergoes rapid degradation by fatty acid amidohydrolase (Cravatt et al., 1996; Beltramo et al., 1997; Hillard et al., 1997; Piomelli et al., 1999). Current evidence suggests that anandamide uptake is a carrier-mediated process that is time- and temperature-dependent, saturable, and inhibited with unique pharmacologic agents (Di Marzo et al., 1994; Beltramo et al., 1997; Hillard et al., 1997; Hillard and Jarrahian, 2000; Rakhshan et al., 2000). Fatty acid amidohydrolase and cannabinoid CB1 receptors that have been shown to be co-localized in brain may indicate fatty acid amidohydrolase's role in anandamide signaling and uptake (Egertova et al., 1998). Thus, chronic ethanol-induced increases in extracellular anandamide could result in a decrease in anandamide influx, an increase in anandamide efflux from the cell, and/or altered intracellular metabolism. In the present study, we have investigated the chronic and acute effects of ethanol on anandamide transport in cerebellar granular neurons. We found that chronic exposure to ethanol leads to increases in extracellular anandamide. After prolonged exposure to ethanol, cells become tolerant to these effects, such that anandamide uptake is no longer inhibited by ethanol. These data suggest that ethanol-induced inhibition of anandamide uptake may in part be responsible for ethanol-induced increase in extracellular anandamide.

2. Materials and methods

2.1. Materials

All culture plastic supplies were purchased from Falcon Labware (VWR Scientific, -Piscataway, NJ). Basal Eagle's medium (BEM), heat-inactivated fetal calf serum (FCS), streptomycin, and penicillin solutions were obtained from Sigma (St. Louis, MO). Liquid scintillation cocktail (INSTA-FLUOR) was purchased from Packard (Meriden, CT). *N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide) (SR 141716A) was a kind gift from Sanofi Pharmaceuticals, (Montpellier, France) and by RBI as part of the Chemical Synthesis Program of the NIMH, contract #1MH30003RBI-NIMH. *N*-(4-hydroxyphenyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (AM404) was purchased from Tocris Cookson (Ellisville, MO). All primers CNKO2 (5′-TGCTTAACTTAGAG TTGAAGGATCC-3′), CNKO3 (5′-AGAACGAGATCAG-CAGCCTCTGTT-3′) and cannabinoid CB1 receptor wild-type (5′-GGATTCAGAATCATGAAGCACTCC-A-3′) were custom made by GIBCO BRL (Rockville, MD). [^{3}H]anandamide (arachidonyl-5,6,8,9,11,12,14,15-^{3}H) (200 Ci/mmol) and [^{3}H]anandamide (ethanolamine1-^{3}H) (10–20 Ci/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO). [^{3}H]arachidonic acid (240 Ci/mmol) was purchased from New England Nuclear (Boston, MA). The polyclonal antibody against the cannabinoid CB1 receptor was a kind gift from Dr. Ken Mackie (University of Washington, Seattle, WA). All other chemicals were obtained from Sigma.

2.2. Primary cultures of cerebellar granular neurons

Cerebellar granular neurons primary cultures were prepared from dissociated cerebella of 8-day-old Sprague–Dawley rat pups or pups from homozygous cannabinoid CB1 receptor knockout mice ($CB1^{-/-}$) and homozygous wild-type mice ($CB1^{+/+}$), as described previously (Oberto et al., 1996; Basavarajappa et al., 2000). Briefly, cells were counted, and plated on poly-L-lysine-coated culture dishes in basal medium Eagle's (BME) supplemented with 10% fetal calf serum (FCS), 25 mM KCl, 200 mM glutamine, penicillin (10,000 U/ml), and streptomycin (10 mg/ml). Approximately, 2×10^6 cells were plated in 2 ml of BME in 35 mm dishes, or 8×10^6 cells were plated in 8 ml of BME in 100 mm dishes. Cells were maintained at 37 °C in a 95% air–5% CO_2 incubator. All of the experimental procedures were performed in accordance with NIH Guidelines for the Care and Use of Laboratory Animals.

2.3. Measurement of anandamide formation in cerebellar granular neurons

The cells were incubated with [^{3}H]arachidonic acid (1 μCi/ml) in BME (without FCS) for 5 h. Media were monitored for uptake and it was found that 85% of the total arachidonic acid was incorporated into the cells within 5 h of incubation. The cells were then washed three times with 0.1% bovine serum albumin (BME) to remove essentially all the free arachidonic acid. The cells were then replenished with the old conditioned media (5 ml) and then subjected to ethanol exposure. The culture conditions for chronic ethanol exposures were similar to those described previously (Basavarajappa and Hungund, 1999a; Basavarajappa et al., 2000). Briefly, ethanol concentration in the media (50, 100, and 150 mM) were maintained by adding appropriate concentrations of absolute alcohol. All

dishes were flushed with 5% CO_2–95% air and sealed with parafilm prior to incubation. The medium was then supplemented at 24 and 48 h with absolute ethanol (3.7 μl/2 ml for 50 mM; 10.6 μl/2 ml for 100 mM; 14 μl/2 ml for 150 mM). Ethanol levels in the media were determined at 72 h by an enzymatic method (Lundquist, 1959) and were found to be 50 ± 2.0, 100 ± 7.5, and 150 ± 20 mM, respectively ($n = 10$ in each group). Appropriate control cultures were similarly maintained in a medium without ethanol. The incubations were terminated by collecting the media. The cells were scraped and then suspended in 10 mM Tris–HCl buffer pH 7.4 containing protease inhibitor cocktail (Sigma). Data (Media and cells) are expressed as the (mean ± S.E.M.) dpm/mg cellular protein.

2.4. Extraction and chromatography

The radioactive lipids from cells and media were extracted using a mixture of chloroform/methanol (2:1, v/v) (Bligh and Dyer, 1959), with 2 μg of unlabeled anandamide included as a carrier. Butylated hydroxytoluene (0.05%) was added to prevent lipid peroxidation. The dried extracts were redissolved in a mixture of chloroform/methanol (2:1, v/v), spotted onto thin layer chromatography (TLC) plates (silica gel 60, 250 μm thickness), and developed with the organic phase of a mixture of iso-octane/ethyl acetate/water/acetic acid (50:110:100:30, v/v) (solvent system A), which allows the separation of 2-arachidonylglycerol (Rf = 0.53) from anandamide (Rf = 0.43) and arachidonic acid (Rf = 0.78). The individual band corresponding to authentic phosphatidylcholine, 2-arachidonylglycerol, anandamide, and arachidonic acid were scraped off the plate into scintillation vials containing 1 ml of chloroform/methanol (2:1). Ten milliliters of scintillation liquid (INSTA-FLUOR, Packard) were added to the samples and the radioactivity was determined by liquid scintillation counting. The identity of anandamide was confirmed by gas chromatography–MS (GC–MS) of the TLC band corresponding to authentic anandamide. The GC–MS was performed, as described before (Basavarajappa et al., 2000) on a Hewlett Packard 5988 mass spectrometer equipped with 5890 series GC and a 7673 automatic injector.

