

**SYNAPTIC ACTION OF ANANDAMIDE AND RELATED
SUBSTANCES IN MAMMALIAN BRAIN**

by

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ABSTRACT

Anandamide and the synthetic cannabimimetic drugs AM 404 and WIN 55,212-2 were found to inhibit the binding of [³H]batrachotoxinin A 20- α -benzoate (BTX) to voltage-gated sodium channels (VGSCs) and also to depress VGSC-dependent release of GABA and L-glutamic acid. These effects occur independently of CB-1 receptor activation since they were not attenuated by AM251 at concentrations known to antagonize CB-1 receptors, although at higher concentrations AM251 inhibited VGSCs also. These results suggest that anandamide and endocannabimimetics have the ability to depress synaptic transmission by reducing the capacity of VGSCs to support action potentials. In other experiments, I observed that anandamide is synthesized by both resting and depolarized synaptoneuroosomes, however, data from filtration and superfusion experiments failed to support the hypothesis that depolarization activates the release of anandamide from these synaptic preparations, even though substantial quantities of anandamide were clearly present in the intracellular compartments. In these investigations, I unexpectedly found that [³H]ethanolamine (a potential precursor of anandamide) is accumulated by synaptosomes and synaptoneuroosomes during extended incubation and released (as [³H]ethanolamine) in a calcium-dependent fashion during a depolarizing challenge with 30 mM KCl. Ethanolamine also stimulates the release of the pH fluoroprobe acridine orange (AO) from synaptosomes and decreases AO release when synaptosomes are depolarized with KCl. However, ethanolamine and other amino alcohols (methylethanolamine and dimethylethanolamine) stimulate resting and KCl-

evoked release of [³H]-D-aspartate from synaptosomes. All amino alcohols rapidly access synaptic vesicles where they sequester protons, thus accounting for AO efflux. Proton sequestration by amino alcohols increases the ATP-dependent transvesicular membrane potential which in turn enhances D-aspartate uptake into synaptic vesicles. My data suggest a potential role for ethanolamine and related amino alcohols in the regulation of synaptic vesicle filling and release of amino acid neurotransmitters. Depolarization evoked release of ethanolamine from synaptosomes supports the idea that this amino alcohol may also have an important postsynaptic role.

Keywords: anandamide; cannabinoids; sodium channels; synaptic vesicles; mammalian brain.

DEDICATION

谨此纪念我的母亲和感谢我的父亲对我的养育之恩。

感谢我的妻子与我相濡以沫，患难以共。

In memory of my mom and thanks to my father
for bringing me up with love and support.

Thanks also to my wife
for always supporting me in hard and happy times.

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GLOSSARY

AEA	Anandamide
2-AG	2-Arachidonoyl glycerol
AM 251	1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(1-piperidyl)pyrazole-3-carboxamide
AM 404	N-(4-Hydroxyphenyl)arachidonoylamide
ATP	Adenosine triphosphate
B _{max}	Maxium concentration of binding sites
BSA	Bovine serum albumin
BTX-B	Batrachotoxinin A 20- α -benzoate
CB1 receptor	Cannabinoid receptor-1
CB2 receptor	Cannabinoid receptor-2
CNS	Central nervous system
DBD-COCl	4-(N-Chloroformylmethyl-N-methyl)amino-7-N,N-dimethylaminosulphonyl-2,1,3-benzoxadiazole
CCCP	Carbonyl cyanide <i>m</i> -chlorophenylhydrazone
DMSO	Dimethyl sulphoxide
DSE	Depolarization-induced suppression of excitation
DSI	Depolarization-induced suppression of inhibition
EtNH ₂	Ethanolamine
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid
FAAH	Fatty acid amino hydrolase
GABA	γ -Aminobutyric acid
5-HT	5-Hydroxytryptamine (serotonin)
nACh-R	Nicotinic acetylcholine receptor
IC ₅₀	Concentration effective in producing 50% inhibition
K _d	Dissociation constant
N-Acyl-PE	N-Acyl phosphatidylethanolamine

NArPE	N-Arachidonoyl phosphatidylethanolamine
PMSF	Phenylmethylsulphonylfluoride
PNS	Peripheral nervous system
SDS	Sodium dodecyl sulphate
THC	Δ^9 -tetrahydrocannabinol
TRPV1-R	Transient receptor potential vanilloid-1 receptor
TTX	Tetrodotoxin
VSSCs	Voltage-sensitive sodium channels
VTD	Veratridine
WIN 55, 212-2	(R)-(+)-[2,3-Dihydro-5-methyl-3-[(4-morpholinyl)methyl-pyrrolo [1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)-methanone

CHAPTER 1

INTRODUCTION

1.1 The discovery of the endocannabinoid system in mammals

The traditional drug, marijuana, has been known for its therapeutic and psychoactive properties for at least 4000 years. It has been used in the treatment of psychoses, convulsions, glaucoma, hypertension, nausea, pain and many other conditions including the relief of pain associated with childbirth (Axelrod and Felder, 1998). The identification of the main active constituent Δ^9 -tetrahydrocannabinoid (THC) in marijuana was accomplished by Gaoni and Mechoulam in 1964 (Gaoni and Mechoulam 1964). Studies on structure activity relationships led to the discovery of other active analogs and collectively, these bioactive natural products from marijuana are called cannabinoids (Melvin and Johnson, 1987). This work, along with observations of marijuana's potential therapeutic applications, opened up research into the medical properties of cannabinoids. Investigations into the cellular actions of THC found that it inhibits adenylate cyclase (Howlett and Fleming, 1984) and binds with high affinity to sites in brain membranes (Devane et al., 1988). These findings indicated that THC most likely binds to a specific receptor in neuronal membranes which may be coupled to adenylate cyclase. In 1990, a discrete receptor for cannabinoids was cloned from rat brain (Matsuda et al., 1990). The following year Gerard and his colleagues cloned a human cannabinoid receptor (the CB1 receptor). This protein which contains 472 amino acid residues, was found to share over 97%

homology with the rat cannabinoid receptor (Gerard et al., 1991). Functional studies on CB1 receptor-transfected fibroblast cell lines (which are devoid of native cannabinoid receptors), confirmed a binding constant ($K_d = 3.3$ nM) to cannabinoid agonists similar to that observed in brain for native CB1 receptors ($K_d = 2.3$ nM). Significantly, activation of the cloned cannabinoid receptor inhibits cAMP formation. CB1 receptors are widely distributed in mammalian brain. They are particularly abundant in the cerebellum, hippocampus, and cerebral cortex. They are also found at high density in the basal ganglia and the nucleus accumbens. CB1 receptors have also been detected at moderate density in the hypothalamus, amygdala, spinal cord, brain stem, central gray, and nucleus of the solitary tract (Herkenham, 1995; Pertwee, 1997; Joy et al., 1999). Since constituents of marijuana, particularly its active component THC can exert a number of effects outside of the central nervous system, such as local analgesia, suppression of inflammation, the alleviation of intraocular pressure in glaucoma and attenuation of vomiting, it was suggested that a cannabinoid receptor may also be present in peripheral tissues. The peripheral cannabinoid receptor was cloned in 1993 (Munro et al., 1993) and was termed the CB2 receptor. The CB2 receptor cloned from a leukaemic cell line was found to have 68% sequence identity within the CB1 ligand binding pocket whereas the remaining structure displayed only 44% overall homology with the CB1 receptor (Axelrod and Felder, 1998). Not surprisingly therefore, the CB2 receptor shows some similar pharmacological properties when compared with the CB1 receptor and there are also some parallels in the mechanism of signal transduction although CB2 receptors do not inhibit Q-type calcium channels or activate inwardly rectifying K^+ channels (Felder et al., 1995). The recent localization of CB2 receptor-

like immunoreactivity in glial cells and neurons in many areas of brain (Gong et al., 2006), suggests that the CB2 receptor may have a more prominent role in the central nervous system than previously anticipated. It is also clear that CB1 receptors are present in the peripheral nervous system, including cells of the immune system, the heart, vascular tissues and the testis (Galiègue et al., 1995; Richardson et al., 1998). While CB1 receptors are evolutionarily conserved in many species (Begg et al., 2005), CB2 receptors appear to be considerably more divergent (Griffin et al., 2000). By analogy with the localization of receptors for opiates in brain which led to the discovery of endogenous opiate-like regulatory substances in the nervous system, the occurrence of discrete receptors for THC in mammals suggests the presence of a regulatory factor (or factors) naturally present in the body capable of binding to cannabinoid receptors and eliciting the various physiological and psychological responses observed with the plant bioactive THC. The first endogenous substance proposed in this regard was the eicosanoid anandamide which has a very different structure from THC, consisting of arachidonic acid coupled to ethanolamine through an amide linkage (Devane et al., 1992). Anandamide was shown to induce similar behavioral (Crawley et al., 1993; Souilhac et al., 1995), pharmacological (Fride and Mechoulam 1993; Smith et al., 1994) and signal transduction effects (Felder et al., 1993) to the classical cannabinoid agonists from marijuana, but relatively high concentrations were required to induce these effects when compared to THC. The name anandamide is derived from an Indian Sanskrit word meaning “bringer of inner bliss and tranquility” (Axelrod and Felder, 1998). Anandamide is widely distributed in the body. Not only is it found in the central and peripheral nervous systems, but also in

kidney, testis, skin, uterus, spleen, blood plasma, and various parts of the cardiovascular system (Martin et al., 1999). Anandamide also displays THC-like effects on behavior and physiological function. Compared to THC which is exogenous, anandamide is an endogenous substance and therefore commonly referred to as an endocannabinoid. Three years after anandamide was discovered, another endocannabinoid contender was isolated from canine gut, spleen, and pancreas (Mechoulam et al., 1995). Its structure was assigned as 2-arachidonoyl-glycerol (2-AG). A short time later 2-AG was also shown to be present in brain (Sugiura et al., 1995). 2-AG binds to both CB1 and CB2 receptors. Other putative endocannabinoid ligands including virodhamine, N-arachidonoyl dopamine, and noladin ether (a more stable ether-linked form of 2-arachidonoyl glycerol) have been reported. All of these compounds appear to bind CB1 receptors (Piomelli, 2003; Di Marzo et al., 1998). Various endocannabinoids have also been reported to retrogradely depress presynaptic release of neurotransmitters in brain. Evidence suggests that endocannabinoids are synthesized, released, taken up, and degraded in neurons by mechanisms similar to those of neurotransmitters. Moreover, anandamide can be released from cultured neurons when they are depolarized by an elevated concentration of K^+ , or when treated with ionomycin or kainic acid (Di Marzo et al., 1994). After anandamide is released into the synaptic cleft, it rapidly binds to presynaptic CB1 receptors, triggering a variety of responses which culminate in inhibition of transmitter release. Signal inactivation is thought to occur when anandamide is taken back up into neurons and hydrolyzed. The fundamental difference between neurotransmitter and endocannabinoid release by the nerve is the direction. Neurotransmitters are released

from presynaptic neurons and exert important excitatory or inhibitory influences on postsynaptic neurons. Endocannabinoids are thought to be released predominately from postsynaptic neurons and then translocate retrogradely to CB1 receptors on the plasma membrane of the nerve ending where, via various mechanisms, they downregulate the responsiveness of the presynaptic nerve ending to incoming stimuli. Neurotransmitter release is controlled by Ca^{2+} influx. Ca^{2+} influx in turn is controlled by at least five distinct types of calcium channels (N, L, T, P and Q) which can respond to depolarizing stimuli. Endocannabinoids can indirectly inhibit Ca^{2+} influx by controlling G-protein activity, by binding to certain specific K^+ channels and, as I have found, by blocking voltage-gated sodium channels (Nicholson et al., 2003). The modulation of these cellular signaling systems by endocannabinoids leads to a decline in neurotransmitter release. There is apparently no evidence at present to suggest that endocannabinoids modulate other mechanisms important for the control of $[\text{Ca}^{2+}]_i$ in neurons, such as $\text{Na}^+/\text{Ca}^{2+}$ exchange. The following sections discuss key aspects of the endocannabinoid system in terms of endocannabinoid synthesis, release, uptake, hydrolysis, physiological function, and mechanisms by which they regulate signal transduction in cells.

1.2 The synthesis of endocannabinoids in brain with particular reference to anandamide and 2-AG.

There are two routes known to be responsible for the synthesis of anandamide in brain. One route requires the presence of Ca^{2+} and another is largely independent of Ca^{2+} (Llano et al., 1991; Schmid et al., 1997; Paria et al., 1996). In brain, two important regulatory phenomena arising from the interaction of anandamide with CB1

receptors are “depolarization-induced suppression of inhibition” (DSI) and “depolarization-induced suppression of excitation” (DSE). Available evidence suggests that endocannabinoids such as anandamide are synthesized and released from postsynaptic neurons as a result of postsynaptic depolarization. Ca^{2+} -dependent endocannabinoid biosynthesis appears to be an important pathway for brain neurons (Di Marzo, et al., 1998). A rise in Ca^{2+} concentration within the postsynaptic neuron is thought to be a crucial initiator of anandamide biosynthesis. Studies in Purkinje cells suggest that DSI can only occur if there is Ca^{2+} influx, since it is not sustained when the inward Ca^{2+} current is terminated (Llano et al., 1991). Further support for this comes from studies with the Ca^{2+} chelators, BAPTA and EGTA, which prevent DSI (Pitler and Alger, 1992). Like DSI, calcium influx is needed for DSE to occur in synapses onto Purkinje cells (Kreitzer and Regehr, 2001). However, it is still unclear as to how much of a rise in the concentration of Ca^{2+} is necessary for endocannabinoid synthesis and depression of presynaptic transmitter release. Some investigations suggest that endocannabinoid synthesis and release requires only modest increases in the concentration of Ca^{2+} . For example in Purkinje cells of the rat cerebellum, a somal increase in free $[\text{Ca}^{2+}]_i$ of 40 nM Ca^{2+} was sufficient to trigger a substantive (50% of maximum) retrograde inhibitory response (Glitsch et al., 2000). In contrast, other researchers suggest that much higher calcium levels in the order of 15 μM in the postsynaptic compartment are required for half-maximal retrograde inhibition in Purkinje cells (Brenowitz and Regehr, 2003) and 4 μM Ca^{2+} is required to induce half maximal DSI in hippocampal neurons (Wang and Zucker, 2001). Clearly, more

investigation is needed to clarify why such large differences in Ca^{2+} concentrations were required to reduce neurotransmission by this retrograde mechanism.

When brain neurons are activated by membrane-depolarizing agents (e.g. ionomycin, kainate and high potassium), membrane depolarization causes Ca^{2+} influx into cells, and activates a calcium-dependent N-acyltransferase which catalyzes the biosynthesis of N-arachidonoyl phosphatidylethanolamine (NArPE). Anandamide can be released intracellularly by the hydrolysis of NArPE which is catalyzed by phospholipase D (Di Marzo et al., 1994). Studies on the regional distribution of NArPE and anandamide in brain demonstrate that the brain stem and striatum contain the highest levels, while levels in the cerebellum and cortex are lower. Thus the levels of NArPE and anandamide in different brain regions show good agreement which supports the idea that NArPE may be of relevance as a precursor of anandamide (Bisogno et al., 1999). The highest amounts of NArPE and anandamide (up to 359 and 87 pmol/g wet weight of tissue respectively) were found in the brain stem and NArPE is generally 3-13 times more abundant than anandamide in all brain regions. These observations confirm that NArPE is present at sufficient levels to act as an effective reservoir for anandamide synthesis. Although the regional distribution of NArPE in brain correlates quite well with that of anandamide there is much less of a correspondence with the distribution of cannabinoid receptors (Bisogno et al., 1999). NArPE can be hydrolysed by N-acyl phosphatidylethanolamine (N-Acyl-PE) phospholipase D (NAPE-PLD) which is involved in the biosynthesis of anandamide and other bioactive N-acylethanolamines (NAEs) (Liu et al., 2002).

Anandamide and other bioactive N-acylethanolamines (NAEs) can be generated by direct cleavage of N-Acyl-PE by phospholipase D (PLD) (Di Marzo et al., 1994; Liu et al., 2002). N-arachidonoyl-PE can also undergo a two step reaction to form anandamide. This first involves hydrolysis of N-arachidonoyl-PE by PLA1 and PLA2 enzymes forming N-arachidonoyl-lyso-PE from which the release of N-arachidonoyl ethanolamine is catalysed by lysophospholipase D (lysoPLD). Both enzymes are found in the brain and the specific activity of lysoPLD is particularly high in cerebrum and cerebellum (Sun et al., 2004). Current evidence indicates that the lysoPLD is distinct from the known N-acyl-PE-hydrolysing PLD (Sun et al., 2004; Bisogno et al., 2005). Another possible route for the biosynthesis of anandamide is by the coupling of arachidonic acid with ethanolamine. Anandamide can be produced *in vitro* by brain tissue homogenates from extraneous arachidonic acid and ethanolamine (Kempe et al., 1996). This biosynthetic reaction does not require Ca^{2+} . Anandamide synthase was partially purified from porcine brain (Ueda et al., 1995) and shown to exhibit the same pH and temperature dependence and inhibition profiles as the enzyme catalyzing anandamide hydrolysis to arachidonic acid and ethanolamine [i.e. anandamide amidohydrolase or fatty acid amide hydrolase (FAAH)]. The levels of FAAH have been reported to be high in the hippocampus, thalamus, striatum, and frontal cortex, whereas pons, cerebellum, and medulla possess the lowest activity. The enzyme was found to be associated with all membrane fractions examined. FAAH activity was highest in cells that were of lower density than 0.8 M sucrose. Such fractions contained synaptic vesicles, microsomes, myelin fragments and synaptosome ghosts. The lowest FAAH activity was observed in the pellet within the 1.2 M sucrose layer which

contains mostly mitochondria and the activities of cytosolic fractions were minimal (Devane, 1994). The FAAH activity was retained after membranes had been extensively washed in saline suggesting the enzyme is strongly bound to membrane components under normal situations (Devane, 1994). The biosynthetic activity of FAAH depends a lot on the concentrations of arachidonic acid and ethanolamine (Maccarrone et al., 1998, 2000). It has been suggested that physiological levels of ethanolamine and arachidonic acid may be too low to allow synthesis of anandamide to occur in brain but FAAH-mediated synthesis clearly occurs in other organs like the uterus (Schmid et al., 1997; Paria et al., 1996). In the uterus there is also evidence from *in vitro* and *in vivo* studies that the synthesis of anandamide dominates over hydrolysis at a stage associated with implantation. It is interesting that phenylmethylsulfonyl fluoride (PMSF), which strongly inhibits amidase activity, has been shown to have no inhibitory activity against the synthase, and in fact, may facilitate the synthesis of anandamide at lower concentrations (Paria et al., 1996). Since the cellular concentrations of arachidonic acid and ethanolamine are considered lower than those necessary for efficient turn over of the synthase enzyme, it was proposed that 'anandamide synthase'-mediated anandamide biosynthesis in brain would likely require activation of the two phospholipases responsible for arachidonic acid and ethanolamine release from membrane phospholipids, [i.e. phospholipase A2 and D] (Di Marzo et al., 1998).

Current evidence suggests that Ca^{2+} entry may not actually be needed for anandamide synthesis. The dopamine D2 receptor agonist quinpirole causes an eightfold increase in anandamide outflow in the rat striatum, which is prevented by the

D2 antagonist raclopride. Moreover, activation of muscarinic acetylcholine receptors and metabotropic glutamate receptors have also been observed to cause endocannabinoid release in hippocampal slices in a Ca^{2+} -independent manner (Piomelli, 2003; Kim, 2002; Varma, 2001). The involvement of Ca^{2+} mobilization from internal stores (Piomelli, 2003) remains to be clarified.

The distribution of 2-AG in brain was found to correlate quite well with that of AEA with levels ranging from 2.0 to 14.0 nmol/g in the diencephalon and brainstem, respectively (Bisogno et al., 1999). A variety of potential pathways leading to 2-AG biosynthesis in neurons have been proposed. These include a primary PLC-mediated hydrolysis of membrane phospholipids, followed by a second hydrolysis of the 1,2-diacylglycerol (DAG) by diacylglycerol lipase, or involvement of PLA1 which generates a lysophospholipid, which, in turn, is hydrolyzed to 2-AG by lysophospholipase C (Piomelli et al., 1998). In addition to the phospholipase-mediated pathways, 2-AG accumulation may occur as a result of breakdown of triacylglycerols which can be catalyzed by a neutral lipase or from the dephosphorylation of lysophosphatidic acid (LPA) (Piomelli et al., 1998). Ca^{2+} appears to be a requirement for the stimulation of 2-AG synthesis by inducing the formation of diacylglycerol (Alger 2002). High frequency stimulation of CA1 neurons has been shown to produce a four-fold increase in 2-AG accumulation compared to the control treatment. This effect can be prevented by the Na^+ channel blocker tetrodotoxin and by eliminating Ca^{2+} from the superfusing solution (Piomelli et al., 1998). Co-release of 2-AG and anandamide has been observed in cortical neurons following ionomycin treatment (Di Marzo et al., 1994, Stella et al., 1997) and 2-AG is 170 times more abundant than

anandamide in rat on a whole brain basis. However, binding studies with [³H]CP-55,940 have demonstrated that the affinity of anandamide for the CB1 receptor is higher than that of 2-AG. In this context anandamide was shown to bind to the CB1 receptor with good affinity (inhibition constant $K_i = 61$ nM) but to have low affinity for the CB2 receptor ($K_i = 1930$ nM) (Lin et al., 1998). By contrast, 2-AG binds weakly to both CB1 ($K_i = 472$ nM) and CB2 ($K_i = 1400$ nM) receptors (Mechoulam et al., 1995). It is unclear how much of an impact 2-AG makes as an extracellular cannabinoid signal because 2-AG also functions as a metabolic modulator of DAG and AA which themselves have important signaling functions (Di Marzo, 1998). Moreover in platelets for example, 2-AG is produced in large quantities but is then rapidly cleaved to arachidonic acid by monoacylglycerol lipase activity (Cabot et al., 1977; Bell et al., 1979). The very short half life of 2-AG suggests that it may serve primarily as a precursor for arachidonic acid release (Lin et al., 1998). Furthermore, since anandamide and 2-AG are both present in the brain and have very similar functions, it may be difficult to assess the contributions arising from anandamide and 2-AG at CB1 receptors and indeed distinguish these from inputs made by other endocannabinoid candidates.

1.3 Anandamide uptake

After anandamide is released, it is rapidly taken up by cells and then quickly hydrolyzed by FAAH. It is generally accepted that uptake of anandamide is an energy-free process. The accumulation of anandamide is clearly not dependent upon ATP or a sodium ion gradient and is not inhibited by ouabain (Hillard et al., 1997). The movement of anandamide into neurons is most likely driven by the concentration

gradient of free anandamide across the plasma membrane. Efficient FAAH-mediated enzymatic cleavage of anandamide inside the cell acts to maintain the inwardly driving concentration gradient (Glaser et al., 2003). So far there is no compelling evidence to indicate whether or not there are specific proteins in the membrane involved in the facilitation of anandamide transport. The evidence required to support the idea of facilitated transport can be summarized as follows: (i) saturation kinetics must occur; (ii) a carrier protein must be demonstrated; (iii) the protein must demonstrate a high degree of specificity and reasonable affinity for the molecule being transported; and (iv) the protein should exhibit sensitivity to inhibitor (Di Marzo et al., 1994; Hillard et al., 1997; Beltramo et al., 1997). The cellular uptake of anandamide is known to be temperature-sensitive and saturable (Hillard et al., 1997). The synthetic cannabinoid AM404 inhibits anandamide uptake in rat cortical neurons and astrocytes and enhances DSI. It was proposed that AM404 likely blocks the membrane carrier for anandamide and therefore increases the persistence of anandamide at CB1 receptors (Beltramo et al., 1997). Ligresti et al. (2004) found that anandamide uptake in neurons or astrocytes is saturable at low concentrations and exhibits time-dependence however, uptake does not saturate at high concentrations. Viridohamine (Porter et al., 2002) and arachidonoylglycerol ether (Fezza et al., 2002) inhibit anandamide uptake competitively suggesting that they interact with the same uptake mechanism. Indeed certain structurally distinct diaryl analogs of anandamide (O-3246 and O-3262) act as potent blockers of anandamide uptake in RBL-2H3 cells (Ligresti et al., 2006). The membrane carrier hypothesis for anandamide is further supported by the finding that uptake and efflux of anandamide is inhibited by specific inhibitors such as VDM1, (N-

4-hydroxy-2-methylphenyl) arachidonoylamide (Ligresti et al., 2004) and that the well known inhibitor of anandamide accumulation, AM404, also inhibits anandamide efflux (Gerdeman et al., 2002). If inhibition of anandamide uptake occurs as a result of competition at intracellular binding sites between anandamide and its inhibitors, or by the inhibitors reducing FAAH activity (both of which could reduce the inward anandamide concentration gradient), it is difficult to understand how either mechanism might bring about inhibition of anandamide efflux. Some selective transport inhibitors do not appear to cross-react with FAAH such as UCM707 (Lopez-Rodriguez et al., 2003), OMDM1, OMDM2 (Ortar et al., 2003) and AM1172 (Fegley et al., 2004) and therefore support the concept of an anandamide membrane transporter in the uptake of anandamide. Moreover, saturable accumulation of anandamide is still observed in FAAH-deficient synaptosomes (Ligresti et al., 2004). Recently, Moore et al. (2005) reported the use of a potent, competitive inhibitor (LY2318912) to block anandamide uptake in RBL-2H3 cells and proposed a high-affinity, saturable transporter binding site for anandamide which is separate from FAAH. In other experiments, LY2318912 was administered into rodents and caused a 5-fold increase in anandamide concentrations in the brain (Moore et al., 2005). However, as anandamide is lipophilic it is a compound that should partition into the plasma membrane easily. Using red cell ghosts as a model, investigation has shown anandamide rapidly passes through membrane (in fact within seconds) however, a carrier is not necessarily required to facilitate this process (Bojesen and Hansen, 2005). Another problem with the carrier hypothesis is that a discrete anandamide carrier has not been identified so far (Bisogno et al., 2005). Many transport protocols used to examine selective inhibition of

anandamide uptake examine time points ranging from 5 to 10 min. This length of time is considered easily sufficient for anandamide to bind to intracellular proteins or to be hydrolyzed intracellularly and both these mechanisms would facilitate uptake. An added complication is that putative inhibitors of anandamide transport such as AM 404 and ADM11 have been found to inhibit FAAH and BSA often incorporated into the assay system may influence the effect of inhibitors on FAAH (Vandevoorde and Fowler, 2005). In other words, current experimental approaches do not discriminate unambiguously between the effects that a carrier in the membrane, intracellular binding proteins or FAAH may have on anandamide uptake (Glaser et al., 2005).

