RESPONSES OF CULTURED NEURONAL NETWORKS TO THE CANNABINOID MIMETIC ANANDAMIDE

THESIS

Presented to the Graduate Council of the
University of North Texas in Partial
Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Samantha I. Morefield, B.S.
Denton, Texas
May, 1998

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The effects of cannabinoid agonists on spontaneous neuronal network activity were characterized in murine spinal cord and auditory cortical cultures with multichannel extracellular recording using photoetched electrode arrays. Different cultures responded reproducibly with global decreases of spiking and bursting to anandamide and methanandamide, but each agonist showed unique minor effects on network activity. The two tissues responded in a tissue-specific manner. Spontaneous activity in spinal tissue was terminated by 1 μM anandamide and 6.1 μM methanandamide. Cortical activity ceased at 3.5 μM and 2.8 μM respectively. Irreversible cessation of activity was observed beyond 8 μM for both tissues and test substances. Palmitoylethanolamide, demonstrated that CB2 receptors were not present or not responsive. However, the data strongly suggested the presence of CB1 receptors.

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CHAPTER 1

INTRODUCTION

1.1 Objective of Study

The main objective of this study was to characterize effects of anandamide on the spontaneous activity of cultured spinal and cortical neuronal networks. Specific questions to be addressed were: (1) Are receptors for this compound present in these cultures? (2) How repeatable and reproducible are the responses? (3) Can a dose-response relationship be established with one or several main features of the activity? (4) Are the responses or dose-relationships different for spinal and cortical tissue (i.e. is there a tissue specificity)? (5) Can reversible and irreversible effects be determined?

The potential use of cannabinoid agonists and antagonists as therapeutic agents has expanded since the discovery of the endogenous ligand, anandamide (Devane, et al., 1992). In order to use these compounds effectively as medicinal substances, extensive research must be performed to evaluate their effects on functional tissue and organ systems. A useful first step in determining the potential use of a compound for development is a rapid prescreening method. Preliminary data have shown that spontaneously active networks in culture may function as effective test systems for the screening of neuroactive compounds (Gross et al., 1997). In this study, monolayer neuronal cultures grown on photoetched multimicro-electrode plates (MMEPs) were used to monitor spontaneous spike production and bursting within a network. These features can be used to evaluate effects of different pharmacological agents, dynamics of cell death, receptor-ligand interactions, and alterations in metabolism. This test approach allows quantitative observations of drug

effects on networks, which are often too complex to be quantified *in vivo*. Neurotoxicity is one such example. The point at which an effect is reversible or not and to what degree it is reversible can be determined in cell culture. However, before screening of new compounds can be done effectively, intra- and inter-culture repeatability must be demonstrated. Thus, the scope of this project focused on the initial steps taken to characterize the response of spinal and auditory cortex neuronal networks to a test substance. The chosen pharmacological compounds were cannabinoid agonists, due to their novelty and potential in neurobiology. This study begins to pave the way for future applications of MMEPs in the area of rapid screening of neuroactive compounds and subsequent drug development.

1.2 Cannabinoids and Their Receptors

The psychotropic and therapeutic effects of plant cannabinoids have been known for centuries. They are known to exert effects on numerous organ systems, such as the nervous, cardiovascular and reproductive systems (Dewey, 1986). In humans, they produce initial euphoria, followed by sedation, alterations in sensory perceptions and interference with the perception of time and short-term memory (Mechoulam et al., 1994). Also associated with cannabinoids are a multitude of effects that have strong therapeutic potentials (Dewey, 1986). These include antiemetic, anticonvulsant, anti-inflammatory and antinociception properties. A further appeal of these compounds for pharmaceutical purposes is their low toxicity and non-addictive qualities. Until recently, the isolation and characterization of cannabinergic compounds have remained scarce. Difficulties often arose in extraction and purification of cannabinoid compounds due to their lipophilic nature. With the advent of more appropriate techniques, these complications are beginning to be resolved. Through these new methods, the biochemical mechanisms triggered by this class of compounds and their pharmacological properties are beginning to be understood.

The first direct evidence for a cannabinoid receptor came in 1988 with the use of a radiolabeled ligand (CP - 55940) that was shown to bind selectively and with high affinity to rat brain membranes (Devane et al., 1988). The anatomical distribution of receptor binding* throughout the brain has been determined by autoradiography (Herkenham et al., 1991). High density of binding was found in the cerebellum, hippocampus, and cortex, while a low density was found in the medullary nuclei. These findings are consistent with the behavioral and physiological effects associated with cannabinoids. The density of the receptors in the spinal cord appears to be less than in the brain (Herkenham et al., 1991). However, the same study showed that the absolute level of binding is quite high compared to the concentration of receptor binding for "classical " neurotransmitters. A study by Martin et al. (1996) suggests the density of cannabinoid receptors exceeds that of opioid receptors in the spinal cord by 10 to 50 times.

Cannabinoid receptors have been found to belong to a superfamily of G protein-coupled receptors (Howlett, 1984). The principal actions of cannabinoids are mediated through these receptors and the subsequent intracellular signaling mechanisms. It has been shown that cAMP is decreased due to the inhibition of adenylyl cyclase. This inhibition results in a slowing of inactivation of the A-type potassium current at high concentrations of cannabinoids (Deadwyler et al., 1995). Other results of the cannabinoid inhibition of adenylyl cyclase activity includes long-term changes in gene expression by inhibition of cyclic AMP response elements and inhibition of anandamide synthesis (Childers and Deadwyler, 1996). Another major action of cannabinoids is the direct G protein-mediated modulation of ion channels. Cannabinoids have been found to inhibit N- and P/Q-type voltage-dependent calcium currents in cultured rat hippocampal neurons (Twitchell et al., 1997). Cannabinoid modulation of the voltage-dependent calcium channels involved in neurotransmitter release suggests that inhibition of neurotransmitter release might be a major mode of the actions of cannabinoids. Recently, cannabinoids have been found to

inhibit glutamatergic synaptic transmission in hippocampal cultures (Shen et al., 1996). In addition to ion channels, other G protein-coupled effectors have been associated with cannabinoid receptors. Most notably, cannabinoid-induced release of arachidonic acid has been observed in cell culture and found to be mediated by both phospholipase activity and G proteins (Shivachar et al., 1996). Thus, the principal actions of cannabinoids are mediated through G protein-coupled receptors. The primary mode of action appears to be via the inhibition of adenyly cyclase, but other effector systems also are activated to produce a variety of responses.

To date, two subtypes of the cannabinoid receptor, CB1 and CB2, have been identified and cloned (Matsuda et al., 1990; Munro et al., 1993). Most work has involved the CB1 receptor since central nervous system (CNS) responses to cannabinoid compounds are believed to be mediated exclusively by this receptor. Cloning of the CB1 receptor was performed in both rat and human. The rat CB1 receptor shares 97.3% sequence homology with the human CB1 receptor, with 100% identity within the transmembrane regions (Gerard et al., 1991). Peripheral effects of cannabinoids, including antiinflammatory actions have been reported. Cloning and *in situ* hybridization have demonstrated a cannabinoid receptor binding protein (CB2) that occurs only in peripheral tissues and is different from the brain cannabinoid receptor (CB1) (Munro et al., 1993). The CB2 receptor exhibits 68% identity to the CB1 receptor within the transmembrane regions and 44% homology throughout the whole protein (Munro et al., 1993). The CB2 receptor was found to be highly concentrated in the spleen, specifically on mast cells. It appears to play a role in the immune system response and possibly in some nonpsychotropic actions of cannabinoids (Facci et al., 1995). The signal transduction mechanism utilized by CB2 receptors is similar to that of the CB1 receptors in that they both are G protein-coupled and lead to the inhibition of adenyly cyclase activity. However, CB2 receptors fail to modulate calcium channels and the inwardly rectifying potassium

channels (Felder et al., 1995). The binding affinities of several cannabinoids for the CB1 and CB2 receptors were investigated and shown to have slight differences. Specifically, anandamide was shown to bind equipotentially at both subtypes (Felder et al., 1995).

1.3 Specific Ligands: Anandamide, Methanandamide and Palmitoylethanolamide

Arachidonylethanolamine, the endogenous ligand of cannabinoid receptors, was named anandamide on the basis of the Sanskrit word "ananda" (meaning bliss) and the chemical nature of the compound, an amide (Devane et al., 1992). This lipid has been found to be a brain constituent and to bind to the CB1 receptor (Vogel et al., 1993) mimicking the effects of Δ9-tetrahydrocannabinol, the active ingredient of hashish and marijuana. Depolarized neurons release anandamide via a mechanism that may require the calcium-dependent cleavage of a phospholipid precursor, N-acylphosphatidylethanolamine (NAPE) in neuronal membranes (Cadas et al., 1997). An alternative biosynthetic pathway involves an anandamide synthase enzyme that would condense arachidonic acid and ethanolamine following their release from phospholipids by phospholipases A2 and D, respectively (Devane and Axelrod, 1984). Extracellular anandamide is thought to be rapidly inactivated by carrier mediated transport back into the cells (Beltramo et al., 1997). Levels of anandamide as measured in the human brain are at equivalent concentrations to those reported for dopamine and serotonin, but are at least 10 times lower than those reported for GABA and glutamate (Felder et al., 1996). Further studies by Felder et al., (1996) isolated anandamide in human and rat spleens, suggesting that it also serves as an endogenous agonist to the CB2 receptor. The absence of anandamide in human serum, plasma, and CSF suggests that anandamide is not hormonal in nature (Felder et al., 1996). The lipid nature of this compound and its ability to permeate membranes suggests that vesicular storage is highly unlikely (Felder et al., 1996), however no studies have

confirmed this speculation. Behaviorally, anandamide has been shown to mimic the psychotropic effects of Δ9-tetrahydrocannabinol. In humans, cannabinoid compounds are associated with heightened sensitivity and euphoria. In rodents, anandamide reduces spontaneous motor activity, produces hypothermia and also exhibits antinociception (Fride and Mechoulam, 1993). Recently, anandamide has been suggested as one of the pharmacological compounds in chocolate responsible for the accompanying transient feeling of well-being upon consumption (di Tomaso et al., 1996). Also, experimental evidence suggests that anandamide may have a role in the mediation of sleep induction (Mechoulam et al., 1997). Other interesting effects of the endogenous lipid compound are continuing to be investigated.

Anandamide has been observed to have effects of short duration, as compared to other cannabinoids. This is thought to result from its rapid breakdown by amidase activity that resides in membrane fractions (Deutsch and Chin, 1993) and/or to its rapid reuptake into the neuron via a transmembrane carrier (Beltramo et al., 1997). In conjunction with this, anandamide binds with only moderate affinity for the receptor (Abadji et al., 1994). Due to the short half-life and metabolic instability of the endogenous ligand, a synthetic compound, R(+)-methanandamide, was manufactured to compensate for these research complications (Abadji et al., 1994). Specifically, methanandamide possesses a higher metabolic stability to amidase hydrolysis. No work has been done that investigates the degree to which methanandamide is taken up via the anandamide transporter. To date, methanandamide has been found to be more potent and have a higher degree of stability than anandamide in rat forebrain membrane binding studies, as well as in rodent behavioral studies (Abadji et al., 1994 & Romero et al., 1996). Like anandamide, methanandamide is thought to bind to the CB1 receptor and elicit the same cellular cascades and behavioral effects (Abadji et al., 1994).

Palmitoylethanolamide, found in both peripheral tissues and central neurons, is an endogenous lipid compound formed from the same precursor as anandamide. This N-acylamide is reported to bind to the CB2 receptor with high affinity, but not to the centrally located CB1 receptor (Facci et al., 1995). Anandamide, however, has been demonstrated to bind to both CB1 and the CB2 receptors with similar potencies (Felder et al., 1995). In lieu of this, palmitoylethanolamide was used in this study as an internal cannabinoid control to confirm the absence of CB2 receptors.

1.4 Networks in Culture: Applications to Neurotoxicology and Drug Development

The CNS is highly affected by toxic substances because functional changes can be as disruptive or lethal to an organism as structural damage. Consequently, a large number of chemicals have potential neurotoxic influences. Animal testing is slow and confounded by secondary physiological responses. Direct effects on CNS function are often difficult to quantify. As simplified representations of parent tissue, spontaneously active monolayer networks in culture can give rapid insights into functional and structural damage following exposure to neuroactive compounds. Grown on thin-film electrode arrays, the cultures represent efficient model systems for the experimental study of neuronal responses to chemicals. If applied as a prescreening method, it can accelerate greatly the rapid detection of neurotoxic substances and enhance drug development. The use of networks, as opposed to single cell recordings, allows the use of network dynamics as a primary test parameter. Single neurons are vulnerable units that cannot reflect the characteristics of a functioning tissue. *In vitro* neuronal networks have been shown to develop spontaneous activity and display complex repertoires of spatio-temporal spike patterns that are highly sensitive to their chemical environment (Gross et al., 1992; Gross, 1995; Gross et al.,1997). These networks, when grown on microelectrode arrays, provide data about

changes in their physical and chemical environments in terms of their burst and spike patterns. The *in vitro* environment allows highly reproducible pharmacological manipulations with no homeostatic interference from other organs. Networks are isolated and do not receive undefined input from other neural tissue (Gross et al., 1997). Thus, monolayer neural networks grown on MMEPs appear to function as unique and highly effective test systems for the evaluation of neuroactive substances. This study begins the characterization of neuronal network responses to cannabinoid agonists in anticipation of future drug development and/or screening of neurotoxic cannabinoid analogs.

CHAPTER 2

MATERIALS AND METHODS

2.1 Fabrication of Electrode Arrays

All fabrication occurred in-house via the Center for Network Neuroscience (CNNS) staff. The techniques used to fabricate and prepare multimicroelectrode plates (MMEPs) have been described elsewhere (Gross, 1979; Gross and Lucas, 1982; Gross et al., 1985; Gross and Kowalski, 1991). Briefly, the MMEPs consisted of an array of photoetched electrodes onto which monolayer neuronal networks were grown (Figure 1). The plates (5 cm x 5 cm) were prepared from 1.2 mm thick indium-tin oxide (ITO) sputtered barrier glass (soda lime glass with a 100 nm quartz layer, Applied Films Corp., Boulder, Co.). The electrode conductor pattern consisted of a central 0.8 mm^2 recording matrix of 64 microelectrodes (4 rows, 16 columns). Photoetching was performed with standard procedures. The plates were spin-insulated with a 2 - 4 μm thick layer polysiloxane resin yielding a shunt impedance of approximately 15 MOhms. The matrix microelectrode sites were deinsulated with single laser pulses (Gross, 1979). This resulted in a 20 μm diameter crater at the end of each 10 μm wide ITO conductor. Low recording crater impedances of 1 - 2 MOhms were achieved by electroplating a thin layer of gold on each exposed ITO tip (Gross et al., 1985).

2.2 Cell Culture

Dissociated tissue cultures were prepared according to the basic method established by Ransom et al. (1977). Spinal cord and cortical tissue was harvested from embryonic 14.5 to 15 day old Hsd:ICR mice. The tissue was dissociated enzymatically and

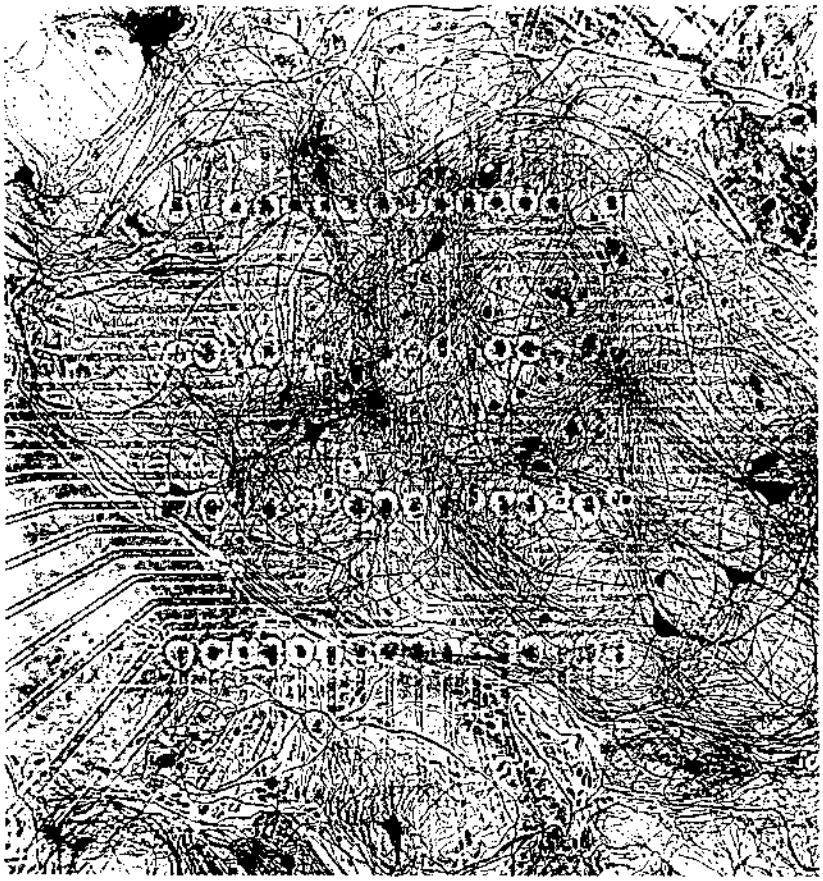


Figure 1: MMEP and monolayer network. High density neuronal monolayer culture composed of approximately 600 neurons growing over a matrix of 64 photoetched electrodes. The recording craters are spaced 40 μm laterally and 200 μm between rows. The transparent indium-tin oxide conductors are 10 μm wide in the recording matrix and are deinsulated at their end points with single laser shots. Tissue, mouse spinal cord; 98 days *in vitro* ; histology, Loots-modified Bodian stain.

mechanically, seeded onto MMEPs, and maintained under Minimal Essential Medium (MEM) supplemented with 10% horse serum and 10% fetal bovine serum. Approximately 1 x 10^6 cells in a 1 mL aliquot were added to each MMEP with the medium confined to a 4 cm^2 area by a silicone gasket.

Before seeding, the MMEPs were prepared to allow for maximum cell adhesion. Butane flaming (1 sec) oxidized the methyl groups of the polysiloxane to hydroxyl groups and formed a hydrophilic surface conducive to cell adhesion. Two adhesion areas, a centrally located recording island (typically 2 - 6 mm in diameter) and a separate, off-center

"medium conditioning area" measuring approximately 1 cm x 2 cm always were formed before seeding of cells. Polylysine and laminin adhered only to the flamed regions and were used to enhance cell attachment and growth.

The cultures were incubated at 37°C in a 10% $C0_2$ atmosphere until ready for use, generally three weeks to three months after seeding. Cells were "fed" twice a week with MEM containing 10% horse serum. In selected cultures 7.5 μM ARA-C, a mitotic inhibitor, was applied to cells to control excessive glial proliferation. Further descriptions of procedures for cell culture and maintenance can be found in previous publications (Gross and Lucas, 1982; Gross et al., 1985; Gross and Kowalski, 1991). All cultures used in this project were provided and maintained by the culture staff of CNNS.

The central island of the MMEP culture, which overlaid the recording electrode matrix, typically developed a confluent glial carpet. Intermixed and atop this carpet, a monolayer neural network formed. Among the network, neuronal somata generally were found on top of the carpet, and axonal processes were found both below and at the surface of the glial layer. Previous unpublished work suggested that basic network morphology is established by 15 days *in vitro* (D.I.V.) and that, on MMEPs, networks can remain viable, stable, spontaneously active, and pharmacologically responsive for more than six months (Gross, 1994).