2.5. Cannabinoid CB1 receptor knock out mice

The present study used homozygous knockout mice ($CB1^{-/-}$), as compared to homozygous wild-type mice ($CB1^{+/+}$). Heterozygous cannabinoid CB1 knockout ($CB1^{+/-}$) mice were generated as described (Steiner et al., 1999), and provided by Dr. Andreas Zimmer (National Institutes of Health, Bethesda, MD). Homozygous knockout mice ($CB1^{-/-}$) and homozygous wild-type mice ($CB1^{+/+}$) were produced by heterozygous intermatings at the Nathan Kline Institute for Psychiatric Research animal facility. Twelve- to 20-week-old male and female mice were used in these experiments. Animals were housed in groups under standard laboratory conditions (12-h light/12-h dark cycle) with food and water available ad libitum. Mice pups were genotyped before the preparation of cerebellar granular neurons by PCR with primers corresponding to CNKO2 (5′-TGCTTAACTTAGAGTTGAAGGATCC-3′), CNKO3 (5′-AAGAACGAGATCAGCAGCCTCTGTT-3′) and cannabinoid CB1 receptor wild-type (5′-GGATTCAGAATCATGAAGCACTCCA-3′). In this analysis, wild-type mice pups exhibited a 1237 bp band, and knockout mice pups exhibited a 1088 bp band.

2.6. Western blotting

Whole brain (-cerebellum) from homozygous cannabinoid CB1 receptor knockout mice ($CB1^{-/-}$) and homozygous wild-type mice ($CB1^{+/+}$) from 8-day-old pups were homogenized in buffer (50 mM Tris–HCl, pH 7.4) containing protease inhibitor cocktail (Sigma) and 1% Triton X-100 and protein concentrations were measured (Smith et al., 1985). Brain homogenate samples were mixed with Laemmli sample buffer and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed essentially, as described by Laemmli (1970). Gels were stained with silver to visualize the proteins. The samples were subjected to electrotransfer on a nitrocellulose membrane in a Genie electroblotter (Idea Scientific Minneapolis, MN). The membranes were incubated with polyclonal antibody to N-terminal 77 amino acids of the cloned cannabinoid CB1 receptor (1:1000) overnight at 4 °C, washed three times for 10 min each in 20 mM Tris-buffered saline with 0.2% Tween, and incubated with alkaline phosphatase-conjugated secondary antibodies for 1 h at room temperature, washed as above, and developed by using CDP star substrate reagent (Tropix, Bedford, MA).

2.7. Measurement of anandamide transport

Anandamide transport assays were performed, as described previously (Beltramo et al., 1997; Hillard et al., 1997). After exposure to ethanol or control conditions, as described before, medium was removed by washing neurons with 3 × 1 ml of a 37 °C buffer A (10 mM HEPES, 118 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, and 2.5 mM $CaCl_2$, pH 7.4). The neurons were then incubated with [^{3}H]anandamide (200 Ci/mmol) at 37 °C in buffer A for up to 3 min. Nonspecific cellular association of [^{3}H]anandamide was determined in the presence of 100 μM AM404 or in samples that were incubated at 4 °C (both conditions resulted similar and comparable nonspecific association). Samples of media were taken at the indicated times and extracted immediately with methanol/chloroform. Incubations were stopped by aspirating the media. The cells were then placed on ice and washed three

times with Hank's balanced salt solution (HBSS) to remove essentially all the free [^{3}H]anandamide. The cells were then scraped into scintillation vials and the amount of radioactivity was determined. Cellular accumulation of [^{3}H]anandamide was calculated as the fraction of the total radioactivity that was retained in the cell lipid extract (subtracted from total anandamide from the media and nonspecific association). SR141716A (5 and 10 μM) (which was used to block cannabinoid CB1 receptors) was added from stock solutions in dimethyl sulfoxide (DMSO). The final concentration of DMSO never exceeded 0.2%, and this concentration had no effect on cell viability.

2.8. Measurement of fatty acid amidohydrolase activity

Anandamide amidohydrolase (enzyme commission 3.5.1.4; arachidonyl ethanolamide amidohydrolase) activity was assayed both in vitro and in intact cerebellar granular neurons, as described previously (Deutsch and Chin, 1993). To measure fatty acid amidohydrolase activity in intact neurons, approximately 2×10^6 cells were incubated either in the presence or absence of ethanol. The cells were then washed and the medium was replaced with fresh BME (high K^+) without FCS and incubated for 30 min with 1 μM anandamide (ethanolamine1-^{3}H) (10–20 Ci/mmol). At the end of the incubation, the media and cells were extracted with two volumes of chloroform/methanol (1:1, v/v) and centrifuged. The amount of radiolabeled anandamide that was hydrolyzed was determined by subjecting 200 μl of the aqueous layer to liquid scintillation counting. The fatty acid amidohydrolase activity was expressed as nmol/h/10^6 cells. To measure the fatty acid amidohydrolase activity in vitro, cell cultures were washed once with PBS and then lysed with mammalian protein extraction reagent (M-PER) (PIERCE, Illinois, USA) containing protease inhibitor cocktail (Sigma). Fifty micrograms of total protein derived from cell lysates were incubated for 30 min at 37 °C in a solution containing 0.1M Tris–HCl, pH 8.0, 2.5 mg/ml bovine serum albumin and 30 μM anandamide (ethanolamine1-^{3}H) (10–20 Ci/mmol) in the presence or absence of 1 mM phenylmethylsulfonyl fluoride (PMSF) in a final volume of 200 μl. After this incubation, samples were extracted and subjected to liquid scintillation counting, as described above. Some samples were incubated in the absence of protein, to determine nonspecific hydrolysis of the substrate. Protein concentration of the cell homogenates was determined by the bicinchoninic acid (BCA) method using bovine serum albumin as the standard (Smith et al., 1985).

2.9. Statistical analysis

Statistical analyses used included the Student's *t*-test and one-way analysis of variance (ANOVA), followed by Dunnett's test using the GraphPad Prism software program (v. 3.01 GraphPad Software, San Diego, CA). Kinetic parameters (e.g., half life and plateau accumulation) were determined by fitting time data to the first-order rate equation by nonlinear regression (GraphPad Software). The K_m and V_{max} values were determined by fitting the concentration–response data to the Michaelis–Menten equation using nonlinear regression (GraphPad Software). Differences were considered to be significant at $P < 0.05$. Data are presented as mean ± S.E.M. from at least three separate experiments run in triplicate.