Both saturable and non-saturable uptake of anandamide has been reported under different conditions in several cell types. For example it has been found that anandamide accumulation by N18TG2 neuroblastoma and C6 glioma cell lines is saturable with steady-state concentrations reached after 5 min. although saturability was not observed with increasing concentrations of anandamide in shorter (< 1 min) incubation times (Glaser et al., 2003). Other uptake investigations using anandamide in RBL-2H3 cells have revealed low concentration saturable, and high concentration non-saturable accumulation (Ligresti et al., 2004). The absolute levels of anandamide (bound + free) in some cells, for example cerebellar granule neurons, is many fold greater than its concentration in the extracellular medium (Hillard and Jarrahian, 2000). A variety of cellular constituents may contribute anandamide accumulation and transport within cells. Firstly, evidence that anandamide associates with membranous compartments comes from the work of McFarland and colleagues who found tritium-enriched subcellular lipid fractions after incubating RBL-2H3 cells with

[³H]anandamide (McFarland et al., 2004). Another possibility is that anandamide is sequestered by intracellular proteins which bind fatty acids, cholesterol and other hydrophobic molecules (Hillard et al., 2003; Stremmel et al., 2001). Clearly therefore, at least some aspects of anandamide uptake and accumulation could be due to anandamide binding to intracellular proteins and not a transmembrane carrier. The involvement of FAAH-mediated anandamide hydrolysis in the regulation of anandamide uptake remains unresolved. For example, saturable anandamide transport can still occur in FAAH knock out cells (Moore et al., 2005; Ligresti et al., 2004). Cells with low FAAH activity, for example cerebellar granule neurons, also demonstrate a robust and saturable accumulation of anandamide (Hillard et al., 1997). But other research suggests that FAAH can significantly increase anandamide uptake. For example, expression of FAAH in HeLa cells increases maximal anandamide transport 2-fold compared to wild-type (no FAAH HeLa cells) (Day et al., 2001). As FAAH can hydrolyze many fatty acid primary amides, other fatty acid amides (for example the putative sleep-inducer oleamide) may reduce uptake of endocannabinoids and therefore augment their physiological effects (Giang et al., 1997). In conclusion it seems reasonable at this time to accept that a variety mechanisms controlling endocannabinoid uptake may occur.

1.4 Anandamide release

How does the anandamide produced within the postsynaptic neuron get released? Is its release facilitated by carriers or does it undergo efflux by simply diffusion? Ronesi et al. (2004) found that in striatal neurons intracellular postsynaptic application of an inhibitor of endocannabinoid uptake (e.g. AM 404 and VDM11) reduces

endocannabinoid-induced long-term depression (LTD). This does not occur if the uptake inhibitor is applied extracellularly. It was concluded therefore that the uptake blocker competes more effectively for the internalized endocannabinoid carrier (Ronesi et al., 2004). Precisely how this transporter-dependent efflux is initiated and maintained, as well as the presynaptic mechanisms of LTD induction, remains to be determined. It has also been reported that CB1 receptor-mediated hippocampal DSI is not inhibited by intracellular blockade of the anandamide membrane transporter (AMT), indicating that differences of postsynaptic endocannabinoid release may exist in different types of neuron (Ronesi et al., 2004; Chevaleyre and Castillo, 2003). Anandamide is a neutral lipophilic molecule with a very limited solubility in water. One would predict that it should be easy for anandamide to associate with specific proteins in the extracellular fluid. In fact, two reports show that serum albumin enhances anandamide efflux from rat and human neurons (Bojesen and Hansen 2003; Giuffrida et al., 2000). BSA has one high-affinity binding site for anandamide. The equilibrium dissociation constant (K_d) of anandamide binding to BSA increases significantly with temperature from 6.87 ± 0.53 nM at 0°C to 54.92 ± 1.91 nM at 37°C (Bojesen and Hansen, 2003). Bojesen and Hansen (2006) found that anandamide efflux from the outer membrane leaflet of red cell ghosts to BSA in the medium depends significantly on the BSA concentration and proposed a model to account for anandamide release (Bojesen and Hansen, 2006). Synaptoneuroosomes consist of a pinched-off nerve ending attached via structural elements of the synaptic cleft region to a pinched-off portion of the postsynaptic neuronal cell body. Since both the pre- and postsynaptic moieties enclose their original content of cytoplasm, synaptoneuroosomes

should represent an ideal synaptic model for studying endocannabinoid function. In Chapter 4 of this thesis I discuss the experiments I carried out to investigate the potential release of anandamide from synaptoneurosomes under resting and depolarized conditions.

1.5 CB1 cannabinoid receptor-mediated signal transduction

CB1 receptors mediate inhibition of excitatory or inhibitory transmission and this occurs widely in the brain. Cannabinoid-induced stimulation of CB1 receptors activate signal transduction pathways via the pertussis toxin (PTX)-sensitive G_i/G_0 (Di Marzo et al., 1998). The mechanisms of downstream intracellular signal transduction however are quite diverse and depend to a large extent on the cell in question. In transfected cells overexpressing CB1 receptors, PTX-sensitive G-proteins allow anandamide to negatively modulate adenylate cyclase and voltage-sensitive Ca^{2+} channels and also to activate inwardly rectifying K^+ channels (Di Marzo et al., 1998). In cerebellar parallel fibre-Purkinje cell synapses two mechanisms for anandamide-dependent inhibition of presynaptic glutamatergic transmission have been reported. The first is mediated by the PTX-sensitive G proteins activating inwardly rectifying K^+ channels which are blocked by Ba^{2+} and tertiapin-Q (Daniel et al., 2004). The second requires similar initial cascades but involves 4-aminopyridine (4AP) and voltage-sensitive K^+ channels as the terminal effector (Daniel and Crepel, 2001). In cerebellar granule neurons, PTX has been found to abolish cannabinoid agonist-mediated inhibition (Huang et al., 2001). The inhibitory effect of the cannabinoid agonist WIN 55212-2 on Ca^{2+} influx was additive with inhibition produced by the P/Q-type calcium channel inhibitor, ω -agatoxin IVA and nifedipine (an L-type Ca^{2+} channel antagonist) but not with the N-

type calcium channel inhibitor, ω -conotoxin GVIA, indicating that N-type voltage sensitive calcium channels (VSCCs) are the primary effector (Nogueron et al., 2001). Using cultured rat hippocampal neurons as a model, Twitchell et al. (1997) found potent inhibition of N- and P/Q-type calcium currents by cannabinoids (Twitchell et al., 1997). The mechanism likely involves decreasing the basal level of cAMP, which activates tyrosine residue phosphorylation and focal adhesion kinase (FAK), which finally lead to inhibitory effects on neurotransmitter release (Di Marzo et al., 1998; Derkinderen et al., 1996).

Although endocannabinoids inhibit both excitatory and inhibitory neurotransmitter release, the sensitivity of different neurotransmitter systems varies considerably. Ohno-Shosaku et al. (2002) examined the effects of cannabinoid agonists on excitatory and inhibitory synapses in rat hippocampal slices and cultures. They found that both DSE and DSI can be induced, but DSI was much more pronounced than DSE. Specifically GABA release was 30-fold more sensitive to suppression. Since neither the induction of DSI nor DSE can be elicited in CB1 receptor knockout mice, it has been concluded CB1 receptors are necessary for DSI and DSE (Ohno-Shosaku et al., 2002). The reasons for the difference in sensitivity to cannabinoids between excitatory and inhibitory transmission requires more study. In rat striatum, anandamide and other cannabinoids significantly reduce forskolin-stimulated cyclic AMP accumulation, resulting in a decrease in electrically-induced dopamine release but not the release of acetylcholine, emphasizing the selective nature of CB1 receptor-coupled effects (Cadogan et al., 1997). In Purkinje cells, the release of endogenous cannabinoids

results in blockade of afferent excitatory inputs as a result of effects on presynaptic calcium entry (Kreitzer et al., 2001).

In the brain, the first characterized cannabinoid receptor signal transduction response was the inhibition of adenylyl cyclase in response to classical or non-classical cannabinoid agonists, such as CP55940 and WIN 55,212-2. Such inhibitory effects can be relieved by CB1 receptor antagonists, such as SR141716A. There are at least nine closely related isoforms of adenylyl cyclase (ACs), each of which is regulated by a different regulatory mechanism (Hanoune and Defer, 2001; Sunahara et al., 1996). The influence of the adenylyl cyclase isoform type on the outcome of the response to cannabinoid agonists has been comprehensively investigated by Rhee et al. (1998). In that study, calmodulin-regulated adenylyl cyclase isoforms 1, 3, and 8, and hormone-stimulated isoforms 5 and 6 were inhibited in response to cannabinoid agonists. In contrast, adenylyl cyclase isoforms 2, 4, and 7 were stimulated. Intracellular Ca^{2+} transients have been shown to be stimulated by the endocannabinoids anandamide and 2-AG, as well as by aminoalkylindole and cannabinoid agonists in undifferentiated N18TG2, neuroblastoma and NG108-15 hybrid cells (Sugiura et al., 1997 and 1999). The C6 astrocytoma did not respond to cannabinoid agonists (Sugiura et al., 1997) suggesting that the CB1 receptor is not coupled to the same signal transduction mechanism that the neuronal cell uses to mediate this response, or alternatively the response is related to other potential binding sites of endocannabinoids which have different structural requirements. With agonists, such as anandamide, WIN55212-2 and CP55940, stimulation of the CB1 receptor produced inhibition of the voltage-gated N-type Ca^{2+} current (I_{Ca}), leading to a decrease in Ca^{2+} influx in 'differentiated' N18 and

NG108-15 cells, which resulted in a decrease in neurotransmitter release (Mukhopadhyay et al., 2002). Stimulation of the CB1 receptor can also activate p42/p44 mitogen-activated protein kinase (MAPK). This has been found to occur through activation of a PTX-sensitive G protein-coupled CB1 receptor in cultured U373MG human astrocytoma (Bouaboula et al., 1995). The specific mechanisms underlying CB1 receptor-mediated MAPK activation have not been fully elucidated as yet. Stimulation of CB1 receptors can also activate MAPK/ERK kinase (MEK) which then increase the activity of the extracellular signal-regulated kinase (ERK) in hippocampal slices (Derkinderen et al., 2003). The strong potential of cannabinoids for inducing long-term alterations in hippocampal neurons through the activation of the ERK pathway may be important for the physiological control of synaptic plasticity and for the general effects of THC in the context of drug abuse (Derkinderen et al., 2003). On balance the available data on CB1 receptor-mediated signal transduction suggests it is highly complex and requires much clarification. An excellent review of the role of endocannabinoids in synaptic signaling has been published by Freund et al. (2003)

Cannabinoid receptors are widely distributed in vertebrate animals (Onaivi et al., 2002) and also occur in some invertebrates, such as sea urchins, hydra, mollusks, and the leech (Salzet and Stefano 2002), but are not apparently found in insects (McPartland et al., 2001). In the rat, the expression of endocannabinoid receptors depend on the developmental stage. For example, mRNA for CB2 receptor in liver was found during the embryonic stages (Day 13–21), after which the levels declined, with no mRNA being detectable in the adult liver (Buckley et al., 1998). CB1 receptors are present at a high density in somatic regions of rat hippocampal neurons at day 5 of

culture. In more mature cultures of these neurons (3-4 wk), the CB1 receptors were greatly enriched along axons and dendrites as well as on presynaptic terminals (Twitchell et al., 1997). Recently, Matyas et al. (2006) investigated the subcellular location of CB1 receptor in the nucleus accumbens, striatum, globus pallidus, substantia nigra, and internal capsule. They observed CB1 receptors on the membrane of the terminal and preterminal regions of axons. The majority of CB1 receptors were located in the axonal plasma membrane (Matyas et al., 2006).

CB2 receptors can modulate adenylyl cyclase and MAPK activity in a similar way to CB1 receptors, through their ability to couple to G_i/G_o proteins. Thus in CHO cells transfected with the CB2 receptor, cannabinoids were shown to inhibit adenylyl cyclase activity in a concentration-dependent manner. This effect was PTX-sensitive suggesting signalling through G_i/G_o proteins (Felder et al., 1995). Cannabinoids also activate p42/p44 MAPK in CB2 receptor expressing cells and these effects are sensitive to inhibition with PTX and the CB2 antagonist SR 144528 (Kobayashi et al., 2001).

1.6 Evidence for other targets for endocannabinoids

Evidence for non-CB1 and non-CB2-mediated actions of anandamide have been clearly demonstrated using CB1 and CB2 knockout mice. Di Marzo and colleagues (2000) tested the ability of anandamide and THC to induce analgesia and catalepsy and decrease spontaneous activity in the CB1 knockout homozygotes (CB1^{-/-}). They found that even though anandamide levels in the hippocampus and the striatum were lower in the CB1 knockout homozygotes (CB1^{-/-}) comparing to the wild-type (CB1^{+/+}), these effects of anandamide were not decreased in CB1^{-/-} mice. Zimmer et

al. (1999) also compared the ability of cannabinoids to induce responses in wild type mice and CB1 knockout mice. They reported that for certain physiological effects induced by cannabinoids (e.g. in the hotplate analgesia test and hypothermia test) CB1^{-/-} are not as sensitive as CB1^{+/+} mice but CB1^{-/-} mice show a similar dose response to THC in the tail-flick test at doses up to 50 mg/kg (Zimmer et al., 1999). This results suggest that part of the cannabinoid effects come from binding of cannabinoids to non-CB1 receptors. The fact that distribution of the endocannabinoids does not match exactly that of the cannabinoid receptor binding sites also suggests that endocannabinoids could bind to other sites (Bisogno et al., 1999). Presence of non-CB1 or non-CB2 receptors for cannabinoids in the central nervous system has been confirmed in several laboratories. WIN 55,212-2 inhibits monosynaptically-evoked EPSCs in CA1 pyramidal cells not only in wild-type but also in CB1 receptor knockout mice (Hajos et al., 2001). Breivogel et al. (2001) reported that anandamide and WIN55,212-2 activate G-proteins in brain membranes in both CB1^{+/+} and CB1^{-/-} mice. By contrast, a wide variety of other compounds that are known to activate CB1 receptors, including CP55940, HU-210, and 9-tetrahydrocannabinol, failed to stimulate G-proteins in membranes derived from CB1^{-/-} mice. Moreover, the CB1 antagonist SR141716A did not reduce G-protein activation by anandamide in CB1^{-/-} mice. These findings strongly suggest the presence of receptors other than CB1 receptors which are capable of binding cannabinoids in nerve cells, and that different cannabinoids have different capacities to bind to these non-CB1 receptors. The presence of non-CB1 or CB2 receptors for endocannabinoids has been reported not only in the central nervous system but also in peripheral tissues. J rai and colleagues demonstrated that

anandamide-induced mesenteric vasodilation persists in CB1 receptor deficient mice and also in mice unable to express CB1 and CB2 receptors (Jarai et al., 1999). Other physiological effects mediated by endocannabinoids via CB1 or CB2-receptor independent mechanisms have been reported. The ability of 2-AG to induce interferon-gamma (IFN-gamma) expression in wild-type as well as in CB1 and CB2 double knockout mice has been investigated. No differences in the magnitude of IFN-gamma suppression by 2-AG in wild-type versus CB1/CB2 null mice were observed. This data suggest that 2-AG suppresses IFN-gamma expression in murine splenocytes in a CB receptor-independent manner (Kaplan et al., 2005). It is also interesting that cannabinoid receptor agonists CP55,940 (EC_{50} , 0.84 μ M), WIN 55,212-2 (EC_{50} , 3.47 μ M), arachidonyl-2-chloroethylamide (ACEA) (EC_{50} , 17.8 μ M), and R-(+)-methanandamide (EC_{50} , 19.8 μ M) can all inhibit glutamate release from hippocampal synaptosomes of rat and CB1 null mutant mouse (Kofalvi, 2003). WIN 55,212-2 displayed the greatest efficacy. SR141716A, AM251 and the vanilloid receptor 1 (VR1) antagonist capsazepine did not antagonize this effect. These data also add weight to the idea that there exist distinct targets of cannabinoids which are independent of CB1 receptors, which when activated inhibit neurotransmitter release (Kofalvi, 2003).

1.6.1 T-type calcium channels

Instead of inhibiting N, P/Q calcium channels via CB1 receptors, Ca^{2+} entry can be controlled by a direct interaction of anandamide with the T-type of calcium channel. In the CNS, T-channels generate low threshold spikes and participate in spontaneous firing (Chemin et al., 2001; Huguenard, 1996). T-channels are also involved in cardiac pacemaker activity (Hagiwara et al., 1988), aldosterone secretion (Rossier et al., 1996)

and fertilization (Arnoult et al., 1996). Ca^{2+} influx in HEK 293 and CHO cells transfected with T-type Ca^{2+} channels was inhibited by anandamide (Chemin et al., 2001). This effect is very similar to that observed in cells with native T-type Ca^{2+} channels. These inhibitory effects were not relieved by CB1 antagonist SR141716A (Chemin et al., 2001).

1.6.2 Potassium channels

Direct cannabinoid-mediated inhibition of certain K^+ channels has also been demonstrated by Maingret and colleagues (2001). The acid-sensitive TASK-1 (TWIK-related acid-sensitive K^+ channel), which regulates background activity of K^+ currents, can be inhibited directly by anandamide as well as certain other cannabinoid agonists such as WIN 55, 212-2. In contrast to other cannabinoids agonists, for example 2-AG, Δ^9 -THC, HU210 and the antagonist SR141617A have no clearcut effect on TASK-1 channels. The inhibitory effects of anandamide and WIN 55,212-2 at TASK-1 channels are independent of CB1/CB2 receptors (Maingret et al., 2001). TASK-1 is differentially expressed in the brain, with high mRNA levels found in cerebellar granule neurons, as well as in brainstem and spinal cord motoneurons (Maingret et al., 2001; Talley et al., 2000). Neurons in these regions are involved in the control and coordination of motor behavior. It is therefore possible that direct block of TASK-1 and subsequent alteration of excitability might be responsible for some of the non-CB1 receptor effects that anandamide has on locomotion in mammals.

The voltage-gated K^+ channel controls repolarization and action potential frequency (Kim et al., 2005). Oliver et al. (2004) demonstrated that anandamide can convert non-

inactivating delayed rectifying voltage-gated K^+ channels into rapidly inactivating A-type K^+ channels. Intriguingly, arachidonic acid has a very similar effect on inducing inactivation of voltage-gated K^+ channels (Oliver et al., 2004), but more work is needed to ascertain whether the primary effect arises from anandamide itself or from a breakdown product of this compound. But clearly, the current data suggest that anandamide or a close metabolite can directly modulate the voltage-gated K^+ channels (Van der Stelt and Di Marzo, 2005).

1.6.3 Vanilloid TRPV1 receptors

Transient receptor potential vanilloid 1 receptors (TRPV1-Rs) are widely distributed in sensory neurons. The TRPV1-R is a membrane protein consisting of six transmembrane domains and a pore-loop. The TRPV1-R protein is classified as a non-specific channel with preference for Ca^{2+} . Activation of the channel induces the release of neuropeptides which can give rise to the sensation of pain, neurogenic inflammation, smooth muscle contraction and cough (Jia et al., 2005). The TRPV1-R channel is activated by vanilloids, capsaicin which is found in chilli peppers and the highly potent diterpene resiniferatoxin (RTX). TRPV1-Rs can also be activated by a number of other stimuli including heat and protons (Jia et al., 2005; Szallasi and Blumberg, 1999). The opening of the ion channel component of the TRPV1-R allows Ca^{2+} influx which increases the frequency of isolated miniature excitatory postsynaptic currents (mEPSCs) in LC neurons (Marinelli et al., 2002; Van der Stelt et al., 2005). Other work shows that anandamide acts as an agonist at TRPV1-Rs expressed in HEK 293 cells and this effect can be inhibited by TRPV1-R antagonist capsazepine (Zygmunt et al., 1999). Vasodilation in arteries can also be induced by anandamide and

is accompanied by release of the calcitonin-gene-related peptide (CGRP). The cannabinoid (CB1) receptor antagonist SR141716A is unable to block this effect (Szallasi and Blumberg, 1999), but the TRPV1-R-selective antagonist capsazepine does inhibit anandamide's vasodilatory effect as well as its effect on CGRP release. Anandamide's effect at TRPV1-Rs is not mimicked by other cannabinoids, such as 2-AG, palmitylethanolamide HU 210, WIN 55,212-2 and CP 55,940 (Zygmunt et al., 1999). Thus it appears that endocannabinoids may have dual roles (i.e. to activate both CB1 receptors and TRPV1-Rs) and there is some evidence to suggest that anandamide brings about depression of transmitter release and stimulation of TRPV1-R at different concentrations. At concentrations of 10 μM and below, anandamide inhibited SR141716A- and PTX-sensitive release of peptides (Nemeth et al., 2003). Higher concentrations of anandamide (10-100 μM) increased release of peptides in a concentration-dependent manner; this could be blocked by capsazepine. As an endogenous ligand of TRPV1-Rs, anandamide may enhance TRPV1 effects by amplifying intracellular Ca^{2+} influx via the TRPV1-R (Van der Stelt et al., 2005) although it is likely that the effects of anandamide on TRPV1-Rs may vary in different tissues and be dependent on prevailing physiological conditions (Jia et al., 2005). As the TRPV1-R can be upregulated by several endogenous regulators such as protein kinases A and C (Numazaki et al., 2002; Bhavé et al., 2002; Bhavé et al., 2003) and certain inflammatory mediators, this may explain anandamide's ability to trigger partial or full TRPV1-R activation in different cell types (Jia et al., 2005).

1.6.4 Serotonin (5-HT) receptors

The 5-HT₃ receptor (5-HT₃-R) is a ligand-gated ion channel which is involved in mood, pain, emesis, some psychiatric disorders, and behaviors associated with drug abuse (Greenshaw, 1993). Fan (1995) found that anandamide, WIN 55,212-2 and CP 55,940 inhibited 5-HT-induced currents in a concentration-dependent manner, IC₅₀ values were 190, 310, and 94 nM for anandamide, WIN 55212-2, and CP55940, respectively. These substances appear not to cause inhibition via CB1/CB2 receptor activation but instead by an agonist-like action at the 5-HT₃-R, since the inward current was blocked by the specific 5-HT₃-R antagonist MDL7222 (Fan, 1995). WIN 55,212-2-induced inhibition was not altered by SR141716A supporting the idea that CB1-Rs do not participate in this effect (Townsend et al., 2002; Barann et al., 2002). Binding studies revealed that WIN55,212-2, CP 55940, anandamide and SR141716A do not modify the binding of the 5-HT₃-R specific ligand [³H]-GR65630 to 5-HT₃-Rs in brain, whereas 5-HT caused a concentration dependent-inhibition. Taken together these results indicate that cannabinoids may act at a site that is separate from but allosterically coupled to the 5-HT₃ receptors or possibly by interfering with coupling of the receptors to ion channel activation (Barann et al., 2002). *In vivo* studies conducted by Godlewski et al. (2003) found even though injection of the 5-HT₃-R agonist phenylbiguanide or the vanilloid VR1-R agonist capsaicin produce immediate decreases in heart rate and mean arterial blood pressure, the phenylbiguanide-induced (but not capsaicin-induced) bradycardia was dose-dependently attenuated by the cannabinoid receptor agonists CP 55,940 and WIN 55,212-2. These data supports the findings of Fan (1995) and Barann (2002) that cannabinoids interact with 5-HT₃ receptors not TRPV1.

1.6.5 Nicotinic acetylcholine (nACh) receptors

Nicotinic acetylcholine receptors (nACh-Rs) containing the $\alpha 7$ subunit common to members of the ligand-gated ion channel superfamily are present in many brain areas where cannabinoids are known to act (Lindstrom et al., 1996). Recently Oz et al. (2004) found that the endocannabinoids, anandamide and 2-AG, inhibit nicotine-evoked currents in *Xenopus* oocytes expressing $\alpha 7$ nACh-Rs, IC_{50} value of 229.7 ± 20.4 nM and 168 nM respectively. Although arachidonic acid also causes inhibition, the inhibitory potencies of 2-AG and anandamide are higher than that of arachidonic acid at $\alpha 7$ -nAChRs (Oz et al., 2004). The inhibition by 2-AG and anandamide was found to be non-competitive and voltage-independent. Since SR141716A was unable to inhibit the effect of endocannabinoids, this supports the idea that inhibitory effects on currents carried by $\alpha 7$ nACh-Rs are not mediated by CB1-Rs. The response to anandamide was insensitive to the CB2 antagonist SR144528, the G-protein inhibitor PTX and 8-bromo-cAMP. The effect of anandamide was also not affected by PMSF or AM 404 which both represent pharmacological manipulations known to increase the persistence of anandamide (Oz et al., 2004).

1.6.6 Voltage-sensitive sodium channels

DSI in the CA1 region of the hippocampus does not occur in the presence of TTX (Alger et al., 2002). TTX is a highly specific voltage-sensitive sodium channel (VSSC) inhibitor. This suggests that VSSCs may be involved in the regulation of transmitter release. Turkanis et al. (1991) found that THC decreased peak inward sodium currents, an indication that THC can block sodium channels. The drug also decreased both the

activation and the inactivation rates of VSSCs. All three effects were apparent at concentrations between 100 nM and 8 μ M (Turkanis et al., 1991).