2.3 Electrophysiological Setup and Recording Procedures

The following arrangement allowed for simultaneous recording of spontaneous extracellular spike activity. The MMEP was placed on a base plate and pressed in place by a silicone 0-ring attached to a stainless steel chamber (Figure 2). The culture was maintained at 37°C by heating the base plate and at a pH of approximately 7.4 under a humidified 10% $C0_2$ atmosphere. A plastic cap covered the chamber and was equipped with lines carrying 10% $C0_2$ and a heating unit to prevent condensation on the cap

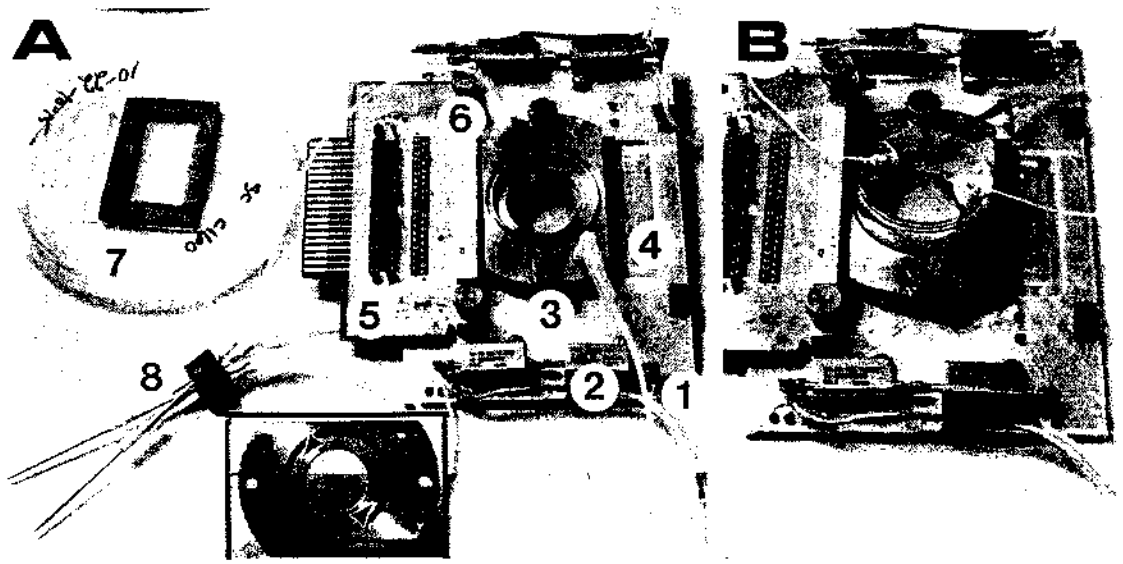


Figure 2: Recording chamber parts and assembly. A: the closed chamber (not used in this study) consists of a base plate (1) with 4 power resistors (2); the chamber cover (3) with the microscope port, medium line connectors, and set screws; two zebra strips (4); and two matched circuit boards (5). Pressure bars (6) press the circuit board against zebra strips. The MMEP is hidden below the chamber cover and zebra strip. A MMEP with gasket and petri dish (normal configuration for maintenance in incubators) is shown to the left (7). Medium pick-up and return lines are also shown (8). The insert at bottom shows the inside of the chamber cover with "O" ring. B: open chamber configuration (employed in study) with microscope window removed and replaced by a plastic cap with CO_2/air gas line.

(Figure 2). This allowed for maintenance of the pH, osmolarity control, and prevention of culture contamination. pH was monitored by a phenol red indicator in the medium. A peach color signified a pH around 7.4, which is optimal for culture survival. Chamber components were sterilized with alcohol and UV light before each experiment. Once the culture was stable, a full medium change using 1 ml of wash medium was performed. All subsequent medium applications originated from the same stock solution. Activity was recorded by a custom multiamplifier system (BAK Electronics) consisting of 36 two stage amplifiers. Total system gain was 10K. Signal output from the amplifiers was distributed to an LED display and to a patch panel. Channels for recording were selected based on the best signal-to-noise ratios. A subset of fourteen channels could be recorded on an analog instrumentation recorder (Racal Store 14 DS) and distributed to a Masscomp 5700

computer. After integration via simple RC circuits (with integration constants of 0.5 sec.), eight channels of data were displayed on a Soltek chart recorder (Figure 3). This spike integration was used as a method of major feature extraction (Figure 4). Integration generated slow voltage changes that were proportional to the spike frequencies recorded and were easily graphed by chart recorders. Integration allowed extraction of bursts, which are generally more easily recognized and measured than spikes. In addition, burst patterns represent a simplified level of activity that often reveal major states or modes of the network activity without massive statistical calculations on very large spike data sets (Gross et al., 1994). If the channel reports a single unit, then the integrated amplitude indicates the instantaneous spike frequency, and the integrated area under the curve represents the total spike production during the burst. However, most channels report more than one unit. Therefore, the integrated burst amplitude is influenced by the number of units, each instantaneous spike frequency, and the action potential size of the various units. Nevertheless, integration provides a useful and informative method of data extraction, especially since there is a strong tendency for units to exhibit approximate synchronization. Further analyses of more subtle aspects of network dynamics would have to incorporate spike separation and spike pattern statistics.

2.4 Data Analysis

Raw spike data was displayed on oscilloscopes. Signal-to-noise ratios were acquired for each channel. During the experiment, 8 channels were monitored on the oscilloscopes and monitored in their integrated form on a chart recorder. Fourteen channels were collected onto the Masscomp 5700 computer. From these data, the burst rate per minute was obtained. For each experiment, the burst rate for each individual channel was plotted during the course of the entire experiment. Also, the mean burst rate for all

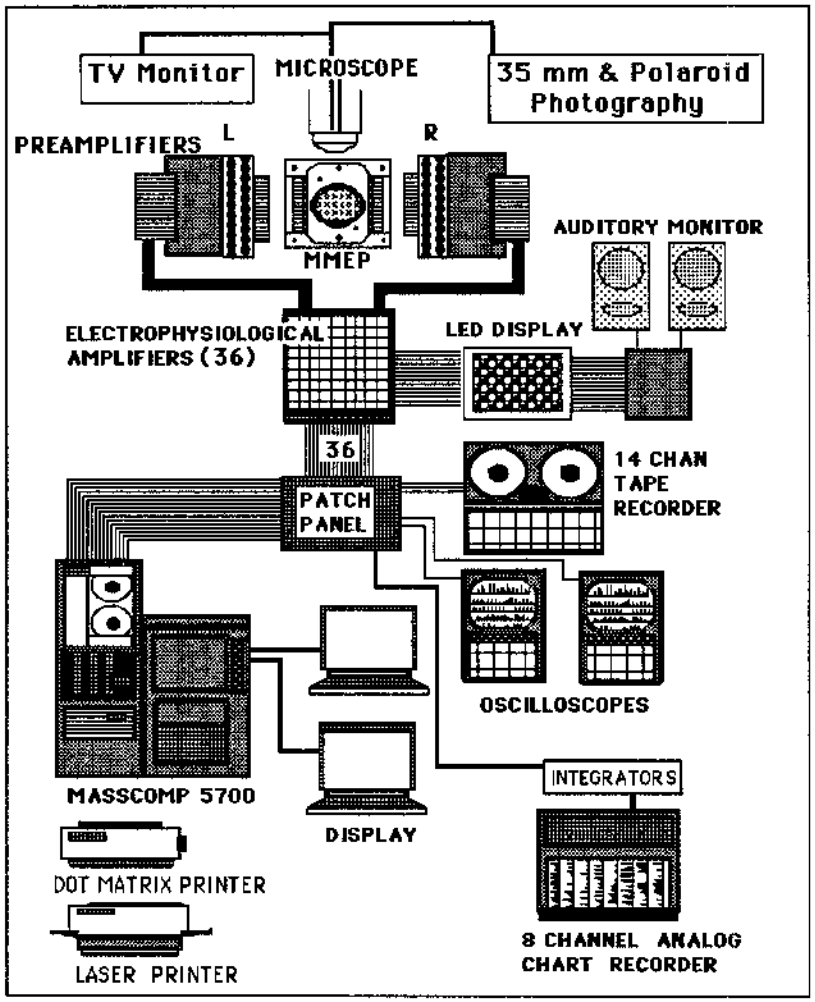


Figure 3: Data acquisition flow chart. Electronic components necessary for data recording and culture life-support.

channels was plotted with the associated standard deviation. For each type of experiment, the mean concentration leading to cessation of activity was determined, as was its associated standard of deviation. Cessation of activity was defined as the point at which the burst rate per minute dropped to ≤ 1 for at least 5 minutes. Washes were expressed as a percent change from the initial native burst rate. If a change of $\pm$ 10% was observed, the system was classified as not returning to a native-like state. If no activity returned over a 2 hour period, the effect was termed irreversible.

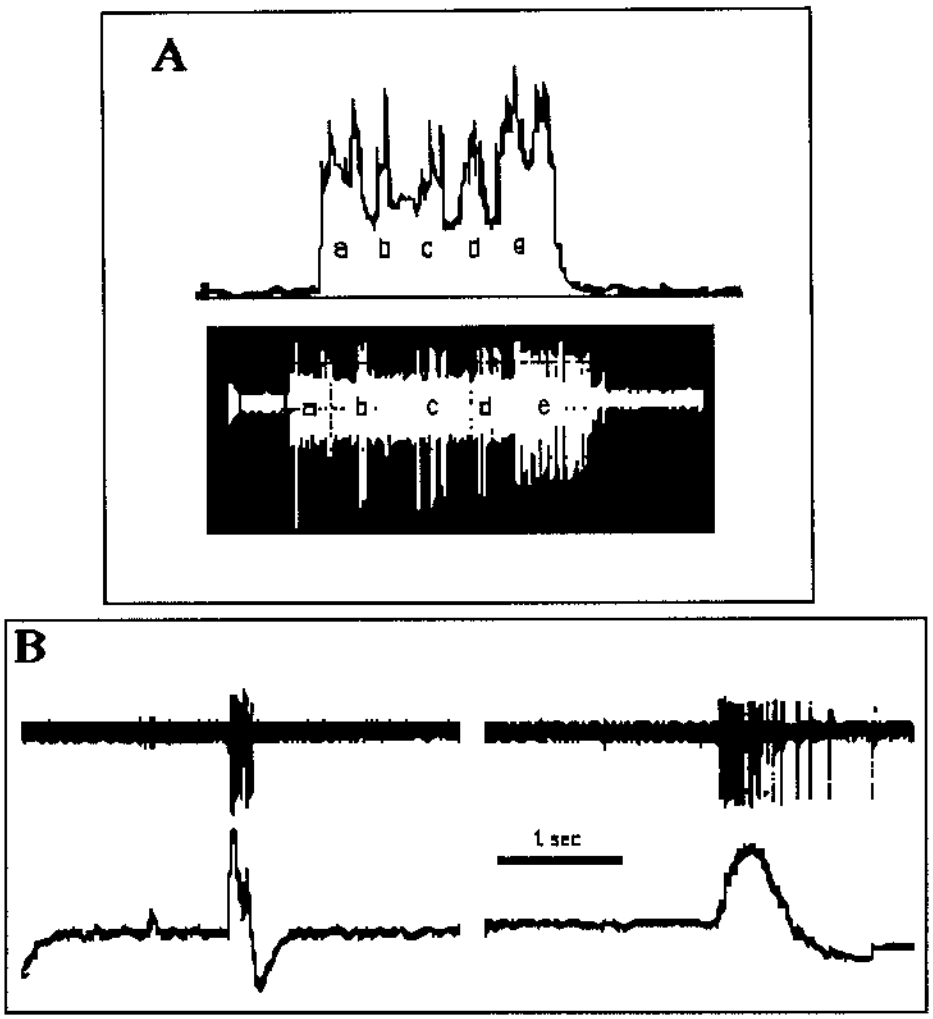


Figure 4: Electrophysiological data. Panel A shows complex burst obtained from five active units recorded simultaneously by one electrode and its analogue integrated profile (time constant: 700 ms). The labels a - e identify major regions of spike density. Panel B shows two separate burst trains with concomitant integrated profiles used for the determination of burst variables.

In all cases, burst rate per minute was used as the primary variable. Burst rate was plotted against time for individual channels as well as for the mean burst rate across all channels for a given experiment. This allowed comparison of the entire network and the individual channels comprising that network. Other major changes in the activity pattern also were noted, such as burst duration and burst amplitude. Spike amplitude of a selected channel was monitored on the oscilloscope during the course of experiments in order to

check for potential effects on action potentials. 50 μM bicuculline was used in 60% of the experiments to simplify data analysis by regularizing and synchronizing the burst rate within the network. Analyses were performed to determine if bicuculline had any confounding effects on the drug responses. Addition of wash medium to the cultures served as a control to assure that the carrier medium elicited no effect. Another source of internal control utilized palmitoylethanolamide. In 20% of the experiments, palmitoyl-ethanolamide, as well as anandamide or methanandamide, was applied to cultures. This compound has been documented not to bind to the CB1 receptor to which anandamide and methanandamide bind (Felder et al., 1995; Skaper et al., 1996), and thus was not expected to produce any effect in the cultures via that receptor.

2.5 Drug Concentration and Administration

Networks on the MMEPs were well-suited for pharmacological analysis due to the ability to maintain a constant environment for long periods of time (several days in the open chamber). Pharmacological manipulations were achieved by drug additions directly to the culture medium bath.

Once the culture was set-up to the recording station and a medium change had been performed, it was allowed to stabilize for approximately 1 hour before any recording proceeded. Base-line activity was recorded for a minimum of 30 minutes before any drugs were applied. Pharmaco- logical test substances were added to the 1 ml bath with Eppendorf micropipettes. Drugs were applied by lifting the chamber cap and gently distributing the compound over the entire bath area. The protocol for all experiments consisted of sequential application of a given test substance ranging from 250 nM - 9.5 μM (10 μL - 100 μL) with simultaneous and continuous recording of the activity. A minimum of 30 minutes between drug applications was observed in order to allow sufficient time for a response. Applications were continued until firing ceased and then were maintained at

that concentration for at least 30 minutes. Next, cultures received 1 to 2 medium washes in order to restore native activity by removing the test substances. Medium washes were conducted by slowly extracting the medium with a 3 mL syringe attached to a 20 gauge needle. Immediately after removal, 1 ml of fresh wash medium was applied using a different syringe. The entire wash procedure took approximately 20 seconds and left the cells exposed under a thin layer of medium for the least amount of time possible. Usually the critical time between removal of medium and arrival of the first part of the new medium was no longer than 10 seconds. Reentry of wash medium was performed gently and with the syringe needle away from the recording matrix in order to minimize cell stress. In most experiments, an exact duplicate of the procedure was repeated on the culture once it reached a stable new baseline. This provided two data sets from a culture and allowed a determination of intraculture variability.

In summary, gross effects on network activity were observed for the minimum dose necessary to produce an observable change (>10%) in burst rate, as well as the minimum dose that led to cessation of activity. Once activity cessation occurred, the degree of reversibility of the effect was evaluated by washing out the drug. This approach allowed for the testing of a range of drug concentrations that defined the minimum and maximum effects on bursting activity. Both anandamide and methanandamide were utilized to observe network response. Although anandamide is the endogenous ligand for the cannabinoid receptors, methanandamide is a more stabile and potent compound (Abadji et al., 1994). Use of this cannabinoid agonist may indicate whether breakdown of anandamide contributed to the observed activity changes. Palmitoylethanolamide also was employed to aid in the determination of the presence of CB2 receptors in the cultures and if the actions of anandamide and methanandamide are receptor or non-receptor mediated

effects. Subsequent work utilizing specific receptor blockers will be necessary to confirm preliminary findings in this area.

In the majority of experiments, 50 μM bicuculline was added to the medium at the onset of the experiment (30 minutes prior to recording) in order to regularize the pattern and facilitate burst counting. The existence of confounding variables associated with bicuculline as an experimental manipulation is still unclear. This study began to look at the network responses to cannabinoid agonists in the presence and absence of bicuculline. This investigation may help facilitate future testing by allowing a simplification of data analysis via regularization of burst rate. However, if significant differences are manifested, this means of simplifying data analysis will need to be eliminated from future protocols. In cases where bicuculline was employed, subsequent washes also contained 50 μM bicuculline.

Anandamide and methanandamide were obtained from RBI (Research Biochemicals International, Natick, MA). Both were supplied as a solution in ethanol. The received stock of anandamide was 14.4 mM and the methanandamide was 13.8 mM. They were stored at -80° C and in amber vials to protect from light exposure. On the experiment day, a 1 mM solution in ethanol was made from the stock. This solution was blown down to dryness under nitrogen and then brought back to a 1 ml volume with wash medium. New working stock (1 mM) was made fresh on each experiment day.

Palmitoylethanolamide initially was provided by Dr. K.D. Chapman's laboratory for preliminary studies. Subsequent supplies of palmitoylethanolamide were obtained from Tocris Chemicals (Ballwin, MO). The compound was supplied as a crystalline solid. A stock solution of 10 mM was made by dissolving the compound in ethanol. This solution was stored at -80° C. On experiment day, a 1 mM solution in ethanol was made from the stock. This solution was blown down to dryness under nitrogen and then brought back to

a 1 ml volume with wash medium. New working stock (1 mM) was made fresh on each experiment day.

Due to the lipophilic nature of the compounds in use, the degree of solubility into the medium was questioned. In order to address this issue, samples were prepared as described above, and given to Dr. K.D. Chapman for gas chromatography analysis. Three samples of each compound were analyzed and the concentrations of each were measured. Table 1 summarizes results obtained from the gas chromatography/flame ionization detector (GC/FID) analysis. The calculated N-acylethanolamine (NAE) concentrations exceeded the measured values by factors of 42.7 - 72.7. All data presented in this study were readjusted based on the correction factors listed in Table 1. Controls, using only wash medium, contained no measurable amounts of NAEs. Concentrations were divided by the corresponding correction factor of the pharmacological compound in medium. An example of anandamide in EtOH can be found in the appendix. NAEs are denoted by the number of carbons in the fatty acid chain and the number of double bonds (i.e. 20:4). Both anandamide and methanandamide are expressed as NAE 20:4.

Briefly, the following procedure was performed for GC/FID analysis: (1) samples were prepared as described above giving a final concentration of 500 μM in a total volume of 500 μL, (2) samples were given to Dr. Chapman and subsequently transferred to glass tubes for extraction of lipids, (3) 9 μL of 0.12 mg/mL NAE 17:0 quantitative standard was added to the samples, representing 1.08 μg original mass, (4) 0.25 mL $CHCl_3$ was added to samples dissolved in EtOH, (5) a mixture of 2 mL propanol and 1 mL $CHCl_3$ was added to samples dissolved in medium, (6) samples were vortexed and left for one hour at 18°C, (7) samples were partitioned with 1 mL $CHCl_3$ and 2 mL of 1M KCl, (8) the organic layer was washed 2 times with 2 mL of 1M KCl, (9) $CHCl_3$ was evaporated off under N_2 and

residue was resuspended in 25 µL bis(Trimethylsilyl)trifluoroacetamide (BTSFA), a derivatizing reagent, (10) samples were capped and incubated at 51°C for 30 minutes, (11) derivitization reagent was evaporated under N_2 and residue was resuspended in 50 µL hexane, (12) samples were stored at -20°C until ready for GC analysis. The TMS-NAEs were chromatographed on a 30 m x 0.32 mm id HP-5 capillary column (HP5890 series II GC). The oven temperature profile was as follows: injection temperature 250°C, gas flow rate 2.3 mL/minute with N_2 as the carrier gas, initial oven temperature 150°C for 1 minute, ramped at 8°C/minute to 280°C and held there for 15 minutes.