3. Results

3.1. Extracellular anandamide

In our previous studies, ethanol-induced increases in the formation of [^{3}H]anandamide in human neuroblastoma cells was shown to be dependent on the dose of ethanol and the duration of exposure (Basavarajappa and Hungund, 1999a). In these studies, most of the synthesized [^{3}H]anandamide (60–70%) was released from the cells and accumulated in the media (Basavarajappa and Hungund, 1999a). In the present study, when cerebellar granular neurons were incubated with various concentration of ethanol for 72 h, the formation of anandamide was enhanced by 100 and 150 mM ethanol. The cell viability was unaffected by any of the treatment conditions used, as determined by the trypan blue exclusion method (data not shown). Approximately 60% of the [^{3}H]anandamide that was synthesized in response to chronic ethanol exposure was released from the cells and accumulated in the culture medium (Fig. 1), which is consistent with our previous observations (Basavarajappa and Hungund, 1999a). To confirm that the radio-

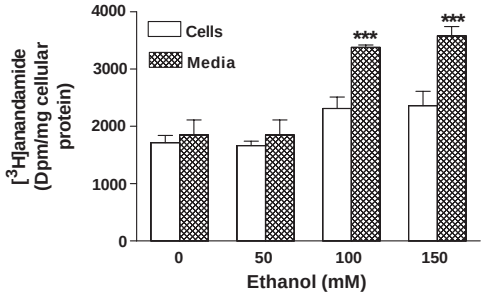

Fig. 1. Effect of chronic ethanol exposure on formation of [^{3}H]anandamide in cerebellar granular neurons. Cells (7 days in vitro) were labeled with [^{3}H]arachidonic acid (1 μCi/ml) in BME (without FCS) for 5 h. Cells were then exposed to various concentrations of ethanol for 72 h, as described in experimental procedures. The [^{3}H]anandamide was extracted from media and cells and separated by TLC, as described in Materials and methods. Each value represents the mean ± S.E.M. ($n = 9$). ***$P < 0.001$ (Student's *t*-test), as compared to control.

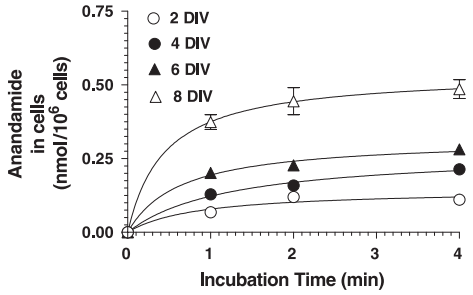

Fig. 2. Anandamide transport in cerebellar granular neurons as a function of days in vitro. Cells were incubated at 37 °C with [^{3}H]anandamide (4 μM) for various times, between 0 and 4 min, as described in experimental procedures. Nonspecific uptake was determined by incubating samples at 4 °C. Non-specific [^{3}H]anandamide association comprised ~5% of the added [^{3}H]anandamide and was subtracted from each data point. Each value represents the mean ± S.E.M. (n = 6).

labeled compound extracted from the cells and media was indeed anandamide, the Rf values of the radioactive samples isolated were compared with those of the authentic anandamide after separation in three different solvent systems. Further characterization of anandamide was achieved by gas chromatography–mass spectrometry (GC–MS) analysis of the trimethylsilyl ether (TMS) derivative of TLC-purified anandamide. Diagnostic ions for anandamide included m/z 419 (M+, molecular ion), 404 $[M-15]^+$, loss of methyl radical, 328 $[M\text{-}91]^+$(data not shown).

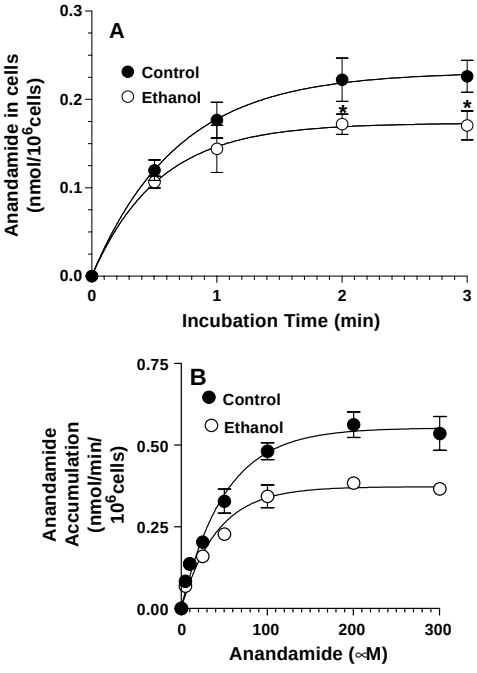

Fig. 3. Kinetic analysis of anandamide transport in cerebellar granular neurons following chronic exposure to ethanol. (A) Time course of anandamide uptake. Chronic ethanol-treated (100 mM, 72 h) neurons were incubated at 37 °C with [^{3}H]anandamide (4 μM) for the times indicated, as described in experimental procedures. Nonspecific uptake was determined by incubating samples at 4 °C and was subtracted from each data point. $T_{1/2}$ value was calculated using the GraphPad program. Data plotted represent mean ± S.E.M. of triplicate determinations, from three separate experiments. (B) Saturation kinetics of anandamide uptake. Chronic ethanol-treated (100 mM, 72 h) neurons were incubated with increasing concentrations of anandamide (with 1 nM [^{3}H]anandamide) for 3 min at 37 °C, as described in experimental procedures. The K_m and V_{max} values were calculated using the GraphPad program. Each value represents the mean ± S.E.M. (n = 6). *P < 0.05; **P < 0.001 (Student's t-test), as compared with control.

3.2. Anandamide transport

Chronic ethanol-induced increase in the extracellular anandamide could be due to a decrease in anandamide uptake. Anandamide uptake in normal cerebellar granular neurons was found to be dependent on the number of days the neurons had been in culture, as shown in Fig. 2. The uptake of anandamide was greater in the older cultures (6–8 days in vitro), than in younger cultures (2–4 days in vitro). Therefore, 7-day cultures were used for subsequent studies.

The time course of anandamide uptake in cerebellar granular neurons is shown in Fig. 3A. Uptake was reduced in neurons exposed to chronic ethanol at 2 and 3 min with a 30% inhibition after 3 min of incubation. Detailed kinetic parameters, including $T_{1/2}$ values are shown in Table 1. Anandamide uptake is saturable with a K_m value of 45 ± 7.8 μM and a V_{max} of 0.65 ± 0.035 nmol/min/10^6 cells. Chronic exposure to ethanol did not alter the K_m, but significantly decreased the V_{max} by 33% (K_m, 32.7 ± 5.5 μM, P> 0.05; V_{max} 0.43 ± 0.009 nmol/min/10^6 cells, P < 0.0001) (Fig. 3B).