Recently in our laboratory we discovered that anandamide and cannabimimetics such as AM 404 and WIN 55,212-2 block veratridine-dependent depolarization of synaptoneuroosomes and veratridine-dependent release of the neurotransmitters glutamate and GABA from synaptosomes isolated from mouse brain (Nicholson et al., 2003). The binding of the site 2-selective radioligand [3 H]batrachotoxinin A 20- α -benzoate (BTX-B) to VSSCs in mouse brain synaptoneuroosomes was also displaced by low to mid-micromolar concentrations of anandamide, AM 404 and WIN 55,212-2. Electrophysiological experiments confirmed that anandamide inhibits VSSC-dependent sustained repetitive firing in cortical neurons *in vitro* and that inhibition is increased by FAAH inhibition (Nicholson et al., 2003). Thus there is compelling evidence to suggest that anandamide targets VSSCs directly to inhibit neurotransmitter release. Chapters 2 and 3 of this thesis describe my contribution to our laboratory's investigation of VSSCs as a target for anandamide and synthetic cannabinoids.

1.7 Inactivation of endocannabinoid effects

As soon as the release of endocannabinoids across the postsynaptic membrane into the synaptic cleft has been activated, these modulators bind to various membrane receptors where they initiate different presynaptic responses. To terminate these effects, endocannabinoids are thought to be translocated into cells where they are rapidly broken down. Although (as previously discussed) it is still the subject of debate whether the uptake of endocannabinoids is mediated by a membrane carrier, it is broadly agreed that endocannabinoid uptake is energy independent. As they

accumulate in neurons endocannabinoids are degraded via two main pathways: hydrolysis and oxidation. Several lines of evidence suggest that fatty acid amide hydrolase (FAAH) is an important enzyme for hydrolysis of endocannabinoids (Deutsch et al., 2002). Anandamide concentrations in FAAH knockout mice brains are 15-fold higher than in wild type mice and knock out mice exhibit intense CB1-R-dependent behavioral responses, including hypomotility, analgesia, catalepsy, and hypothermia (Cravatt et al., 2001). FAAH is widely distributed in the central and peripheral nervous system and this enzyme has a broad substrate specificity including the ability to hydrolyze oleamide (a sleep modulator). Competition between oleamide and endocannabinoids for FAAH may reduce the rapid intracellular degradation of endocannabinoids. 2-AG appears to be a better substrate for FAAH compared to anandamide. In fact, the hydrolysis of 2-AG takes place about 4-fold faster than that of anandamide in rat COS-7 cells overexpressing FAAH (Goparaju et al., 1998). However, a large part of the 2-AG degradative activity could be attributed to a monoacylglycerol lipase-like enzyme that did not hydrolyze anandamide (Goparaju et al., 1999). These findings suggest that at least two enzymes feature in the hydrolysis of 2-AG. The concentration of 2-AG is more than 170-fold higher than that of anandamide in cells, therefore more than one enzyme may be necessary for the efficient termination of 2-AG's effect.

Another important mechanism for endocannabinoid degradation is oxidation. Anandamide and 2-AG both contain the arachidonic acid moiety which is sensitive to attack by oxygenases, such as cyclooxygenases, lipoxygenases, and various cytochrome P₄₅₀s (Kozak and Marnett, 2002). Cyclooxygenases (COX) catalyze a

critical step in prostaglandin (PG) and thromboxane (TX) biosynthesis by bis-dioxygenating arachidonic acid. COX-2 can oxygenate anandamide with lower affinity than for arachidonic acid. Lipoxygenase-mediated oxidation of endocannabinoids has been demonstrated *in vitro* and intact tissues and much of this activity resides in the microsomal fraction (Kozak and Marnett, 2002). The relative significance of oxidation versus FAAH degradation of endocannabinoids *in vivo* remains to be resolved.

1.8 The regulatory role of ethanolamine

Hydrolysis of anandamide by FAAH produces ethanolamine and arachidonic acid. Ethanolamine is an important substrate for phospholipid synthesis required in the construction of membrane systems (Lipton et al., 1990). Ethanolamine can be directly incorporated into existing phospholipids by a Ca^{2+} -dependent base exchange reaction or transformed via CDP-ethanolamine which then combines with 1,2-diacylglycerol. A third pathway relating to phosphatidylethanolamine synthesis involves the decarboxylation of phosphatidylserine, a reaction which occurs predominantly within mitochondria (Lipton et al., 1990). Ethanolamine also plays important regulatory roles at the synapse. It increases both glutamate-dependent excitation and GABA-dependent inhibition of avian tectal neurons (Wolfensberger et al., 1982). Bostwick et al. (1989) found ethanolamine enhances choline accumulation which then stimulates acetylcholine synthesis. Compared to what is known about the regulatory role of anandamide, the cellular functions of ethanolamine are poorly understood. In my studies to ascertain whether we could demonstrate depolarization-dependent release of [^3H]anandamide from synaptic fractions preloaded with [^3H]ethanolamine, we found that depolarization of preloaded synaptosomes or synaptoneuroosomes causes release of

[³H]ethanolamine, supporting the idea that this amino alcohol may play a regulatory role in synaptic function. This work is the focus of Chapter 5 of my thesis. We also observed that although ethanolamine does not depolarize synaptosomes (Liao and Nicholson, 2005) it strongly activates the output from synaptosomes of acridine orange (AO) a fluorescent indicator of synaptic vesicle exocytosis or proton concentration within the synaptic vesicles. Chapter 6 of this thesis describes the experiments I undertook to investigate in more detail the mechanisms underlying these presynaptic phenomena.

1.9 Conclusion

Endocannabinoids are widely distributed in the central nervous system, peripheral nervous system and other tissues of the body. In the nervous system, endocannabinoids are thought to be released from postsynaptic neurons and move retrogradely to depress neurotransmitter release from the presynaptic neuron. Endocannabinoids are known to bind to a number of cellular sites or receptors including CB1 receptors, CB2 receptors, VSSCs, several types of voltage-gated calcium channels, potassium channels and vanilloid TRPV1 receptors. CB1 receptors have been shown to activate at least 6 subtypes of G_i and G_o proteins which are involved in modulating a variety of physiological responses, including nociception, appetite enhancement, locomotion, memory, analgesia and catalepsy (Breivogel et al., 2001) as well as embryo development (Mechoulam et al., 2001). As a variety of different endocannabinoid targets may be present in the same cell, it is unclear how different effector proteins interact to achieve the ultimate response. Like many neurotransmitters, the influence of endocannabinoids can be terminated by enzymic degradation. But metabolites of

endocannabinoids may still have important roles in regulating cellular function, for example my results demonstrate that ethanolamine, (which arises in part from anandamide hydrolysis) may play an important role in physiological function. Thus the endocannabinoid system is a complex and important cellular regulatory system with profound effects on physiological outcome.

CHAPTER 2

INHIBITION OF VOLTAGE-SENSITIVE SODIUM CHANNELS BY ANANDAMIDE AND CANNABIMIMETIC DRUGS

2.1 Abstract

In view of previous findings with the sleep inducing hormone *cis*-oleamide, this study was undertaken to investigate the potential interaction of anandamide (a structurally similar fatty acid amide) and related cannabimimetics with voltage-sensitive sodium channels (VSSCs) in mouse brain synaptic preparations. My experiments demonstrate that anandamide, AM404 and WIN55,212-2 inhibit veratridine- (but not KCl-) dependent release of L-glutamic acid (L-glu) and GABA from synaptosomes [IC₅₀s: 5.1 μM (L-glu) and 16.5 μM (GABA) for anandamide; 1.6 μM (L-glu) and 3.3 μM (GABA) for AM 404; and 12.2 μM (L-glu) and 14.4 μM (GABA) for WIN 55,212-2]. The binding of [³H]batrachotoxinin A 20-α -benzoate to VSSCs was also inhibited by low to mid micromolar concentrations of anandamide, AM 404 and WIN55,212-2. Significantly, none of the inhibitory effects demonstrated on VSSCs were attenuated by the cannabinoid (CB1) receptor antagonist AM251 (2 μM). Inhibition of fatty acid aminohydrolase by PMSF enhanced anandamide's ability to displace [³H]BTX-B. Scatchard analyses and kinetic experiments found that anandamide, WIN 55,212-2 and AM404 bind to a site that is distinct from, but allosterically coupled to site 2 on VSSCs. Experiments conducted by other researchers involved in this study demonstrated that anandamide and cannabimimetics blocked

TTX-sensitive sustained repetitive firing in cortical neurons and veratridine-induced depolarization of the neuronal membrane. These results suggest that VSSCs participate in a novel signalling pathway involving endocannabinoids. Such a mechanism has potential to depress synaptic transmission in brain by reducing the capacity of neurons to support action potentials and release excitatory and inhibitory neurotransmitters.

2.2 Introduction

A variety of low molecular weight neurotoxins, CNS drugs and neuroactive pesticides interact with discrete binding sites on VSSCs to produce wide ranging and profound effects on channel operation (Willow and Catterall, 1982; Postma and Catterall, 1984; Strichartz et al., 1987; Hill et al., 1989; Deecher et al., 1989; Sheldon et al., 1994; MacKinnon et al., 1994; Ratnakumari and Hemmings, 1996). Changes in phosphorylation status of VSSCs by protein kinases such as protein kinase C (Numann et al., 1991), cAMP-dependent protein kinases (Li et al., 1982), tyrosine kinase (Hilborn et al., 1998) and phosphatases (Chen et al., 1995; Ratcliffe et al., 2000) are important physiological regulators of VSSC function. However, the seemingly huge innate capacity for remote modulation of sodium channels by direct acting, low molecular weight, neurohumoral factors has led to the identification of very few potential candidates. Nicholson and his colleagues have demonstrated that the sleep-inducing lipid *cis*-9,10-octadecenoamide (cOA) exerts a depressant effect on the nervous system by inhibiting sodium channels (Nicholson et al., 2001). Since cOA bears a structural similarity to endocannabinoids and also shows cannabinoid agonist activity in rat behavioral models (Mendelson and Basile, 1999), the possibility arose

that more potent cannabimimetic substances, such as the endocannabinoid anandamide, may have VSSC-inhibitory properties.

In this part of the thesis I present my experimental evidence that sodium channels are inhibited by anandamide, AM 404 and WIN 55,212-2. From a mechanistic standpoint, the inhibitory effects of the study compounds occur in ways that resemble those reported for sodium channel-directed antiarrhythmics, anticonvulsants, local and general anesthetics and neuroprotectants. Our results suggest the presence of a cannabinoid binding region on sodium channels through which depressant effects of anandamide in neuronal networks may be mediated.

2.3 Methods

2.3.1 Animals

All experiments were conducted with male CD1 mice (20–25 g) purchased from Charles River Laboratories (Quebec). Animals were maintained in group cages at the Simon Fraser University Animal Care Facility under standardized environmental conditions (21 °C; 55% relative humidity; 12 h light/dark cycle) and were permitted unlimited access to food and water. Mice were sacrificed for experiments by cervical dislocation and decapitation. All procedures associated with animal care and experimentation were carried out according to Canadian Council on Animal Care guidelines and with formal approval of the Simon Fraser Animal Care Committee.

2.3.2 Isolation of synaptosomes and determination of endogenous L-glutamic acid and GABA release

Synaptosomes (pinched-off nerve endings) were generated from the whole brains of male CD1 mice (20–25 g). Synaptosomes were purified on a Percoll step gradient according to Dunkley et al. (1986). Synaptosomes retain many of the functional properties of the nerve ending *in situ* including the maintenance of normal resting potentials, uptake of neurotransmitters and release of neurotransmitters in response to depolarizing or other stimuli. They are therefore ideal for the proposed study on the pre-synaptic effects of endocannabinoids. Investigations using synaptosomes do suffer from the disadvantages that the preparation represents a mixture of many transmitter-specific nerve endings and does not give much insight into how nerve cells integrate signaling and communicate at the local and higher levels. Highly purified synaptosomes were used to eliminate possible indirect effects of anandamide and cannabimimetics on release via postsynaptic entities or extrasynaptosomal membranes. Synaptosomes (fractions 3 and 4) were pooled, pelleted, then suspended in saline (in mM) NaCl 128, KCl 5, MgCl₂·7H₂O 1.2, NaHCO₃ 5, CaCl₂·2H₂O 0.8, glucose 14, HEPES 20 (buffered to pH 7.4 with Tris) to a concentration of 7 mg protein/ml and held on ice prior to assay.

Cannabinoids were formulated immediately prior to assay by vigorously injecting them as DMSO solutions (2 µl) into 1 ml saline, followed by sonication and vortexing. TTX was added to saline in buffer (1 µl) and formulated without sonication. Synaptosomal suspensions (100 µl) were added to saline (250 µl; to which either vehicle control, cannabinoids or TTX, as necessary had been added), then vortexed gently and incubated for 15 min at 30 °C. Activators, veratridine (final concentration 2 µM; added

in 5 μ l saline) or KCl (final concentration 29 mM), were then introduced, followed by rapid mixing. After a further 5-min incubation, samples were centrifuged (Beckman Microfuge E; 40 s), and 284 μ l of each supernatant removed and acidified (perchloric acid; 6 M; 6 μ l). Just prior to analysis by high performance liquid chromatography (HPLC), samples were centrifuged and aliquots (80 μ l) of each supernatant added to borate buffer (246 μ l; 0.1 M). Next, *o*-phthaldialdehyde reagent (OPA; 60 μ l; Kilpatrick (1991) was added and after 1 min at room temperature, 40 μ l was injected onto the column. HPLC was carried out using a Hewlett Packard 1050 Series Chromatograph and a C18 column (15 cm \times 4.6 mm i.d.; particle size 5 μ m). The mobile phase consisted of buffer A (80% 0.05 M sodium phosphate buffer + 20% methanol; pH 5.7) and buffer B (20% 0.05 M sodium phosphate buffer + 80% methanol; pH 5.7). Chromatography was started with buffer A (85%) + buffer B (15%). The buffer A component was reduced to 15% over 16 min and after this, column clean-up and reequilibration was achieved by a 4 min elution with buffer A (85%) + buffer B (15%). Effluent from the column was routed to an HP 1046A programmable fluorescence detector [330 nm (excitation) and 450 nm (emission)] and OPA-amino acid peak areas were quantified and traces recorded with an HP 3396 Series II integrator. OPA-derivatives of L-glutamic acid and GABA in synaptosomal extracts were identified by comparison with authentic OPA standards and gave complete separation from other OPA-amino acids that were present. Linear fluorescence responses to L-glutamic acid and GABA standards were observed over a wide concentration range.

2.3.3 Isolation of synaptoneuroosomes and [³H]batrachotoxinin A 20- α -benzoate binding assays

Synaptoneuroosomes was prepared from CD1 male mice based on the procedure of Harris and Allen (1985). One mouse brain was homogenized in a teflon/glass homogenizer (five excursions) with Na⁺-free saline (in mM): choline chloride 130, KCl 5.4, MgSO₄.7H₂O 0.8, glucose 5.5 and HEPES 50, adjusted to pH 7.4 with Tris base. The homogenate was diluted with 15 ml of the same saline and filtrated through two layers of nylon mesh (100 μ m). The filtrate were subjected to an additional filtration through a polypropylene filter (10 μ m; Gelman Sciences) and centrifuged at 1000 x g for 10 min. The pellet was washed again by centrifugation and the synaptoneuroosomes were then resuspended in 900 μ l saline. Binding assays were conducted at 35 °C in Na⁺-free saline containing 1 mg/ml BSA (Catterall et al., 1981) with modifications (Deecher et al., 1991). The binding reaction was started by adding synaptoneuroosomes (20 μ l; 250 μ g protein) to Na⁺-free saline (140 μ l) containing [³H]BTX-B (final assay concentration 1–160 nM; 46 Ci/mmol; Perkin-Elmer Life Sciences Inc., Boston, MA, USA), scorpion (*Leiurus quinquestriatus*) venom (ScV; 30 μ g; Sigma, St Louis, MO, USA) and inhibitors or solvent control, as appropriate. Prior to addition of radiolabel and ScV, the inhibitors (in 2 μ l DMSO) were incorporated into 1 ml Na⁺-free saline as described in the previous sections to achieve the required concentration of study compound and a final DMSO concentration of 0.08%. After an incubation of 50 min, 3 ml of ice-cold wash saline (in mM): choline chloride 163, MgSO₄.7H₂O 0.8, CaCl₂.2H₂O 1.8 and HEPES 5 (adjusted to pH 7.4 with Tris base) was added to the membranes and the suspension then filtered under vacuum (Whatman GF/C filters; Hoefer FH 225V filtration manifold). Filters were immediately washed

with three 3-ml rinses of ice-cold saline and radioactivity associated with filters was measured using liquid scintillation counting. Non-specific binding, determined in the presence of veratridine (0.3 mM) was $8.97 \pm 0.29\%$ of total binding with [^3H]BTX-B at 6 nM. To investigate possible effects of anandamide on the association of [^3H]BTX-B with site 2 on VSSCs, synaptoneurosomes were preincubated with this substance for 10 min. [^3H]BTX-B (6 nM) was then introduced and the increase in specific binding measured up to 40 min. For dissociation experiments, synaptoneurosomes were equilibrated with [^3H]BTX-B (6 nM), then either a saturating concentration of veratridine (plus solvent control) or veratridine plus the study compound was added and specific binding monitored over a 20-min time course. Binding experiments were conducted in the presence of phenylmethsulphonyl fluoride (PMSF) where indicated.

2.3.4 Data analysis

In these investigations, curve fitting, determination of binding parameters and IC_{50} values were carried out using Prism software (GraphPad, San Diego, CA, USA). Statistical analyses used Student's t-test (unpaired; two tailed).

2.4 Results

2.4.1 Anandamide and cannabimimetics selectively block veratridine-induced release of L-glutamic acid and GABA from synaptosomes

I examined the ability of the endocannabinoid anandamide and the cannabimimetics AM404 and WIN 55,212-2, to inhibit veratridine-induced release of endogenous transmitters from synaptosomes. My results show that veratridine-evoked release of L-glutamic acid and GABA is inhibited by anandamide (Fig. 1). These effects occurred over concentration ranges similar to those needed to inhibit

veratridine-induced changes to the membrane potential of synaptoneurosomes (experiments conducted by Laurence S. David in our lab; Nicholson et al., 2003). The inhibition of veratridine-evoked release of L-glutamic acid by anandamide is approximately 3 times greater than that of GABA release, as indicated by IC_{50} values of 5.1 and 16.5 μM , respectively. TTX (14 μM) fully suppressed veratridine-evoked release of both L-glutamic acid and GABA (data not shown). Veratridine-stimulated release of L-glutamic acid and GABA are also inhibited by AM404 and WIN 55,212-2 at low micromolar concentrations; however, the differences between release of excitatory and inhibitory transmitters were not significant [IC_{50} values: AM404 = 1.6 μM (L-glutamic acid), 3.3 μM (GABA); WIN 55,212-2 = 12.2 μM (L-glutamic acid), 14.4 μM (GABA)], (Figs. 2 and 3). K^+ -induced release of each transmitter was only weakly inhibited by TTX (14 μM) and inhibited by anandamide (25 μM) and AM404 (10 μM) to much lesser extents (Fig. 4). WIN 55,212-2 (25 μM) inhibited K^+ -induced release of L-glutamic acid and GABA with a similar efficacy to TTX (Fig. 4). Cannabinoid antagonist AM 251 failed to relieve the inhibitory effects of anandamide and cannabimimetics on veratridine-induced release (Fig. 5).

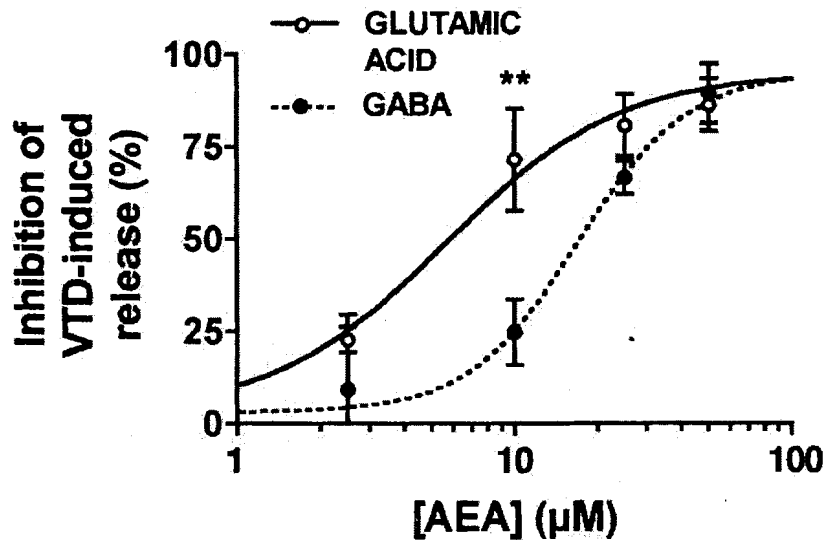


Fig 1. Inhibition of veratridine (VTD)-evoked release of L -glutamic acid and GABA from mouse brain synaptosomes by AEA. IC_{50} s: 5.1 μ M (L -glutamate), 16.5 μ M (GABA). Data points represent mean \pm S.E.M. of at least three independent determinations; **Significant difference in % inhibition of L -glutamate and GABA release at 10 μ M ($P < 0.01$). TTX (14 μ M) completely suppressed VTD-dependent release of L-glutamic acid and GABA and AEA alone (25 μ M) had no discernible effect on basal release of these transmitters. VTD (2 μ M) increased the release of GABA and L -glutamic acid from synaptosomes by 450 ± 56 and 740 ± 98 pmol/mg protein.

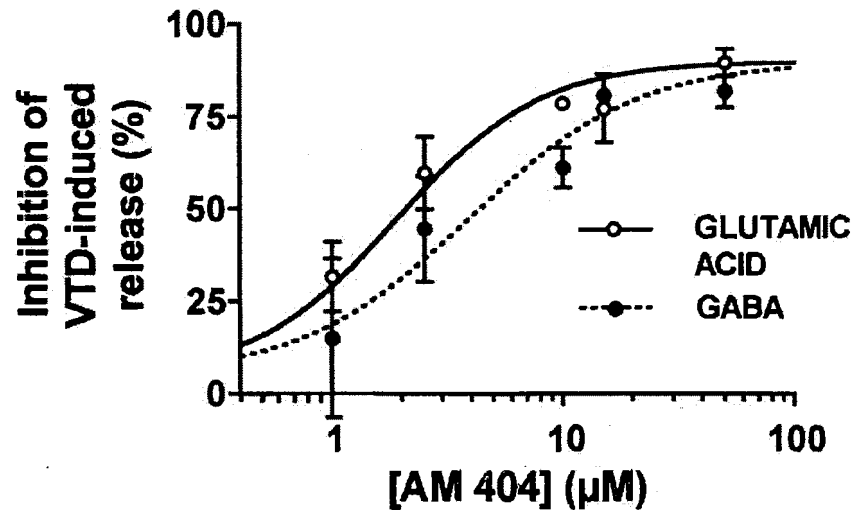


Fig 2. Inhibition of veratridine (VTD)-evoked release of L -glutamic acid and GABA from mouse brain synaptosomes by AM 404. IC_{50} s: 1.6 μ M (L-glutamate), 3.3 μ M (GABA). Data points represent mean \pm S.E.M. of 3–4 independent determinations. Basal release of L-glutamic acid and GABA was unaffected by 10 μ M AM 404 .

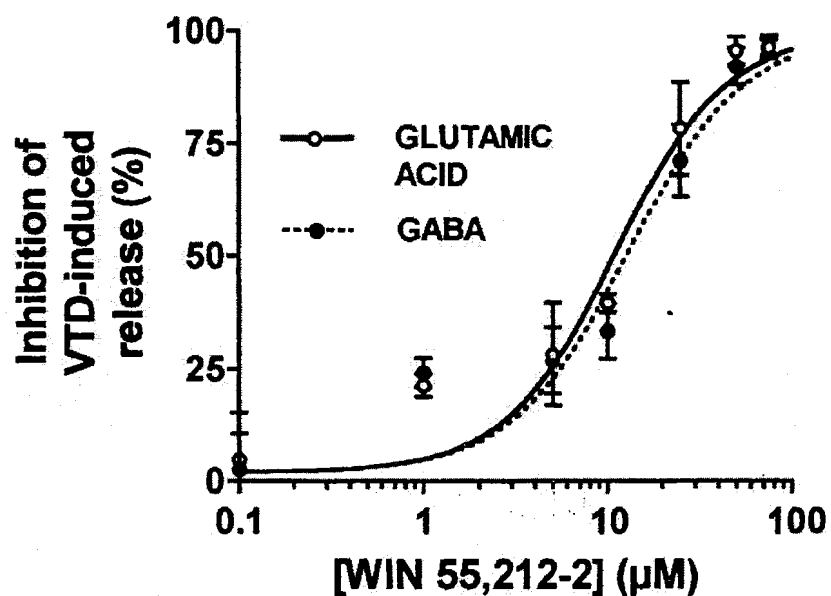


Fig 3. Inhibition of veratridine (VTD)-evoked release of L-glutamic acid and GABA by WIN 55,212-2.

IC₅₀s: 12.2 µM (L-glutamate), 14.4 µM (GABA). Data points represent mean ± S.E.M. of 3–6 independent determinations. WIN 55,212-2 alone (25 µM) had no effect on basal release of L -glutamic acid or GABA.