Table 1: Measured Drug Concentrations per GC Analysis

Sample Description	N	Calculated Concentration (µM of NAE)	Mean Measured Concentration (µM of NAE)	S.D.	Correction Factor
Anandamide (EtOH)	3	500	8.94	0.29	55.9
Anandamide (Medium)	3	500	7.18	0.43	69.6
Methanandamide (EtOH)	3	500	7.08	0.45	70.6
Methanandamide (Medium)	3	500	11.7	0.49	42.7
Palmitoylethanolamide (EtOH)	3	500	6.88	0.38	72.7
Palmitoylethanolamide (Medium)	3	500	11.2	0.18	44.6

N = sample size S.D. = standard deviation

CHAPTER 3

RESULTS

3.1 Normal Spontaneous Activity and Regularization of Bursting with Bicuculline

Spontaneous activity obtained from cultured spinal cord networks ranged from apparent stochastic spiking to organized bursting. However, most networks displayed burst patterns that were coordinated among some or all of the channels selected for analysis. The initial part of Figure 5 shows integrated spike activity of a spinal cord culture. Coordinated bursting and "random" spiking can be seen among the 5 channels shown. Regardless of the initial native activity, organized coordinated activity can be obtained in almost all cases via disinhibition with bicuculline. This response, which is reversible, results in stable oscillatory behavior. In 65% of the spinal cord cultures, 50 μM bicuculline was applied to regularize network burst activity. This enabled bursts to be discriminated more easily and led to an overall simplification of data analysis. Also, because integrated bursts become similar in amplitude, duration, and general shape (i.e., spike patterns are similar), the use of burst rate as a primary variable is more reasonable. Without bicuculline, bursts used for the determination of burst rate may vary substantially in shape. Figure 5 shows the integrated burst activity for 5 channels for a total of 3.1 minutes. 50 μM bicuculline, added at the arrow, resulted in an immediate change in the burst activity. Similar integrated burst amplitudes and durations were observed among all channels. The mean burst rate across all channels stabilized within 4 minutes after application of the bicuculline to 14 ± 2 bpm.

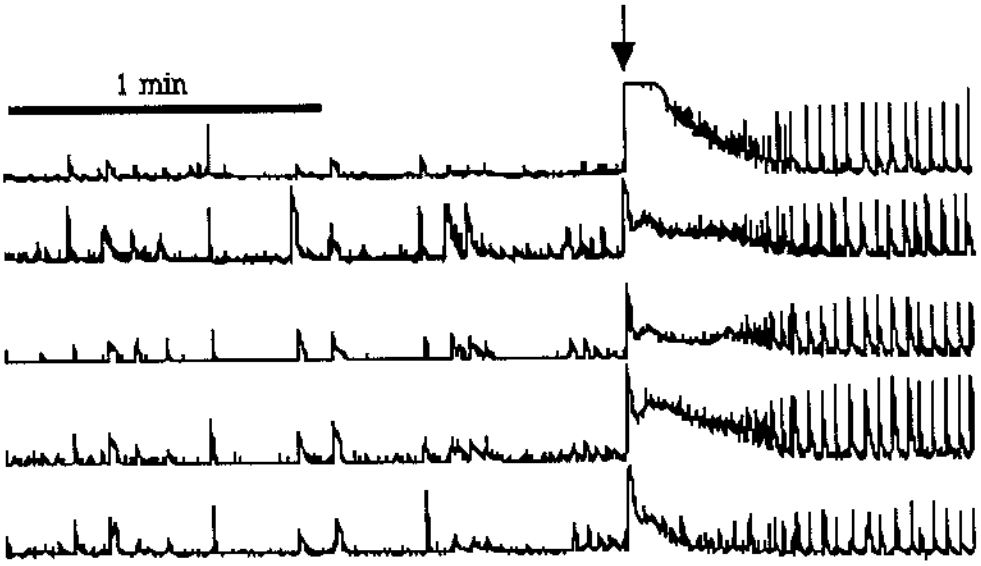


Figure 5: Regularization of burst rate and integrated burst envelope upon addition of 50 μM bicuculline (spinal network). Integrated burst activity of 5 channels is displayed for a 3.1 minute period. The initial 2 minutes shows spinal cord spontaneous activity. Burst rate increased upon addition of 50 μM bicuculline (at arrow). Also, individual channels became more coordinated and regularized (i.e. similar spike patterns in bursts). Within 4 minutes after bicuculline application, network activity stabilized to 14 +/- 2 bpm.

Similar responses were seen with auditory cortical cultures. Normal spontaneous activity displayed random spiking as well as organized bursting. In general, cortical cultures showed a greater amount of native coordination among channels than did spinal networks. As with spinal cultures, disinhibition of the network with bicuculline was followed by enhanced organization into coordinated activity. In 65% of the auditory cortex cultures, 50 μM bicuculline was applied to regularize burst activity. As stated previously, this manipulation enabled a more simplified approach to data analysis. Figure 6 shows 5 channels from a cortical culture prior to and during bicuculline exposure. The initial 2.4 minutes show spontaneous activity. The arrow denotes addition of 50 μM bicuculline. Following this addition, burst rate became more coordinated among the channels. Burst amplitude displayed a large increase and burst duration appeared uniform across all

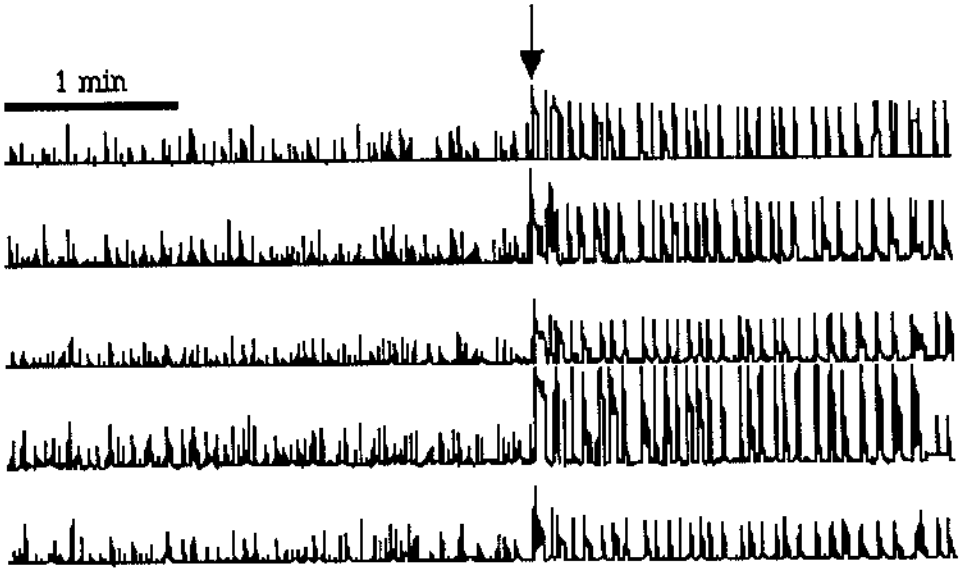


Figure 6: Regularization of burst rate and integrated burst envelope upon addition of 50 μM bicuculline (auditory cortical network). Integrated burst activity of 5 channels is displayed for a 4.3 minute period. The initial 2.4 minutes shows spontaneous activity. Burst rate regularized upon addition of 50 μM bicuculline (at arrow). The greatest effect of disinhibition appeared to be increase of burst amplitude. Within 4 minutes after bicuculline application, network activity stabilized.

channels. As in the spinal cord cultures, stabilization of network activity occurred within 4 minutes of bicuculline administration.

Although the addition of bicuculline aids in data analysis, there is some concern as to whether this manipulation alters the effect of pharmacological test agents. This study looked at the potential for confounding results when employing both bicuculline and cannabinoid agonists. These results are discussed in Section 3.6.

3.2 Effects of Cannabinoid Agonists on Spinal Networks

Table 2 lists the cultures used in experiments investigating the effects of cannabinoid agonists on spinal networks. In all cases, the seeding density onto the MMEPs was 10^6 cells per a 1 ml aliquot.

Table 2: Data Set of Spinal Cord Cultures

Exp	Drug	Bicuc.	Seed Date	Age (DIV)	Treat-ment	Avg. S:N	Max S:N	% Active Channels
SM47	Anand	0	02-27-97	90	ARA-C	3.6:1	6:1	77
SM52	Anand	0	02-27-97	111	-	5.1:1	25:1	69
SM41	Anand	50 μM	01-30-97	79	-	4.8:1	19:1	58
SM42	Anand	50 μM	01-30-97	83	ARA-C	3.6:1	12:1	31
SM27	MethA	0	01-02-97	41	-	2.8:1	10:1	61
SM31	MethA	50 μM	01-23-97	62	-	3.4:1	8:1	89
SM32	MethA	50 μM	01-23-97	70	-	3.1:1	10:1	69
SM37	MethA	50 μM	01-30-97	73	ARA-C	2.8:1	7:1	22

Anand, anandamide; MethA, methanandamide; DIV, days *in vitro* ; ARA-C, cytosine β-D-Arabinofuranoside; S:N, signal-to-noise ratio.

Application of anandamide in spinal cord cultures showed a decrease in the burst rate for all channels recorded. Upon increasing doses of the drug, spike amplitudes either remained the same or increased slightly. No decrease of amplitude occurred indicating that the compound did not act as a metabolic poison. Figure 7 shows spike amplitudes of channel 1 from a spinal cord culture exposed to anandamide. Three distinct states are shown: (A) native activity, (B) application of 850 nM anandamide, and (C) wash x2. The amplitude increased slightly (8%) from native activity to exposure of anandamide. Washing further increased the amplitude, possibly due to extra glucose associated with application of fresh medium. Based upon observations as represented in Figure 7, lack of

decreased spike amplitude indicates that anandamide did not affect the mebrane potential of the cell.

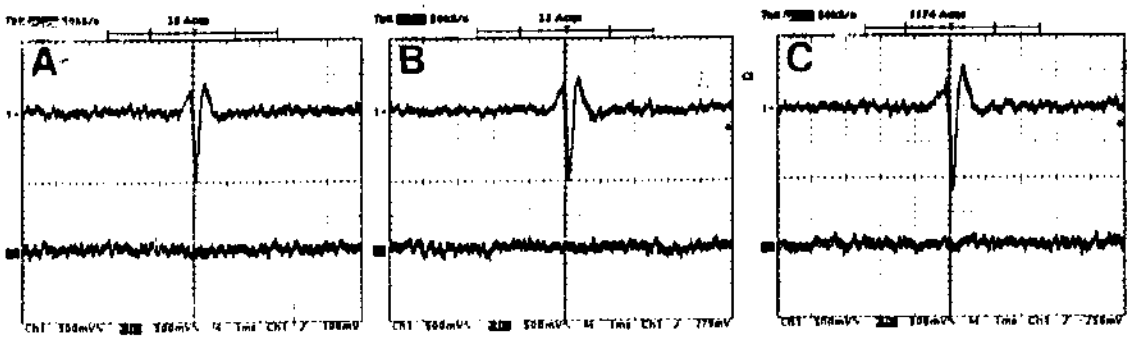


Figure 7: Spike amplitudes of a spinal cord culture exposed to anandamide. Channel 1 from SM47 shows: (A) native activity, (B) 850 nM anandamide, and (C) wash x2. Amplitude was seen to increase slightly upon exposure to anandamide. Washing further increased the spike size. Lack of any amplitude decrease indicated that anandamide did not target the metabolism of the cell.

In all spinal cord cultures, an initial decrease in activity occurred upon application of 150 nM anandamide, and cessation of burst activity occurred between 850 nM - 1.5 μM. Removal of the pharmacological agent was achieved by performing 2 complete medium changes. These "washes" resulted in a return to reference-like burst activity in 100% of the experiments. Subsequent application of the previous dose that lead to burst cessation also resulted in cessation of activity in 100% of the experiments. Further washing (x2) produced the same effect of a return to reference-like bursting. This type of repetition showed a high degree of intraculture repeatability.

Figure 8 displays typical response of a spinal cord culture to anandamide. The mean burst rate ± standard deviation across 12 channels is plotted against time. Experimental manipulations to the culture are listed above the corresponding data segments. In this particular culture, 50 μM bicuculline was added to regularize burst activity (denoted as reference activity). All subsequent washes contained 50 μM bicuculline. Reference burst activity began to stabilize at 40 minutes and was monitored for an additional 30

minutes. At this point, 150 nM anandamide was applied to the culture and an immediate decrease of 5 bpm occurred. While under this concentration, burst rate remained fairly constant at 10 bpm. Subsequent application of anandamide totaling 850 nM immediately decreased burst rate. Within 8 minutes, cessation of bursting had occurred (< 1 bpm) and remained as such until medium changes were performed. Two medium changes were performed to ensure that the majority of unbound drug was removed from the culture. Upon the first washing (medium change) activity commenced. The second wash reestablished reference-like activity after 30 minutes. This return to reference activity was found each time anandamide was removed from the system. The same degree of coordination among the channels was also seen following medium changes as indicated by the standard deviations. Additional applications of 850 nM anandamide continued to bring about cessation of bursting within 12 minutes and remained effective until medium changes removed the compound. In spinal cord cultures exposed to anandamide, manipulation of the decline, cessation and return to reference-like activity showed great repeatability between and among cultures.

In one spinal cord culture out of four, partial recovery of burst rate was observed during an application of 150 nM in a culture without bicuculline exposure (Figure 9). An immediate decrease in activity occurred upon application of 150 nM anandamide. Over the 30 minutes of 150 nM exposure, a rise in burst rate, approaching native activity, was observed. This effect was labeled "accommodation". The rate at which accommodation occurred was 5 bpm/30 minutes. Interestingly, this 5 bpm/30 minutes slope was again seen with the second medium washes. As with other spinal cord cultures exposed to anandamide, native-like activity was restored after these medium changes. This figure further shows the consistency of 850 nM anandamide as the cessation concentration for spinal networks. In this particular case, a subsequent application of anandamide, leading to a final concentration of 1.5 μM in the medium bath, tested for possible toxic effects. The

first wash following this dose did not regain any activity. A second medium change was required for bursting to resume. Within 60 minutes of washing, native burst rates were reestablished at 13 bpm.

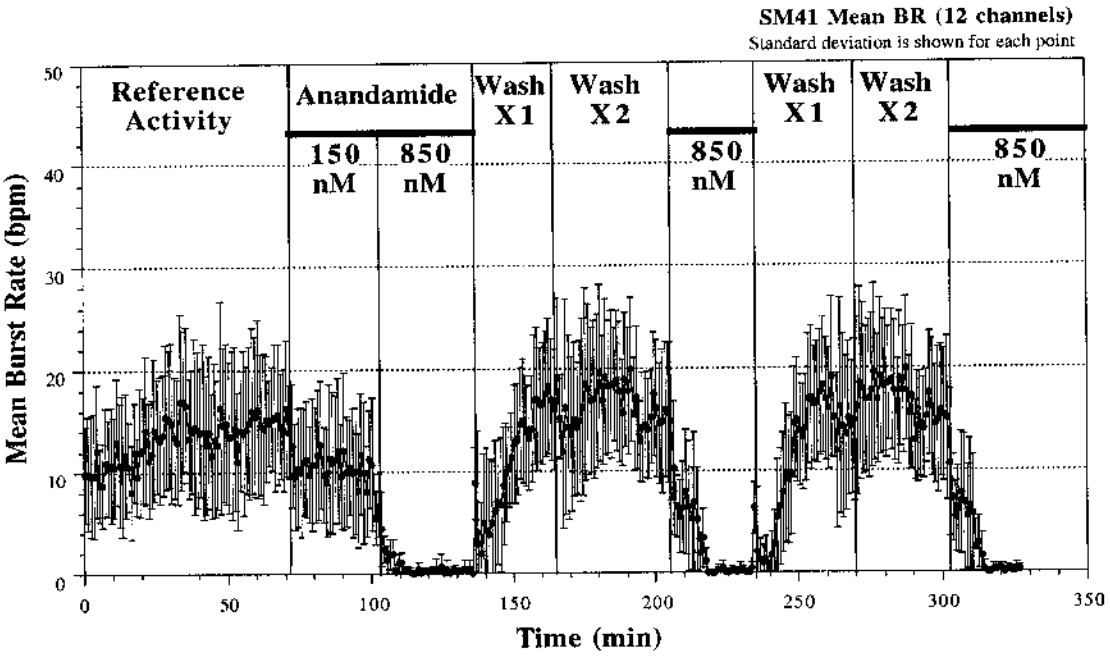


Figure 8: Response of spinal cord culture to anandamide. Network activity is depicted in terms of mean burst rate (bpm) +/- standard deviation for 12 channels. Burst activity was regularized with the addition of 50 μM bicuculline (reference activity). Initial decrease of activity occurred at 150 nM anandamide with cessation at a total of 850 nM. Two complete medium changes brought about a return to reference activity. Intraculture repeatability was shown by the second addition of 850 nM anandamide followed by a return to reference-like activity with two complete medium changes. A third addition of 850 nM anandamide again resulted in cessation of activity.

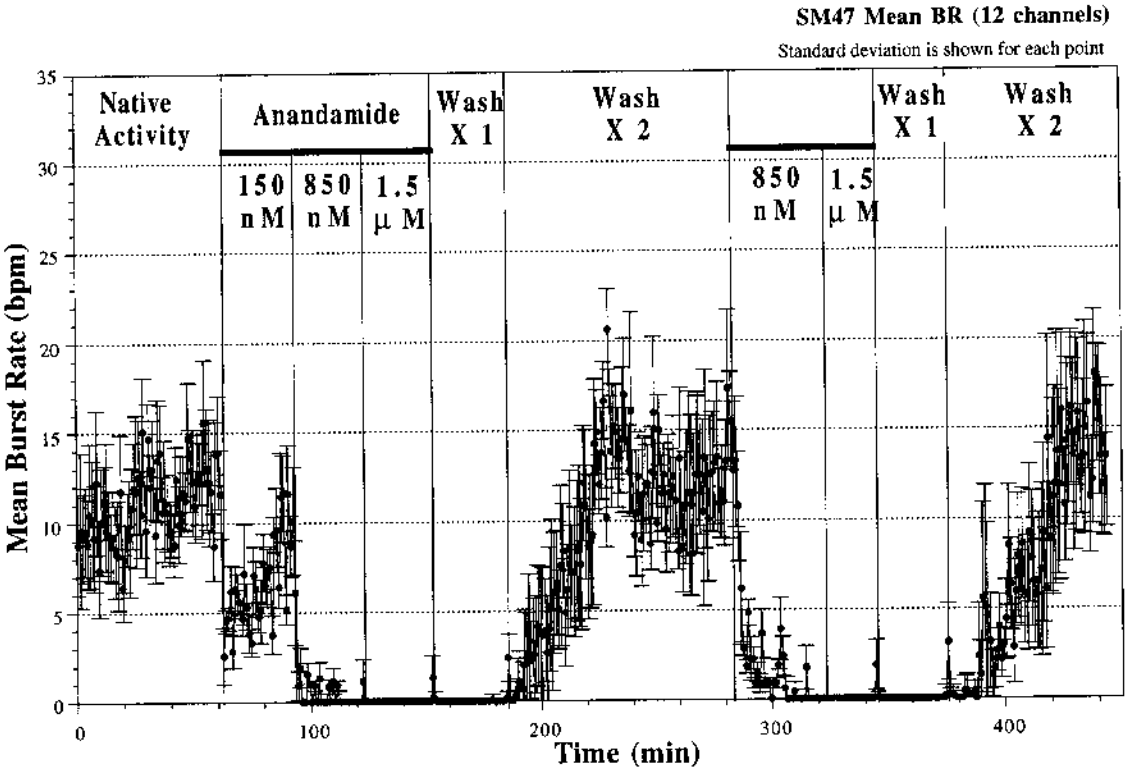


Figure 9: Response of spontaneous native activity (spinal cord culture) to anandamide. Network activity is depicted in terms of mean burst rate (bpm) +/- standard deviation for 12 channels. Burst activity decreased rapidly after application of 150 nM anandamide to a constant bath medium of 1.0 ml. Note the partial recovery of bursting (accommodation) during the 150 nM (total concentration) application. Cessation of bursting occurred at a total concentration of 850 nM. A subsequent 1.5 µM concentration was tested for toxic effects. Drug washout was slow and required 2 complete medium changes. Intraculture repeatability was demonstrated with the second set of anandamide applications and washes.