Table 1
Effect of chronic ethanol exposure on kinetic parameters of anandamide transport in cerebellar granular neurons

	Control	Ethanol
Rat		
K_m (μM)	45.0 ± 7.8	32.7 ± 5.5
V_{max} (nmol/min/10^6 cells)	0.65 ± 0.035	0.43 ± 0.009***
$T_{1/2}$ (s)	28.32 ± 6.4	22.14 ± 3.80
Mice CB1 receptor wild type		
V_{max} (nmol/min/10^6 cells)	0.75 ± 0.09	0.49 ± 0.084*
$T_{1/2}$ (s)	82.32 ± 9.0	59.4 ± 6.0*
Mice CB1 receptor knockout		
V_{max} (nmol/min/10^6 cells)	0.72 ± 0.11	0.55 ± 0.1***
$T_{1/2}$ (s)	79.0 ± 8.7	60.12 ± 5.1**

Kinetic analysis of anandamide transport in cerebellar granular neurons. Neurons (2 or 8 × 10^6 cells) were treated with or without chronic ethanol (100 mM, 72 h) and incubated at 37 °C with [^{3}H]anandamide as described in experimental procedures. Nonspecific transport was determined in the presence of 100 μM AM404 or incubation at 4 °C and was subtracted from each data point. Data represent apparent K_m, V_{max}, and $T_{1/2}$ values as a means ± S.E.M. of duplicate or triplicate determinations from four separate experiments. *P < 0.05; **P < 0.001; ***P < 0.0001 compared with the control.

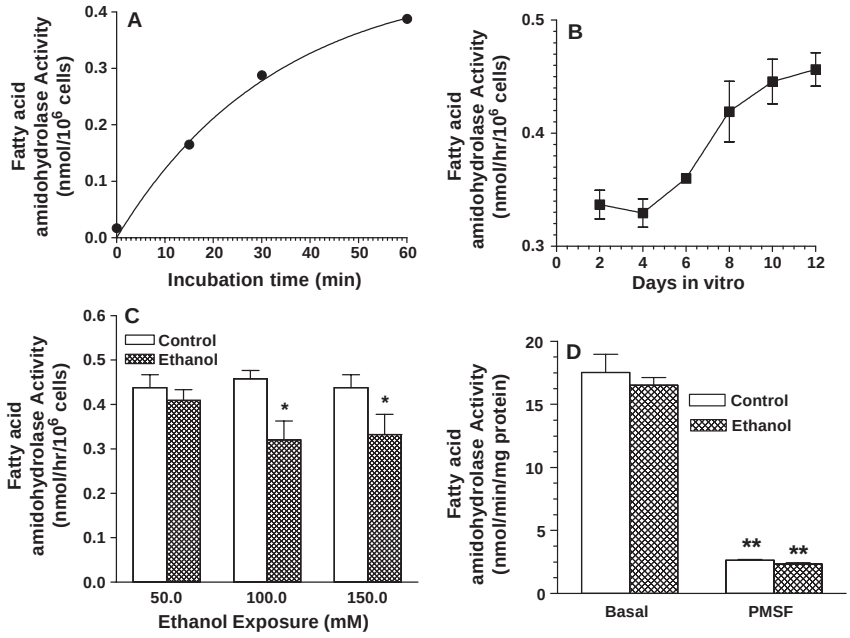

Fig. 4. Effect of chronic ethanol exposure on fatty acid amidohydrolase activity in cerebellar granular neurons. (A) Neurons (2×10^6 cells) were incubated with anandamide (1 μM, [ethanolamine-3H) in BME (high K^+) without FCS for the indicated times. The media and cells were extracted and the amount of ethanolamine-^{3}H in the aqueous phase was determined as described in experimental Procedures. (B) Effect of age (days in vitro) on fatty acid amidohydrolase activity in cerebellar granular neurons. Cells, 2×10^6, from various days in vitro were incubated with anandamide (1 μM, [^{3}H]-Ethanolamine) in BME (high K^+) without FCS for 60 min. (C) Neurons (2×10^6 cells) were exposed to various concentrations of ethanol, as shown, for 72 h and incubated with anandamide (ethanolamine1-^{3}H) (1 μM) in BME (high K^+) without FCS for 60 min. (D) Neurons (8×10^6 cells) were exposed to 100 mM ethanol for 72 h and fatty acid amidohydrolase activity was determined in vitro. Samples of cell lysates containing 50 μg of protein were incubated in 0.1 M Tris–HCl, pH 8.0, 2.5 mg/ml bovine serum albumin and 30 μM anandamide (ethanolamine-^{3}H) for 30 min at 37 °C in the absence or presence of 1 mM phenylmethylsulfonyl fluoride, as described in experimental procedures. The fatty acid amidohydrolase activity was expressed as nmol/h/10^6 cells or nmol/min/mg protein. Each value represents the mean ± S.E.M. ($n = 9$). *$P < 0.05$; **$P < 0.001$, as compared with control.

3.3. Effects of chronic ethanol on fatty acid amidohydrolase

Fatty acid amidohydrolase metabolized anandamide in a time-dependent manner. It was found that the reaction proceeded linearly for at least 30 min with maximum hydrolysis of anandamide observed at 60 min (Fig. 4A). Furthermore, fatty acid amidohydrolase activity was higher in the older cultures (6–12 days in vitro) than in younger cultures (2–4 days in vitro) (Fig. 4B). The amount of fatty acid amidohydrolase activity found in the neurons after 60 min incubation was significantly reduced (by about 30%) in the neurons that were exposed chronically (72 h) to 100 or 150 mM ethanol, as compared to controls (Fig. 4C). Furthermore, in order to determine whether the observed effect was due to a direct effect of ethanol on fatty acid amidohydrolase activity or on the anandamide transport, we determined fatty acid amidohydrolase activity in vitro in cell lysates derived from neurons either treated or untreated with chronic ethanol. There was no statistical difference in fatty acid amidohydrolase activity between lysates derived from neurons treated with or without chronic ethanol ($P > 0.05$). In lysates from both control and ethanol-exposed neurons, fatty acid amidohydrolase activity was inhibited by phenylmethylsulfonyl fluoride ($P < 0.001$), a known fatty acid amidohydrolase inhibitor (Fig. 4D).

3.4. Effect of chronic ethanol on AEA transport in cannabinoid CB1 receptor knockout neurons

We have extended our studies to clarify whether cannabinoid CB1 receptors have any role in anandamide transport and chronic ethanol has any influence on this process in cerebellar granular neurons. SR141716A did not alter anandamide transport either in control or in the neurons exposed to ethanol (Fig. 5A). In the PCR analysis, wild-type mice exhibited a 1237-bp band and knockout mice exhibited a 1088-bp band (Fig. 5B). A predominant band of ~ 54 kDa was observed in cannabinoid CB1 receptor wild-type mice brain samples and it was absent in knockout mice (Fig. 5B). The ~ 54 kDa band is consistent with the predicted size of the cannabinoid CB1 receptor (Matsuda et al., 1990).