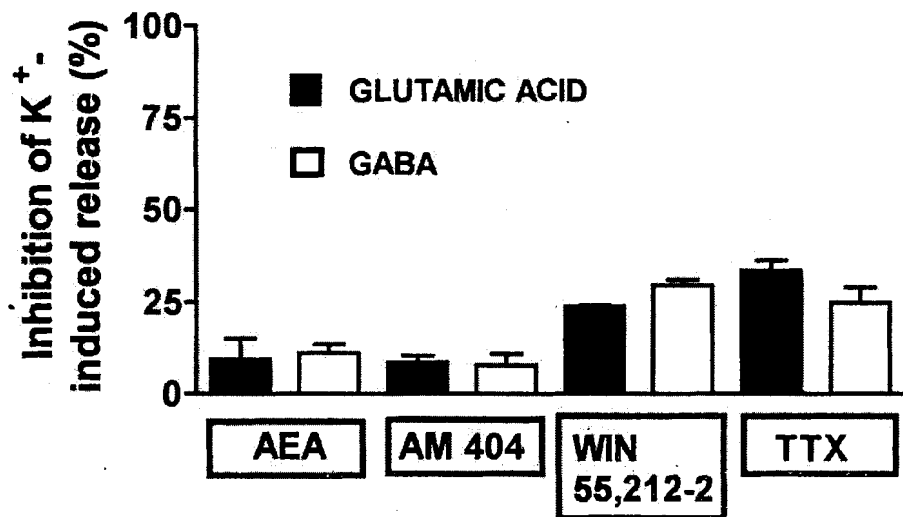


Fig 4. Inhibitory effects of AEA (25 μ M), AM 404 (10 μ M), WIN 55,212-2 (25 μ M) and TTX (14 μ M) on KCl-induced release of L-glutamic acid and GABA from synaptosomes.

Bars represent means and error bars S.E.M. of three independent experiments. KCl (29 mM) increased the release of GABA and L-glutamic acid from synaptosomes by 770 ± 112 and 1470 ± 291 pmol/mg protein, respectively.

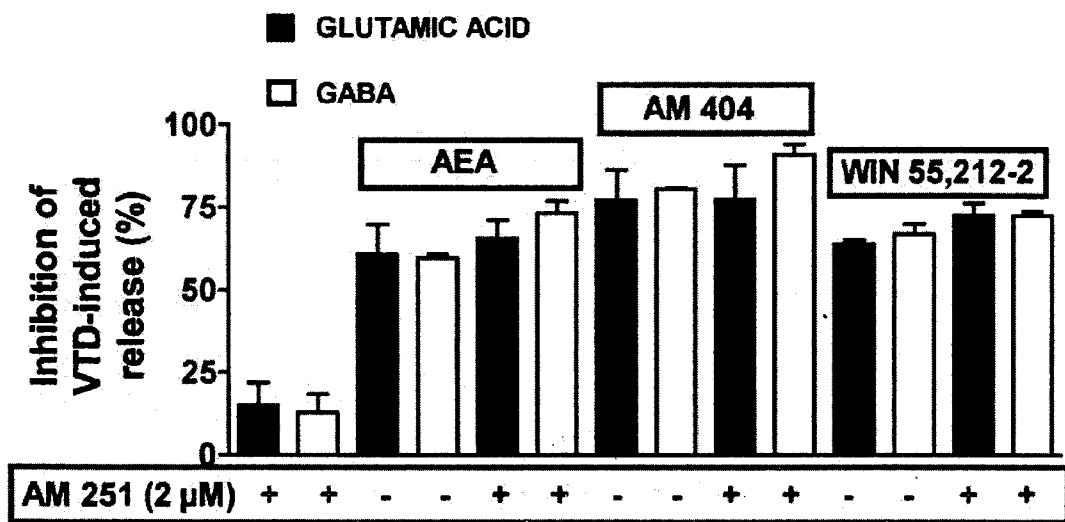


Fig 5. AM 251 (2 μM) does not attenuate the inhibitory effects of AEA (25 μM), AM 404 (15 μM) and WIN 55,212-2 (25 μM) on sodium channel-dependent release of L -glutamic acid and GABA. AM 251 had no effect on basal release at 25 μM. Bars represent means and error bars S.E.M. of three independent experiments respectively.

2.4.2 Anandamide and cannabimimetics inhibit the binding of [³H]batrachotoxinin A 20- α -benzoate to VSSCs

To investigate the possibility that a binding regions for anandamide and other cannabimimetics may be present on VSSCs, I examined their ability to interfere with the binding of [³H]batrachotoxinin A 20- α -benzoate ([³H]BTX-B). BTX-B, in common with veratridine, binds selectively to site 2 on VSSCs (Catterall et al., 1981; Strichartz et al., 1987). I found that the binding of [³H]BTX-B to VSSCs was inhibited by anandamide, AM 404 and WIN 55,212-2 (Fig. 6). Since FAAH is capable of hydrolyzing anandamide and AM404 (Fegley et al., 2004), I examined the effect of the FAAH inhibitor phenylmethylsulfonyl fluoride (PMSF) on the abilities of anandamide and AM 404 to inhibit the binding of BTX-B to VSSCs. PMSF substantially increases the inhibitory potency of both anandamide and AM404, indicating that PMSF prevents their degradation during the protracted incubation of the [³H]BTX-B binding assay. PMSF did not affect total [³H]BTX-B binding or the ability of veratridine to displace [³H]BTX-B in control incubations. The synthetic CB1 receptor agonist WIN 55,212-2 (IC₅₀ = 19.1 μ M) did not show an increase in inhibitory effect with PMSF. Scatchard analysis of equilibrium saturation data demonstrates that anandamide reduces the number of binding site on VSSCs available to [³H]BTX-B without affecting the affinity of radioligand for the remaining sites (Fig. 7). In contrast, AM404 and WIN 55,212-2 reduce the affinity of VSSCs for radioligand and binding capacity remains unchanged (Figs. 7 and 8). Kinetic experiments showed anandamide to cause a concentration-dependent increase in the rate of dissociation of [³H]BTX-B over and above that of a saturating concentration of veratridine (Fig. 9). In accordance with

observations in the membrane potential (Nicholson et al., 2003) and transmitter release experiments, the CB1 antagonist AM 251 (2 μ M) did not prevent anandamide, AM 404 or WIN 55,212-2 from displacing radioligand (Fig. 10).

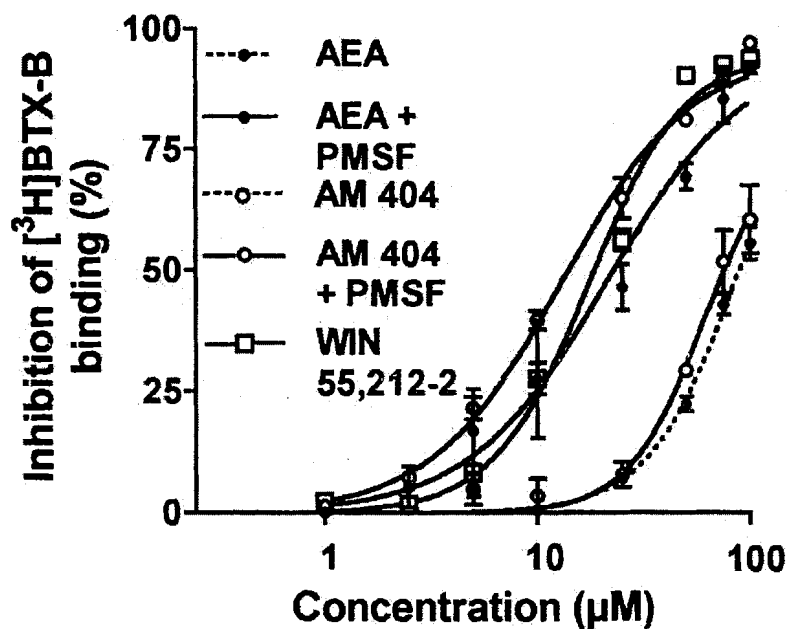


Fig 6. Anandamide (AEA), AM 404 and WIN 55,212-2 inhibit the binding of [3 H]BTX-B to voltage sensitive sodium channels in mouse brain synaptoneurosomes. PMSF (50 μ M) produces a left shift in the displacement curves for AEA (IC₅₀s = 88.7 μ M no PMSF vs. 23.4 μ M plus PMSF) and AM 404 (IC₅₀s = 77.0 μ M no PMSF vs. 14.6 μ M plus PMSF). WIN 55,212-2 displaces [3 H]BTX-B binding (IC₅₀s = 19.5 μ M). Within each experiment, binding determinations were conducted in duplicate. Data represent means and error bars the SEMs of 3-4 independent experiments.

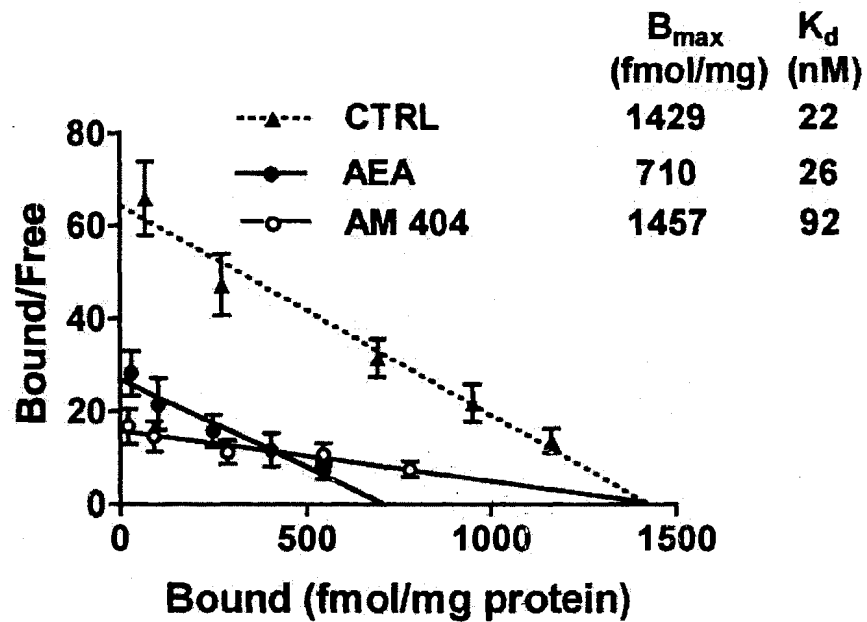


Fig 7. Scatchard analyses of the specific binding of [3 H]BTX-B to mouse brain voltage-sensitive sodium channels in the absence and presence of AEA (15 μ M) and AM 404 (10 μ M). PMSF (50 μ M) was included in these experiments.

Within each experiment, binding determinations were conducted in duplicate. Data points represent means and error bars the SEMs of three separate experiments.

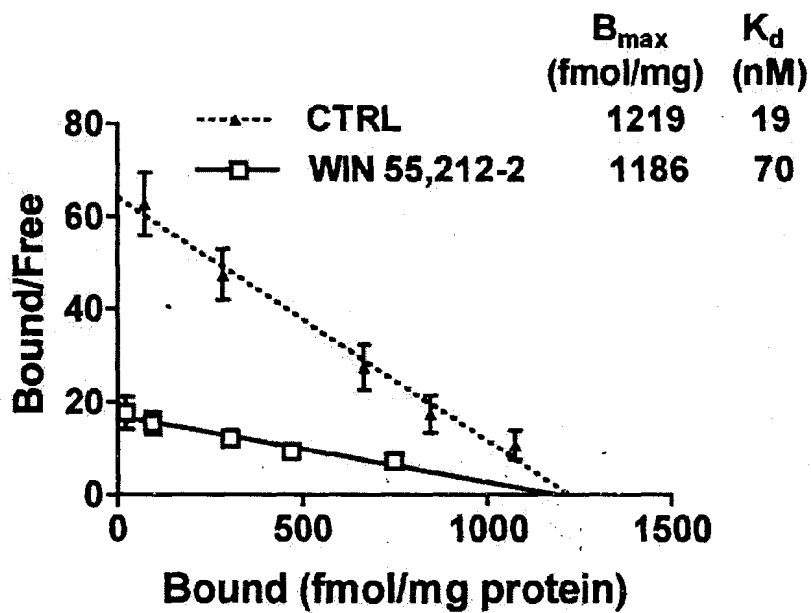


Fig 8. Scatchard analysis of [³H]BTX-B binding to mouse brain voltage-sensitive sodium channels in the absence and presence of WIN 55,212-2 (15 μM). Within each experiment, binding determinations were conducted in duplicate. Data points show the means and error bars the SEMs of three independent experiments.

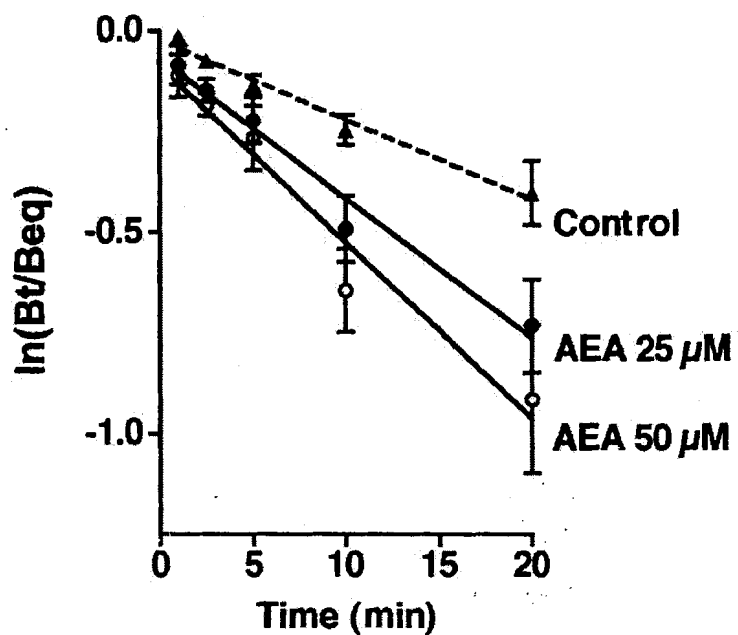


Fig 9. AEA increases the rate of dissociation of the [^3H]BTX-B/sodium channel complex.

The control represents veratridine alone (300 μM). Within each experiment, binding determinations were conducted in duplicate. Data points represent means and error bars the SEMs of three independent experiments.

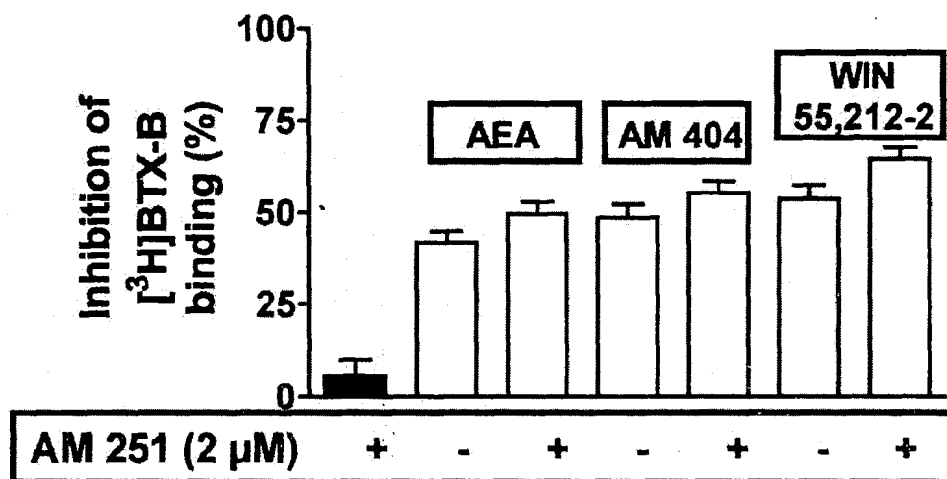


Fig 10. AM 251 (2 μM) does not prevent AEA (75 μM), AM 404 (75 μM) or WIN 55,212-2 (25 μM) from displacing the specific binding of [³H]BTX-B to voltage-sensitive sodium channels of mouse brain.

Within each experiment, binding determinations were conducted in duplicate. Columns represent means and error bars the SEMs of three different experiments.

2.5 Discussion

This part of my research provides evidence to support the hypothesis that the endocannabinoid anandamide inhibits VSSCs in low to mid micromolar concentrations in neuronal preparations. This property is also shared by the cannabimimetics AM404 and WIN 55, 212-2. Clearly the cannabinoid antagonist AM 251 does not attenuate VSSC inhibition by these substances. Together with electrophysiological evidence that these substances also inhibit depolarization of VSSCs in a way that is insensitive to AM 251 (Nicholson et al., 2003), our findings argue strongly against indirect mediation of VSSCs inhibition via CB1 receptor activation.

The idea that sodium channels are inhibited by anandamide and related substances is supported by evidence from other investigators. TTX is a specific inhibitor of VSSCs and Alger (2002) reported that depolarization-induced suppression of inhibition (DSI) is not expressed in the presence of TTX in hippocampal neurons of CA1. Turkanis et al. (1991) found that the potent botanical THC decreased the peak inward sodium current, an indication that THC can block sodium channels. The drug also decreased both the activation and the inactivation rates of sodium channels and these effects were concentration-related (100 nM to 8 μ M). Kim et al. (2005) examined the effect of anandamide on tetrodotoxin-sensitive (TTX-S) and tetrodotoxin-resistant (TTX-R) Na⁺ currents in rat dorsal root ganglion neurons. Anandamide inhibited both TTX-S and TTX-R Na⁺ currents in a concentration-dependent manner. The sleep inducing factor cOA which is similar in structure to anandamide allosterically inhibits [³H]BTX-B binding to VSSCs (Nicholson et al., 2001). The results of my experiments provide further weight to the hypothesis that anandamide and the synthetic

cannabimimetics AM404, WIN 55,212-2 operate allosterically to inhibit BTX-B binding to VSSCs and that this interaction can regulate transmitter release.

The ability of anandamide and the synthetic agents to displace the binding of [³H]BTX-B to synaptoneuroosomes and to display classical non-competitive (anandamide) and competitive (AM 404 and WIN 55,212-2) behavior with respect to sodium channel/neurotoxin binding site 2 shows a distinct parallel with the actions of Class I antiarrhythmics and other neuroactive drugs including anticonvulsants, local and general anesthetics as well as neuroprotectants, all of which are directly inhibitory to sodium channels (Willow and Catterall, 1982; Postma and Catterall, 1984; Hill et al., 1989; Sheldon et al., 1994; MacKinnon et al., 1996; Ratnakumari and Hemmings, 1996; Crevling et al., 1990; Sheldon et al., 1989). Since the binding region on sodium channels influenced by anandamide is intimately associated with site 2, it suggests that certain of these neuroactive and cardiac agents, in particular those which show non-competitive behavior with respect to [³H]BTX-B binding such as procaine derivatives (Crevling et al., 1990), lidocaine (Hill et al., 1989), RH 3421 (Deecher et al., 1991) and amiodarone (Sheldon et al., 1989), may produce inhibition by acting on an endocannabinoid signaling pathway involving sodium channels. Dissimilar Scatchard profiles clearly exclude the possibility that anandamide and the synthetic cannabinoids inhibit sodium channels through mobilization of a common second messenger. Given the competitive inhibition of [³H]BTX-B binding observed for the endogenous sleep inducer cOA (Nicholson et al., 2001) and AM 404, both of which are closely related in structure to anandamide, and the fact that cOA (Nicholson et al., 2001) also increases

the dissociation of the radioligand/sodium channel complex, it is very likely that anandamide binds sodium channels directly.

Furthermore, since the Scatchard plots for anandamide and AM 404 are different, our results do not support the idea that AM 404 mediates its inhibitory effect on sodium channels by augmenting anandamide levels. AM 404 has been reported to selectively inhibit anandamide transport in astrocytes ($IC_{50} = 5 \mu M$) and neurons ($IC_{50} = 1 \mu M$) (Beltramo et al., 1997). This has led to its use as a potentiator of anandamide action in whole animal studies and in *in vitro* experiments on synaptic signaling where an increase in extraneuronal levels of endocannabinoids is required. Recently, AM 404 has been found to mimic anandamide at vanilloid receptors (Zygmunt et al., 2000), raising further questions about its selectivity for endocannabinoid transport in certain preparations. In the present study, we found that in agreement with my binding results, AM 404 blocks sodium channel-dependent depolarization (L. S. David's data), sustained repetitive firing (data from Dr. Lee's group) and release of transmitters from purified nerve endings at low micromolar concentrations. It is also apparent that WIN 55,212-2 exhibits a very similar pharmacological profile. Our results suggest strongly that the potential for AM 404 and WIN 55,212-2 to block sodium channels should be taken into account when they are used as pharmacological tools to explore synaptic actions of endocannabinoids.

Endocannabinoids are becoming increasingly recognized for their ability to mediate retrograde signaling in brain via CB1 receptors, this phenomenon ultimately leading to reduced neuronal excitability and suppression of transmitter release (Huang et al., 2001; Kreitzer and Regehr, 2001; Levenes et al., 1998; Ohno-Shosaku et al.,

2001; Wilson and Nicoll, 2001; Gerdeman et al., 2002). CB1 receptor activation inhibits presynaptic release of L-glutamic acid and GABA from hippocampal neurons (Shen et al., 1996; Katona et al., 1999; Ledent et al., 2000; Hajos et al., 2001; Wilson and Nicoll, 2001), and L-glutamic acid from striatal (Huang et al., 2001) and spinal dorsal horn (Morisset and Urban, 2001) neurons. Various studies indicate that CB1 receptor-mediated inhibition of transmitter release can occur via inhibition of N- and P/Q-type calcium channels and activation of an inwardly rectifying K⁺ conductance (Felder et al., 1993; Mackie et al., 1995; Twitchell et al., 1997; Huang et al., 2001; Nogueuron et al., 2001). Inhibition of transmitter release is also considered to be a consequence of direct endocannabinoid-mediated inhibition of T-type calcium channels (Chemin et al., 2001). The interaction of anandamide with neuronal cannabinoid receptors has been demonstrated at nanomolar concentrations (Felder et al., 1993; Nogueuron et al., 2001), although some preparations require up to 20 μM to achieve robust or maximal response (Felder et al., 1993; Huang et al., 2001; Luo et al., 2002). T-type calcium channels are also inhibited over a similar range (Chemin et al., 2001). Our results suggest sodium channel inhibition may represent a novel mechanism of anandamide action in the nervous system. Since sodium channels are receptive to concentrations of anandamide in the low to mid micromolar range, this could constitute a low affinity damping mechanism within the various excitation suppression pathways that anandamide and anandamide-related endocannabinoids control in brain. The idea that such sites are present directly adjacent to post-synaptic release sites (Kreitzer et al., 2001; Wilson et al., 2001) makes it likely that sufficient concentrations can be achieved, but only locally at synaptic terminals. The significance

of sodium channel inhibition by anandamide lies primarily in the potential to limit the capacity of the nerve ending to support incoming action potential volleys and in consequence, relax the drive on evoked release of L-glutamic acid and GABA. Our experiments also reveal that anandamide differentially inhibits the release of L-glutamic acid and GABA from a mixed population of isolated nerve endings. A more effective block of excitatory than inhibitory transmitter release could therefore act to further reduce the level of downstream postsynaptic excitation in neural networks. However, it should be recognized that a reduction in glutamate-mediated synaptic transmission will reduce feed forward and feedback inhibition mediated by GABA-ergic interneurons and that the sum of these effects in combination with a reduction in GABA release is very difficult to predict. Nevertheless, there is clear potential for sodium channel inhibition to decrease responsiveness to incoming stimuli in somatic or axonal regions of the neuron where they lie in close proximity to sites of anandamide release. My results, together with electrophysiological evidence and membrane potential data of my co-authors (Nicholson et al., 2003), suggest that sodium channels may be involved in a novel signaling pathway for the endocannabinoid anandamide with a capacity for anterograde, retrograde and local suppression of neuronal excitability. It is significant that recent studies in our laboratory found that other endocannabinoids, specifically, 2AG, noladin ether and N-arachidonoyl dopamine all inhibit [³H]BTX-B binding and veratridine-induced release of the neurotransmitters GABA and L-glutamic acid at similar concentrations (Duan et al., unpublished observations). In retrospect, it would have been of potential interest to examine whether the effects of anandamide (or other cannabimimetics) and TTX are additive.

Following our study, other researchers reported that AM404 depresses Ca^{2+} spiking in hippocampal neurons by directly inhibiting VSSCs (Kelley and Thayer, 2004). Subsequent to this Kim et al. (2005) confirmed that anandamide blocks VSSC function. Also in agreement with my results, both these effects occurred in the absence of CB1 receptor activation.

In summary, my research suggests a novel mechanism for endocannabinoid depression of transmitter release. Humoral modulators of sodium channels are relatively rare, but this is now a recognized feature for neuromodulatory substances e.g. dopamine (Cantrell et al., 1999). As well as advancing our fundamental knowledge, a greater understanding of the mechanism and consequences of sodium channel inhibition by endocannabinoids and how the different cannabinoid targets integrate to achieve the regulation of neurotransmission in the brain should also give insights into the development of safer, more effective, drugs for management of anxiety, sleep disorders, anesthesia, epilepsy, coma and cardiac arrhythmias.

Acknowledgment

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CHAPTER 3

INHIBITION OF VOLTAGE-SENSITIVE SODIUM CHANNELS BY THE CANNABINOID 1 RECEPTOR ANTAGONIST AM 251 IN MAMMALIAN BRAIN

3.1 Abstract

The CB1 receptor antagonist AM 251 is known to block the inhibitory effects of endocannabinoids and synthetic cannabinoid agonists on transmitter release through an action at presynaptic CB1 receptors in brain. I examined the ability of AM 251 to inhibit sodium channel-dependent transmitter release and the binding of [³H]batrachotoxinin A 20- α -benzoate to sodium channels in mouse brain synaptic preparations. Veratridine-dependent (tetrodotoxin suppressible) release of L-glutamic acid and GABA from synaptosomes was reduced by AM 251 [IC₅₀s = 8.5 μ M (L-glutamic acid), 9.2 μ M (GABA)]. The binding of the radioligand [³H]batrachotoxinin A 20- α -benzoate to site 2 on sodium channels was displaced by AM 251 (IC₅₀ = 11.2 μ M). Scatchard analysis of binding showed that at its IC₅₀, AM 251 increased (by 2.3 fold) the K_d of radioligand without altering B_{max}, suggesting a competitive mechanism of inhibition by AM 251. Kinetic experiments indicated that AM 251 inhibits equilibrium binding by allosterically accelerating the dissociation of the [³H]batrachotoxinin A 20- α -benzoate:sodium channel complex. My data suggest that micromolar concentrations of AM 251 are capable of reducing neuronal excitability and inhibiting release of excitatory and inhibitory transmitters through blockade of voltage-sensitive sodium channels in brain.