Methanandamide, a synthetic cannabinoid agonist which is not enzymatically broken down, was employed in this study for comparison of anandamide effects on spinal network activity. Like anandamide, methanandamide ultimately led to a decrease in burst activity. However, the concentrations and manner in which activity stopped were different between the two compounds. In all spinal cord cultures, an initial increase in burst activity was seen at methanandamide concentrations of 250 nM - 4.5 µM. Such increases were not observed with anandamide. Initial decrease in activity occurred between 4.5 - 7.0 µM with cessation of burst rate at 4.5 - 9.5 µM. Two complete medium changes led to a return of

reference-like burst rate in only 25% of the cultures. The other 75% had either a slightly increased or decreased burst rate after washing as compared to reference burst rate. The burst coordination among the channels was less synchronous after removal of the pharmacological agent in 75% of the cultures. In all spinal networks, subsequent application of methanandamide at doses that previously stopped bursting led to a more rapid bpm decrease and earlier cessation of bursting. Further washings showed a delay of return of any activity for up to 30 minutes.

Figure 10 depicts a typical response of spinal networks to methanandamide. Addition of 50 μM bicuculline (reference activity) was given 20 minutes after initial recording of native activity. This application resulted in more coordination of bursting among the individual channels as indicated by smaller standard deviation bars. An increase in burst rate of 6 bpm occurred over the course of the 250 nM, 1.5 μM and 2.5 μM applications. Upon administration of 4.5 μM, burst rate decreased to $\leq$ 1 bpm. Medium changes, which contained 50 μM bicuculline, led to a burst rate which was approximately 4 bpm greater than the reference burst rate. Though the burst rate returned to a similar reference activity, the coordination among the channels was significantly altered. Initial activity exposed to 50 μM bicuculline had a S.D. of +/- 4 bpm. After 2 complete medium changes, the S.D. increased to +/- 12 bpm. Further application of 4.5 μM led to a more rapid cessation of bursting (by at least 15 minutes) than the previous 4.5 μM exposure. Once cessation occurred, it was not recoverable within 30 minutes of washing with bicuculline-supplemented medium.

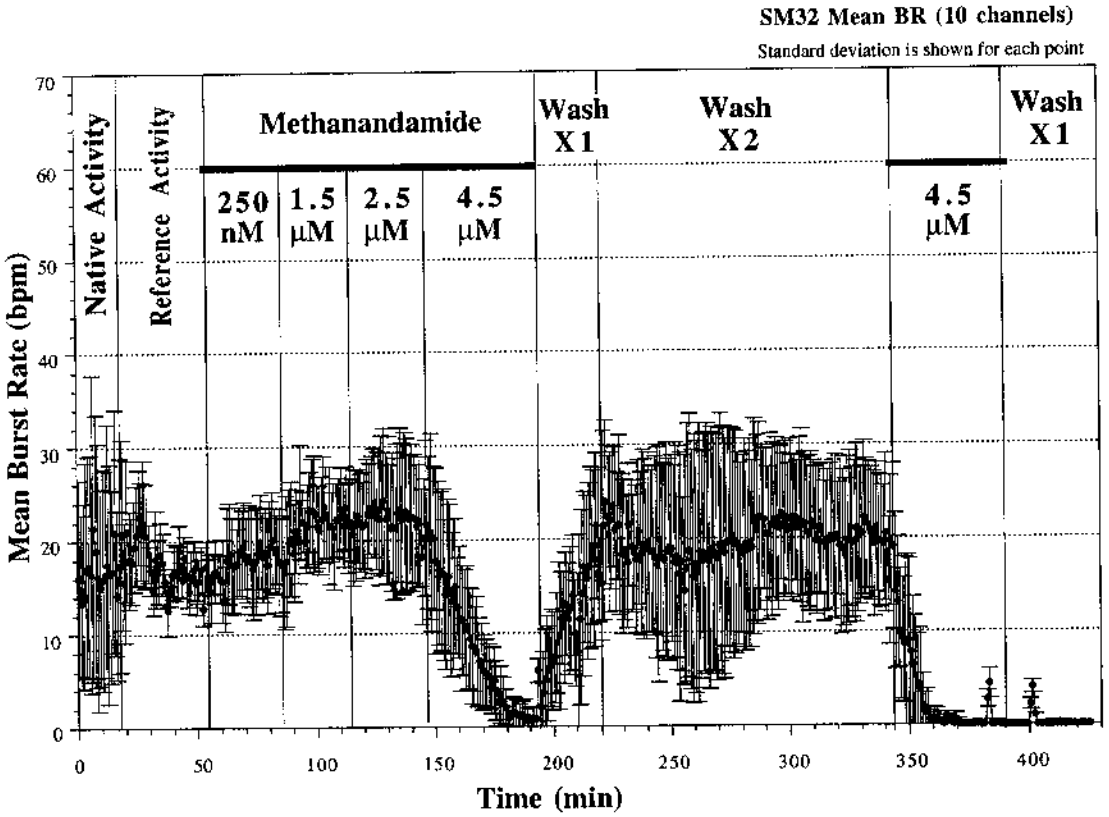


Figure 10: Response of spinal cord culture to methanandamide [mean burst rate (bpm) +/- S.D. (n = 10 channels)] Regularization of burst activity is shown with the addition of 50 µM bicuculline (reference activity), resulting in a reduced S.D. Initial decrease and cessation of activity occurred at 4.5 µM methanandamide. Two complete medium changes contained 50 µM bicuculline. The second medium change led to similar reference burst rate, but with a significantly larger S.D. indicating less burst coordination among channels. A second application of 4.5 µM led to a more rapid decrease and cessation of bursting. No recovery occurred within 30 minutes of the second medium change.

Figure 11 shows a dramatic effect of washing on the coordination between individual channels. 50 µM was applied to regularize bursting. This "reference activity" began to decrease 6 minutes after addition of 4.5 µM methanandamide with cessation of activity ($\leq$ 1 bpm) after 25 minutes. During this time, burst rate decreased with no change in the coordination of the 7 channels. The following medium changes, also with 50 µM bicuculline, disrupted synchronicity among the channels for the first 30 minutes after the wash. After 30 minutes of the second medium change, coordination stabilized. The burst

rate required 100 minutes for stabilization to occur. This figure also displays the differences in the rate at which subsequent doses effect the network. A second application of 4.5 μM led to an immediate and complete cessation of bursting. Return of activity did not occur for 20 minutes after the first wash. The second wash showed the same effect of asynchrony as previously observed.

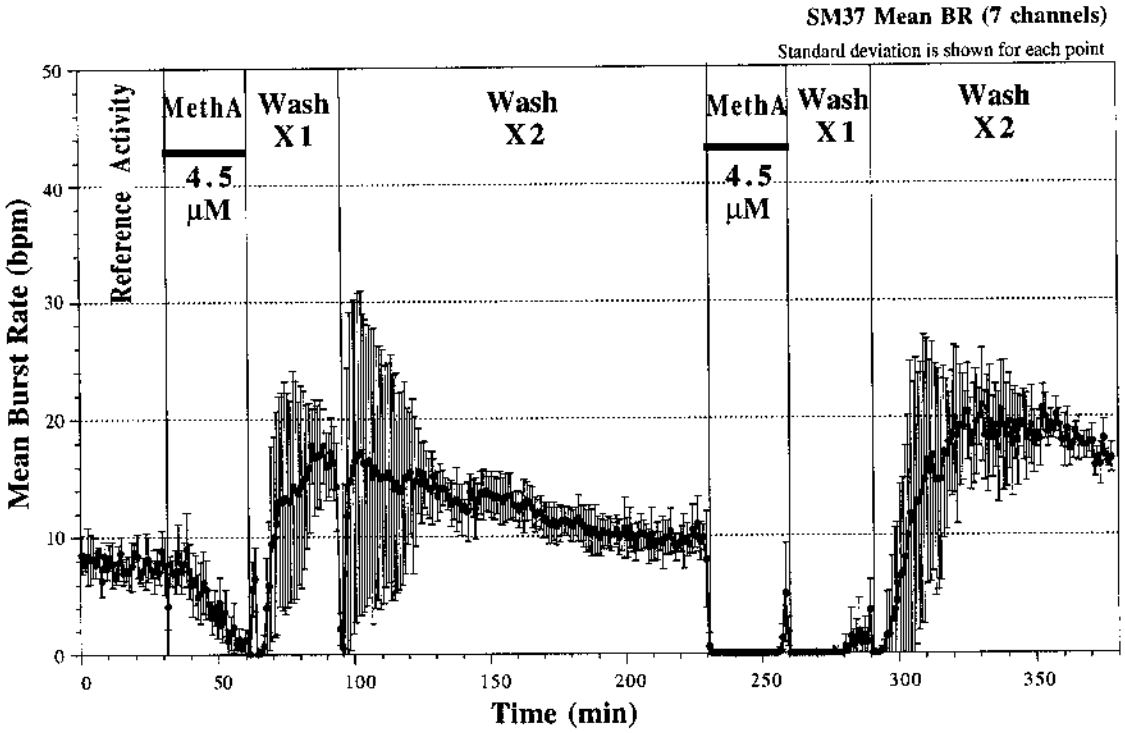


Figure 11: Response of spinal cord culture to methanandamide [mean burst rate (bpm) +/- S.D. (n = 7 channels)] 50 μM bicuculline was applied to regularize burst activity (reference activity). Exposure to 4.5 μM methanandamide (MethA) for 30 minutes followed by 2 complete medium changes led to a slightly higher burst rate. The first application of 4.5 μM took longer to stop activity and recovered at a faster rate than the second application. In both cases, medium changes led to an initial phase of asynchrony between the channels (large S.D.). After washings, burst rate was greater than the previous reference state. All washes contained 50 μM bicuculline.

Table 3 summarizes the experiments in which spinal cord cultures were exposed to anandamide and methanandamide. Three distinct phases of burst activity are viewed: initial decrease in burst rate, 50% of the reference activity (EC_{50}), and cessation of burst rate. The concentrations of anandamide and methanandamide for the activity states are

shown. The individual experiments for each drug are averaged to give mean and standard deviations for the three phases of burst rate. From this table it is evident that the concentrations required to bring about these changes in burst rate are considerably greater for methanandamide than for anandamide. Table 4 further summarizes gross effects of the cannabinoid agonists on spinal cord networks. Again, differences between the compounds were observed. However, in all of the spinal cord cultures exposed to the two compounds, burst rates were eventually decreased to the point of cessation. Intra- and inter- culture repeatability of spinal network responses to specific concentrations of cannabinoid agonists was demonstrated.

Table 3: Summary of Cannabinoid Agonists on Burst Rate of Spinal Networks

Exp	Drug	Bicuc.	Initial Decrease of BR	EC_{50} (50% of Reference BR)	Cessation of BR
SM47	Anand	0	150 nM	150 nM	850 nM
SM52	Anand	0	150 nM	150 nM	850 nM
SM41	Anand	50 µM	150 nM	850 nM	850 nM
SM42	Anand	50 µM	150 nM	850 nM	1.5 µM
SM27	MethA	0	4.5 µM	4.5 µM	6.0 µM
SM31	MethA	50 µM	7.0 µM	9.5 µM	9.5 µM
SM32	MethA	50 µM	4.5 µM	4.5 µM	4.5 µM
SM37	MethA	50 µM	4.5 µM	4.5 µM	4.5 µM
Mean (n = 4) +/- S.D.	Anand		150 nM 0.0	500 nM 400	1.0 µM .33
Mean (n = 4) +/- S.D.	MethA		5.1 µM 1.2	5.8 µM 2.5	6.1 µM 2.4

Table 4: Occurrence of Agonist Induced Effects on Spinal Network Activity

	Anandamide	Methanandamide
Accommodation of response	25% (n = 4)	0% (n = 4)
Return to reference-like BR after cessation & washing*	100% (n = 4)	25% (n = 4)
Return to reference-like synchronicity (S.D.) after cessation & washing*	100% (n = 4)	25% (n = 4)
Sensitization**	50% (n = 4)	100% (n = 2)

* Return to +/- 10% of the reference activity

** BR decreases faster and/or cessation occurs faster to subsequent applications of compound following washes.

3.3 Effects of Cannabinoid Agonists on Auditory Cortex Networks

Table 5 lists the cultures used in experiments investigating the effects of cannabinoid agonists on auditory cortical networks. In all cases, the seeding density onto the MMEPs was 10^6 cells per a 1 ml aliquot.

Table 5: Data Set of Auditory Cortex Cultures

Exp	Drug	Bicuc.	Seed Date	Age (DIV)	Treat-ment	Avg S:N	Max S:N	% Active Channels
SM51a	Anand	0	04-24-97	48	-	5.8:1	10:1	100
SM46	Anand	50 µM	04-24-97	27	-	2.9:1	5:1	81
SM51b	Anand	50 µM	04-24-97	48	-	5.8:1	10:1	100
SM57	MethA	0	04-24-97	65	-	3.1:1	10:1	74
SM62	MethA	50 µM	04-10-97	82	-	2.3:1	5:1	17
SM63a	MethA	50 µM	04-24-97	75	-	4.7:1	25:1	74

Anand, anandamide; MethA, methanandamide; DIV, days *in vitro* ; S:N, signal-to-noise ratio.

In all auditory cortex cultures, initial decreases in activity were observed at a concentration of 850 nM anandamide, and cessation of burst activity occurred between 3.0 - 4.5 µM. In all cortical networks exposed to anandamide, return of activity was observed with only 1 complete medium change. However, for a return to reference-like activity, 2 complete medium changes were required. The cortical networks displayed greater sensitivity to further applications of anandamide. This was demonstrated by a faster rate to cessation of activity and/or a lesser dosage of the compound being required than was previously used (see Figure 12B). It should be noted that these subsequent doses were applied at one time as opposed to sequential applications of the drug leading up to the final concentration. Application of single doses may have different effects on the network than

the sequential applications which were typically employed in this investigation. However, regardless of the manner of drug application, 2 complete medium changes consistently produced a return to reference-like activity in these cultures. In conjunction with a reference-like return of the burst rate upon washings, coordination of bursting among individual channels within a given network was also recoverable.

Figure 12A displays typical cortical network responses to anandamide. Upon application of 850 nM anandamide, burst rate immediately decreased and then accommodation was observed. Additional applications of anandamide totaling 3.0 μM were required to stop activity. Two complete medium changes were performed and a return to native-like activity occurred, as well as a return of the synchronicity between the channels. Comparing responses of spinal cord and cortical cultures (Figures 9 &12A) shows differences in the concentrations necessary to bring about network changes. Another difference between the spinal cord and cortical networks was the rate of increase observed with accommodation. Figure 12A shows accommodation occurred at 25 bpm/ 15 minutes, a much faster "recovery" than 5 bpm/ 30 minutes seen with the spinal network. However, in the cortical culture, the decrease in activity leading to cessation of bursting was a gradual process requiring a final concentration of 3.0 μM.

In 2 out of 3 cortical cultures exposed to anandamide, accommodation was observed. Figure 12B shows this effect for not only the 850 nM application, but also smaller effects at 1.5 and 2.0 μM. This graph is a continuation of Figure 12A. Burst rates at 250 minutes began to stabilize to 30 bpm. The addition of 50 μM bicuculline showed a dramatic reduction in variability of the burst rate among the 12 channels, but also revealed a decrease to a new reference burst rate of 12 bpm. This is not unusual as bicuculline causes burst stretching and a decrease in burst rate for cultures with high initial bursting. In this particular case, an accommodation rate of approximately 10 bpm / 25 minutes occurred during the 850 nM exposure. The degree of accommodation lessens with increasing doses

of 1.5 and 2.0 μM to 4 bpm / 10 minutes and 6 bpm / 5 minutes. Another interesting effect seen in this figure is the immediate decrease of activity associated with the second application of 2.0 μM. The dramatic effect coupled with this concentration may be the result of the drug being applied in a single large dose as compared to the gradual increase of concentration previously employed. Also, the first medium change (wash) following this single 2.0 μM application resulted in no recovery of activity. A second medium change took 60 minutes for stabilization and a return to reference-like activity.

Methanandamide was also used to observe cortical network responses. Like anandamide, it led to decrease of activity in all cortical cultures. An initial decrease in activity occurred upon application of 250 nM to 1.5 μM methanandamide, and cessation of of burst activity occurred between 2.5 - 3.5 μM. Two complete medium changes did not result in a return to reference-like activity. Instead, the burst rate was significantly increased or decreased (greater than 30%) and coordination between channels was less synchronous than native or reference activity. Additional applications of methanandamide at cessation-producing concentrations, led to a more rapid cessation of bursting indicating a possible sensitization of the network. Further washing again showed less coordinated bursting among channels and a non reference-like burst rate. These overall effects of methanandamide on cortical cultures were similar to those produced in spinal networks with the exception of the concentration necessary to bring about responses. Spinal cultures required greater amounts of methanandamide to produce effects than cortical cultures.