In these studies, anandamide transport was not altered in cerebellar granular neurons derived from cannabinoid CB1 receptor knockout mice, as compared to neurons derived from wild-type mice. However, chronic exposure to ethanol significantly reduced anandamide transport in neurons derived from both knockout and wild-type mice (Fig. 5B).

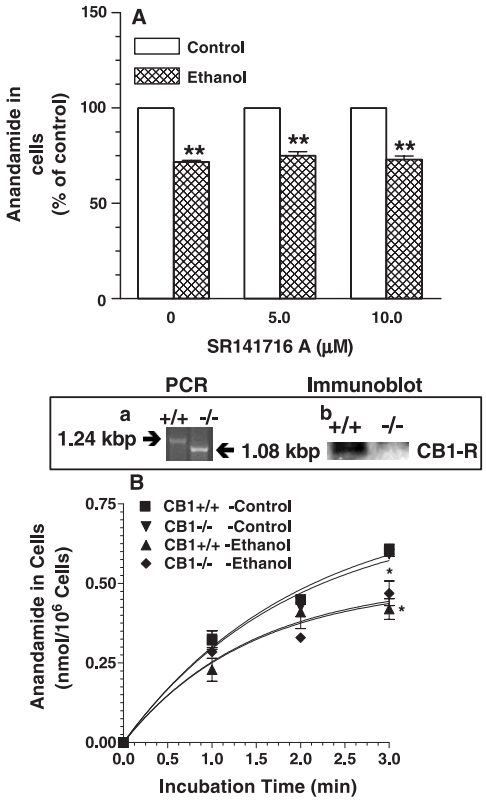

Fig. 5. Effect of cannabinoid CB1 receptor antagonist and targeted mutation of cannabinoid CB1 receptors on anandamide transport in cerebellar granular neurons following exposure to chronic ethanol. (A) Neurons were pre-incubated at 37 °C with various concentrations of SR141716A for 10 min. Cells were then further incubated with [^{3}H]anandamide (4 μM) for an additional 3 min, as described in experimental procedures. (B) Effect of chronic ethanol (100 mM, 72 h) on anandamide uptake in cerebellar granular neurons derived from cannabinoid CB1 receptor wild-type and knockout mice. Neurons were incubated at 37 °C with [^{3}H]anandamide (4 μM) for the indicated times, as described in experimental procedures. Nonspecific uptake was determined by incubation at 4 °C and was subtracted from each data point. Average control value was 0.35 ± 0.05 nmol/10^6 cells/min. Results are expressed as average percentage of control ± S.E.M. from four independent determinations. Inset: (a) Tail DNA from cannabinoid CB1 receptor wild-type ($CB1^{+/+}$) and knockout ($CB1^{-/-}$) mice pups was analyzed by PCR, as described in experimental procedures. (b) Western blot analysis of brain (-cerebellum) from wild-type ($CB1^{+/+}$) and knockout ($CB1^{-/-}$) mice demonstrating the selective absence of cannabinoid CB1 receptor protein in knockout mice ($CB1^{-/-}$). $^*P<0.05$;$^{**}P<0.00,1$ as compared with control.

Detailed kinetic parameters, including V_{max} and $T_{1/2}$ are shown in Table 1.

3.5. Effects of acute ethanol on anandamide transport

The acute effect of ethanol on anandamide transport in cerebellar granular neurons was also evaluated. Acutely, ethanol inhibited anandamide uptake in a time- and dose-dependent manner (Fig. 6A and B). In order to determine whether the anandamide transport plays a role in cellular tolerance to ethanol, we also exposed neurons to chronic

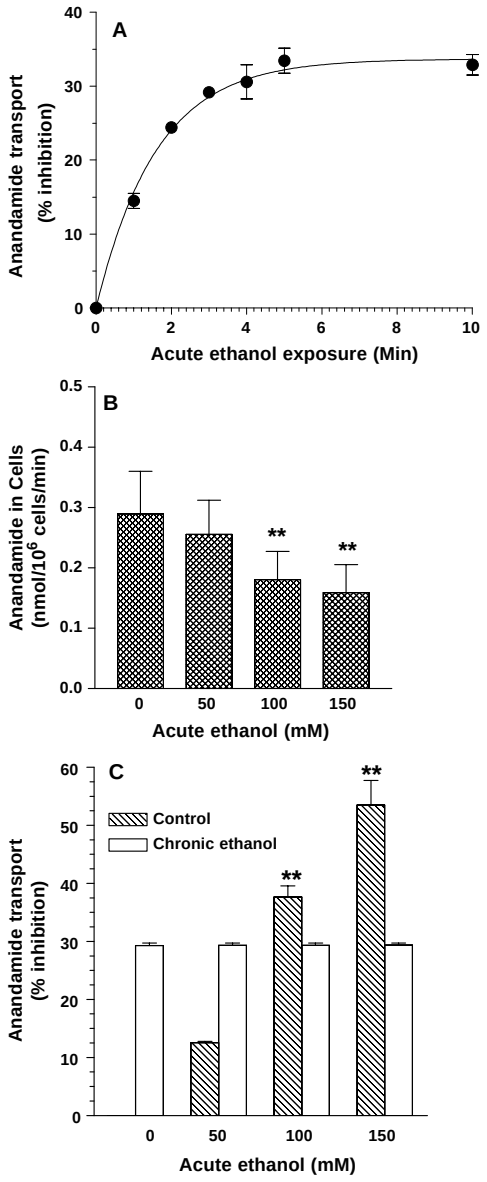

Fig. 6. Effect of acute ethanol on anandamide uptake in cerebellar granular neurons following exposure to chronic ethanol. (A) Time course of anandamide uptake in neurons in the presence or absence of acute ethanol. Neurons were pre-incubated at 37 °C with 100 mM ethanol for the times indicated and further incubated with [^{3}H]anandamide (4 μM) for an additional 3 min as described in experimental procedures. (B) Dose-dependent inhibition of anandamide uptake by various concentrations of acute ethanol. Neurons were pre-incubated at 37 °C with various concentrations of ethanol for 5 min and further incubated with [^{3}H]anandamide (4 μM) for an additional 3 min. (C) Effect of acute ethanol on anandamide uptake in neurons exposed to with or with out chronic ethanol. Neurons were exposed to 100 mM ethanol for 72 h (chronic ethanol) and pre-incubated at 37 °C with various concentrations of ethanol for 5 min (acute ethanol) and further incubated with [^{3}H]anandamide (4 μM) for an additional 3 min as described in experimental procedures. Nonspecific uptake was determined by incubation at 4 °C and was subtracted from each data point. Value for control is 0.35 ± 0.05 nmol/10^6 cells/min. Results are expressed as mean ± S.E.M. of four independent determinations done in duplicates. $^{**}P<0.001$ as compared with control.

ethanol (100 mM for 72 h) prior to the acute ethanol treatment (100 mM for 10 min) and analyzed anandamide uptake for 3 min. After prolonged exposure to ethanol, the acute effect of ethanol on anandamide uptake in neurons was abolished (Fig. 6C).