3.2 Introduction

The CB1 receptor antagonist AM 251, (N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide) inhibits the binding of both CB1 agonists (e.g. [³H]CP-55,940) and antagonists (eg [³H]SR141716A) to rat brain membranes at nanomolar concentrations (Thomas et al., 1998; Lan et al., 1999). Wilson and Nicoll (2001) reported that 2 μM AM 251 effectively reduces the inhibitory effects of endocannabinoids on presynaptic release of GABA in hippocampus, while in cerebellum, 1 μM AM 251 provides virtually complete (Kreitzer and Regehr, 2001), or full (Kreitzer et al., 2002) antagonism of endocannabinoid-mediated depression of interneuron firing.

The results described in Chapter 2 of this thesis demonstrate that low micromolar concentrations of CB1 receptor agonists, specifically the endocannabinoid anandamide and WIN 55,212-2, inhibit the binding of [³H]batrachotoxinin A 20- α -benzoate ([³H]BTX-B) to voltage-sensitive sodium channels. I also found that anandamide and WIN 55,212-2 were effective blockers of veratridine-(sodium channel-)dependent release of endogenous L-glutamic acid and GABA from highly purified synaptosomes. In this investigation, my co-workers found that anandamide and WIN 55,212-2 fully suppress both tetrodotoxin-sensitive sustained repetitive firing in cultured cortical neurones and depolarization of mouse brain synaptoneuroosomes by veratridine (Nicholson et al., 2003). Significantly, none of the observed inhibitory effects are antagonized by pretreatment with 1-2 μM AM 251, supporting the idea that CB1-independent depressant effects of anandamide on sodium channels can occur.

During the course of these studies, I observed that 2 μ M AM 251 produces a consistent (albeit extremely weak) inhibition of both veratridine-dependent transmitter release and [3 H]batrachotoxinin A 20- α -benzoate binding (Figs. 5 and 10). AM 251 may therefore have inherent sodium channel blocking activity at concentrations very close to those used by us (Nicholson et al., 2003) and others (Kreitzer et al., 2002; Wilson and Nicoll, 2001) to block activation of CB1 receptors. Veratridine and batrachotoxinin A 20- α -benzoate activate voltage-gated sodium channels by binding to site 2 which represents one of five well-characterized neurotoxin binding sites on this complex (Strichartz et al., 1987). The objective of this part of the investigation was, therefore, to explore this putative interaction of micromolar concentrations of AM 251 with neuronal voltage-sensitive sodium channels in relation to channel sensitivity and mechanism.

3.3 Methods

3.3.1 Quantitation of endogenous amino acid release from synaptosomes

Highly purified synaptosomes were prepared from the whole brains of two male CD1 mice (20-25g) using the method of Dunkley et al., (1986) as previously described in Chapter 2. Synaptosomes were then suspended in saline [(composition in mM: NaCl 128, KCl 5, MgCl₂ 1.2, NaHCO₃ 5, CaCl₂ 0.8, glucose 14, HEPES 20 (buffered to pH 7.4 with Tris)] to a concentration of 7 mg protein/ml. Prior to assay, AM 251 was formulated by rapid injection (of a DMSO solution; 2 μ l) into 1 ml saline. Synaptosomes (100 μ l) were introduced into saline (250 μ l; containing either vehicle control or AM 251 as necessary) and vortexed gently. After 15 min. preincubation at 30 $^{\circ}$ C, veratridine (added in 5 μ l saline; 2 μ M final concentration) was introduced and

samples mixed quickly. At $t = 20$ min., samples were quickly centrifuged (Beckman Microfuge E; 40 sec.), and their supernants (284 μ l) acidified with perchloric acid (6 M; 6 μ l). Samples were kept at -20 °C until HPLC analysis could be performed. The derivatization with *o*-phthaldialdehyde and HPLC analysis to determine release of L-glutamic acid and GABA has already been described in detail (Chapter 2).

3.3.2 Binding of [3 H]BTX-B to mouse brain sodium channels

Synaptoneurosomes were isolated using the method described in Chapter 2. Binding reactions were initiated by added synaptoneurosomes (20 μ l; 250 μ g protein) to Na^+ -free saline (140 μ l) containing [3 H]BTX-B (6 nM for displacement and kinetic experiments; 1-160 nM for Scatchard assay; 46 Ci/mmol; PerkinElmer Life Sciences Inc, Boston, MA, USA), scorpion (*Leiurus quinquestriatus*) venom (ScV; 30 μ g; Sigma, St. Louis, MO, USA), 1 mg/ml BSA and AM 251 or solvent control, as appropriate. PMSF (50 μ M) was included in some binding assays. Prior to addition of radiolabel and ScV, AM 251 (in 2 μ l DMSO) or solvent control (DMSO) was incorporated in to 1 ml Na^+ -free saline as detailed in the previous section. At $t = 50$ min., binding assays were terminated and radioactivity associated with the membranes quantitated as described in Chapter 2. Non-specific binding to membranes was determined in the presence of 300 μ M veratridine. To investigate possible effects of AM 251 on the kinetics of association of [3 H]BTX-B with site 2 on VSSCs, synaptoneurosomes were preincubated with AM 251 or solvent control for 10 min. [3 H]BTX-B (6 nM) was then introduced and the increase in specific binding measured up to 20 min. For dissociation experiments, synaptoneurosomes were equilibrated with [3 H]BTX-B (6 nM) for 50 min., then either a saturating concentration of veratridine

(plus solvent control) or veratridine plus AM 251 was added and specific binding monitored over 20 min. time course.

3.3.3 Analysis of data

Curve fitting, determination of IC_{50} , binding parameters, and statistical analyses were performed using Prism software (GraphPad Software Inc., San Diego, CA, USA).

3.4 Results

3.4.1 Release of neurotransmitters L-glutamic acid and GABA from synaptosomes

Veratridine stimulated the release of transmitter from synaptosomes by 0.45 ± 0.06 nmol mg^{-1} (L-glutamic acid) and 0.74 ± 0.10 nmol mg^{-1} (GABA) respectively. Both these responses are totally abolished by 16 μM tetrodotoxin. The inhibitory effects of AM 251 on veratridine-evoked release of neurotransmitters (L-glutamic acid and GABA) from synaptosomes are shown in Fig. 11. Maximum inhibition (glutamic acid release, $80.1 \pm 4.5\%$; GABA release, $71.8 \pm 5.6\%$) was observed between 30 and 50 μM AM 251 and IC_{50} s for inhibition of glutamic acid and GABA were established at 8.5 μM (95% confidence interval = 6.5 to 11.2 μM) and 9.18 μM (95% confidence interval = 6.93 to 12.16 μM) respectively. Background release of L-glutamic acid from polarized (control) synaptosomes was 0.65 ± 0.12 nmol mg^{-1} , and control release of GABA was very close to, or below the detection limit, making quantitation difficult. However, control release of L-glutamic acid was not significantly affected by AM 251 ($P > 0.05$) and no discernable effect of AM 251 on control release of GABA was observed.

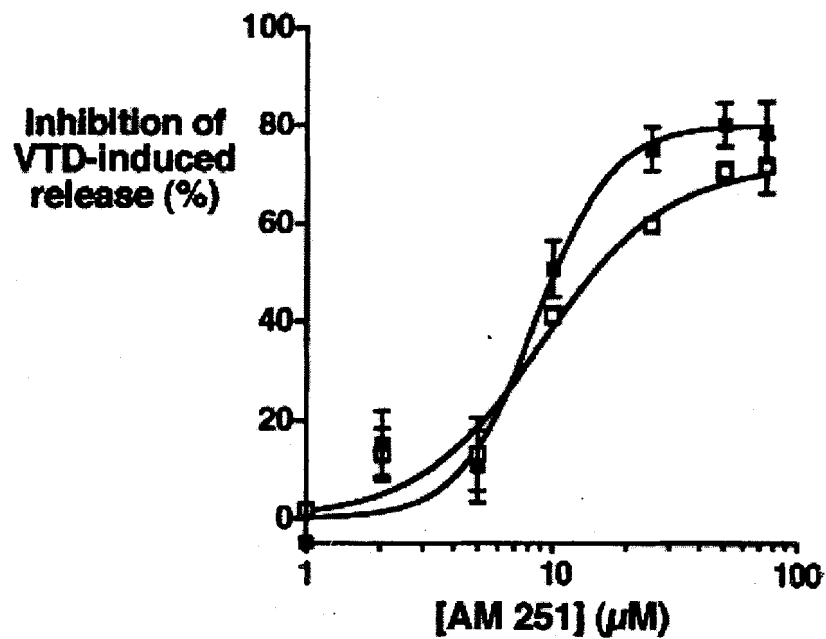


Fig 11. AM 251 inhibits veratridine-dependent release of endogenous L-glutamic acid (■) and GABA (□) from mouse brain synaptosomes in a concentration-dependent fashion.

Data points show means \pm S.E.M. of 3 independent experiments.

3.4.2 [³H]BTX-B binding assay

The specific binding of [³H]BTX-B to synaptoneurosomal membranes, determined as the fraction of binding eliminated in the presence of a saturating concentration of veratridine (300 μM), averaged > 90% of total binding using a standard radioligand concentration of 6 nM. Specific binding of [³H]BTX-B to mouse brain sodium channels was inhibited in a concentration-dependent fashion by AM 251 to a maximum of $81.3 \pm 3.5\%$ and the IC_{50} was calculated at 11.2 μM (95% confidence limits 10.0 to 12.5 μM; Fig. 12). In parallel experiments, the inhibitory effect of 15 μM AM 251 on [³H]BTX-B binding was not modified ($P > 0.05$) when experiments were conducted in the presence of 50 μM phenylmethylsulfonyl fluoride, a potent inhibitor of fatty acid amidohydrolase. The mechanism of inhibition by AM 251 of [³H]BTX-B binding to sodium channels under equilibrium conditions was investigated using Scatchard analysis. The effect of AM 251 on the concentration dependence of [³H]BTX-B binding is displayed in Fig. 13. At 10 μM, AM 251 increased the K_d of radioligand binding from 19.0 ± 1.3 nM to 42.7 ± 6.4 nM without affecting B_{max} (Control, 1220 fmol mg⁻¹; AM 251, 1189 fmol mg⁻¹). AM 251 did not reduce the rate of association of [³H]BTX-B with sodium channels (K_{OBS} values: Control, 0.079 ± 0.001 min⁻¹; 25 μM AM 251, 0.080 ± 0.001 min⁻¹; 50 μM AM 251, 0.084 ± 0.003 min⁻¹; Fig. 14), however, this compound did increase the rate of dissociation of radioligand over and above that produced by a saturating concentration of veratridine (K_{-1} values: Control, -0.025 ± 0.001 ; 25 μM AM 251, -0.033 ± 0.002 ; 50 μM AM 251, -0.035 ± 0.001 ; Fig. 15).

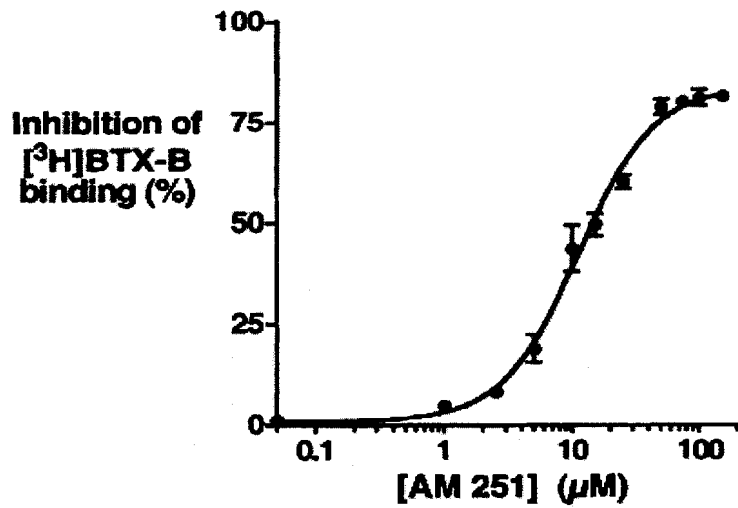


Fig 12. Inhibition by AM 251 of the specific binding of [³H]BTX-B to sodium channels in the mouse brain synaptoneurosomal fraction.

All data points represent means \pm S.E.M. of three independent experiments apart from those for the three lowest concentrations of AM 251 which are the means of two determinations. Assays within each experiment were performed in duplicate.

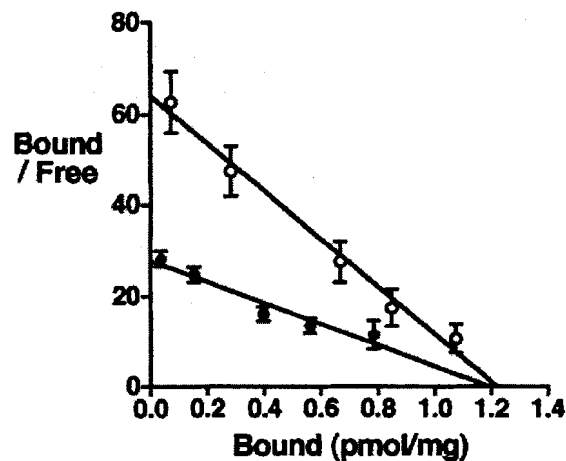


Fig 13. Scatchard analysis of [³H]BTX-B binding to sodium channels in the absence (○) and presence (●) of 10 μM AM 251.

Results are means \pm S.E.M. of three independent experiments, each performed in duplicate.

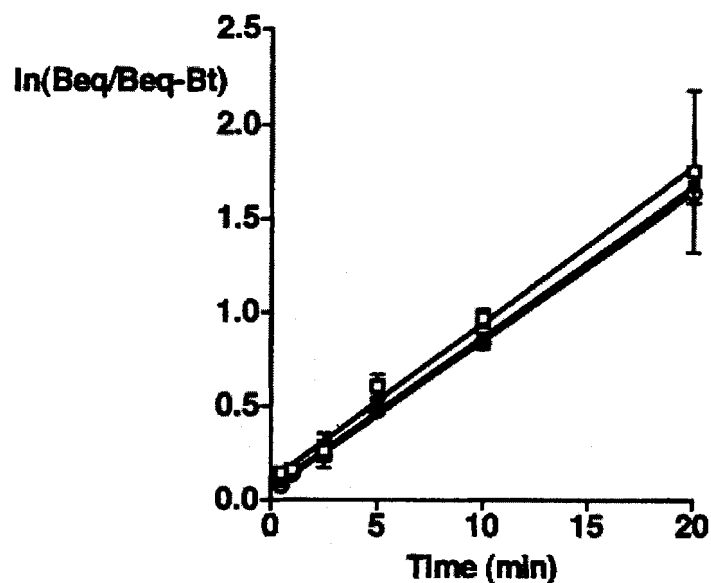


Fig 14. AM 251 (25 μ M \square ; 50 μ M \blacksquare) does not reduce the association of [3 H]BTX-B with mouse brain sodium channels.

All data points represent means \pm S.E.M. of three to six independent experiments, each performed in duplicate (\circ = control).

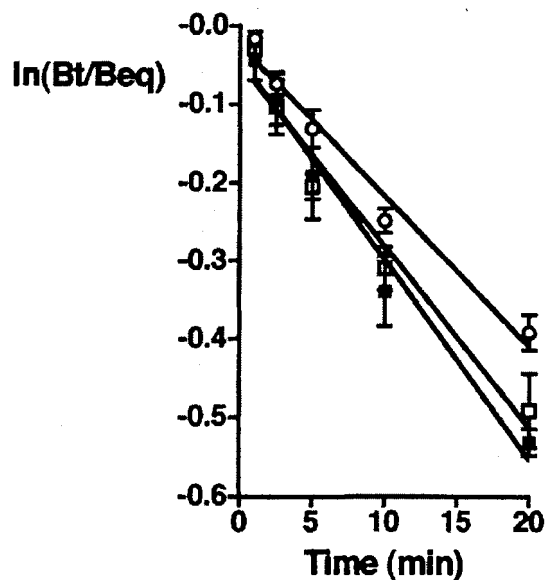


Fig 15. Dissociation of the [3 H]BTX-B:sodium channel complex (initiated by 300 μ M veratridine) in the absence (\circ) and presence of 25 μ M (\square) and 50 μ M (\blacksquare) AM 251. All data points represent means \pm S.E.M. of three independent experiments, each performed in duplicate.

3.5 Discussion

As pharmacological tools, AM 251 and its analogs have provided unique insight into our understanding of cannabinoid CB1 receptor pharmacology and the role of endocannabinoids in synaptic signalling in the brain (Gifford and Ashby, 1996; Lan et al., 1999; Rinaldi-Carmona et al., 1994; Thomas et al., 1998; Wilson and Nicoll, 2001; Ohno-Shosaku et al., 2001; Kreitzer and Regehr, 2001; Kreitzer et al., 2002). However, the neurotoxic alkaloids veratridine and batrachotoxin are highly specific activators of neuronal voltage-gated sodium channels (Catterall, 1980; Catterall et al., 1981; Crevling et al., 1983) and the observation that AM 251 suppresses veratridine-dependent depolarization of synaptoneuroosomes (Liao et al., 2004; data obtained by L.S. David), veratridine-dependent release of neurotransmitters from synaptosomes and inhibits the binding of [³H]BTX-B to synaptoneurosomal membranes, provides compelling evidence that at micromolar concentrations this compound blocks voltage-gated sodium channels at the synaptic level in brain. There was good agreement both between the inhibitory potencies of AM 251 in each of the three assays, as judged by IC₅₀ values (range 8.5 - 11.2 μM), and in the maximum percentage inhibition achieved (range 60.3 - 81.3), indicating that AM 251 is acting on sodium channels in a very similar fashion in both the functional and receptor binding systems. Furthermore, veratridine-induced depolarization (Liao et al., 2004; data obtained by L.S. David), and veratridine-induced release of L-glutamic acid and GABA are blocked fully by tetrodotoxin, in line with previous reports (Nicholson and Merletti, 1990; Eells et al., 1992; Nicholson, 1992; Zhang et al., 1996), adding weight to the idea that the response to veratridine is primarily a function of sodium channel activation.

Since my binding experiments demonstrate displacement by AM 251 of specific binding of [³H]BTX-B, I conclude that the blockade of sodium channel-dependent depolarization and transmitter release in synaptic preparations occur as a result of AM 251 affecting site 2 on the sodium channel. Saturation binding experiments conducted with [³H]BTX-B in the presence of 10 μM AM 251, a concentration very close to the IC₅₀ (11.2 μM), revealed that this compound increases the dissociation constant (K_d) of [³H]BTX-B binding without affecting the number of binding sites (B_{max}) available to the radioligand. Such a profile suggests AM 251 to be a competitive ligand for site 2 on voltage-gated sodium channels. Subsequent investigation of the influence of AM 251 on the kinetics of [³H]BTX-B binding demonstrated that AM 251 is unable to slow the association of radioligand with site 2 showing that this is not a simple competitive mechanism. However, in the presence of excess veratridine, this pyrazole is able to produce an increase in the rate of dissociation of the [³H]BTX-B:sodium channel complex above that seen with veratridine alone. My results are consistent with AM 251 binding to a site that is distinct from, but coupled to the radioligand binding site. Such a profile suggests that AM 251 can be classified as an allosteric competitive inhibitor of [³H]BTX-B binding. The pharmacology of AM 251 is similar to those of sodium channel-directed anticonvulsant, antiarrhythmic and local anesthetic drugs (Catterall, 1981; Willow and Catterall, 1982; Postma and Catterall, 1984), however, I cannot exclude the possibility that AM 251 binds to a macromolecular complex other than the sodium channel (such as a kinase) to achieve inhibition indirectly. The inhibition maxima achieved with AM

251 in both functional and binding assays amounted to 60-80% suggesting this compound is not a full antagonist of sodium channels.

The stability of modulators containing an amide linkage can be quite low in *in vitro* assays due to the presence of fatty acid amidohydrolase (FAAH). This can lead to difficulties in establishing true potency values and inclusion of an inhibitor of FAAH such as phenylmethylsulfonyl fluoride will circumvent this problem (Childers et al., 1994; Adams et al., 1995). Since AM 251 contains an amide linkage and the [³H]BTX-B binding assay involved a 50 min incubation, I carried out a series of experiments in the presence of 50 μM PMSF. The inhibitory effect of an IC₅₀ concentration of AM 251 on [³H]BTX-B binding was not changed by PMSF, confirming that FAAH does not interfere with inhibitory potency estimations for AM 251 in our assays.

In view of the very close structural similarity between AM 251 and SR141716A, it is likely that SR141716A will show a similar pharmacological profile and in this context, it is noteworthy that the insecticidal dihydropyrazole, RH 3421, which blocks sodium channel-dependent release of GABA from synaptosomes (Nicholson and Merletti, 1990; see also for RH 3421 structure) through an interaction with site 2 on the sodium channel (Deecher et al., 1991), bears a structural resemblance to AM 251.

In conclusion, using *in vitro* biochemical approaches, I have demonstrated that AM 251 has the ability to depress neuronal excitability at concentrations around 2-5 μM and higher through sodium channel blockade. Such responses are qualitatively similar to those observed with CB1 cannabinoid receptor agonists at synapses and as

such should be taken into account when AM 251 is used in the low micromolar range to selectively antagonize CB1 receptor function.

Acknowledgment

Sections reprinted from *Basic and Clinical Pharmacology and Toxicology* C.Liao, J. Zheng, L.S. David and R.A. Nicholson, Inhibition of voltage-sensitive sodium channels by the CB1 receptor antagonist AM 251 in mammalian brain, 94, pages 73-78, © 2004, with permission Nordic Pharmacological Society.

CHAPTER 4

THE EFFECT OF DEPOLARIZATION ON ANANDAMIDE LEVELS, ANANDAMIDE SYNTHESIS AND ANANDAMIDE EFFLUX IN SYNAPTONEUROSOMAL PREPARATIONS FROM MOUSE BRAIN

4.1 Abstract

The effect of depolarization on the levels of anandamide in synaptoneurosomes was investigated using DBD-COCl derivatization followed by HPLC analysis. The biosynthesis of anandamide was also examined by conducting incubations with the anandamide precursor [³H]ethanolamine. Release of [³H] from synaptoneurosomes preloaded with [³H]anandamide was also examined using filtration and superfusion assays representing assessment of shorter term and longer term release respectively. A consistent theme to the results of these experiments is that a depolarizing challenge with 35 mM KCl did not enhance anandamide production, moreover, depolarization does not lead to an increase in release of this endocannabinoid from synaptoneurosomes.

4.2 Introduction

It is widely accepted that anandamide is biosynthesized in synaptic regions upon demand as part of a negative feedback loop which serves to reduce excessive release of neurotransmitters. Studies indicate that depolarization stimulates anandamide synthesis within the postsynaptic neuron and newly synthesized anandamide then diffuses

retrogradely to presynaptic nerve endings where it inhibits release of not only excitatory and but also inhibitory transmitters (Maejima et al., 2001, Vincent et al., 1992). These phenomena are respectively termed depolarization-induced suppression of excitation (DSE) (Maejima et al., 2001) and depolarization-induced suppression of inhibition (DSI) (Llano et al., 1991; Vincent et al., 1992; Pitler and Alger, 1994). CB1-receptor agonists have been found to induce DSI in hippocampus and cerebellum (Katona et al., 1999; Takahashi and Linden, 2000) and CB1 receptor antagonists block DSI (Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001) providing strong evidence that CB1 receptors mediate this effect. Likewise, cannabinoid agonists promote DSE in the cerebellum and hippocampus and CB1 antagonists reverse it (Takahashi and Linden, 2000; Kreitzer and Regehr, 2001). Further support for such a mechanism comes from the studies of Ledent et al. (1999) and Wilson and Nicoll (2002) which found that DSI and DSE cannot be demonstrated in certain neurons of CB1 receptor knockout mice. However, there appears to be no direct evidence showing that endocannabinoids (for example anandamide) are produced and released from postsynaptic somata or indeed synthesized exclusively in postsynaptic regions of the synapse. Also, it is unclear whether anandamide is produced locally at the synapse or in the neuron as a whole. Two main routes have been documented for the biosynthesis of anandamide in nerve. The first, a Ca^{2+} -independent route, involves fatty acid amide hydrolase (FAAH) which catalyzes the formation of anandamide from ethanolamine and arachidonic acid (Kempe et al. 1996). Since the K_d of anandamide for FAAH is high, the reverse reaction is generally favored. The second process is Ca^{2+} -dependent and involves N-acyltransferase and phospholipase D (Axelrod and Felder, 1998). At

present there is no direct evidence to show that enzymes for anandamide biosynthesis are present in postsynaptic areas (Wilson and Nicoll 2002). Several investigations suggest Ca^{2+} is important for anandamide biosynthesis. DSI is known to be dependent on Ca^{2+} influx (Lenz et al., 1998) and Di Marzo et al. (1994) found that synthesis and the release of [^3H]anandamide is stimulated by the Ca^{2+} ionophore (ionomycin) and depolarizing agents (including KCl) in primary cultures of rat brain striatal or cortical neurons following preincubation with [^3H]ethanolamine. Anandamide synthesis has been confirmed in human and rat brain slices and the tissue levels of this endocannabinoid increase after depolarization (Steffens et al., 2003). However, all of the reports to date concerning anandamide synthesis in functional systems rely on intact nerve cells or brain tissue and neither of these provide insight into the specific role of postsynaptic and/or presynaptic regions of the synapse in this process.