Figure 13 displays typical responses of an auditory cortical network exposed to methanandamide. In this particular experiment, 50 μM bicuculline was added to regularize burst activity. This 70 minute episode was used as the reference activity. Palmitoyl-ethanolamide (PAE) was applied at concentrations of 1.0, 3.5, and 6.5 μM to serve as an internal control. This compound was used to exclude the possibility of non-receptor mediated actions or of CB2 mediated responses. No significant change in burst rate

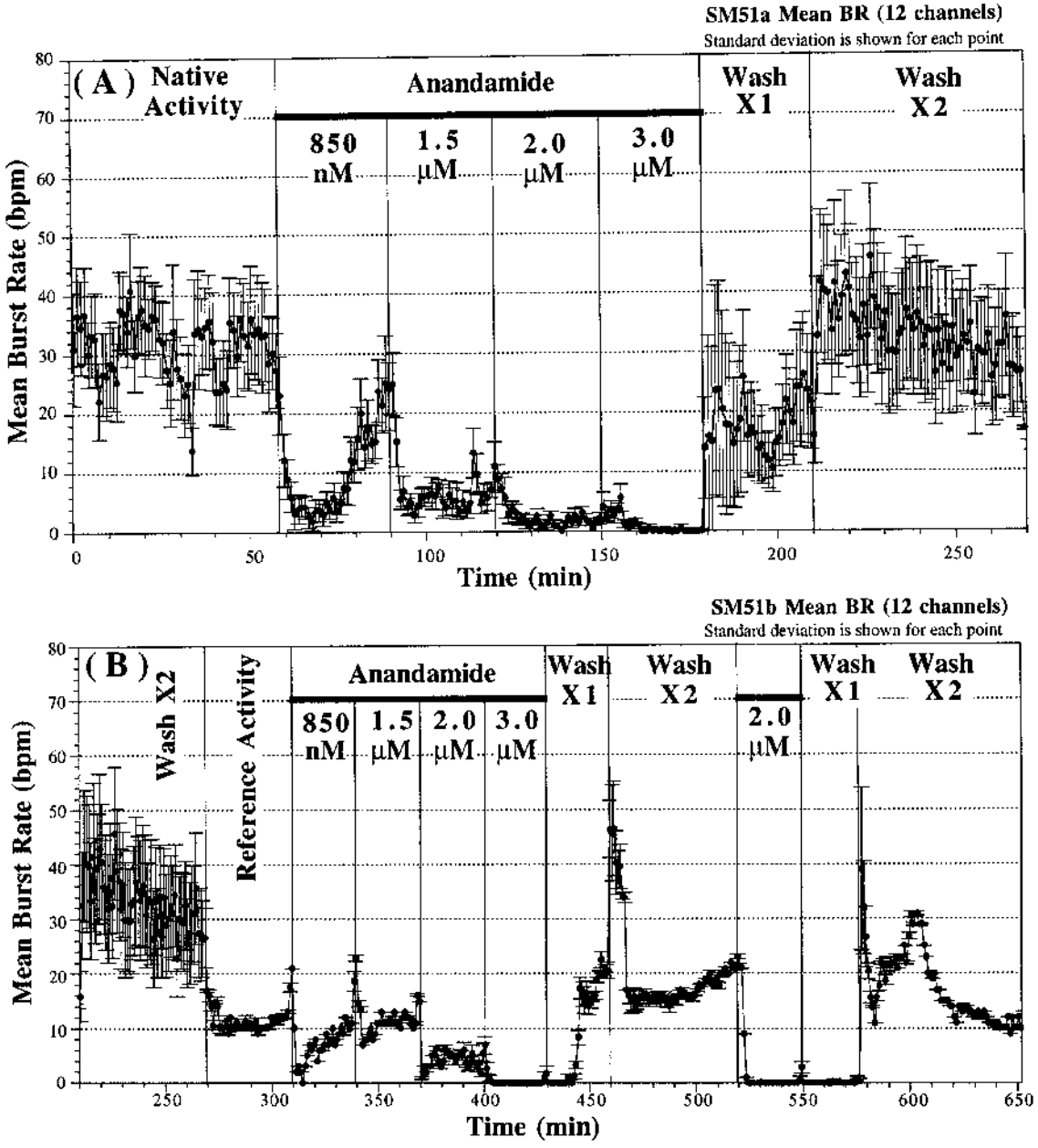


Figure 12: Response of auditory cortex to anandamide [mean burst rate (bpm) +/- S.D. for 12 channels]. Figure A represents activity not influenced by bicuculline. At 850 nM burst rate decreased and accommodation was observed. Two medium changes (wash) restored native-like burst rate. Figure B shows addition of 50 µM bicuculline (reference activity). Concentrations leading to a decrease in burst rate were similar in Figures A &B.

or coordination of individual channels was observed. A complete medium change was performed to remove this drug with no alterations in burst rate. For further experiments

employing PAE refer to section 3.5 (Control Experiments). Methanandamide was then administered and a decrease in activity was observed at 1.5 μM. A 2.5 μM application was necessary to bring about cessation of activity. A subsequent medium change containing 50 μM bicuculline did not result in a return of activity. A second medium change was required for bursting to be regained. However, after 60 minutes neither the burst rate nor coordination among channels was restored. Other cortical networks exposed to methanandamide showed the same lack of restoration of the reference burst rate and synchronicity after washing.

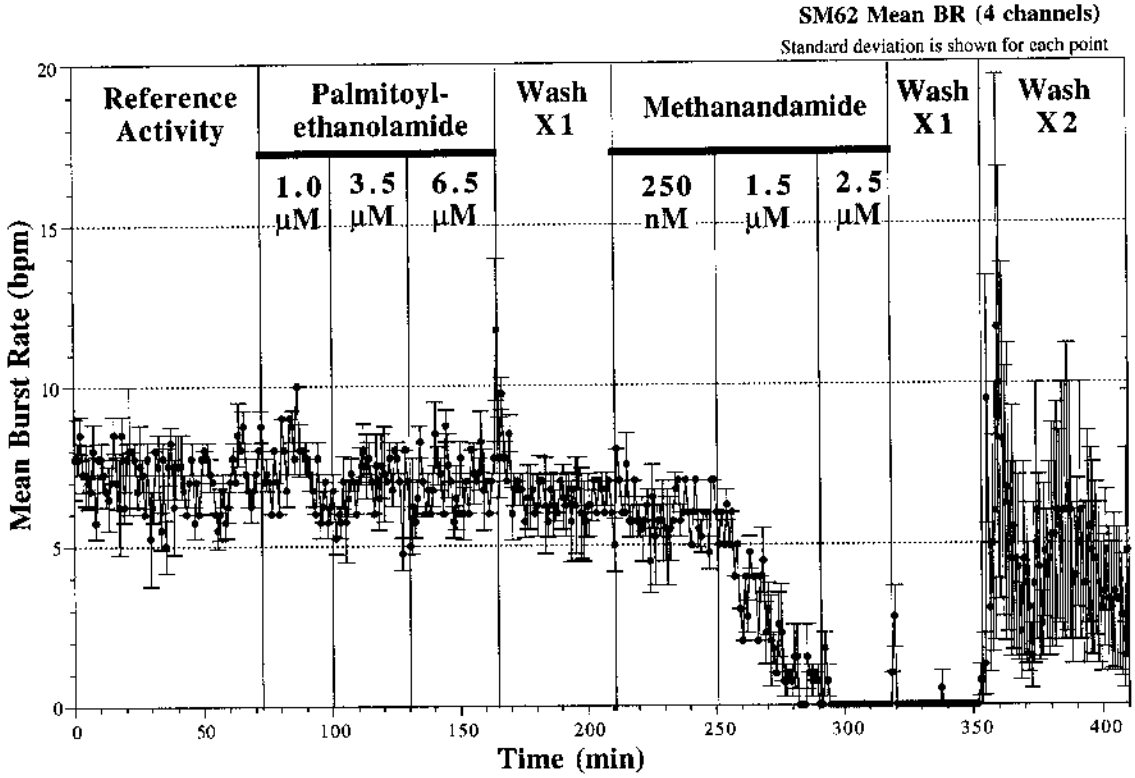


Figure 13: Response of auditory cortex culture to palmitoylethanolamide and methanandamide [mean burst rate (bpm) +/- S.D. (n = 4 channels)] 50 μM bicuculline was added to regularize burst activity (reference activity). Palmitoylethanolamide (PAE), a lipid-soluble compound having the same precursor as anandamide, was used as an internal control to determine non-receptor mediated and non-CB1 receptor effects. A wash was performed to remove PAE and methanandamide was applied. Two complete medium changes containing 50 μM bicuculline returned activity to a lower and less coordinated bursting rate than native.

Table 6 summarizes the results from auditory cortical cultures exposed to anandamide and methanandamide. Three distinct phases of burst activity are viewed: initial decrease in burst rate, 50% of the native activity (EC_{50}), and cessation of burst rate. Concentrations of anandamide and methanandamide for the activity states are shown. The individual experiments for each compound are averaged to give mean and standard deviations for the three phases of burst rate. From this table it is evident that the concentrations required to bring about these changes in burst rate are similar between the two compounds. Slightly less anandamide was needed to decrease burst rate. Cessation of bursting required more anandamide than methanandamide. Table 7 further summarizes gross effects of the cannabinoid agonists on auditory cortical networks. Differences between the compounds were observed. However, in all of the auditory cultures exposed to the two compounds, burst rate was eventually decreased to the point of cessation. Intra- and inter- culture repeatability of cortical network responses to specific concentrations of cannabinoid agonists was demonstrated.

Table 6: Summary of Cannabinoid Agonists on Burst Rate of Cortical Networks

Exp	Drug	Bicuc.	Initial Decrease of BR	EC_{50} (50% of Reference BR)	Cessation of BR
SM51a	Anand	0	850 nM	850 nM	3.0 µM
SM46	Anand	50 µM	850 nM	2.0 µM	4.5 µM
SM51b	Anand	50 µM	850 nM	850 nM	3.0 µM
SM57	MethA	0	250 nM	250 nM	2.5 µM
SM62	MethA	50 µM	1.5 µM	1.5 µM	2.5 µM
SM63a	MethA	50 µM	1.5 µM	2.5 µM	3.5 µM
Mean (n = 3) +/- S.D.	Anand		850 nM 0.0	1.2 µM .66	3.5 µM .87
Mean (n = 3) +/- S.D.	MethA		1.1 µM .42	1.4 µM 1.1	2.8 µM .58

Table 7: Occurrence of Agonist Induced Effects on Cortical Network Activity

	Anandamide	Methanandamide
Accommodation of response	65% (n = 3)	0% (n = 3)
Return to reference-like BR after cessation & washing*	65% (n = 3)	0% (n = 3)
Return to reference-like synchronicity (S.D.) after cessation & washing*	100% (n = 3)	0% (n = 3)
Sensitization**	100% (n = 2)	100% (n = 2)

* Return to +/- 10% of reference-like activity.

** BR decreases faster and/or cessation occurs faster to subsequent applications of compound following washes.

3.4 Tissue Specificity

From data obtained, an interesting and paradoxical tissue specificity for both anandamide and methanandamide was observed. In 86% of the experiments, anandamide produced a reduction in burst rates at lower concentrations in the spinal cord cultures than in the auditory cortex cultures. Conversely, methanandamide terminated activity at lower concentrations in the auditory cortex cultures than in the spinal cord. In the spinal cord, anandamide decreased activity at 150 nM and stopped activity between 850 nM - 1.5 μM; methanandamide decreased activity between 4.5 - 7.0 μM and stopped activity between 4.5 - 9.5 μM. In the auditory cortex cultures, anandamide decreased activity at 850 nM and stopped activity between 3.0 - 4.5 μM; methanandamide decreased activity between 250 nM - 1.5 μM and stopped activity between 2.5 - 3.5 μM. Figure 14 illustrates the differences between the three activity states and mean concentration of the drugs. In each of the graphs, the spinal cord showed the greatest separation between the actions of anandamide and methanandamide. Clearly, anandamide was more much potent in the spinal tissue than was methanandamide. The cortical cultures displayed a lesser degree of separation between the two drugs.

Figure 15 further shows the tissue specificity associated with the two compounds. Figure 15A displays spinal cultures being more sensitive to anandamide than cortex cultures. Figure 15B shows a great separation between the two tissues with the cortex being far more sensitive to methanandamide than the spinal cord. In these spinal cord cultures exposed to methanandamide, a substantial increase in activity occurred with doses ranging from 250 nM - 3.5 μM. This increase in burst rate over multiple drug applications was not observed in the other cortical cultures exposed to methanandamide nor in either of the tissues exposed to anandamide.

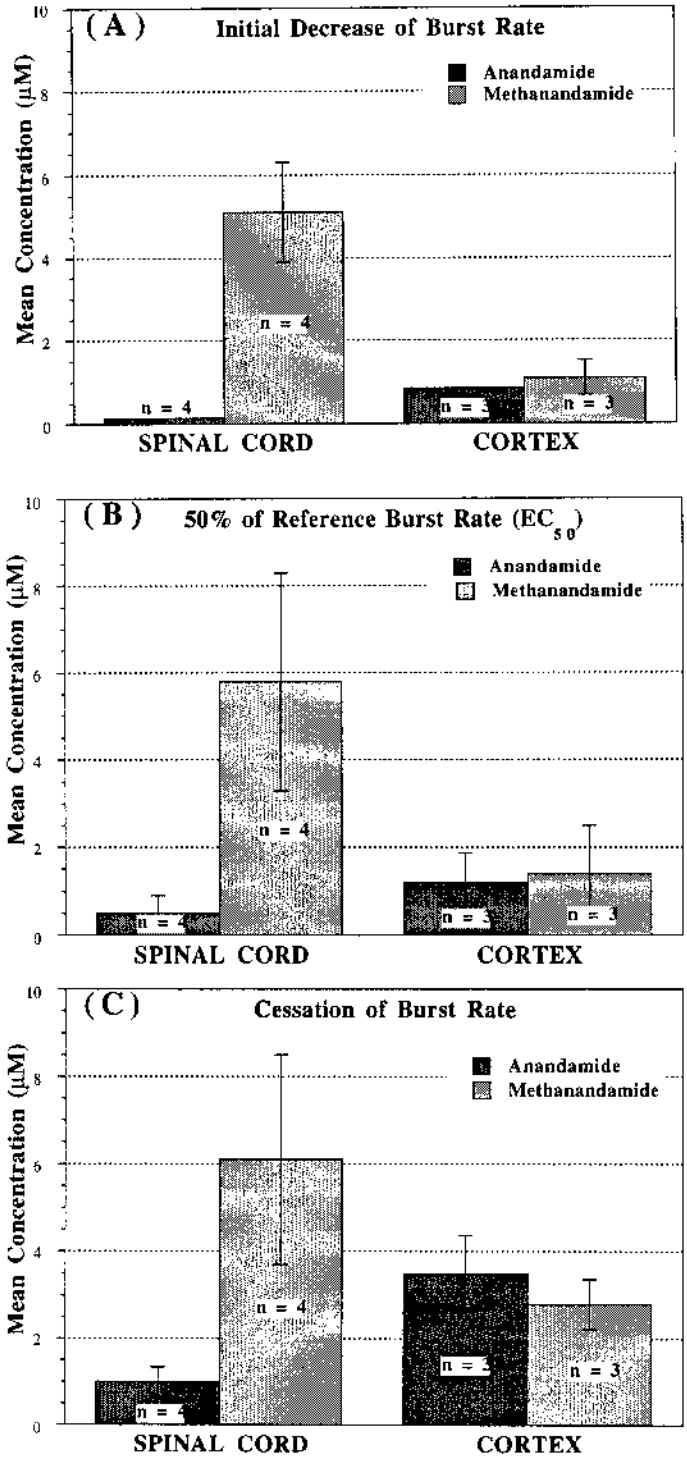


Figure 14: Summary graphs of mean concentration of anandamide & methanandamide and their effects on spinal cord and auditory cortex cultures. In all cases, anandamide produced effects at lower concentrations in the spinal cord cultures than in the auditory cortex cultures. Conversely, methanandamide terminated activity at lower concentrations in auditory cortex than in spinal cultures.

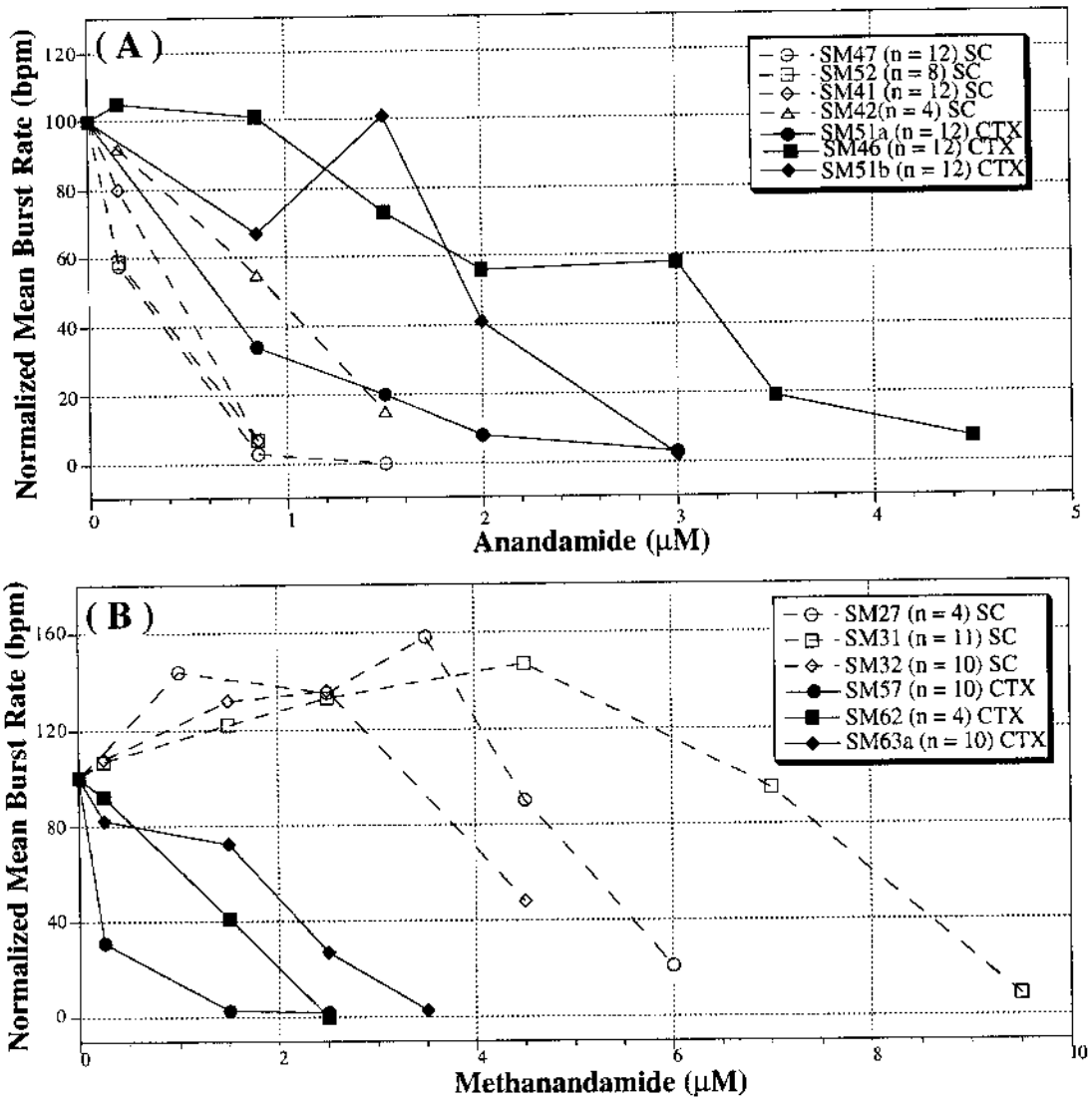


Figure 15: Comparison of effects of anandamide and methanandamide on spinal cord and auditory cortical cultures. Panel "A" shows the effects of anandamide on 4 spinal cord (SC - open symbols) and 3 cortical (CTX - closed symbols) cultures. Panel "B" shows the effects of methanandamide on SC and CTX cultures. Anandamide was observed to decrease activity at lower concentrations in spinal cord cultures than cortical cultures. Conversely, methanandamide displayed a clear distinction between spinal and cortical cultures by requiring less concentration in cortical networks than in spinal networks. Also, methanandamide had an excitatory effects on all spinal cord cultures.