4. Discussion

We have recently demonstrated that chronic ethanol exposure increases the extracellular levels of endocannabinoids in human neuroblastoma cells and in primary cultures of cerebellar granular neurons (Basavarajappa and Hungund, 1999a; Basavarajappa et al., 2000). In the present study, we investigated the effect of ethanol on anandamide transport processes as a possible mechanism underlying ethanol-induced increases in extracellular anandamide levels.

Anandamide transport was higher in older cerebellar granular neurons cultures than in younger cultures, suggesting that the expression of the anandamide transporter may be higher in older cultures. These results are consistent with transport processes that are protein-mediated and suggest that protein expression, or lack thereof, is likely to play a major role in anandamide accumulation by various cell types. This has been previously demonstrated with 2-arachidonylglycerol, which is a second putative endocannabinoid that competes with anandamide for transport into neurons. The accumulation of 2-arachidonylglycerol was higher in older cultures, as compared to younger cultures (Basavarajappa et al., 2000).

Since it was previously shown that chronic ethanol treatment increases extracellular anandamide levels in a time- and dose-dependent manner in human neuroblastoma cells, with a maximal effect at 100 mM ethanol after 72 h (Basavarajappa and Hungund, 1999a), we used these conditions in the present study with cerebellar granular neurons. Chronic exposure to ethanol enhanced the formation and increased the extracellular levels of [^{3}H]anandamide in neurons, consistent with our previous observations in neuroblastoma cells (Basavarajappa and Hungund, 1999a). This is the first observation where chronic ethanol exposure has been found to decrease anandamide uptake by inhibiting its influx in neurons. This decrease in anandamide uptake appears to account for the chronic ethanol-induced increase in extracellular anandamide. Interestingly, a similar phenomenon was also observed on adenosine uptake in response to acute ethanol (Nagy et al., 1990). The basal K_m and V_{max} values observed in rat cerebellar granular neurons in the present investigation more closely resembled those reported in cerebellar granular neurons by others (41 ± 15 μM and V_{max} 0.61 ± 0.04 nmol/min/10^6 cells) (Hillard et al., 1997), than the kinetic parameters reported for cortical neurons (K_m, 1.2 μM and V_{max} 0.091 nmol/min/mg protein) (Beltramo et al., 1997), cortical astrocytes (K_m, 0.32 μM and V_{max} 0.171 nmol/min/mg protein) (Beltramo et al., 1997) or CHP100 cells (K_m, 0.2 ± 0.02 μM and V_{max} 0.03 ± 0.003 nmol/min/mg protein) (Maccarrone et al., 1998). It should be noted that anatomical studies of endocannabinoid transport are greatly limited by the lack of specific markers for the anandamide transport processes. Nevertheless, biochemical experiments have documented anandamide uptake in the somatosensory, motor, and limbic areas of the cortex, striatum, hippocampus, thalamus, septum, substantia nigra, amygdala, and hypothalamus (Giuffrida et al., 2001), brain regions also known to express cannabinoid CB1 receptors.

Chronic ethanol-induced inhibition of anandamide uptake was time-dependent and saturable in cerebellar granular neurons. These findings are consistent with a protein carrier-mediated transport process. These observations suggest that chronic ethanol prevents anandamide from inactivation and thereby magnifies the biological effects of this short-lived lipid mediator. This may represent the mechanism by which cannabinoid CB1 receptors are downregulated in the brains of mice chronically exposed to ethanol (Basavarajappa et al., 1998; Basavarajappa and Hungund, 1999b).

We have extended our current observations to identify the other targets that might be involved in the enhanced accumulation of extracellular anandamide in cerebellar granular neurons by chronic ethanol, such as fatty acid amidohydrolase. The catalytic properties of fatty acid amidohydrolase, the major enzyme responsible for the metabolism of fatty acid amides, have been thoroughly investigated (Di Marzo et al., 2000). When anandamide is added to cultured cerebellar granular neurons, it is taken up and hydrolyzed to arachidonate in a time-dependent manner. The kinetic results that we observed are similar to those obtained for the brain and neuronal fatty acid amidohydrolase (Di Marzo et al., 1994; Omeir et al., 1995; Ueda et al., 2000). Fatty acid amidohydrolase activity is dependent on the age of the cerebellar granular neurons in culture. Fatty acid amidohydrolase activity levels were higher in older cultures than in younger cultures. It has previously been shown that fatty acid amidohydrolase expression increases dramatically during the postnatal stages of rat brain development (Thomas et al., 1997), a time at which synapse formation is occurring rapidly (Eayrs, 1966). Fatty acid amidohydrolase was shown to be widely expressed in neurons throughout the brain (Thomas et al., 1997). The most prominent expression was observed in neurons of the neocortex, hippocampus, amygdala, and cerebellar cortex, the regions have been implicated in sleep, motivational, and emotional activities of the brain (Thomas et al., 1997). The amount of fatty acid amidohydrolase activity found in the intact neurons after 60 min incubation was significantly reduced by exposure to 100 or 150 mM ethanol for 72 h. Although chronic ethanol inhibited the fatty acid amidohydrolase activity in intact cells, no such

inhibition was observed in cell lysates, which seems to suggest that the observed increase in extracellular anandamide is not due to inhibition of fatty acid amidohydrolase activity by chronic ethanol but due to inhibition of anandamide transport. The alternative explanation for this observation is that ethanol may be inhibiting fatty acid amidohydrolase activity in intact neurons by indirect mechanism that may be abolished in cell lysates conditions. Although these studies clearly suggest a role for anandamide transporter in ethanol-induced accumulation of extracellular anandamide in cerebellar granular neurons, these observations do not completely rule out the involvement of fatty acid amidohydrolase and other enzymes involved in the metabolism of anandamide. This leads us to believe that the mechanism for increased accumulation of anandamide in chronic ethanol-treated cells could also be due to ethanol's ability to inhibit fatty acid amidohydrolase activity and stimulation of anandamide synthesis. Inhibition of both the anandamide transport and fatty acid amidohydrolase activity could contribute to the observed changes in anandamide accumulation. We are currently evaluating other possible mechanisms using fatty acid amidohydrolase knockout mice and other available chemical tools.