It is first important to investigate whether the endocannabinoid anandamide is synthesized in synaptic regions. Synaptoneuroosomes are synaptic elements individually consisting of a pinched-off nerve ending linked via the synaptic region to a pinched-off portion of the cell body and would appear to offer an ideal functional model for the study of anandamide synthesis in resting and depolarized states. Also amenable to study in this preparation is whether depolarization stimulates the release of [^3H]anandamide from synaptoneuroosomes previously loaded with [^3H]ethanolamine. After its release anandamide is considered to be taken up rapidly by synaptic elements and this serves to extinguish its inhibitory effect on transmitter release. Some support for this is provided by analyses which show that in cerebellar neurons the concentration of anandamide is many fold greater than its concentration in the

extracellular media (Hillard and Jarrahian, 2000). This suggests anandamide is avidly accumulated by neurons, although it is unclear at present whether any accumulated anandamide can be stored for release at a later stage. Facilitated transport and simple diffusion represent two hypotheses seeking to describe how anandamide may pass through the neuronal plasma membrane. The observation that anandamide analogs interfere with the uptake of [³H]anandamide has been interpreted as evidence for facilitated transport (Beltramo et al., 1997). However, a specific integral membrane carrier for anandamide has not been identified yet. The second hypothesis is simple diffusion. However, since anandamide is a very lipophilic compound, its capacity to diffuse will be greatly influenced by the distribution of protein and lipid molecules and structures within the cell, the plasma membrane and in the extracellular space. Using the red blood cell membrane model it has been shown that after loading with [³H]anandamide this substance passes rapidly (within seconds) from the cell interior through the membrane and into the extracellular medium, but only if protein (e.g. BSA) is present as an extracellular binding substrate to drive this redistribution. These results indicate that a carrier to specifically facilitate translocation is not necessarily essential (Bojesen and Hansen, 2005). Although it remains unresolved whether the translocation of anandamide across the plasma membrane is governed by simple diffusion or protein carriers, it is commonly accepted that anandamide translocation is independent of any direct energy input (Bojesen and Hansen, 2006). This chapter describes my investigations using synaptoneurosomes to investigate 1) whether increased levels of endogenous anandamide can be demonstrated during depolarization, 2) whether depolarization-induced release of organosoluble [³H] can be detected after incubation

of synaptoneurosomes with the anandamide precursor [³H]ethanolamine and 3) whether depolarization stimulates the release of exogenously-loaded [³H]anandamide.

4.3 Methods

4.3.1 Preparation of synaptoneurosomes and determination of anandamide levels

Synaptoneurosomes were prepared as previously described (Liao and Nicholson, 2005). Synaptoneurosomes from 4 mice were distributed between 4 tubes and incubated for 1 minute at 35 °C. KCl (50 mM final concentration) was added as appropriate and membrane depolarization continued for 3 minutes. Phenylmethylsulfonylfluoride (PMSF; 100 μM) was routinely added to prevent hydrolysis of anandamide. To terminate the incubation, synaptoneurosomal suspensions were rapidly centrifuged at 10,000 x g and supernatants removed. Anandamide extraction from pellets and supernatants and HPLC analysis was conducted using the method of Steffents et al. (2003) with minor modifications.

4.3.2 Extraction of anandamide from synaptoneurosomes and saline supernatants

20 ml hexane/isopropanol (3:2 v/v) was added to the synaptoneurosomal pellet or 1 ml of the supernatant obtained after centrifugation of the synaptoneurosomal suspension, and mixed vigorously for 5 min. Aqueous and organic phases were separated by centrifugation (10,000 x g, 10 min). The upper phase was collected and the lower aqueous phase was washed again using a hexane/isopropanol mixture. The organic phases were then pooled and dried under N₂. The residue was resuspended in 1900 μl of 6% ethanol in n-hexane and applied to an aminopropylsilica column

(Chromabond-NH₂, 3 ml, 500 mg; Macherey-Nagel, Duren, Germany). All columns were equilibrated with 2 × 2 ml n-hexane, prior to loading with anandamide solution. After washing with 3 × 2 ml of n-hexane the lipid fraction containing anandamide was eluted with 2 × 2 ml 10% EtOH in n-hexane and evaporated to dryness.

4.3.3 Derivatization of anandamide

The extracted anandamide fraction was re-dissolved in CH₃CN. DBD-COCl (4-(N,N-dimethylaminosulfonyl)-7-(N-chloroformylmethyl-N-methyl-amino)-2,1,3-benzoxadiazole) in CH₃CN (10 mM, 40 µl) was added and the mixture vortexed vigorously. The mixture was then heated at 60 °C for 2 hr to convert anandamide to its DBD-CO derivative. After stopping the reaction by adding 100 µl CH₃CN/H₂O (1:1, v/v), the mixture was centrifuged at 10,000 × g for 2 min. An aliquot of the solution (100 µL) was then subjected to high performance liquid chromatography (HPLC) analysis.

4.3.4 HPLC analysis

HPLC was carried out using a Hewlett Packard 1050 series chromatograph and a C₁₈ reverse-phase silica column (15 cm x 4.6 mm i.d.; particle size 5 µm). DBD-CO anandamide peak areas were quantified by their fluorescence at 560 nm using an excitation wavelength of 450 nm. The mobile phase was (CH₃CN/H₂O, 70:30, v/v). Each injection contained 80 µl of the derivatized anandamide solution. To assess linearity, recovery and precision of the HPLC measurements, stock solutions of anandamide (10 nM - 10 µM) were prepared and stored at -20 °C until use. Calibration

curves were prepared using anandamide standards (0.3 - 120 pmol) and either derivatized directly or derivatized after addition to synaptoneurosomes.

4.3.5 Determination of anandamide release from synaptoneurosomes following incubation with [³H]anandamide

4.3.5.1. Filtration assay (shorter term release)

After 5 min preincubation at 32 °C, 0.5 ml synaptoneurosomes were mixed with PMSF (10 µM), 5 µCi [³H]anandamide (specific activity 204 Ci/mmol, PerkinElmer Life Sciences Inc.) and 50 µM unlabeled anandamide (INC Biomed) and then incubated at 32 °C for 25 min. Alcohol was routinely removed from anandamide stock solutions under a stream of N₂. After completing the loading with anandamide, the synaptoneurosomal samples were transferred to ice. 20 µl of synaptoneurosomes were then transferred to 2 ml normal saline (in mM) NaCl 128, KCl 5, MgCl₂·7H₂O 1.2, NaHCO₃ 5, CaCl₂·2H₂O 0.8, glucose 14, HEPES 20 (buffered to pH 7.4 with Tris) to a concentration of 1.2 ± 0.3 mg protein/ml or 2 ml saline containing 35 mM KCl (both with 0.1% BSA present). After various times (0-5 minutes) the suspensions were filtered under vacuum (Whatman GF/C filters; Hoefer FH 225V filtration manifold). Filters were immediately washed with 5 ml ice-cold saline (without BSA). The filter discs were placed in 20 ml vials and 1 ml 1% SDS was added followed by 12 ml counting scintillation solution. Radioactivity was quantitated using a liquid scintillation counter.

4.3.5.2. Superfusion assay (longer term release)

To 0.5 ml synaptoneurosomes was added to 5 µCi [³H]anandamide (specific activity 204 Ci/mmol, PerkinElmer Life Sciences Inc.) and unlabeled anandamide

(final anandamide concentration 50 μ M) and incubated at 32 °C for 50 min. 1 mM PMSF was present to reduce anandamide degradation and loading was terminated by resuspending synaptic particles in 10 ml ice-cold saline (NaCl 128 mM, KCl 5 mM, Na₂HPO₄ 1 mM, CaCl₂ 0.8 mM, MgCl₂ 1.2 mM, glucose 14 mM and HEPES 20 mM buffered to pH 7.4 with Tris) and centrifugation (1,000 x g; 10 min). Pellets were resuspended in 800 μ l of ice-cold saline and synaptoneuroosomes (0.90 \pm 0.16 mg protein) were placed in each of ten Swinnex filtration units (Millipore) containing Whatman GF/B filters. Superfusions were conducted as described previously (Nicholson and Merletti, 1990).

Each filter unit was continuously supplied with saline containing 0.1% BSA from above by a reservoir (30 ml syringe barrel). Saline superfusates were then routed to an LKB 2070 Ultrarac fraction collector modified to collect 10 superfusates simultaneously. The flow rate was held at 1 ml/min by a 10-channel peristaltic pump (Watson Marlow 503S) placed between the filtration units and the fraction collector. The collection of fractions (10 x 3 ml) was started after the [³H]anandamide-loaded synaptoneuroosomes had been superfused with 10 or 30 ml control saline containing 0.1% BSA. The radioactivity in each fraction was quantitated by liquid scintillation counting. Addition of depolarizing treatments normally coincided with fraction 4. In all experiments where salines containing elevated KCl were used, the NaCl was reduced by a corresponding amount to maintain isosmotic conditions. Superfusion experiments were carried out at room temperature 24–28 °C.

4.3.6 Synthesis of organosoluble substances in the presence of [³H]ethanolamine

The preparation of synaptoneurosomes was carried out as already described. Synaptoneurosomes obtained from one mouse brain were suspended in 1.2 ml saline. 10 μ Ci [³H]ethanolamine hydrochloride (specific activity 23 Ci/mmol; Amersham Biosciences UK Ltd) was added and synaptoneurosomes were then incubated at 32 °C for 10 min. Each 0.1 ml (2.3 mg \pm 0.51 mg protein) synaptoneurosomes was introduced into 2 ml saline containing various treatments and incubated at 32 °C for 50 min. The synaptoneurosomes were pelleted by centrifugation (8,000 x g, 5 min) and organosoluble materials in supernatants and pellets were then extracted using methanol and chloroform (Bligh and Dyer, 1959). Extracts were dried at 28 °C overnight and radioactivity then quantitated by liquid scintillation counting.

4.4 Results

4.4.1 Anandamide levels in synaptoneurosomes

Anandamide was detected in both resting and 50 mM KCl-stimulated synaptoneurosomes using HPLC analysis (Fig. 16a and b). The quantities of anandamide released into the saline from synaptoneurosomes were 6.85 \pm 3.12 pmol/mg and 9.66 \pm 3.87 pmol/mg for the control and KCl depolarized treatments respectively, whereas the levels of anandamide in the synaptoneurosomal pellets were 7.21 \pm 2.28 pmol/mg in resting synaptoneurosomes and 11.20 \pm 2.98 pmol/mg in the KCl depolarized synaptoneurosomes. There were no significant differences between the levels of anandamide present in resting and KCl-depolarized synaptoneurosomes, moreover no significant differences ($P > 0.05$) were observed in

the supernatants derived from control and KCl-depolarized synaptoneurosomes. Other experiments to investigate anandamide biosynthesis in synaptoneurosomes and the distribution of anandamide between synaptoneurosomes and extracellular saline were then performed with the anandamide precursor [³H]ethanolamine. Ethanolamine is very soluble in water and as its conversion to the lipophilic anandamide proceeds the radioactivity becomes highly organosoluble. I found no evidence for significant increases in organosoluble radioactivity in either the synaptoneurosomal pellet or the saline supernatant (the latter indicative of released ³H) as a result of depolarization with KCl (Fig. 17a and b). In these experiments BSA was included in the incubation medium to encourage release of [³H]anandamide.

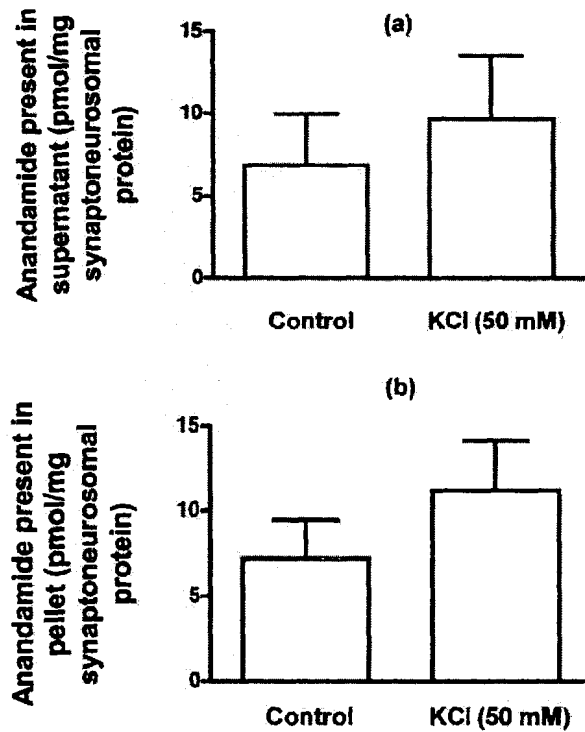


Fig 16. Levels of anandamide in synaptoneurosomes and the surrounding saline under resting and depolarizing conditions as determined by HPLC.

The depolarizing treatment consisted of incubation with KCl (50 mM) for 3 minutes. a) Levels of anandamide released into medium. b) Anandamide present in the synaptoneurosomal pellet. Values represent means \pm SEM of 3-7 determinations. Differences between resting and depolarizing treatments were not significant ($P > 0.05$).

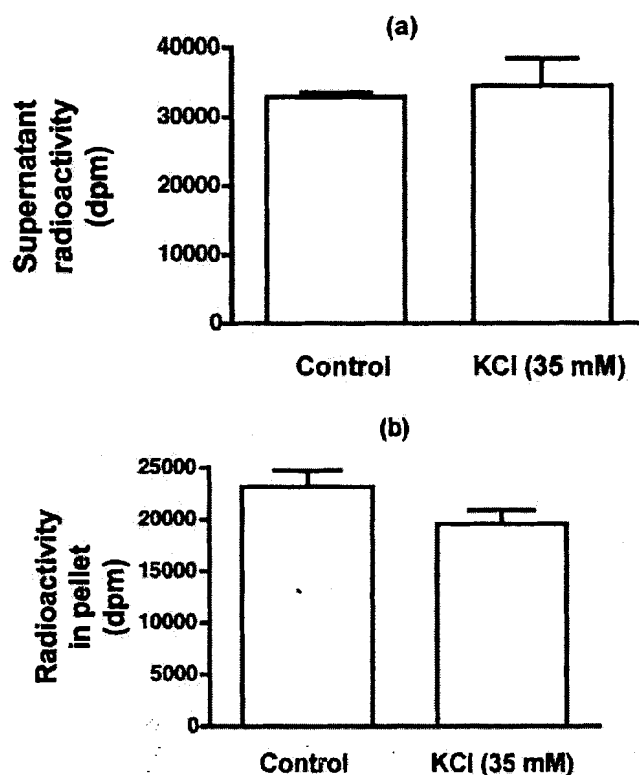


Fig 17. Amount of organosoluble ^3H present in a) the supernatant and b) the synaptoneurosomal pellet after centrifugation.

Synaptoneurosomes pre-incubated with [^3H]ethanolamine and then challenged with 35 mM KCl. BSA was present throughout at 1 mg/ml. Values represent means \pm SEM of 4 assays. Differences between the control and KCl treatments in a) and b) were not significant ($P > 0.05$).

4.4.2 Studies on release of [^3H] material(s) after preloading synaptoneurosomes with [^3H]anandamide

The results of the filtration experiments (Fig. 18) confirm that [^3H] material(s) can be released from synaptoneurosomes preloaded with [^3H]anandamide, however depolarization with 35 mM KCl failed to produce an increase in the release of radiolabeled materials from synaptoneurosomes over the short term. Likewise, in the superfusion experiments (Fig. 19a and b), radiolabeled material was released but there

was no convincing evidence for depolarization-stimulated release of radioactivity over longer time periods either. It was also clear that at the end of these superfusion experiments, a substantial amount of [^3H] material was still present in synaptoneurosomes (Fig. 20a and b). In agreement with the release profiles was no reduction in radioactivity associated with synaptoneurosomes receiving the KCl challenge. The ^3H associated with synaptoneurosomes (Fig. 20) was approximately 40 and 6 times the amount of ^3H present in the final fractions collected after the shorter and longer superfusions respectively (Fig. 19).

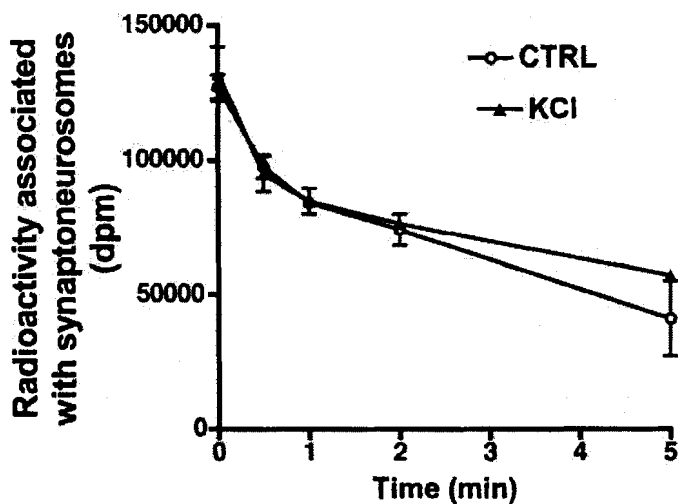


Fig 18. The lack of effect of depolarizing treatment (35 mM KCl) on release of ^3H from synaptoneurosomes pre-equilibrated with [^3H]anandamide as measured using a filtration assay.

Values represent mean dpms present on GF/C filters \pm SEM for 3 experiments. Where error bars cannot be seen, they are within the data points.

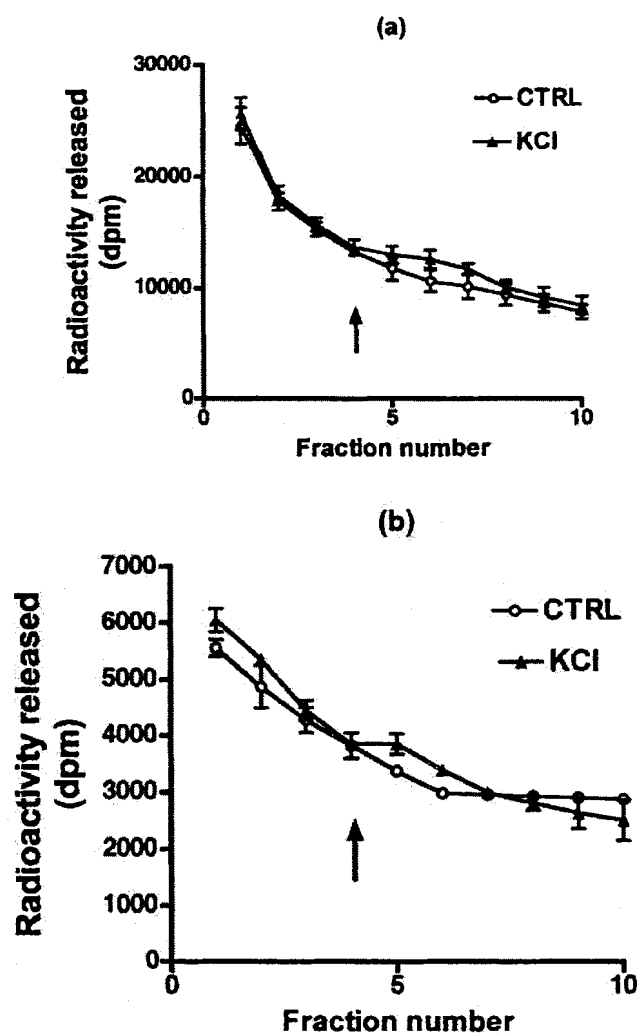


Fig 19. Release of [³H]material from superfused synaptoneurosomes after preloading with [³H]anandamide. KCl (35 mM) was introduced at the arrows (fraction 4) and remained present until the end of the superfusion.

Profiles obtained after subjecting [³H]anandamide-loaded synaptoneurosomes to a 10 min wash before starting the collection of fractions and b) profiles obtained after subjecting [³H]anandamide-loaded synaptoneurosomes to a 30 min wash before starting the collection of fractions. The flow rate was 1 ml/min and BSA (1 mg/ml) was present throughout and the volume of each fraction was 3 ml. The data points represent means of 3 different experiments.

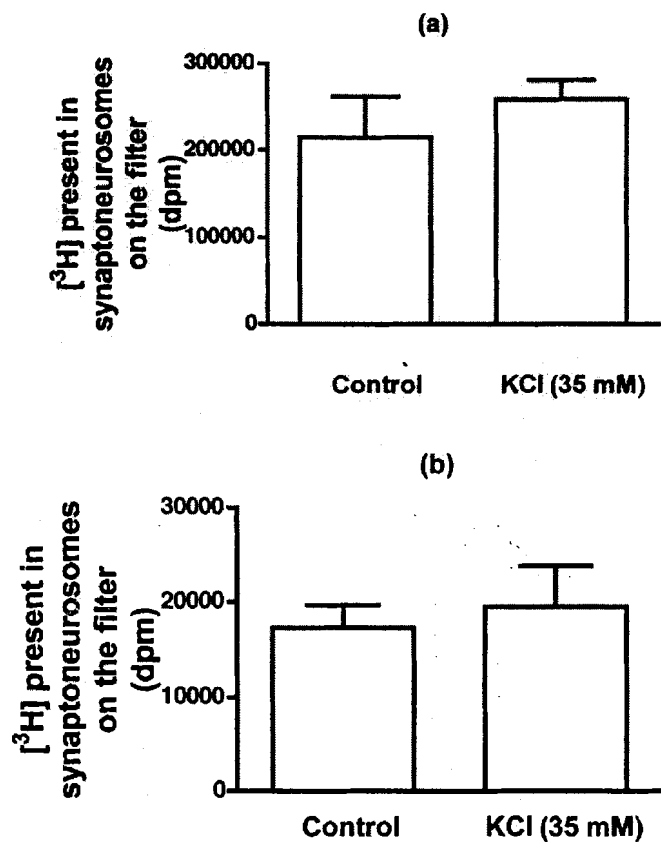


Fig 20. Comparisons of ^3H remaining in synaptoneurosomes (on filters) immediately after collection of the final fraction.

a) Synaptoneurosomes that received the 10 ml wash before starting the collection of fractions and b) Synaptoneurosomes that received the 30 ml wash before starting collection of fractions. Each column represent mean \pm SEM of 3 assays.

4.5 Discussion

4.5.1 Levels of anandamide

The HPLC investigations show that anandamide can be detected in synaptoneuroosomes at levels ranging from 6.85 ± 3.12 to 11.20 ± 2.98 pmol/mg synaptoneurosome protein (Fig 16). A number of methods have been developed to measure anandamide including GC-MS (Giuffrida and Piomelli, 1998), HPLC-MS (Koga et al., 1997) and HPLC-fluorimetric quantitation (Arai et al., 2000; Yagen et al., 2000). As a prelude to my experiments on anandamide I compared the dansyl chloride with the DBD-COCl derivatization method (Arai et al., 2000; Steffens et al., 2003; Yagen and Burstein, 2000). I found both of them to give linear responses with an anandamide standard, however the DBD-COCl derivatization method was preferred because the dansyl method was less sensitive and more complicated because water must be totally eliminated from the derivatization step and sometimes multiple peaks occurred. The amounts of anandamide detected in the saline were not found to change significantly when the 35 mM KCl depolarizing treatment was used. Likewise, no significant depolarization-related differences in the levels of anandamide remaining in synaptoneuroosomes were detected. If the biosynthesis of anandamide is increased by depolarization one might expect its synaptoneurosomal levels to rise, however clearly this did not occur. Alternatively, one could argue that any anandamide generated above the resting level might exit the synaptoneurosomal compartment rapidly, leaving intracellular levels unchanged. Again there was no evidence for this based on measurement of endogenous levels of anandamide. It must be stressed that although anandamide can be detected routinely in synaptoneuroosomes using DBD-COCl

derivatization, the technique may be unsuitable for measuring small differences which may exist between treatments. Another part of this study involved performing experiments with the anandamide precursor [^3H]ethanolamine. Ethanolamine has been shown to be a useful substrate for anandamide synthesis in neuronal cell cultures (Di Marzo et al., 1994). The amount of organosoluble ^3H (generated from [^3H]ethanolamine) in synaptoneurosomes can be used as an indicator of [^3H]anandamide biosynthesis, while organosoluble ^3H in the saline can provide a measure of anandamide release. My results (Fig. 17a and b) show that organosoluble ^3H derived from [^3H]ethanolamine is clearly present both in synaptoneurosomes and the saline, however in agreement with my HPLC analyses, no depolarization-dependent enhancement of [^3H]anandamide formation (measured as organosoluble ^3H) from [^3H]ethanolamine in synaptoneurosomes was observed. Moreover, I found no evidence for depolarization-enhanced release of organosoluble ^3H from synaptoneurosomes into the surrounding saline (which contained BSA). Although organosoluble ^3H is produced in significant quantities during incubation, it represents only 5~8% of the total ^3H . Thus under these conditions, > 90% of [^3H]ethanolamine is not metabolized to anandamide by synaptoneurosomes. Note that (as described in Chapter 5) when synaptoneurosomes and synaptosomes are pre-incubated with [^3H]ethanolamine and then superfused, marked depolarization-induced increases in the release of exclusively [^3H]ethanolamine can be demonstrated (Liao and Nicholson, 2005). In conclusion therefore, anandamide (measured as organosoluble ^3H) is capable of being synthesized from [^3H]ethanolamine in resting synaptoneurosomes but only to a limited extent. Depolarization (using 50 mM KCl) does not significantly

enhance anandamide synthesis in synaptoneurosomes or accumulation in the surrounding medium as measured in synaptoneurosomal suspensions. Depolarization activates Ca^{2+} influx, which is necessary for Ca^{2+} -dependent synthesis of anandamide in other systems (Glitsch et al., 2000; Di Marzo et al., 1994). More recent evidence indicates that Ca^{2+} entry may not be necessary for anandamide synthesis (Kim et al., 2002). Thus the Ca^{2+} supply for anandamide synthesis may be stored in organelles, such as mitochondria or endoplasmic reticulum which are present in structures like synaptoneurosomes. Other work indicates that FAAH may be involved in the synthesis of anandamide in brain under specific conditions such as post-mortem accumulation of anandamide (Patel et al., 2005). The mechanism of anandamide biosynthesis in the resting nerve requires more investigation.