3.5 Control Experiments

In order to show that the network did not undergo large fluctuations of burst activity as a result of several medium additions over the course of several hours, three control experiments were performed. The medium, which was used as a carrier source for the pharmacological compounds, was applied to cultures at 30 minute intervals for a total of 240 minutes. Basically, the procedure of applying drugs was mimicked, except that only medium (control) was added to cultures. Figure 16 displays a typical control experiment. Native activity had an average burst rate of 9 bpm. This mean burst rate remained constant until the addition of 350 μL medium. At this point, burst rate decreased by half for 10 minutes and then regained a mean of 9 bpm. Other small "dips" in burst rate were observed at the onset of each addition of medium. This initial decrease in burst rate was transitory, lasting approximately one minute. This artifact was often seen in other experiments and not perceived to be "an effect" of a given compound. The volumes used in this control experiment exceeded normal volumes during drug application where typical values ranged from 10 - 100 μL. Thus, the volumes and subsequent results in the control experiments are well within the typical ranges of the pharmacological experiments. From the three external control experiments performed, burst activity was seen to be stable over the time course of a typical experiment as demonstrated by Figure 16.

Also, palmitoylethanolamide (PAE), was employed as an internal control during the course of three experiments. It was administered in both cultures exposed to bicuculline and those cultures without bicuculline. In all cases, the compound exhibited neither an excitatory nor inhibitory effect on network activity at concentrations that reached a maximum of 6.5 μM. As seen in Figure 13, the culture underwent one medium change following PAE application without disrupting network activity. Also, palmitoyl-ethanolamide showed no signs of affecting the subsequent activity of the culture when exposed to methanandamide or anandamide. While under PAE exposure, no significant

changes in burst rate or coordination among the channels occurred. Figure 17 shows minimal fluctuations (<10%) of the burst rate for all cultures exposed to palmitoylethanolamide.

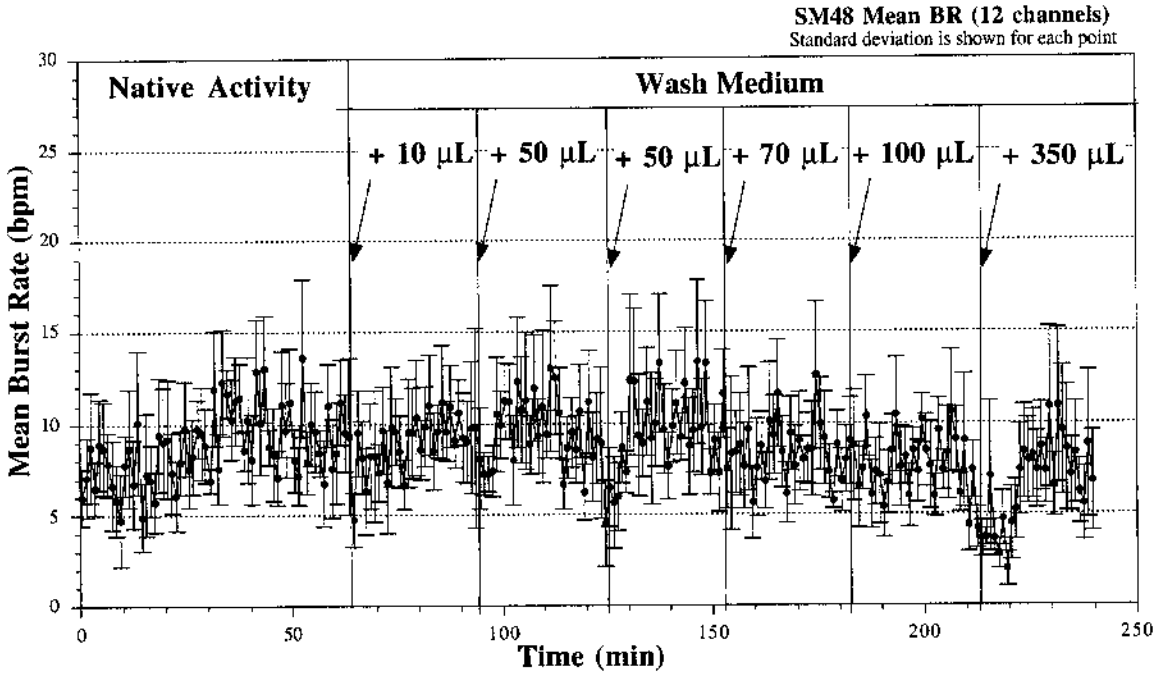


Figure 16: Response of spinal cord culture to applications of wash medium [mean burst rate (bpm) +/- S.D. (n = 12 channels)] Wash medium was used in all experiments as the carrier of the drugs and for medium changes (washings). No significant changes in burst rate occurred as a result of medium applications until an application of 350 µL. At this time, burst rate dropped for approximately 10 minutes and then recovered. Typical drug applications ranged from 10 - 100 µL. Small dips in burst rate are associated with the initial application of medium. These are transitory artifacts. All medium applications were made sequentially t a 1 ml bath.

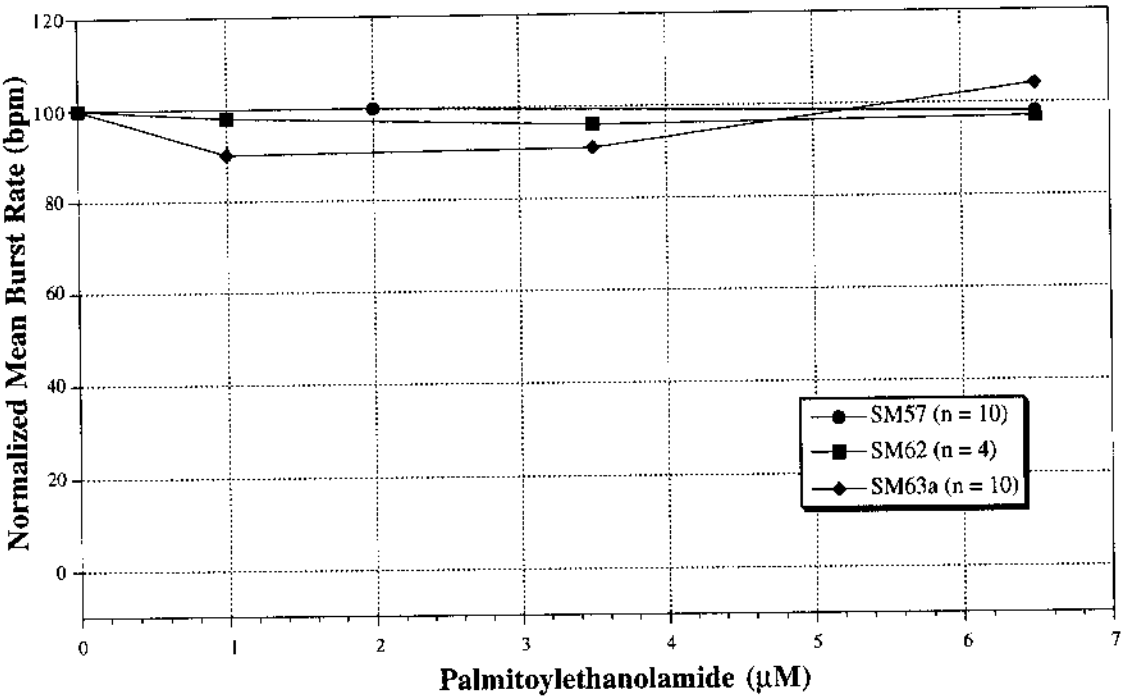


Figure 17: Effects of palmitoylethanolamide on the burst rate. Normalized mean burst rate is shown for 3 experiments (n = number of channels). Standard error (not shown) was minimal for all experiments. Palmitoylethanolamide was applied in doses corresponding to those used for anandamide and methanandamide (1.0 - 6.5 μM). No significant effect in the burst rate was observed. Palmitoylethanolamide was used as an internal control. Palmitoylethanolamide is a lipid cannabinoid agonist at the peripheral CB2 receptor.

3.6 Bicuculline Influences on Network Activity

As previously shown (Section 3.1), bicuculline disinhibited network activity which resulted in a greater level of organized and coordinated burst activity. In view of the use of bicuculline for burst pattern regularization, it was necessary to assess whether bicuculline also influenced the pharmacological results. The following two graphs, Figure 18 A & B, show the effects of cannabinoid agonists on spinal cord networks. With each agonist, those cultures not treated with bicuculline (open symbols), displayed the same general trends as those cultures with bicuculline. The graphs show that bicuculline tends to keep activity at a higher level at all drug concentrations. In the cultures exposed to anandamide, concentrations of 150 nM caused a decrease in activity for all the cultures. However, those networks not treated with bicuculline experienced a greater decrease in burst rate than those exposed to bicuculline. The point at which cessation of activity occurred was similar, but not identical, among all cultures. Similarly, the spinal cultures exposed to methanandamide displayed a greater degree of change in burst rate upon low concentrations of the drug. In this case the burst rate initially increased. Again, cessation of burst rate between the two types of cultures was similar.

In regards to the addition of bicuculline, similar trends were observed with auditory cortical cultures as with spinal cultures. In all cases, bicuculline tended to keep network activity elevated. Thus, those cultures not treated with bicuculline showed a greater decrease of burst rate than those with bicuculline. Figures 19 A & B show the general trend of decreasing burst rate to be the same for cortical cultures exposed to anandamide and methanandamide. In each of these cases, cultures treated with bicuculline showed a "robustness" to the effects of the cannabinoid agonists. Those cultures not exposed to bicuculline displayed a lesser resistance to the effects of the drugs. In bicuculline treated cultures, concentrations leading to cessation of activity were slightly than those in non-bicuculline treated cultures.

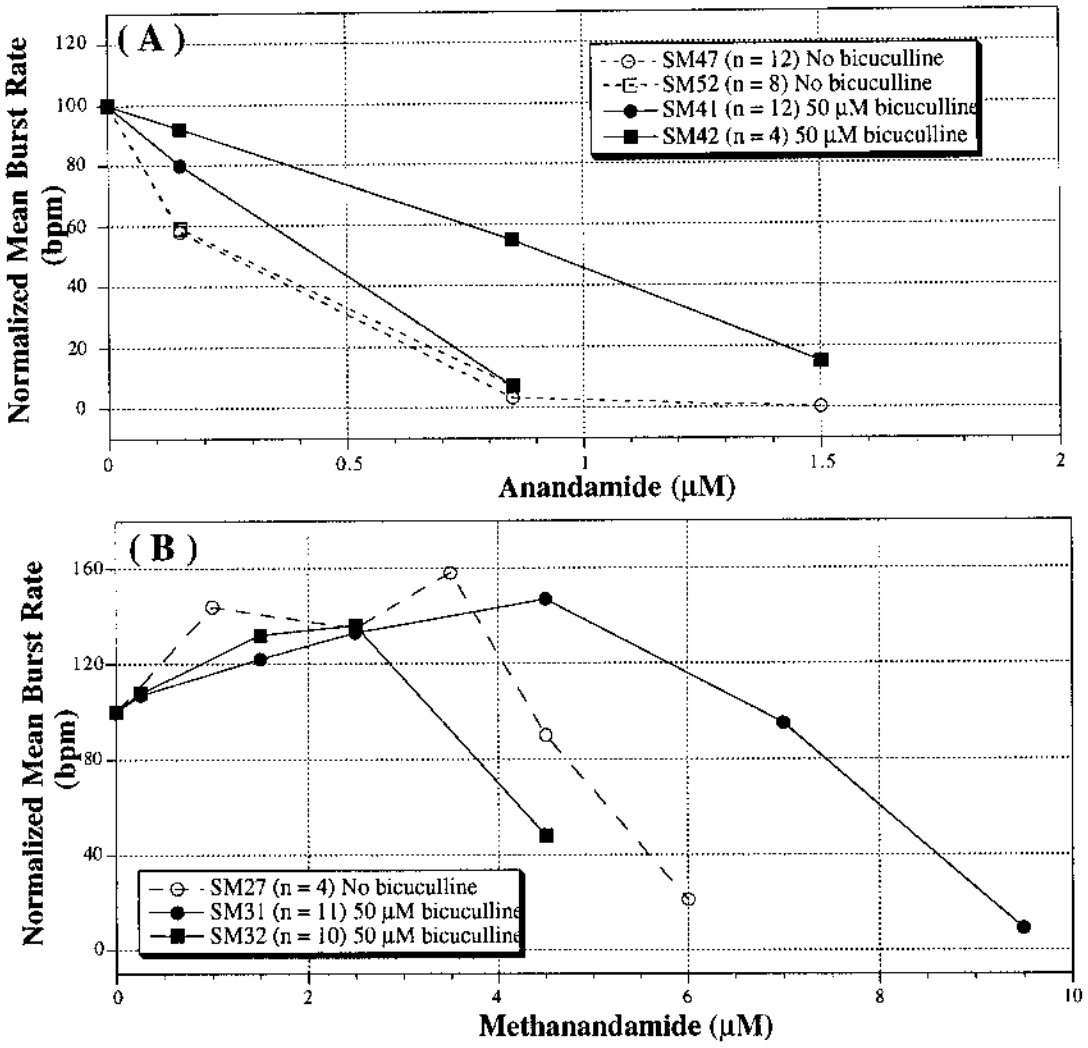


Figure 18: Comparison of effects of anandamide and methanandamide on spinal cord cultures exposed to 50 μM bicuculline and without bicuculline. Normalized mean burst rate was plotted against drug concentration. The cultures without bicuculline (dashed lines) showed a greater initial decrease with anandamide (A) and a greater initial increase of burst rate with methanandamide (B) than those cultures treated with bicuculline.

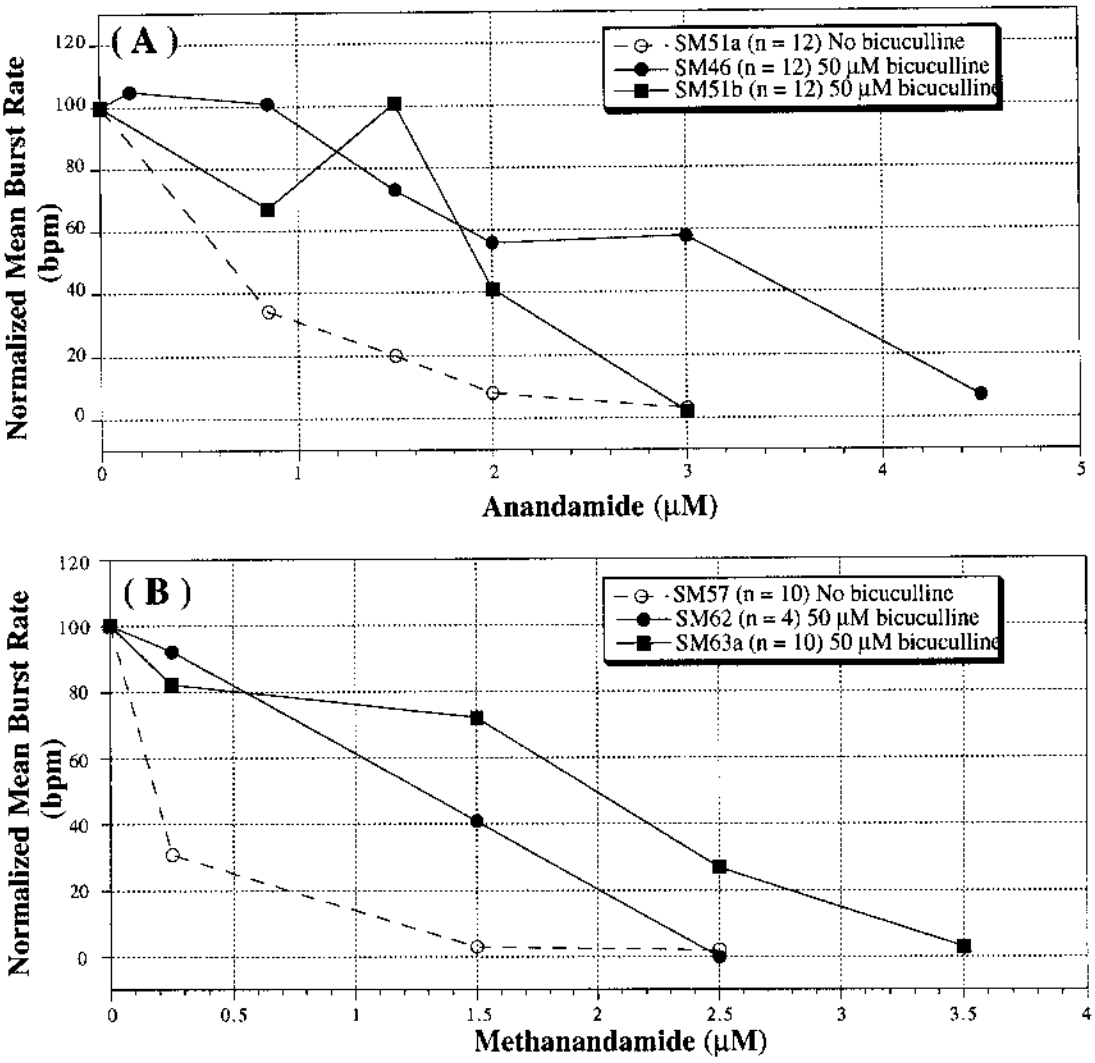


Figure 19: Comparison of effects of anandamide and methanandamide on auditory cortical cultures exposed to 50 µM bicuculline and on those without bicuculline. Normalized mean burst rate was plotted against drug concentration. The cultures not treated with bicuculline showed a greater decrease of burst rate at lower concentrations of the agonists than those cultures exposed to bicuculline. Cessation of bursting occurred at slightly higher concentrations among bicuculline treated cultures.

3.7 Toxicity of Anandamide and Methanandamide

Preliminary studies in the neurotoxic effects of anandamide and methanandamide on spinal cord cultures were also examined. Neurotoxicity was defined as irreversible damage to the bursting of the network. Irreversibility was defined as any major change of native activity that could not be reversed by 2 medium changes and a recovery period of at least 2 hours.

It was observed that anandamide exposure of 850 nM for 30 minutes and 1.5 μM for 1000 minutes led to quick return of activity within 1 minute and complete return to reference-like activity within 60 minutes. When the concentration of anandamide exposure was increased to 8.5 μM for 30 minutes, no recovery of any burst activity occurred within a 2 hour period. Figure 20 shows recovery of the culture after exposures to 850 nM for 30 minutes and subsequent irreversible shut-down when exposed to 8.5 μM anandamide for 30 minutes. Each 850 nM application resulted in cessation of network bursting, but was readily regained upon medium changes. Native burst rate and coordination among channels was recovered. However, medium changes following the 8.5 μM application were unable to restore any bursting within 2 hours. Thus, this anandamide exposure led to irreversible network shut-down.