We have extended our studies to clarify whether cannabinoid CB1 receptors have any role in anandamide transport and whether chronic ethanol has any influence on this process. The potent cannabinoid receptor antagonist SR141716A did not inhibit anandamide transport in cerebella granular neurons, suggesting that cannabinoid receptors are not directly involved in anandamide uptake. These results are in agreement with previous observations that the potent cannabinoid CB1 receptor agonists and antagonist did not significantly affect the cellular accumulation of anandamide (Hillard et al., 1997; Rakhshan et al., 2000). Furthermore, for the first time, the current study evaluated anandamide transport in cerebellar granular neurons derive from cannabinoid CB1 receptor knockout mice to understand the functional interaction of cannabinoid CB1 receptors in ethanol induced inhibition of anandamide transport. Anandamide transport was not altered in cerebellar granular neurons from cannabinoid CB1 receptor knockout mice, as compared to wild-type. These results clearly demonstrate that anandamide transport processes are independent of the cannabinoid CB1 receptor. Similarly, chronic exposure to ethanol significantly reduced anandamide transport in neurons derived from both cannabinoid receptor knockout and wild-type mice which suggest that chronic ethanol modulates anandamide transport processes. It is possible that ethanol may modify the conformation of the protein carrier in such a way that the anandamide transporter cannot efficiently recognize and translocate anandamide. It has been suggested that the recognition and translocation of substrates by the anandamide transporter are governed by distinct conformational preferences (Piomelli et al., 1999). When more specific molecular probes for the anandamide transport processes become available, it will be possible to directly determine the mechanism by which chronic ethanol exposure alters the anandamide transport.

Acute ethanol treatment inhibited anandamide uptake in cerebellar granular neurons in a time- and dose-dependent manner. This inhibitory effect of acute ethanol was absent in neurons that had been chronically exposed to ethanol. These findings suggest that the development of cellular tolerance to ethanol might be due to modification of the anandamide transport in cerebellar granular neurons. Similar cellular tolerance to ethanol has been observed in the adenosine transport system (Nagy et al., 1990).

Anandamide has been shown to mediate many of the functions of cannabinoid CB1 receptors (for references, see Basavarajappa and Hungund, 2002). Thus, it is possible that the chronic ethanol-induced downregulation of cannabinoid CB1 receptors and its intracellular signaling pathways (Basavarajappa et al., 1998; Basavarajappa and Hungund, 1999b) are partially caused by inhibition of the anandamide transport followed by enhanced accumulation of extracellular anandamide and 2-arachidonylglycerol (Basavarajappa and Hungund, 1999a; Basavarajappa et al., 2000). Ethanol-induced changes in the anandamide transport processes could account for many of the adaptive responses to ethanol. Furthermore, these findings may relate to the role of the endocannabinoid signaling system in regulation of ethanol drinking behavior (Hungund et al., 2003; Wang et al., 2003). Finally, ethanol-induced regulation of anandamide transport may represent a common brain pathway that mediates the reinforcing properties of various drugs of abuse, including alcohol. Further in vivo studies will clearly be required to test this hypothesis.

In summary, we have found that anandamide transport processes are inhibited by EtOH in primary cultured cerebellar granular neurons. This inhibition is likely to represent the partial mechanism by which chronic ethanol exposure increases the extracellular accumulation of anandamide. However, it does not exclude other mechanisms including fatty acid amidohydrolase activity. Anandamide transport processes and chronic alcohol action on anandamide transport are independent of cannabinoid CB1 receptors. A better understanding of the molecular mechanisms underlying the effects of prolonged exposure to ethanol may facilitate the development of improved pharmacological strategies to treat alcoholism. Further studies of the roles played by endocannabinoids, fatty acid amidohydrolase, and anandamide transport in the long-term effects of ethanol represent an important step in this direction.

Acknowledgements

This study was supported in part by funds from New York State Psychiatric Institute and NIH grant, AA13003 and NARSAD independent investigator award.

References

Arnone, M., Maruani, J., Chaperon, F., Thiebot, M., Poncelet, M., Soubrie, P., Le Fur, G., 1997. Selective inhibition of sucrose and ethanol intake by SR 141716, an antagonist of central cannabinoid (CB1) receptors. Psychopharmacology 132, 104–106.

Basavarajappa, B.S., Hungund, B.L., 1999a. Chronic ethanol increases the cannabinoid receptor agonist, anandamide and its precursor *N*-arachidonyl phosphatidyl ethanolamine in SK-N-SH cells. J. Neurochem. 72, 522–528.

Basavarajappa, B.S., Hungund, B.L., 1999b. Down-regulation of cannabinoid receptor agonist-stimulated [^{35}S] GTPγS binding in synaptic plasma membrane from chronic ethanol exposed mouse. Brain Res. 815, 89–97.

Basavarajappa, B.S., Hungund, B.L., 2002. Neuromodulatory role of the endocannabinoid signaling system in alcoholism: an overview. Prostaglandins Leukot. Essent. Fat. Acids 66, 287–299.

Basavarajappa, B.S., Cooper, T.B., Hungund, B.L., 1998. Chronic ethanol administration down-regulates cannabinoid receptors in mouse brain synaptic plasma membrane. Brain Res. 793, 212–218.

Basavarajappa, B.S., Saito, M., Cooper, T.B., Hungund, B.L., 2000. Stimulation of cannabinoid receptor agonist 2-arachidonylglycerol by chronic ethanol and its modulation by specific neuromodulators in cerebellar granule neurons. Biochem. Biophys. Acta 1535, 78–86.

Beltramo, M., Stella, N., Calignano, A., Lin, S.Y., Makriyannis, A., Piomelli, D., 1997. Functional role of high-affinity anandamide transport, as revealed by selective inhibition. Science 277, 1094–1097.

Bligh, E., Dyer, W., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–920.

Colombo, G., Agabio, R., Fa, M., Guano, L., Lobina, C., Loche, A., Reali, R., Gessa, G., 1998. Reduction of voluntary ethanol intake in ethanol-preferring sP rats by the cannabinoid antagonist SR-141716. Alcohol Alcohol. 33, 126–130.

Colombo, G., Serra, S., Brunetti, G., Gomez, R., Melis, S., Vacca, G., Carai, M.M., Gessa, L., 2002. Stimulation of voluntary ethanol intake by cannabinoid receptor agonists in ethanol-preferring sP rats. Psychopharmacology (Berl) 159, 181–187.

Cravatt, B.F., Giang, D.K., Mayfield, S.P., Boger, D.L., Lerner, R.A., Gilula, N.B., 1996. Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. Nature 384, 83–87.

Deutsch, D.G., Chin, S.A., 1993. Enzymatic synthesis and degradation of anandamide, a cannabinoid receptor agonist. Biochem. Pharmacol. 46, 791–796.

Devane, W.A., Hanus, L., Breuer, A., Pertwee, R.G., Stevenson, L.A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A., Mechoulam, R., 1992. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 258, 1946–1949.