4.5.2 Efflux of [^3H] from synaptosomes preloaded with [^3H]anandamide

Several reports have appeared describing the cellular uptake of anandamide and it is commonly accepted that anandamide translocation is energy-independent (Mechoulam and Deutsch, 2005). However, little is known about the fate of anandamide following uptake and in particular whether depolarization is capable of evoking the release of sequestered anandamide. To investigate this possibility, I loaded synaptoneurosomes with [^3H]anandamide and measured resting and depolarization-evoked release over the short term (filtration assay) and longer term (superfusion assay). My data (Figs. 18 and 19) suggest that anandamide is steadily released from synaptoneurosomes over time and that depolarization of synaptoneurosomes with KCl does not stimulate efflux of anandamide in the early stages (Fig. 18) or later (Fig 19) when significant quantities of anandamide still remain internalized (Fig. 20a and b). In

as much as these results strongly suggest that anandamide is not accumulated by synaptic vesicles and released exocytotically they also show convincingly that extraneuronally sequestered anandamide is not released from synaptoneurosomes by any other mechanism activated by depolarization. To facilitate depolarization-evoked release from synaptoneurosomes, I routinely used 0.1% BSA in the assays, since others have reported its utility in trapping extracellularly released anandamide (Bojesen and Hansen, 2003; Giuffrida et al., 2000). In my experiments anandamide release continues for more than 20 minutes and even after this significant amounts of [³H]anandamide remain in synaptoneurosomes. The avid sequestration of exogenously applied [³H]anandamide by synaptoneurosomes is consistent with the findings of Hillard and Jarrahian (2000).

In summary, the efflux of [³H]anandamide from resting synaptoneurosomes occurs readily. This process appears largely driven by diffusional run down of anandamide from a higher concentration to lower concentration assisted by binding to BSA in the saline. This efflux process is not significantly affected by depolarization in the early or late phases and, at the end of the time course, substantial levels of residual [³H]anandamide are still observed in synaptoneurosomes. As PMSF was routinely added to prevent anandamide degradation, it may not represent a truly physiological situation since the activity of FAAH would be very low. However, it is unlikely that [³H]anandamide is trapped within the synaptoneurosomal compartment as a result of inhibition of a PMSF-sensitive process since similar results were observed in initial experiments where PMSF was absent. This investigation demonstrates that anandamide (as assessed by HPLC and organosoluble ³H) is synthesized in

synaptoneuroosomes and, accordingly, the necessary enzyme(s) and substrate(s) must be present. However, the results strongly suggest that the amounts of anandamide synthesized in synaptoneuroosomes during rest and during depolarization are similar and that KCl-induced depolarization does not enhance release. While synaptoneuroosomes support many of the processes attributed to the synapse *in situ* it is possible that this sub-cellular neuronal preparation, may not retain functional carrier-mediated pathways for anandamide release although simple diffusional efflux should not be impaired.

CHAPTER 5

DEPOLARIZATION-INDUCED RELEASE OF ETHANOLAMINE FROM BRAIN SYNAPTIC PREPARATIONS *IN VITRO*

5.1 Abstract

The research described in this chapter focuses on the release of the anandamide metabolite ethanolamine from mouse brain synaptosomes and synaptoneuroosomes. Depolarizing treatments [veratridine (50 μ M), KCl (35 mM) and 4-aminopyridine (2 mM)] increased the release of [3 H]ethanolamine from superfused synaptosomes preloaded with this substance. Strong inhibitory effects of tetrodotoxin (2 μ M) on veratridine- and 4-aminopyridine-stimulated release of [3 H]ethanolamine from synaptosomes were observed but KCl-evoked and resting release was not affected. In the absence of calcium, a reduction in the resting release of [3 H]ethanolamine occurred and release evoked by veratridine, and KCl was also reduced. 5 mM ethanolamine (but not 5 mM serine or 5 mM choline) increased the efflux of preloaded [3 H]ethanolamine from synaptosomes, although this was not accompanied by membrane depolarization. When these experiments were performed using synaptoneuroosomes, qualitatively similar results were obtained. However, the resting and evoked release of [3 H]ethanolamine in synaptoneuroosomes was approximately 2.5-fold higher compared to synaptosomes on a brain equivalent amount, suggesting that uptake and release occur at other sites in addition to the nerve ending. My data are consistent with the idea that a significant amount of ethanolamine accumulates presynaptically and a

proportion of this undergoes calcium-dependent release upon depolarization possibly via classical exocytosis. In contrast, ethanolamine-induced release of [³H]ethanolamine likely involves mostly diffusional exchange across the neuronal membrane rather than base exchange. The present results add support to the concept that ethanolamine may play a role as a synaptic signalling molecule in mammalian brain.

5.2 Introduction

After the endocannabinoid anandamide is taken up into nerve cells and hydrolyzed by FAAH, ethanolamine is released. Breakdown of membrane phospholipids such as phosphatidylethanolamine represents another important mechanism for the intracellular release of ethanolamine (Spanner et al., 1978). Ethanolamine has been detected in human serum at approximately 12 μ M (Baba et al., 1984), although the serum levels of this substance range from 5-50 μ M in other species (Houweling et al., 1992; Spanner et al., 1978). In marked contrast to this, the intracellular concentrations of ethanolamine in liver for example are close to 0.5 mM (Houweling et al., 1992). Moreover, Ellison and co-workers observed the resting levels of ethanolamine in various regions of mammalian brain to lie between 0.2 and 0.9 mM (Ellison et al., 1987). Base exchange reactions are strongly implicated in the incorporation of extracellular ethanolamine into phosphatidylethanolamine in the membrane of nerve endings (Holbrook and Wurtman, 1988) and such reactions also appear to be responsible for the release of ethanolamine accompanying ischemic episodes in brain (Buratta et al., 1998). However, in contrast to these findings Perschak et al., (1986) observed no significant change in the concentrations of serine and choline when ethanolamine efflux increased more than 3-fold after stimulation of cortico-pontine

fibers. While there is no dispute that ethanolamine is released both intracellularly and extracellularly, base-exchange reactions alone cannot explain the phenomena. In fact, Kiss et al. (1999) could find no evidence that base-exchange pathways contribute significantly to the intracellular and extracellular release of ethanolamine.

It has been suggested that much of the ethanolamine present in neurons arises from the transport of free ethanolamine from the extracellular compartment (Massarelli et al., 1986) and uptake is known to proceed by high affinity mechanisms both in the retina (Pu and Anderson, 1984) and cerebral neurons in culture (Massarelli et al., 1986).

After loading rabbit retina photoreceptor cells with [^3H]ethanolamine, Pu and Anderson (1984) were unable to demonstrate any increase in efflux of radiolabel upon exposure to high [K^+] medium. Similarly, using a push-pull cannula method, Van der Heyden et al. (1979) reported no increase in the release of ethanolamine from the substantia nigra in response to depolarizing concentrations of [K^+]. However, electrical stimulation was found to cause the concentration of ethanolamine in perfusates from the avian optic tectum to rise (Wolfensberger et al., 1982). Ethanolamine release from rat pontine nuclei is also enhanced during electrical stimulation of cortical afferents (Pershak et al., 1986), and mechanisms involving membrane phospholipid precursors, base exchange and /or release of free ethanolamine from intraneuronal stores have been proposed. Additionally, ethanolamine is calcium-dependently release from hair cell cluster within the trout macula in response to challenge with an elevated [K^+] (Drescher et al., 1987).

Evidence for a regulatory role of ethanolamine at the synapse is provided by the observations that this substance intensifies both glutamate-dependent excitation and

GABA-dependent inhibition of avian tectal neurons. This latter effect appears to be in part related to ethanolamine's ability to inhibit GABA aminotransferase in brain (Loscher, 1983). Moreover, like phosphoethanolamine, ethanolamine appears to promote acetylcholine production in developing neurons by enhancing choline accumulation (Bostwick et al., 1989). In many tissues ethanolamine appears to affect cellular growth regulation (Kiss et al., 1999).

Previously in Chapter 4, I described my investigations into the possible release of anandamide from superfused synaptoneurosomes following extended incubation with a potential precursor [³H]ethanolamine. My experiments demonstrated enhanced release of radiolabel under depolarizing conditions, however, chloroform-methanol partitioning data revealed that unlike anandamide (which is chloroform soluble), the released radioactivity was exclusively methanol-water soluble. Since these initial superfusion experiments indicated a release pattern reminiscent of conventional neurotransmitters, the present investigation was undertaken to characterize this phenomenon further and clarify some of the mechanisms involved.

5.3 Methods

Male CD1 mice (20-25g) were used for these experiments and the isolation of synaptosomes and synaptoneurosomes was performed as described in Chapters 2 and 3.

5.3.1 Superfusion experiments

Synaptoneurosomes or synaptosomes (500 μ l) were preincubated for 5 min. and then 10 μ Ci [1-³H]ethanolamine hydrochloride (specific activity 23 Ci/mmol; Amersham Biosciences UK Ltd) was added. Incubations with radiolabel were carried

out at 32 °C for 50 min and were terminated by resuspending synaptic particles in 10 ml ice-cold saline and centrifuging (1000 x g; 10 min). Pellets were resuspended in 800 µl of ice-cold saline, and synaptosomes (0.82 ± 0.19 mg protein) or synaptoneuroosomes (0.90 ± 0.16 mg protein) were placed in each of ten Swinnex filtration units (Millipore) containing Whatman GF/B filters. Superfusions were conducted as described previously (Chapter 4). Briefly, each filter unit was continuously supplied with standard saline (containing 0.1% BSA) from above by a reservoir (30 ml syringe barrel). Saline superfusates were then routed to an LKB 2070 Ultrorac fraction collector modified to collect 10 superfusates simultaneously. The flow rate was held at 1 ml/min by a 10-channel peristaltic pump (Watson Marlow 503S) placed between the filtration units and the fraction collector. The effects of various treatments were examined after nerve preparations had been superfused with 20 ml control saline to reduce extracellular radioactivity and establish consistent rates of release. At this point, sample collection was started. Ten 3 ml fractions were collected from each filtration unit, and radioactivity in each was quantitated by liquid scintillation counting. Interaction of depolarizing treatments and other drugs with the synaptic preparations normally coincided with fraction 4. Water insoluble drugs were introduced into the saline in dimethyl sulfoxide (DMSO). In these situations, the concentration of DMSO did not exceed 0.1%, and appropriate amounts were present in control superfusions. Where salines containing elevated [KCl] were used, the [NaCl] was reduced by a corresponding amount to maintain isoosmotic conditions. Superfusion experiments were carried out at ambient temperature 23–25 °C.

5.3.2 Depolarization assay

The plasma membrane potential of synaptosomes and synaptoneuroosomes was measured on the basis of fluorescence changes to the voltage-sensitive probe rhodamine 6G as we have previously described (Nicholson et al., 2003). Rhodamine 6G is very well-suited to these studies since Mandala et al. (1999) have shown that this fluoroprobe is specific for the plasma membrane potential and is not influenced by treatments known to change the mitochondrial membrane potential. We consider any interference with our measurements of plasma membrane potential due to rhodamine 6G associating with/dissociating from intrasynaptosomal or intrasynaptoneurosomal mitochondria to be minimal, since blockade of sodium channels with TTX completely eliminates any depolarizing influence of veratridine. Calibrations were performed in the presence of different concentrations of external $[K^+]$ and the reduced Goldman equation was used to calculate membrane potentials.

5.3.3 Analysis of radioactivity in superfusates

To investigate the nature of the radioactivity released from nerve fractions, samples of their superfusates were subjected to 1) precipitation using TCA, 2) partitioning using the chloroform:methanol lipid extraction procedure of Bligh and Dyer (Bligh and Dyer, 1959) and 3) paper chromatography (Whatman, 3 mm) using n-butanol:acetic acid:water (120:30:50) and isopropanol:ethanol:concentrated HCl:water (75:75:5:45). Appropriate regions of the paper chromatogram were cut out, and, after addition of water, radioactivity was measured by liquid scintillation counting. Ethanolamine and phosphoethanolamine standards were visualized using ninhydrin and molybdate

reagents respectively. Choline, acetylcholine, methylcholine and dimethylcholine were detected using iodine vapor.

5.3.4 Protein assay

Protein assays were conducted according to the method of Lowry as adapted by Peterson (Peterson, 1977).

5.3.5 Analysis of results

Results are expressed as mean \pm standard error (SEM) with the level replication defined in fig legends. One-way ANOVA and Student's t test used in statistical evaluation of data employed Prism 3 software (Graphpad, CA, USA). P values < 0.05 were taken as significant.

5.4 Results

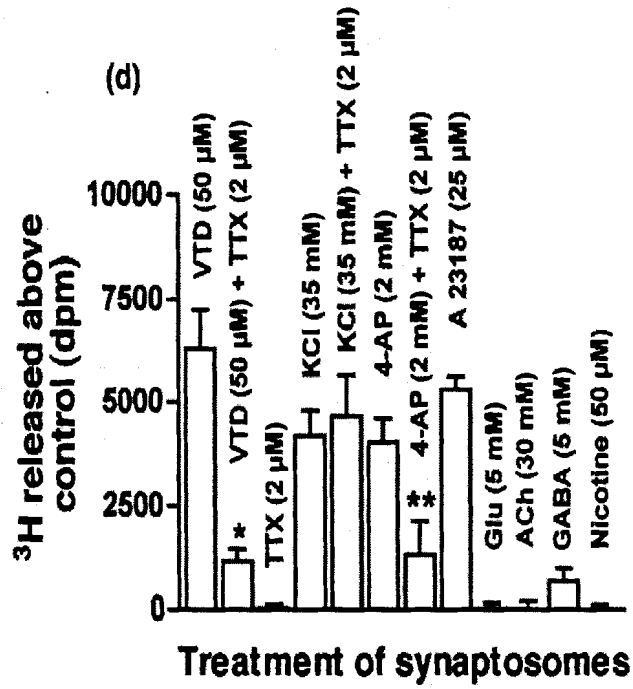
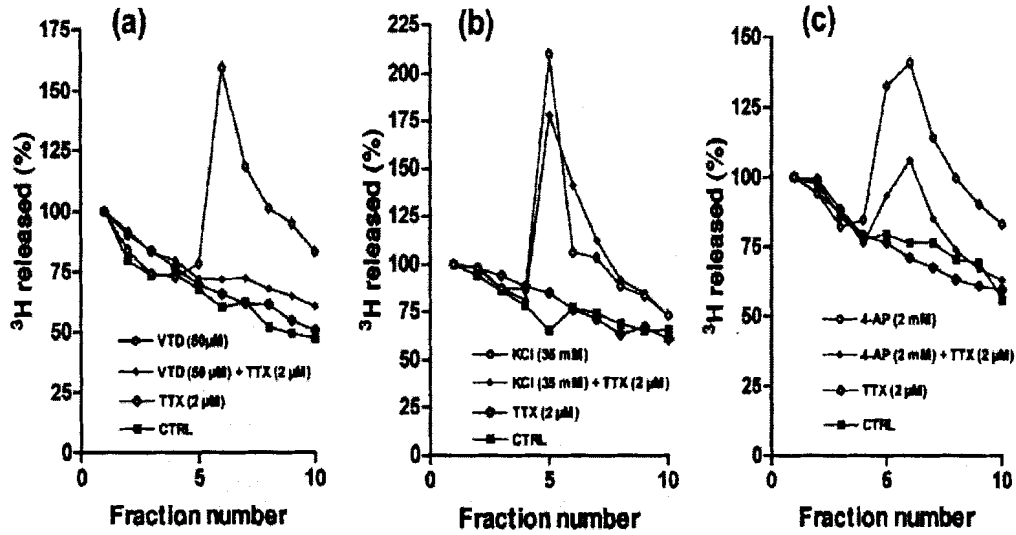
5.4.1 Release of ethanolamine from synaptosomes and synaptoneuroosomes

In synaptosomal preparations preloaded with [^3H]ethanolamine, both veratridine (50 μM), 4-aminopyridine (2 mM) and saline containing 35 mM KCl produced a significant increase in the rate of release of radioactivity (identified as [^3H]ethanolamine, see later) above control values (Fig. 21a-d). The results also demonstrate that veratridine- and 4-aminopyridine-induced release is markedly sensitive to inhibition by tetrodotoxin (2 μM), in clear contrast to the KCl response, and that the calcium ionophore A23187 (25 μM) also stimulates release. No significant effect on synaptosomal release of radiolabel was observed with L-glutamate (5 mM), acetylcholine (30 mM), GABA (5 mM) or nicotine (50 μM) (Fig. 21d). The basal

efflux of [³H]ethanolamine from synaptosomes was found to be reduced (circa 20%) when calcium was omitted from superfusing saline containing 2 mM EGTA (Fig. 22a), and the ability of veratridine or KCl to stimulate release was also attenuated in the absence of calcium (Fig. 22b). When [³H]ethanolamine-loaded synaptosomes were challenged with serine (5 mM), choline (5 mM) or ethanolamine (5 mM), increased release was only observed upon challenge with ethanolamine. The latter response displayed dependency on external calcium (Fig. 23) but, in contrast to the effects of KCl and veratridine, did not involve membrane depolarization (data summarized in Table 3, Chapter 6).

Fig 21. Release of [³H]ethanolamine from superfused synaptosomes.

In (a), (b) and (c), representative profiles illustrate the stimulatory profiles of 50 μ M veratridine (VTD), 35 mM KCl and 2 mM 4-aminopyridine (4-AP) and the inhibition of VTD and 4-AP responses by 2 μ M tetrodotoxin (TTX). Values represent ³H released as a percentage of ³H in fraction 1. The histogram (d) summarizes the full datasets of treatments shown in (a–c) together with the stimulatory effect of A23187 (25 μ M) and failure of L-glutamate (Glu), acetylcholine (ACh), GABA and nicotine to elicit release. Vertical columns represent mean increases in release above control values, and bars the SEM of 3–6 independent experiments (* = significantly different from VTD alone ($P < 0.01$); ** = significantly different from 4-AP alone ($P < 0.05$); TTX had no effect on KCl-induced release, $P > 0.05$).



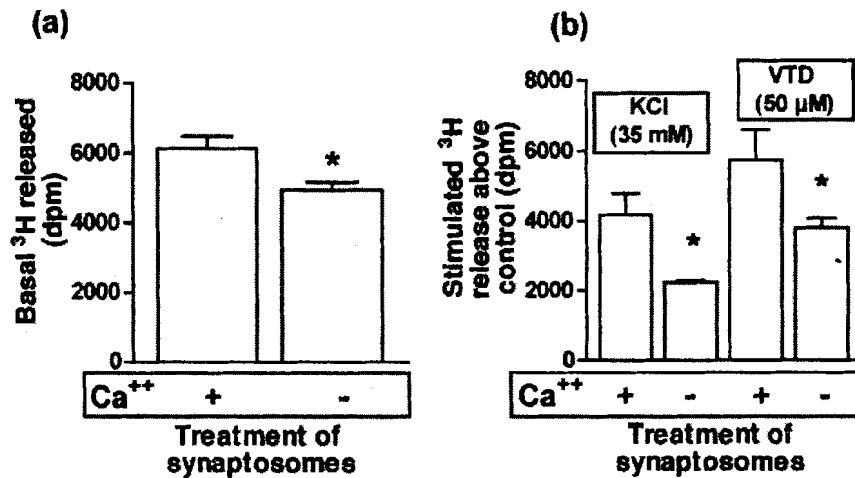


Fig 22. Effect of ionic calcium on basal (a) and chemically evoked (b) release of [³H]ethanolamine from superfused synaptosomes.

Data points represent means \pm SEM of 4–11 independent determinations. Asterisks signify statistically significant differences. In (a), basal release was significantly lower ($P < 0.015$), and, in (b), KCl- and VTD-evoked release was significantly reduced ($P = 0.017$ and $P = 0.034$ respectively), when Ca^{2+} was omitted from the saline.

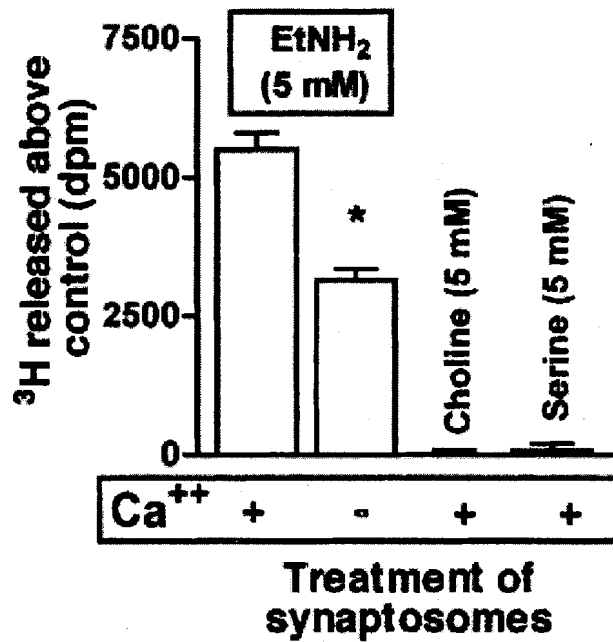


Fig 23. Challenge with 5 mM ethanolamine (EtNH₂), but not the nitrogenous bases serine (5 mM) or choline (5 mM), causes efflux of [³H]ethanolamine from superfused synaptosomes.

Ethanolamine-induced release is partially dependent on the presence of Ca²⁺ in the superfusing saline (* = significantly different from release by ethanolamine with Ca²⁺ present (P < 0.01). Vertical columns represent means ± SEM of 3 experiments.

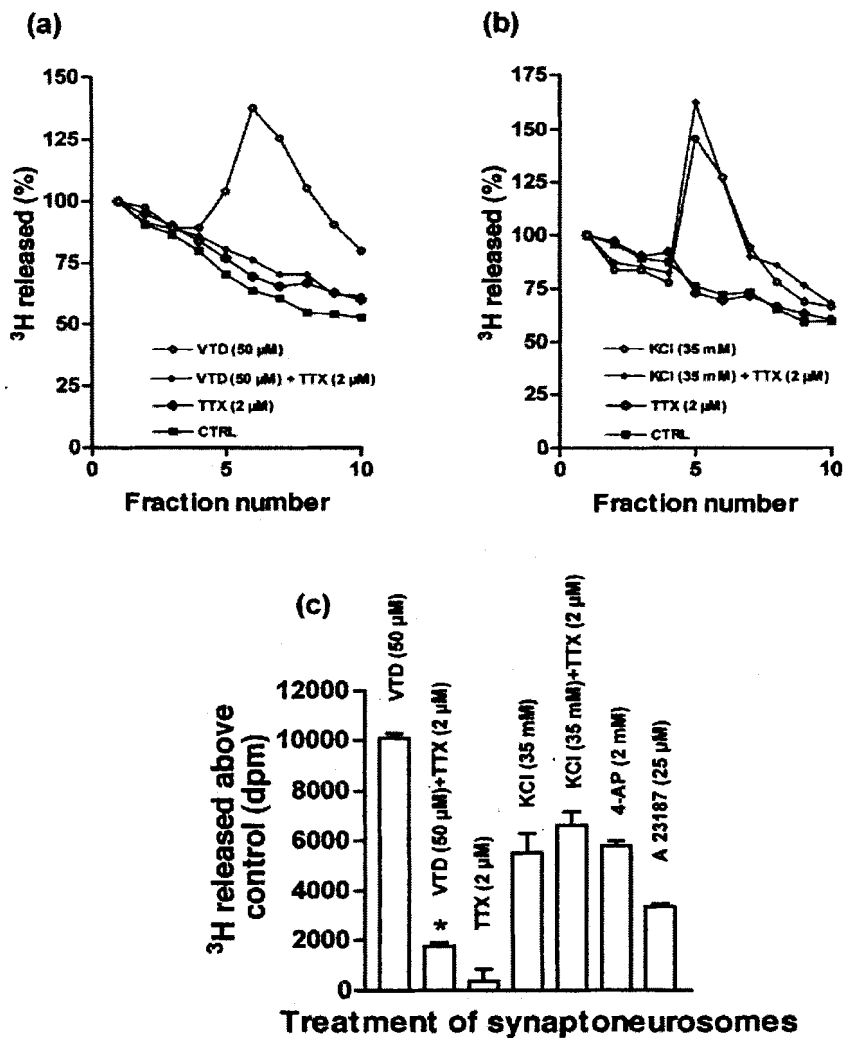


Fig 24. Release of [³H]ethanolamine from superfused synaptoneuroosomes.

In (a) and (b), representative stimulatory profiles for 50 μM veratridine (VTD) and 35 mM KCl in the presence and absence of 2 μM tetrodotoxin (TTX) are shown. Values represent ³H released as a percentage of ³H present in fraction 1. The histogram (c) shows full datasets of treatments (a) and (b) and also the stimulatory effects of 4-aminopyridine (4-AP) and A23187 (25 μM). Vertical columns represent mean increases in release above control values, and bars the SEM of 3–6 independent experiments (* = significantly different from VTD alone (P < 0.01)).