Methanandamide also displayed irreversible effects. Exposures of 4.5 μM for 30 minutes resulted in a cessation of activity for 20 minutes. At that time, activity was restored with medium changes. However, nativity activity did not return within 2 hours; thus, the application of methanandamide resulted in irreversible changes in the burst activity of the network. Although direct comparison can not be made between the reversible anandamide effects and this methanandamide application, it is interesting to note that cultures treated with methanandamide (4.5 μM) required 20 minutes for a return of activity. The anandamide (1.5 μM) exposed culture regained activity within 1 minute. Increasing concentration of methanandamide to 9.5 μM for 1350 minutes and 25 μM for 40 minutes

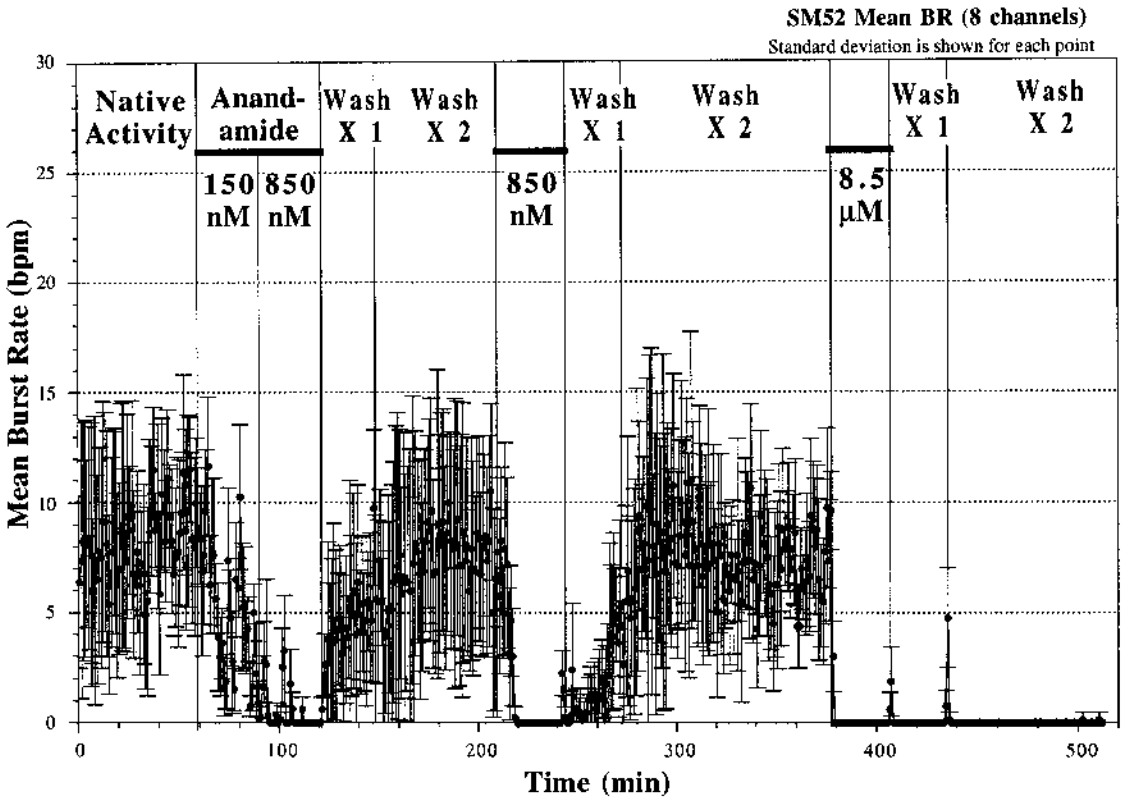


Figure 20: Reversible and irreversible effects of anandamide on spinal cord culture [mean burst rate +/- S.D. for 8 channels]. Anandamide was shown to reversibly stop activity at exposures of 850 nM for 30 minutes. Subsequent medium changes restored native activity. Irreversible effects are seen at exposures of 8.5 µM for 30 minutes. Medium changes did not restore any activity within a 2-hour period.

resulted in no recovery of bursting for either culture within a 2-hour period. Figure 21 shows the dramatic irreversible effects of 25 µM methanandamide on a spinal network. Upon application of the drug, burst rate sharply increased by 18 bpm / 5 minutes and then steadily decreased to zero at a rate of 38 bpm / 10 minutes. The drug remained on the culture for an additional 25 minutes without any occurrence of bursting. Subsequent medium changes showed no indication of network recovery within 2 hours.

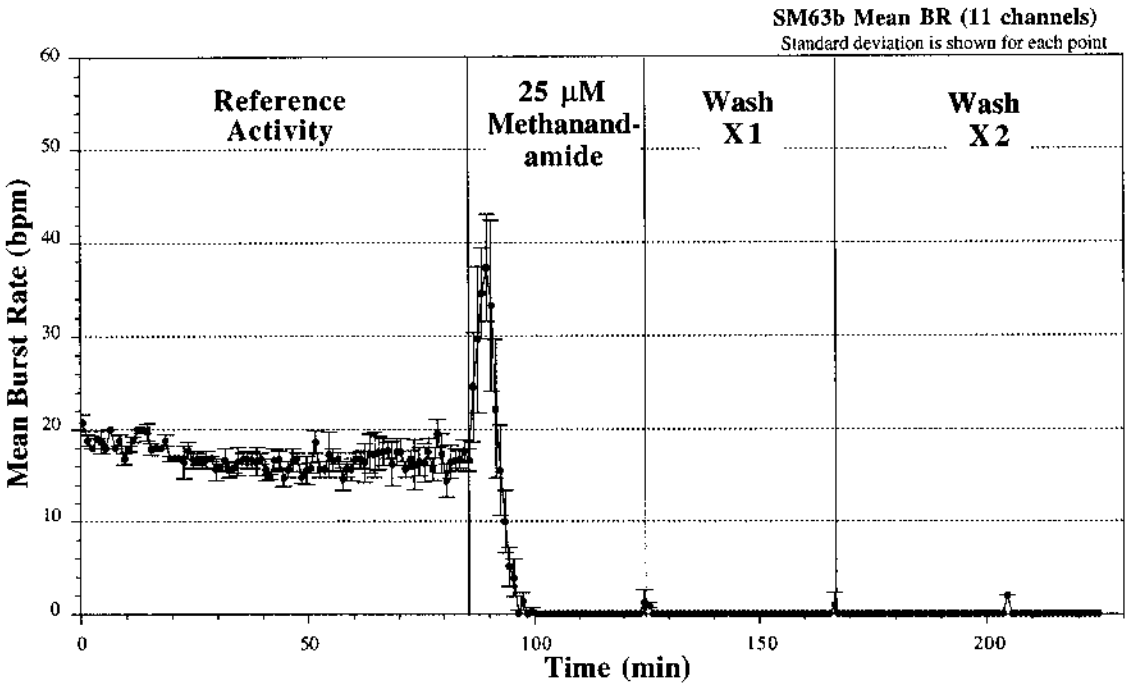


Figure 21: Irreversible loss of activity after exposure of spinal cord culture to 25 µM methanandamide [mean burst rate +/- S.D. for 11 channels]. 50 µM bicuculline was added to regularize burst activity (reference activity). Exposure to 25 µM methanandamide for 40 minutes stopped activity for at least 135 minutes. This may be defined as an irreversible effect, as it clearly would challenge the survival of any organism.

Figures 22 and 23 show overnight exposures to anandamide at 1.5 µM and to methanandamide at 9.5 µM. The culture exposed to methanandamide does not regain burst activity. The culture exposed to anandamide ceased bursting at 1.5 µM, but then regained activity while still under the influence of this drug application. Upon washing, network activity returned. Direct comparison of these two experiments, however, can not be made due to the different concentrations of drug applications. It is interesting to note that based on data obtained from this study, the decrease and cessation of bursting in the spinal cord was more responsive to anandamide than to methanandamide. However, from these experiments, it was observed that the spinal cord displayed a lesser degree of recovery when exposed to methanandamide.

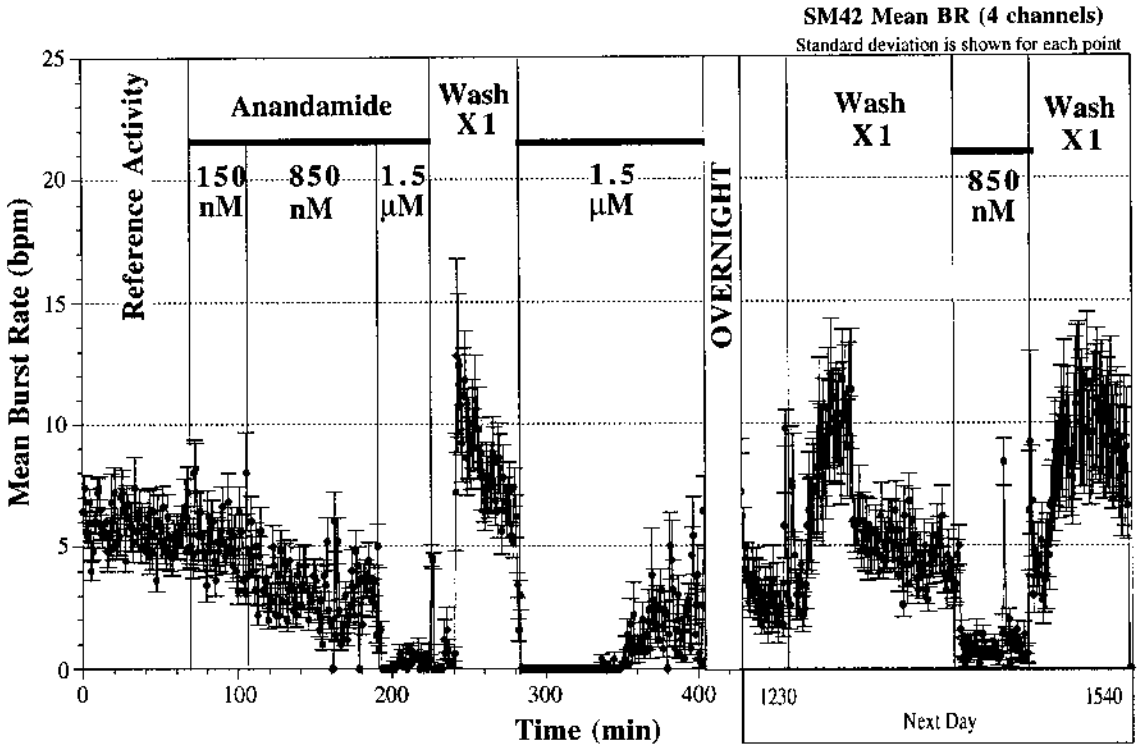


Figure 22: Response of spinal cord culture to an overnight exposure of anandamide [mean burst rate +/- S.D. for 4 channels]. Reference activity indicates the addition of 50 µM bicuculline to culture in order to regularize spontaneous network activity. Increasing doses of anandamide decreased burst rate. A full medium was applied, followed by a second application of 1.5 µM anandamide resulting in cessation of burst rate for 60 minutes. During this exposure, burst activity began and continued overnight, but remained at a lower level than reference activity. Subsequent washing containing 50 µM bicuculline led to an initial increase in burst rate for 30 minutes, and then reference bursting was restored. Further application of 850 nM decreased activity but did not cease bursting. Final medium change, also with 50 µM bicuculline, led to an increase of bursting with substantial loss of coordination among channels.

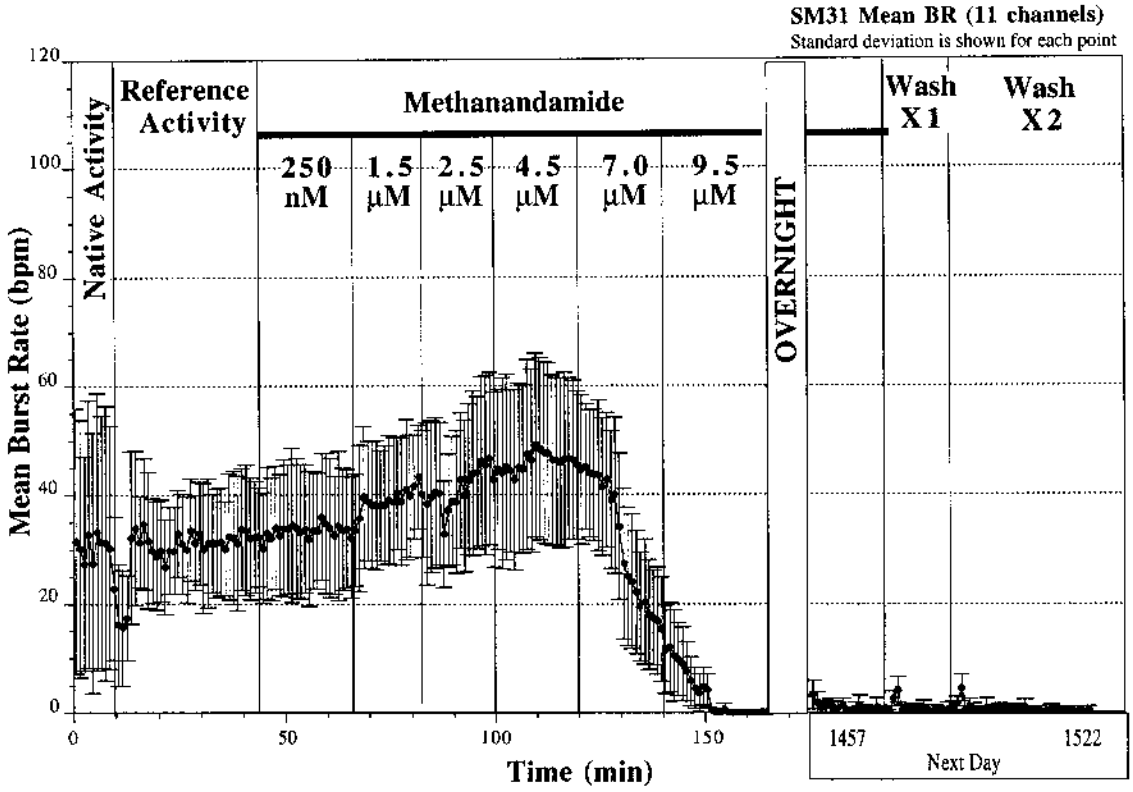


Figure 23: Response of spinal cord culture to overnight exposure of methanandamide [mean burst rate +/- S.D. for 11 channels]. 50 µM bicuculline was added to regularize bursting (reference activity) as seen by reduced standard deviations. Application of methanandamide led to a gradual increase in burst rate. Application of 7.0 µM led to a decrease of burst rates, and 9.5 µM resulted in cessation of bursting. This dose was left on the culture overnight. The next day, while still under the influence of the drug, spiking was observed. Two complete medium changes with 50 µM bicuculline did not restore any network burst activity.

CHAPTER 4

DISCUSSION

4.1 Cannabinoid Receptors

The initial aims of this project were to investigate the network responses of murine spinal cord and auditory cortical cultures to cannabinoid agonists. The data presented suggests that CB1 receptors were present in both tissue types. Both the spinal cord and auditory cortex cultures responded to anandamide and methanandamide in a dose-dependent manner (Figures 8 - 13). Previous autoradiographic studies have identified cannabinoid receptors localized within the spinal cord and auditory cortex (Herkenham et al., 1991). More recent immunohistochemistry work revealed that the CB1 receptor is widely distributed in the forebrain with less distribution in the hindbrain and spinal cord (Tsou et al., 1998).

In conjunction with the CB1 receptor, the CB2 receptor also has been implicated as having a binding site for anandamide and methanandamide (Felder et al., 1995). In order to determine whether the spinal cord and auditory cortex cultures contained CB2 receptors, palmitoylethanolamide was employed. This compound binds with high affinity to the CB2 receptor, but not to the central CB1 receptor. Based upon the lack of response in network activity associated with palmitoylethanolamide (Figures 17 & 39), it can be concluded that no CB2 receptors were present in the tissues used in this study. This finding confirmed previous work demonstrating that CB2 receptors exist only in peripheral tissues, not in brain nor spinal cord (Facci et al., 1995). Palmitoylethanolamide served a second purpose. Due to the lipophilic nature of the cannabinoids, non- receptor mediated perturbation of membrane lipids were thought to be a potential source of the compounds' actions (Gill and

Lawrence, 1976). If this were correct, use of palmitoylethanolamide, at concentrations comparable to those of anandamide and methanandamide, should elicit a response. As noted, palmitoylethanolamide did not alter network activity. Thus, non-receptor mediated responses were not a mode of action with these compounds.

4.2 Response of Networks to Cannabinoid Agonists

Anandamide and methanandamide were shown to produce similar effects on the spontaneous network activity of spinal cord and auditory cortical cultures. Each compound ultimately led to a cessation of burst activity. For anandamide it was shown that initial activity decreased burst rate at mean concentrations of 150 nM in the spinal cord and 850 nM in the cortex; EC_{50} occurred at 500 nM (spinal cord) and 1.2 μM (cortex); and cessation was reached at 1.0 μM (spinal cord) and 3.5 μM (cortex). Methanandamide, while also decreasing network activity, produced results at very different concentrations than anandamide. Mean concentration of methanandamide leading to initial decrease of activity was 5.1 μM in the spinal cord and 1.1 μM in the auditory cortex; EC_{50} occurred at 5.8 μM (spinal cord) and 1.4 μM (cortex); and cessation was reached at 6.1 μM (spinal cord) and 2.8 μM (cortex). This overall effect of decreasing network activity is consistent with previous evidence. Activation of CB1 receptors inhibit adenylyl cyclase activity (Howlett, 1984), which leads to a further decrease in cAMP. Additionally, CB1 leads to inactivation of N- and P/Q-type calcium channels associated with neurotransmitter release (Mackie et al., 1993). Cannabinoids have been reported to presynaptically inhibit the release of glutamate from cultured neurons (Shen et al., 1996). Finally, CB1 activation can enhance A-type potassium currents, which can lead to hyperpolarization of the membrane (Deadwyler et al., 1993). All of these cannabinoid-induced actions ultimately result in inhibition of activity.

In evaluating the effectiveness of cultured neurons as platforms for rapid prescreening of neuroactive compounds and for drug research and development, the effective concentrations of the drugs administered to the network environment must be physio-logically relevant. Comparisons of *in vitro* and *in vivo* studies have shown that the placement and density of cannabinoid receptors in culture appears to parallel the situation in mature brain, where cannabinoid receptors are greatly enriched along axons and dendrites and on presynaptic terminals (Herkenham et al., 1991). Much work has been done employing *in vitro* techniques and a range of cannabinoid concentrations can be found. Previous *in vitro* studies in other laboratories have shown: (a) IC_{50} of 200 nM anandamide for inhibition of cAMP in Chinese hamster ovary cells transfected with rat cannabinoid receptor employing whole single cell recording techniques (Vogel et al., 1993), (b) EC_{50} of 71 nM for Ca spiking inhibition using patch clamping (Shen et al., 1996), (c) IC_{50} of 20 nM for Ca channels inhibition in N18 cells (Mackie et al., 1993), (d) Ki of 543 nM for displacement of [^{3}H]-CP 55,940 (a synthetic cannabinoid agonist) from cloned cannabinoid receptors expressed in murine Ltk (L)-cells (Felder et al., 1993), and (e) decrease in cAMP and neurotransmitter release with 10 μM in rat striatal and cortical slices (Cadogan et al., 1997). The range of cannabinoid concentrations (150 nM - 9.5 μM) used in this project to determine network failures is close to and in some cases identical to those found effective in previous *in vitro* studies.

In order to bridge the gap between the culture environment and the intact animal, cannabinoid dosages must be comparable among *in vitro* and *in vivo* responses. In rats, the classical cannabinoid behavioral pattern is defined by a series of four behavioral tests: analgesia, hypomotility, lowered body temperature, and catatonia. Anandamide was shown to exert potent but relatively short-lived behavioral and physiological effects when administered intravenously to freely behaving rats (Stein et al., 1996). Intravenous anandamide caused immediate dose-related decreases in rat behavior at concentrations

between 3 and 30 mg/kg (90 - 900 nM assuming that 1kg = 1L) and lasted about 15 minutes (Stein et al., 1996). As with the *in vitro* studies, concentrations employed in this project are comparable to those in the intact animal. When binding affinities of cannabinoids to brain receptors in culture are compared to *in vivo* potencies in eliciting behavioral patterns in mice and rats, highly significant correlations have been observed. The conclusions from all of these studies are that the relationships of the binding studies correlate well with both *in vivo* pharmacology and the cultured network environment.