Di Marzo, V., Fontana, A., Cadas, H., Schinelli, S., Cimino, G., Schwartz, J.C., Piomelli, D., 1994. Formation and inactivation of endogenous cannabinoid anandamide in central neurons. Nature 372, 686–691.

Di Marzo, V., Bisogno, T., De Petrocellis, L., 2000. Endocannabinoids: new targets for drug development. Curr. Pharm. Des. 6, 1361–1380.

Eayrs, J.T., 1966. Thyroid and central nervous development. Sci. Basis Med. Annu. Rev. 1, 317–339.

Egertova, M., Giang, D.K., Cravatt, B.F., Elphick, M.R., 1998. A new perspective on cannabinoid signalling: complementary localization of fatty acid amide hydrolase and the CB1 receptor in rat brain. Proc. R. Soc. Lond., B Biol. Sci. 265, 2081–2085.

Freedland, C.S., Sharpe, A.L., Samson, H.H., Porrino, L.J., 2001. Effects of SR141716A on ethanol and sucrose self-administration. Alcohol., Clin. Exp. Res. 25, 277–282.

Gallate, J.E., McGregor, I.S., 1999. The motivation for beer in rats: effects of ritanserin, naloxone and SR 141716. Psychopharmacology 142, 302–308.

Gallate, J.E., Saharov, T., Mallet, P.E., McGregor, I.S., 1999. Increased motivation for beer in rats following administration of a cannabinoid CB1 receptor agonist. Eur. J. Pharmacol. 370, 233–240.

Giuffrida, A., Beltramo, M., Piomelli, D., 2001. Mechanisms of endocannabinoid inactivation: biochemistry and pharmacology. J. Pharmacol. Exp. Ther. 298, 7–14.

Hillard, C.J., Jarrahian, A., 2000. The movement of *N*-arachidonoylethanolamine (anandamide) across cellular membranes. Chem. Phys. Lipids 108, 123–134.

Hillard, C.J., Edgemond, W.S., Jarrahian, A., Campbell, W.B., 1997. Accumulation of *N*-arachidonoylethanolamine (anandamide) into cerebellar granule cells occurs via facilitated diffusion. J. Neurochem. 69, 631–638.

Hungund, B.L., Szakall, I., Adam, A., Basavarajappa, B.S., Vadasz, C., 2003. Cannabinoid CB1 receptor knockout mice exhibit markedly reduced voluntary alcohol consumption and lack alcohol-induced dopamine release in the nucleus accumbens. J. Neurochem. 84, 698–704.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lundquist, F., 1959. The determination of ethyl alcohol in blood and tissue. Meth. Biochem. Anal. 7, 217–251.

Maccarrone, M., van der Stelt, M., Rossi, A., Veldink, G.A., Vliegenthart, J.F., Agro, A.F., 1998. Anandamide hydrolysis by human cells in culture and brain. J. Biol. Chem. 273, 32332–32339.

Matsuda, L.A., Lolait, S.J., Brownstein, M.J., Young, A.C., Bonner, T.I., 1990. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346, 561–564.

Mechoulam, R., Fride, E., 1995. In Cannabinoid Receptors. Academic Press, NY, pp. 233–258.

Mechoulam, R., Ben-Shabat, S., Hanus, L., Ligumsky, M., Kaminski, N.E., Schatz, A.R., Gopher, A., Almog, S., Martin, B.R., Compton, D.R., et al., 1995. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. Biochem. Pharmacol. 50, 83–90.

Nagy, L.E., Diamond, I., Casso, D.J., Franklin, C., Gordon, A.S., 1990. Ethanol increases extracellular adenosine by inhibiting adenosine uptake via the nucleoside transporter. J. Biol. Chem. 265, 1946–1951.

Oberto, A., Marks, N., Evans, H.L., Guidotti, A., 1996. Lead (Pb+2) promotes apoptosis in newborn rat cerebellar neurons: pathological implications. J. Pharmacol. Exp. Ther. 279, 435–442.

Ohno-Shosaku, T., Maejima, T., Kano, M., 2001. Endogenous cannabinoids mediate retrograde signals from depolarized postsynaptic neurons to presynaptic terminal. Neuron 29, 729–738.

Omeir, R.L., Chin, S., Hong, Y., Ahern, D.G., Deutsch, D.G., 1995. Arachidonoyl ethanolamide-[1,2-^{14}C] as a substrate for anandamide amidase. Life Sci. 56, 1999–2005.

Piomelli, D., Beltramo, M., Glasnapp, S., Lin, S.Y., Goutopoulos, A., Xie, X.Q., Makriyannis, A., 1999. Structural determinants for recognition and translocation by the anandamide transporter. Proc. Natl. Acad. Sci. U. S. A. 96, 5802–5807.

Rakhshan, F., Day, T.A., Blakely, R.D., Barker, E.L., 2000. Carrier-mediated uptake of the endogenous cannabinoid anandamide in RBL-2H3 cells. J. Pharmacol. Exp. Ther. 292, 960–967.

Rodriguez de Fonseca, F., Roberts, A.J., Bilbao, A., Koob, G.F., 1999. Cannabinoid receptor antagonist SR141716A decreases operant ethanol self administration in rats exposed to ethanol-vapor chambers. Zhongguo Yaoli Xuebao 20, 1109–1114.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76–85.

Steiner, H., Bonner, T.I., Zimmer, A.M., Kitai, S.T., Zimmer, A., 1999. Altered gene expression in striatal projection neurons in CB1 cannabinoid receptor knockout mice. Proc. Natl. Acad. Sci. U. S. A. 96, 5786–5790.

Stella, N., Schweitzer, P., Piomelli, D., 1997. A second endogenous cannabinoid that modulates long-term potentiation. Nature 388, 773–778.

Sugiura, T., Kondo, S., Sukagawa, A., Nakane, S., Shinoda, A., Itoh, K., Yamashita, A., Waku, K., 1995. 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. Biochem. Biophys. Res. Commun. 215, 89–97.

Thomas, E.A., Cravatt, B.F., Danielson, P.E., Gilula, N.B., Sutcliffe, J.G., 1997. Fatty acid amide hydrolase, the degradative enzyme for anandamide and oleamide, has selective distribution in neurons within the rat central nervous system. J. Neurosci. Res. 50, 1047–1052.

Ueda, N., Puffenbarger, R.A., Yamamoto, S., Deutsch, D.G., 2000. The fatty acid amide hydrolase (FAAH). Chem. Phys. Lipids 108, 107–121.

Wang, L., Liu, J., Harvey-white, J., Zimmer, A., Kunos, G., 2003. Endocannabinoid signaling via CB1 receptors is involved in ethanol preference and its age-dependent decline in mice. Proc. Natl. Acad. Sci. U. S. A. 100, 1393–1398.