4.3 Tissue Specificity

An interesting, but surprising, finding from this study was the indication that the two different tissue responses displayed a specificity for the compounds. This observation was unexpected due to several previous findings. One study confirmed a larger number of receptors in the cortex as compared to the spinal cord (Herkenham et al., 1991). Another study indicated that methanandamide was a more potent and stable cannabinoid (Abadji et al., 1994) than anandamide. Anandamide has been described as a partial agonist (Mackie et al., 1993), meaning that, although the compound has bound to the receptor, it may not elicit a cellular cascade. Finally, to date, only one central cannabinoid receptor has been isolated (Matsuda et al., 1990). Taken together, these facts suggest that, for a given response, more anandamide would be required than methanandamide in both tissues and that greater amounts of each drug would be necessary to elicit responses in the spinal cord. However, the data obtained were not as straightforward as expected. Anandamide showed a distinction between the spinal cord and auditory cortical cultures by decreasing burst rates at lower concentrations in spinal cord cultures than in auditory cortical cultures (Figure 15A). To decrease activity initially, 5.5 times more anandamide was required in the cortex than in the spinal cord (Figure 14). This separation in effective concentrations continued until cessation of burst activity. The dosages of methanandamide necessary to bring about

network changes also were different between the spinal cord and the cortex (Figure 15B). Interestingly, methanandamide displayed the opposite effect by decreasing burst rates at lower concentrations in auditory cortical cultures than in spinal cord cultures. To decrease activity initially, 5 times more methanandamide was required in the spinal cord than in the auditory cortex (Figure 14). Again, these differences continued until burst activity ceased. To date, these effects have not been shown in the literature.

Comparing the concentrations of anandamide and methanandamide in the spinal cord shows a substantial variation. To decrease activity initially, 34 times more methanandamide was required. The EC_{50} was 12 times greater for methanandamide than anandamide, and the dosage leading to cessation of burst rate was 6 times greater for methanandamide than for anandamide (Figure 14). However, the auditory cortex does not display this distinction. The concentrations necessary to produce network effects are within 1.3 times of each other. The cortical cultures show that concentrations of anandamide and methanandamide were comparable to elicit the same responses. If the cortex is taken to display "normal" cannabinoid behavior, then the spinal cord is blatantly unique to all data presented to date. Tables 2 and 5 list the culture variables for each experiment. In comparing conditions (age, seeding date, treatment, % active channels) for spinal cord cultures exposed to anandamide and methanandamide, no correlations were found that could account for the dramatic differences between spinal cord responses to the two compounds. Therefore, the paradoxical effect found in the spinal cord cultures does not appear to be an artifact.

Possible explanations for this tissue specificity could be differential affinities of the compounds to the CB1 receptor located in the spinal cord. The affinities may be more similar in the cortex. Studies have shown interesting differences between receptor binding and G-protein activation with cannabinoids, i.e., relatively little G-protein activation (compared to receptor binding) was seen in thalamus, while relatively little receptor binding

(compared to G-protein activation) was seen in cortex (Sim et al., 1995). These differences suggest that some brain regions may be more catalytically active than others. Another explanation may be differences in receptor density in the spinal cord and cortex. Cannabinoid receptors in the spinal cord are sparse compared to other brain regions. The greatest concentration of these receptors has been found in the cortical structures (Herkenham et al., 1991). The arguments presented suggest that a novel receptor subtype may be the best explanation for the observed differences between anandamide and methanandamide in the spinal cord. It has been observed that cannabinoid receptors are located presynaptically as well as postsynaptically. Work by Kirby et al. (1997) suggested that SR141716A, a cannabinoid antagonist, binds with differential affinity to the presynaptic and postsynaptic receptors in rat hippocampal slices. Possibly, anandamide and methanandamide also bind differentially to these two subtypes. In addition to the receptor affinities, receptor densities may be unequal in the spinal cord. If anandamide binds to the subtype with the greatest density and methanandamide binds to the other subtype, this difference in the spinal cord responses might be explained.

4.4 Specific Effects of Anandamide on Network Activity

In both the spinal cord and cortical cultures, anandamide displayed specific characteristics that were not observed with methanandamide. In 3 out 7 experiments, cultures exposed to anandamide displayed "accommodation" (Figures 9 & 12). Accommodation was defined as a partial return (increase) of burst rate while under the influence of a specific drug concentration. Similar findings were observed *in vivo*. Behavioral work has found that the effects of anandamide, as compared to THC, are of shorter duration, likely due to its rapid breakdown by amidase activity (Romero et al., 1996). The breakdown or removal of the compound could be a plausible explanation for accommodation. Anandamide, being the endogenous ligand, is much more susceptible to

metabolism and reuptake than is methanandamide, a synthetic agonist. In conjunction with this fact, accommodation was not observed with any culture exposed to methanandamide. A carrier-mediated transport mechanism for anandamide removal has been postulated (Beltramo et al., 1997). This study has shown that the process is rapid, reaching 50% of its maximum ability within 4 minutes in both neurons and astrocytes. Beltramo et al. (1997) suggests that neurons and astrocytes may act synergistically in the brain to dispose of extracellular anandamide. Removal of anandamide from the synaptic cleft would result in a return of burst rate.

4.5 Specific Effects of Methanandamide on Network Activity

Methanandamide also exhibited characteristic effects on the network activity of both spinal cord and cortical cultures, which were not observed with anandamide. Six out of seven cultures exposed to the drug displayed a lack of return to a reference-like burst rate or synchronicity among channels following cessation of activity and medium changes (Figures 11 & 13). This effect was dramatically different from anandamide, which displayed 86% return of burst rate and 100% return of coordination between channels after washings. *In vivo* results partially support this observation. In behavioral tests on mice, effects of methanandamide are more prolonged than those of anandamide. Explanation of this observation may be attributable to the higher potency and greater stability to amidase hydrolysis (Romero et al., 1996). Also, methanandamide differs structurally from anandamide by a single methyl group. This extra component gives the compound a greater degree of membrane solubility. Methanandamide may be more difficult to remove, making the membrane a "reservoir" for the drug and resulting in a low background level of methanandamide that continues to affect synaptic activity, thus disenabling the network to return to its baseline activity.

Another fascinating characteristic observed only with methanandamide was an initial increase in activity for all spinal cord cultures tested (Figures 15B & 32). The increase in burst rate accompanied dosages ranging from 250 nM - 4.5 μM with subsequent decreases occurring between 4.5 - 7.0 μM. Nearly 25 years ago, Paton and Pertwee (1973) observed a biphasic response of ΔTHC^9 with low doses being a stimulant and "depressant action becoming increasingly important as dose increases". In 1998, Sulcova et al. showed similar responses of anandamide on physiological and behavioral assays performed with mice. High doses (10 - 100 mg/kg) displayed stereotypical inhibitory effects while low doses (0.01 mg/kg) led to stimulatory actions. Possible explanations for this are a differential involvement of a Gs and a Gi protein activated at low and high doses or activation of a specific subtype of receptor (i.e., located presynaptically vs. postsynaptically) by low doses of methanandamide. The latter explanation could possibly tie together the puzzling nature of the disparity between anandamide and methanandamide in the spinal cord. Previous work has implicated CB1 receptor coupling with not only Gi proteins but also Gs proteins (Glass and Felder, 1997). However, this work was looking at CB1 receptors and D2 receptors on primary culture striatal neurons.

4.6 Bicuculline Effects on Network Activity

In addition to regularizing and coordinating spontaneous activity, bicuculline additions tended to shift the dose-response curves to higher values for both anandamide and methanandamide. In 4 out of 5 cases, those cultures not treated with bicuculline showed a greater decrease of burst rate than those with bicuculline (Figures 18 & 19). The concentrations leading to cessation of activity were similar, but not identical, in cultures with and without bicuculline. Those exposed to bicuculline had a slightly greater shut-off concentrations than those not treated. A similar effect was observed by Jordan (1992) in his investigations of GABA effects on spontaneous spinal cord activity. Until better data

processing methods are established, the improved burst detection and evaluation provided by pattern regularization may justify the use of bicuculline. However, these alterations in dose-response curves must be taken into account, especially for quantitative analyses of other burst parameters, such as burst duration, spike frequencies in bursts and total spike production per burst. It is clear from these studies that burst regulation via bicuculline does not generate large changes in network response and does not alter the overall inhibition of the networks to anandamide.

Cannabinoids have been implicated in modulating GABA reuptake. Activated cannabinoid receptors located on GABAergic neurons in the rat striatum were accompanied by a reduction in neurotransmitter uptake, thus prolonging the presence of GABA into the synaptic cleft and decreasing activity (Romero et al., 1998). Blocking these GABA receptors with bicuculline would tend to increase the activity from its previous inhibited state. Obviously, this is not the only system affected by the cannabinoids, but its involvement may explain the slight differences seen between bicuculline and non-bicuculline treated cultures.

4.7 Toxicity of Anandamide and Methanandamide

Preliminary work was conducted on the concentrations and exposure times leading to irreversible effects of cannabinoid agonists on network activity. Irreversibility was defined as cessation of activity that could not be reversed by 2 medium changes during a 2-hour period. Both anandamide and methanandamide displayed irreversible effects at concentrations exceeding 8.5 μM for a minimum of 30 minutes (Figures 20 - 23). Most of the previous work in the area of cannabinoids and neurotoxicity have been performed with THC and intact animals with exposure times of days to months. No data has implicated that death or severe damage has accompanied an organism exposed to THC. The majority of recent work employing cannabiniods have focused on mechanisms and mode of actions.

Further work need to be explored to substantiate the findings from this portion of the project. It is important to note that even in the absence of cell death, the suppression of network activity alone can challenge the survival of an organism. The observation that both bursting and spiking were essentially stopped at the concentrations indicated suggest that a general system failure can lead to death.

4.8 Networks in Culture

Neuronal networks *in vitro* develop spontaneously activity and are highly sensitive to their chemical environment. The MMEP allows for the simultaneous recording of 64 electrodes from a neuronal network which, pharmacologically, functions as a histiotypic representative of the parent tissue. Potential functions of recording from spontaneously active monolayer networks in culture are applicable to areas of neurotoxicology, drug development and biosensors. A primary goal of this study was to evaluate the reproducibility of the network to cannabinoid agonists. Both interculture and intraculture repeatability were demonstrated for each tissue type. Figure 8 shows typical interculture repeatability. With anandamide, the cultures were able to be taken to cessation of bursting and returned to a reference-like state after washing over the course of several cycles. Cultures exposed to methanandamide also displayed interculture repeatability with drug concentrations consistently eliciting the same effects (Figure 10). However, methanandamide did not allow a return of reference-like activity in 86% of the cultures. Once again, this was a highly reproducible phenomenon. In conjunction with the reproducibility to the cannabinoid agonists is the potential for employing network responses for "fingerprinting" of compounds. Specific differences in tissue responses and the concentrations of drug leading to a particular effect may enable a compound to be identified. Clearly, this would require further work, but is attainable based upon findings from this and other related projects. Overall, this study demonstrated that spontaneously

active neuronal networks have characteristic responses to cannabinoid agonists that are highly reproducible in cell culture. In the future, cultured networks may serve as efficient, functional and reproducible environments in the areas of rapid prescreening of neuroactive compounds, drug development and biosensors. The potential for parallel, automated network analysis make this methodology very attractive to basic and applied research in neurobiology.

APPENDIX

Table 8: Summary of Thesis Experiments

EXP	EXP. DATE	SEED DATE	AGE (DIV)	MMEP #	TISSUE TYPE	TREAT MENT	AVG S:N (X:1)	MAX S:N (X:1)	% ACTIVE CHANNELS
SM26	02-05-97	12-05-96	61	F202rp	SC	ARAC	2.6	7	36
SM27	02-12-97	01-02-97	41	L380	SC	-	2.8	10	61
SM28	02-19-97	01-02-97	48	L459	SC	-	3.1	10	36
SM29	02-25-97	01-16-97	40	C163rp11	SC	ARAC	-	-	0
SM30	02-26-97	01-16-97	40	C26rp11	SC	ARAC	2.4	3	22
SM31	03-26-97	01-23-97	62	C289rp1	SC	-	3.4	8	89
SM32	04-02-97	01-23-97	70	F374rp11	SC	-	3.1	10	69
SM33	04-08-97	03-07-97	32	Y139	CTX	-	-	-	0
SM34	04-08-97	01-23-97	75	L296rp1	CTX	-	-	-	0
SM35	04-12-97	01-16-97	88	C590rp11	SC	ARAC	-	-	20
SM36	04-12-97	01-30-97	73	P48	SC	ARAC	-	-	0
SM37	04-14-97	01-30-97	73	P68	SC	ARAC	2.8	7	22
SM38	04-16-97	01-16-97	90	L336rp1	SC	ARAC	-	-	8
SM39	04-16-97	01-16-97	90	L452rp	SC	ARAC	-	-	0
SM40	04-16-97	01-30-97	76	P84	SC	ARAC	5.8	17	22
SM41	04-19-97	01-30-97	79	L292	SC	-	4.8	19	58
SM42	04-22-97	01-30-97	83	L324rp11	SC	ARAC	3.6	12	31
SM43	05-14-97	04-03-97	41	C210rp11	CTX	-	-	-	0
SM44	05-14-97	04-10-97	34	Y132	CTX	-	-	-	0
SM45	05-14-97	01-23-97	111	P61rp	SC	ARAC	2.8	8	69
SM46	05-21-97	04-24-97	27	P31rp	CTX	-	2.9	5	81
SM47	05-28-97	02-27-97	90	C21rp11	SC	ARAC	3.6	6	77
SM48	05-29-97	02-27-97	91	C21rp11	SC	ARAC	3.4	8	75
SM49	05-30-97	02-27-97	92	C21rp11	SC	ARAC	3.8	8	83
SM50	06-11-97	04-17-97	55	Y210	CTX	ARAC	-	-	0
SM51	06-11-97	04-24-97	48	L260rp	CTX	-	5.8	10	100
SM52	06-18-97	02-27-97	111	X644rp	SC	-	5.1	25	69
SM53	06-23-97	02-27-97	116	Y154	SC	ARAC	2.1	4	5
SM54	06-23-97	03-27-97	88	L266rp1	SC	-	4.1	10	54
SM55	06-28-97	04-03-97	86	P183	CTX	-	-	-	0
SM56	06-28-97	03-27-97	93	L485rp	CTX	-	1.8	3	22
SM57	06-28-97	04-24-97	65	C220rp1	CTX	-	3.1	10	74
SM58	06-30-97	04-17-97	78	P43rp	CTX	-	-	-	0
SM59	06-30-97	04-03-97	88	P9rp1	CTX	-	2	3	17
SM60	06-30-97	03-27-97	95	L111rp1	CTX	-	-	-	0
SM61	07-01-97	04-24-97	68	F134rp11	CTX	-	1.7	2	9
SM62	07-01-97	04-10-97	82	Y144	CTX	-	2.3	5	17
SM63	07-08-97	04-24-97	75	C26rp111	CTX	-	4.7	25	74
SM64	02-13-98	01-15-98	29	Y619	SC	FDU	2.5	3	20
SM65	02-13-98	11-29-97	76	Y264	SC	-	3.5	10	63

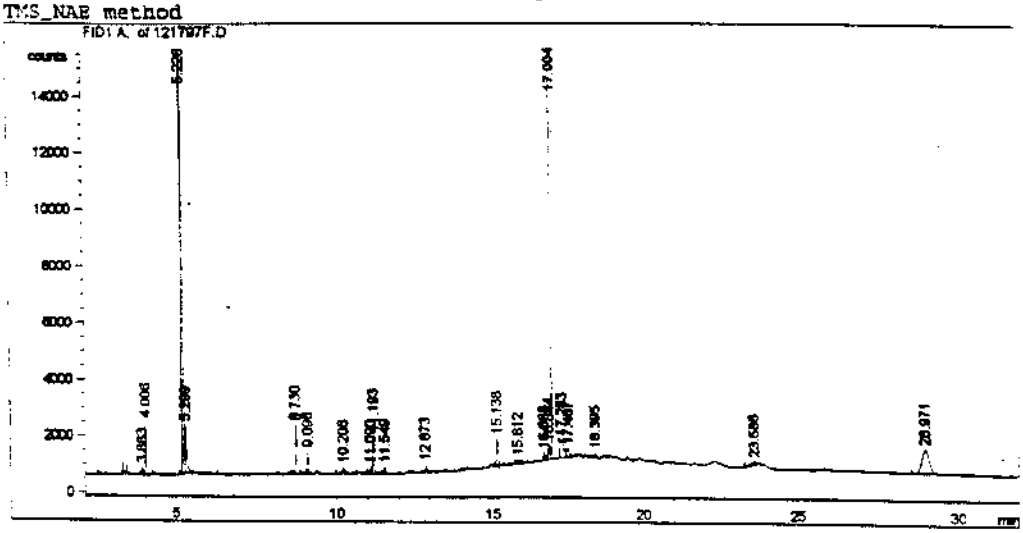
Figure 24: Sample of raw data via GC/FID analysis.

Samantha's samples NAes / Anandamid in EtOH

Injection Date : 12/17/97 2:01:14 PM Seq. Line : -
Sample Name : An A Vial : 1
Acq. Operator : K.Chapman Inj : -
Inj Volume : Manually

Method : C:\HPCHEM\1\METHODS\NAE_TMS2.M
Last changed : 12/17/97 12:12:15 PM by K.Chapman
(modified after loading)

TMS_NAE method



Area Percent Report

Sorted by Signal
Multiplier : 1.000000
Dilution : 1.000000
Sample Amount : 1.000000 [ng/ul] (not used in calc.)

Signal 1: FID1 A,

Peak #	RT [min]	Type	Width [min]	Area	Height	Area %
1	3.883	BV	0.035	547.52386	244.95854	0.1461
2	4.006	VB	0.036	4258.36865	1857.76208	1.1360
3	5.226	BV	0.040	59344.01562	23754.35352	15.8305
4	5.299	VB	0.052	5269.87842	1681.12903	1.4058
5	8.730	VV	0.058	6757.50488	1732.74707	1.8026
6	9.096	PB	0.050	2443.10937	776.76794	0.6517
7	10.208	BV	0.039	440.55728	193.55766	0.1175
8	11.090	BV	0.050	791.95465	246.63232	0.2113
9	11.193	VV	0.054	4521.75830	1341.03589	1.2062
10	11.549	BB	0.049	833.00140	269.74139	0.2222
11	12.873	BB	0.056	978.23737	268.25174	0.2610
12	15.138	PV	0.056	3482.98071	979.76575	0.9291
13	15.812	PV	0.090	1029.17542	163.16119	0.2745
14	16.668	VV	0.098	1875.38647	276.33060	0.5003
15	16.844	VV	0.064	2044.96399	506.04855	0.5455
16	17.004	VV	0.059	255992.57812	66723.08594	68.2879
17	17.283	VV	0.120	5902.09570	641.31293	1.5744
18	17.487	VV	0.092	1891.56140	304.73575	0.5046
19	18.395	BV	0.082	1066.14392	182.48552	0.2844
20	23.586	BB	0.207	1822.62036	121.38503	0.4862
21	28.971	BB	0.211	13578.88867	851.69275	3.6223
Totals :				374872.31250	103116.93750	

*** End of Report ***

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