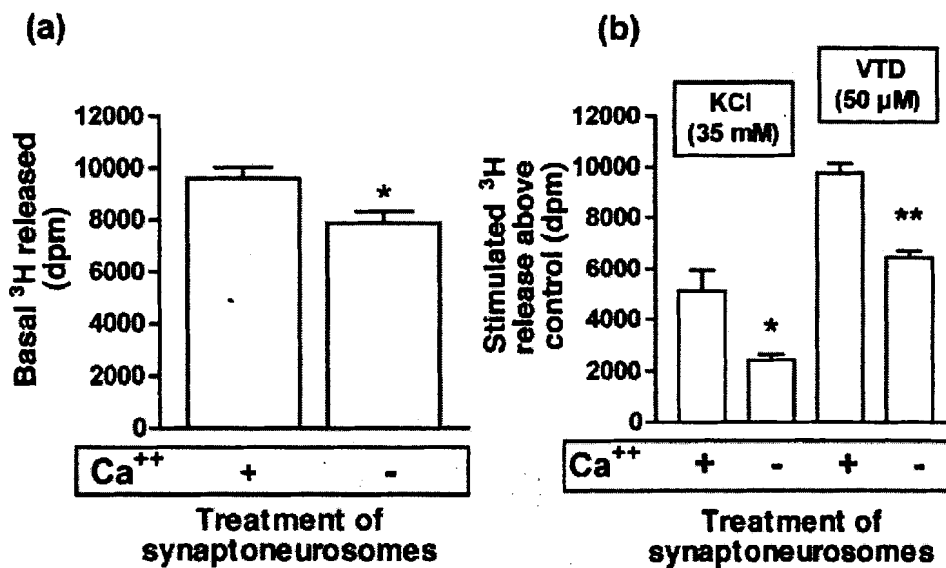


Fig 25. Effect of Ca^{2+} on (a) basal and (b) KCl- and VTD-evoked release from superfused synaptoneurosomes preloaded with [^3H]-ethanolamine.

Values as mean \pm SEM of 4–8 experiments. In (a), * signifies $P = 0.02$. In (b), * and ** signify P values of 0.02 and <0.01 respectively compared to the Ca^{2+} replete situation.

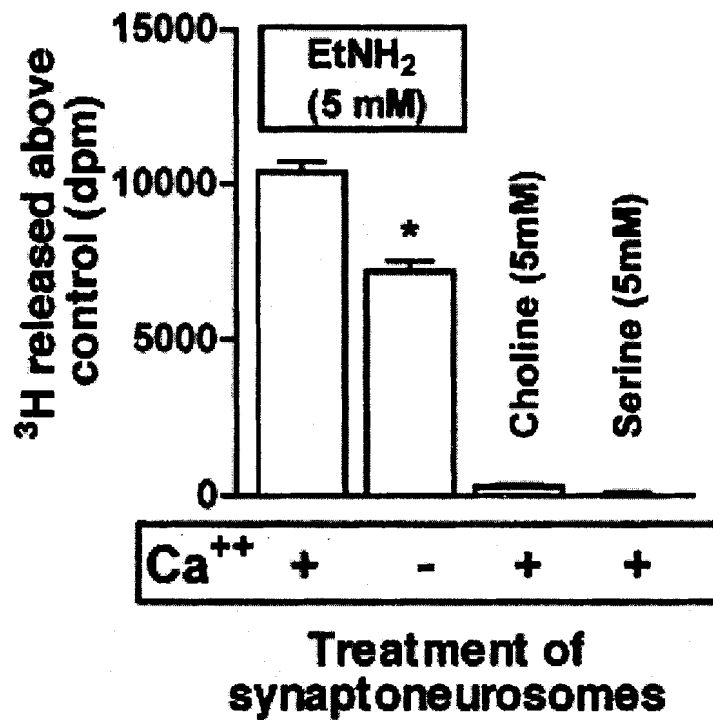


Fig 26. Effect of the nitrogenous bases ethanolamine (EtNH₂), serine and choline (all 5 mM) on release of [³H]ethanolamine from superfused synaptoneuroosomes. Columns represent means, and error bars the SEM of 3 experiments; *Indicates significantly lower release (P = 0.012) compared to when Ca²⁺ is present.

In experiments with synaptoneuroosomes, very similar pharmacological profiles were observed (Figs. 24, 25 and 26), although, on a brain equivalent basis, the absolute level of KCl- or veratridine-stimulated [^3H]ethanolamine release was greater (circa 2.5-fold) compared with synaptosomes. KCl-evoked [^3H]ethanolamine release above control (as a percentage of total [^3H] recovered in the 10 superfusate fractions plus radioactivity remaining on the filter) averaged $5.14 \pm 0.99\%$ (synaptosomes) and $5.63 \pm 0.67\%$ (synaptoneuroosomes).

5.4.2 Nature of radioactivity released from synaptic preparations under resting and depolarized conditions

When radioactivity present in the superfusate samples was partitioned with chloroform and methanol (Bligh and Dyer, 1959) the increase in radioactivity after stimulation with KCl and veratridine was exclusively restricted to the water:methanol phase (Table 1). Treatment with TCA effectively precipitated the bovine serum albumin contained in the superfusion saline, but, in contrast, the levels of radioactivity in control and stimulated superfusate samples were not reduced by precipitation treatment (Table 2). Superfusate samples obtained from synaptosomal experiments at peak stimulation together with matched controls were also compared quantitatively using paper chromatography in two separate solvent systems (Fig. 27). In each solvent system, the radioactivity migrated as a single peak coincident with authentic ethanolamine standard, and its level was significantly higher in KCl and veratridine peak stimulation samples compared to respective controls (Fig. 27). Superfusates from synaptoneuroosomes subjected to these treatments yielded very similar ^3H profiles to those shown for synaptosomes in Fig. 27 after chromatography (data not shown).

Phosphoethanolamine ($R_F = 0.18$), a potential contender for release, was clearly separated from ethanolamine ($R_F = 0.44$) using *n*-butanol:acetic acid: water (120:30:50). In the isopropanol:ethanol:concentrated HCl:water (75:75:5:45) solvent system, choline ($R_F = 0.54$), acetylcholine ($R_F = 0.57$), methylethanolamine ($R_F = 0.55$) and dimethylethanolamine ($R_F = 0.55$) were sufficiently more mobile than ethanolamine ($R_F = 0.44$) (and even more so after a double development) to conclude that none of these potential metabolites could account for the radioactivity released during basal and stimulated release.

Table 1. Partitioning of radioactivity from peak release and control supernatants into chloroform:methanol

Treatment	Fraction	n	Radioactivity recovered (dpm)	
			H ₂ O:MeOH fraction	CHCl ₃ fraction
Control	SS	7	1500 ± 144	264 ± 204
KCl (35 mM)	SS	4	2664 ± 96 (<i>P</i> < 0.01)	120 ± 168
VERATRIDINE (50 μM)	SS	3	3072 ± 216 (<i>P</i> < 0.01)	552 ± 144
Control	SN	4	2064 ± 336	120 ± 138
KCl (35 mM)	SN	4	4320 ± 456 (<i>P</i> < 0.01)	696 ± 192
VERATRIDINE (50 μM)	SN	3	5040 ± 1176 (<i>P</i> < 0.05)	24 ± 141

Values represent mean total radioactivity ± SEM; P values indicate significant difference from respective controls; SS = synaptosomes, SN = synaptoneuroosomes.

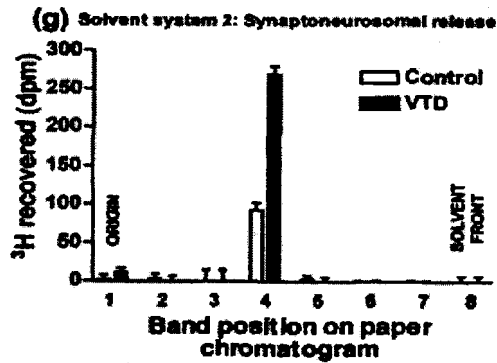
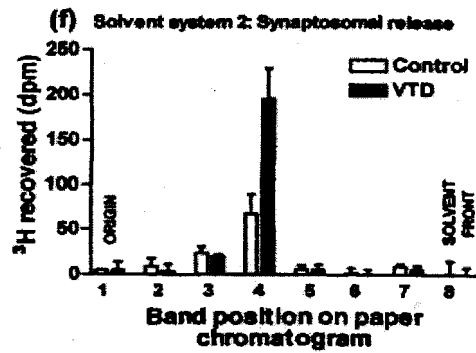
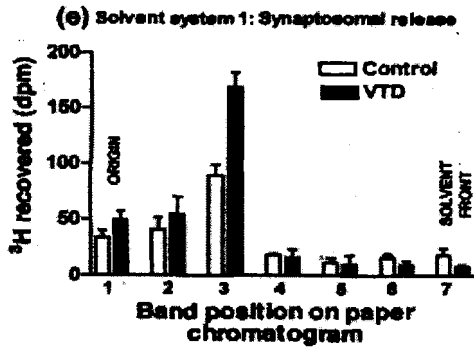
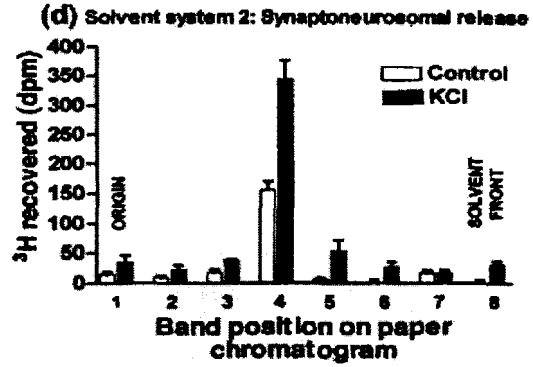
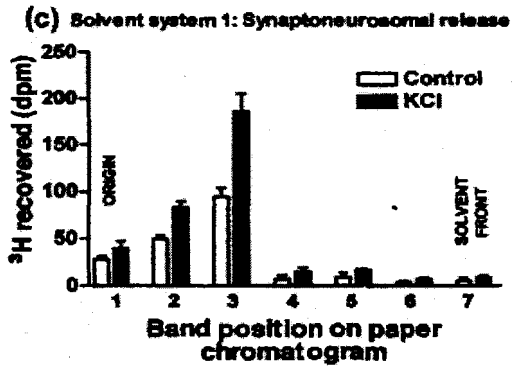
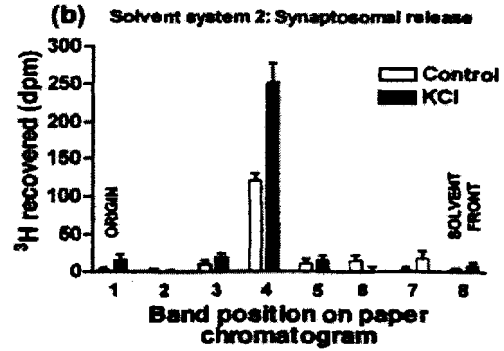
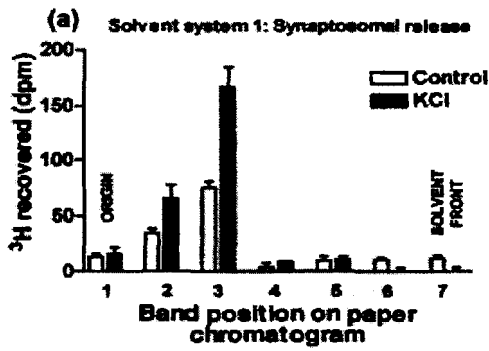
Table 2. Treatment of control and peak release supernatants with trichloroacetic acid (TCA)

Treatment	Fraction	n	³ H in original superfusate sample (dpm/ml)	³ H in sample after TCA pptn. (dpm/ml)	³ H in precipitate (dpm)
Control	SS	3	580 ± 43	472 ± 31	16 ± 24
KCl (35 mM)	SS	3	968 ± 36	1006 ± 55	13 ± 10
VERATRIDINE (50 μM)	SS	3	1376 ± 15	1309 ± 61	25 ± 9
Control	SN	3	577 ± 30	640 ± 14	24 ± 10
KCl (35 mM)	SN	3	1389 ± 60	1402 ± 30	79 ± 19
VERATRIDINE (50 μM)	SN	3	1465 ± 188	1306 ± 23	28 ± 27

Values represent mean radioactive concentrations ± SEM; SS = synaptosomes, SN = synaptoneuroosomes.

Fig 27. Paper chromatography of peak stimulation and control superfusate samples using n-butanol:acetic acid:water (120:30:50) (solvent system 1) and isopropanol:ethanol: concentrated HCl:water (75:75:5:45) (solvent system 2).

Ethanolamine standard was exclusively associated with band 3 (solvent system 1) and band 4 (solvent system 2), and statistically significant differences between control and stimulated treatments ($P < 0.05$) were only observed in these bands. Results show chromatograms of superfusates from KCl-stimulated synaptosomes (a and b) in each solvent system. In (c) and (d), chromatograms from veratridine-stimulated synaptosomal fractions are shown. RFs of ethanolamine and other related nitrogen-containing compounds are provided in the Results section. In solvent system 1, phosphoethanolamine resided in band 2. In solvent system 2 choline, acetylcholine, monomethylethanolamine and dimethylethanolamine were all located in bands 4 and 5 with at least 50% of each standard (as determined by spot area) always migrating in band 5 ahead of ethanolamine. All values represent means \pm SEM ($n = 3$).



5.5 Discussion

The present work demonstrates that exogenously applied [^3H]ethanolamine is taken up by synaptosomes and synaptoneurosomes alike and can be rapidly released by a depolarizing challenge during the course of superfusion. Several lines of evidence support the idea that free ethanolamine is released. The finding that the radioactivity released by veratridine and KCl is aqueous:methanol soluble excludes the possibility that the released material is an ethanolamine-coupled fatty acid such as anandamide or a phospholipid such as phosphatidylethanolamine, both of which can be generated from ethanolamine in neuronal preparations (Di Marzo, 1994; Holbrook and Wurtman, 1988). The inability of trichloroacetic acid to precipitate the released radioactivity suggests this material is not a peptide or protein to which [^3H]ethanolamine has been linked, as has been previously described (Tisdale and Tartakoff, 1988). Lastly, my chromatographic results have excluded the possibility that the released radioactivity is phosphoethanolamine, methylethanolamine, dimethylethanolamine, choline or acetylcholine, all of which can be produced from ethanolamine in mammalian brain (Andriamampandry et al., 1989; Massarelli et al., 1982).

After washing and initial superfusion of these preparations, the retention of sufficient [^3H]ethanolamine to generate the depolarization-induced increases in release during superfusion suggests that this substance is stored effectively within an intracellular compartment (or compartments). Identification of the nature of storage is beyond the scope of the present investigation. However, since the nitrogenous bases choline and serine did not induce [^3H]ethanolamine release via the classical base exchange reaction (Holbrook and Wurtman, 1988; Porcellati et al., 1971), the

possibility that the [³H]ethanolamine released originates from a [³H]ethanolamine-coupled phospholipid is not supported, although a phospholipase, capable of removing the ethanolamine base moiety from phospholipids such as phospholipase D (Pershak et al., 1986), cannot be ruled out. The failure of GABA or L-glutamic acid to stimulate [³H]ethanolamine release makes involvement of a heterocARRIER type release mechanism analogous to that reported by others (Bonanno et al., 1993; Fassio et al., 1996) unlikely. Moreover, the lack of effect of nicotine and acetylcholine on ethanolamine efflux indicates the absence of an nAChR-activated release pathway such as that established for GABA in mouse brain synaptosomes (Lu et al., 1998).

The results of the present study suggest a presynaptic release pattern for ethanolamine similar in some respects to that of a neurotransmitter or releasable signaling molecule since [³H]ethanolamine release can be evoked by veratridine, elevated [K⁺] or 4-aminopyridine. All three pharmacological manipulations are known to activate exocytotic release of neurotransmitter from synaptosomes (Sanchez-Prieto et al., 1987; Tibbs et al., 1989) as a result of rapid and sustained activation of voltage-gated sodium channels (veratridine) (Ohta et al., 1973), inhibition of K_A⁺ channels leading to oscillatory bursts of sodium channel activity (4-aminopyridine) (Kapoor et al., 1997) and direct membrane depolarization (KCl) (Blaustein and Goldring, 1975). The sensitivity of veratridine- and 4-aminopyridine-induced [³H]ethanolamine release to inhibition by tetrodotoxin shows clearly that nerve depolarization is a critical initiator of release. While 4-aminopyridine and KCl are considered the more “physiological” of the depolarizing agents employed in my investigation, the relative contributions of classical exocytosis of ethanolamine compared to depolarization-

induced reversal of the plasma membrane ethanolamine transporter remain unresolved. Both mechanisms have been reported to operate in the case of GABA release from synaptosomes undergoing chemical depolarization (Raiteri et al., 2002). Nevertheless, my finding that depolarization-induced release of [³H]ethanolamine from synaptosomes exhibits a significant dependency on extracellular Ca²⁺ lends support to the idea that exocytotic release of this substance occurs.

It is also apparent that Ca²⁺ is needed to maximize release of radiolabel following challenge with 5 mM ethanolamine, a treatment which does not affect membrane potential. Under these circumstances, a large proportion of the [³H]ethanolamine efflux is Ca²⁺-independent and probably involves a concentration gradient-driven transmembrane exchange of the unlabeled ethanolamine with an intracellular radiolabeled pool.

It is noteworthy that in the majority of my experiments we observed more than a 2-fold greater responsiveness of synaptoneuroosomes to depolarizing agents on a brain equivalent basis compared to synaptosomes indicating that the nerve ending is not the only site of release, although axonal release is considered unlikely (Pershak et al., 1986). It must also be pointed out that the absolute quantities of [³H]ethanolamine released in my experiments are relatively low, and this may argue against a classical neurotransmitter role where levels approaching the millimolar range can be required to exert a postsynaptic response.

In conclusion, this investigation highlights a novel mechanism for the release of ethanolamine from superfused nerve terminals and synaptoneuroosomes isolated from mammalian brain. The role of presynaptically released ethanolamine may be restricted

to indirect augmentation of postsynaptic amino acid neurotransmitter responses (Loscher, 1983; Wolfensberger, 1982), as previously noted, however, the possibility that a discrete receptor exists postsynaptically for ethanolamine or that this substance participates in some form of depolarization-activated regulation of presynaptic function warrants careful investigation. In this latter context, my studies (Chapter 6) identify a mechanism involving ethanolamine which appears to be of relevance to presynaptic regulation.

Acknowledgment

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CHAPTER 6

ETHANOLAMINE AND RELATED AMINO ALCOHOLS INCREASE BASAL AND EVOKED RELEASE OF [³H]-D-ASPARTIC ACID FROM SYNAPTOSOMES BY ENHANCING THE FILLING OF SYNAPTIC VESICLES

6.1 Abstract

This part of my research examines the effects of ethanolamine and other amino alcohols on the dynamics of acridine orange, oxonol V, and [³H]-D-aspartic acid in synaptic preparations isolated from mammalian brain. Ethanolamine concentration-dependently enhanced acridine orange release from synaptosomes. Similar effects were observed with methylethanolamine and dimethylethanolamine, but not choline. The enhancement of acridine orange efflux by ethanolamine was independent of extrasynaptosomal calcium (in contrast to KCl-induced acridine orange efflux), was unaffected by tetrodotoxin and did not involve depolarization of the synaptosomal plasma membrane. KCl was unable to release acridine orange from synaptosomes following exposure to ethanolamine, however ethanolamine and other amino alcohols were found to enhance both basal and KCl-evoked release of [³H]-D-aspartic acid from synaptosomes. Using isolated synaptic vesicles I demonstrate that amino alcohols are able to 1) abolish the ATP-dependent intravesicular proton concentration (i.e. stimulate efflux of acridine orange) in a similar way to CCCP, 2) increase the ATP-supported transvesicular membrane potential (i.e. quench oxonol V fluorescence) in contrast to CCCP and 3) enhance intravesicular uptake of [³H]-D-aspartic acid. These results suggest that positively charged, membrane impermeant amino alcohol species are

generated within synaptic vesicles as they sequester protons. Cationic forms of these amino alcohols boost the transvesicular electrical potential which increases transmitter uptake into synaptic vesicles and facilitates enhancement of basal and evoked release of transmitter. My data suggest a potential role for ethanolamine and related amino alcohols in the regulation of synaptic vesicle filling. These findings may also have relevance to neuropathophysiological states involving altered production of ethanolamine.

6.2 Introduction

The resting levels of ethanolamine in mammalian brain are known to be in the low millimolar range (Ellison et al., 1987) and, despite a considerable amount of research, the role that this amino alcohol plays in normal brain function and neuropathological states remains to be fully elucidated. Studies indicate that ethanolamine facilitates both amino acid and cholinergic neurotransmission in the brain. For example, Wolfensberger et al. (1982) found that ethanolamine augments glutamate-dependent excitation and GABA-dependent inhibition of avian tectal neurons. The enhancement of GABA-induced depression by ethanolamine in these experiments was suggested to be related to the capacity of this amino alcohol to reduce GABA breakdown via inhibition of GABA aminotransferase (Loscher, 1983). Ethanolamine markedly increases the levels of the amino acid neurotransmitters aspartic acid and glutamic acid in microdialysates from the anterior hippocampus (Buratta et al., 1998). Similarly, ethanolamine and other amino alcohols were found to selectively stimulate K^+ -evoked acetylcholine release from hippocampal slices, which was attributed to activation of

calcium entry through L-type calcium channels (Bostwick et al., 1992; Bostwick et al., 1993).

In other studies, high affinity uptake of ethanolamine has been demonstrated in the retina (Pu and Anderson, 1984) and in cultured cerebrocortical neurons (Massarelli et al., 1982) observations which accord with ethanolamine's function as an important metabolic precursor molecule for acetylcholine and phosphatidylcholine (Corazzi et al., 1986) and the involvement of ethanolamine in phospholipid metabolism (Porcellati et al., 1971).

Several investigations show that ethanolamine release in a number of brain regions is associated with electrical or chemical depolarization. For example, the levels of ethanolamine rise in perfusates of the avian optic tectum as a result of electrical stimulation (Wolfensberger et al., 1982), and release of this substance from rat pontine nuclei can be electrically evoked (Pershak et al., 1986). Enhanced levels of ethanolamine were also demonstrated in dialysates of rat striatum during challenge with an elevated concentration of K^+ (Korf and Venema, 1985), however, the same depolarizing treatment failed to increase ethanolamine levels in perfusates from rat substantia nigra (Van der Heyden et al., 1979). In our laboratory we found that synaptosomes and synaptoneuroosomes release [3H]ethanolamine during superfusion in a calcium-dependent fashion in response to a KCl challenge (Liao and Nicholson, 2005) and suggested that depolarization-evoked release of [3H]ethanolamine from the nerve ending may occur via classical exocytosis. Associated experiments using the pH-sensitive dye acridine orange demonstrated that ethanolamine rapidly accesses synaptic vesicles within the nerve ending. I now report on these and subsequent observations

which led me to develop and test the hypothesis that ethanolamine modifies presynaptic release of [³H]-D-aspartic acid by affecting synaptic vesicle function.

6.3 Methods

6.3.1 Chemicals and radiochemicals

Ethanolamine, methylethanolamine, dimethylethanolamine, choline, carbonylcyanide-*m*-chlorophenylhydrazone (CCCP), veratridine, acridine orange and tetrodotoxin were obtained from Sigma-Aldrich Canada. Rhodamine 6G was purchased from Eastman Kodak, Rochester, NY, USA. Oxonol V was supplied by Molecular Probes Inc., Eugene, OR, USA. [³H]-D-aspartic acid (specific activity 23.9 Ci/mmol) was from Perkin Elmer:NEN, Boston, MA, USA.

6.3.2 Isolation of synaptosomes from mouse brain

The whole brain material from three mice (acridine orange experiments) or one mouse ([³H]-D-aspartic acid release experiments) was cooled rapidly in ice-cold 0.32 M sucrose (adjusted to pH 7.4 with Tris base), and then chopped into small pieces. The purified synaptosomal fraction was isolated according to the method of Hajos (1975) with minor modifications. Brain tissue was first homogenized in ice-cold buffered 0.32 M sucrose (20 ml) to generate synaptosomes using 6 excursions of a motor driven pestle. The homogenate was centrifuged (1,500 x g; 10 min.) and the supernatant retained on ice. The pellet was dispersed in sucrose and centrifuged again. Supernatants were combined, centrifuged (9,000 x g; 20 min.) and the crude synaptosomal pellet (P₂) was then gently resuspended in 0.32 M sucrose (5 ml). The P₂ fraction was then divided equally and each portion carefully run onto the surface of 0.8

M sucrose (20 ml. pH 7.4) in a centrifuge tube. The two tubes were then centrifuged (9,000 x g; 26 min). Material in each 0.8 M layer was removed and diluted (over 30 min. with continuous mixing) to the equivalent of 0.32 M with ice-cold distilled water. After centrifugation (9,000 x g; 20 min) the purified synaptosomal pellet was suspended in calcium-free saline (NaCl 128 mM, KCl 5 mM, Na₂HPO₄·7H₂O 1 mM, MgCl₂·7H₂O 1.2 mM, EGTA 100 μM, glucose 14 mM and HEPES 20 mM buffered to pH 7.4 with Tris base) and held on ice.

6.3.3 Experiments using synaptosomes and the pH-sensitive dye acridine orange

Experiments with the pH-sensitive fluorescent indicator acridine orange (AO) were conducted according to published methods (Zoccarto et al., 1999; Melnik et al., 2001). A 200 μl aliquot of purified synaptosomes (0.47 ± 0.03 mg protein) was added to 2.8 ml calcium-free saline containing BSA (1 mg/ml) and AO (5 μM final concentration) and then incubated at 35 °C with gentle shaking for 20 minutes. The suspension of AO-loaded synaptosomes was then transferred to a stirred quartz fluorescence cuvette thermostated at 35 °C, and, using an excitation wavelength of 490 nm, the fluorescence emission intensity was measured continuously at 530 nm in a Perkin-Elmer LS-50 fluorescence spectrophotometer. Slit widths were each 3 nm. Immediately after starting the recording Ca²⁺ (1.2 mM) or TTX (10 μM) was added if required, additions of study compounds were then made from approximately 100 seconds onwards and assays were normally terminated at 400 seconds. Ethanolamine, methylethanolamine, dimethylethanolamine, and choline were added in saline (10 μl). Veratridine, CCCP were added in DMSO (2 μl). Neither addition of control carriers to AO-loaded

synaptosomes nor addition of the study compounds to saline containing AO (in the absence of biological material), had any effect on the level AO fluorescence.

6.3.4 Isolation of synaptoneuroosomes and their use in the depolarization assay

The synaptoneurosomal fraction, isolated from the whole brain of a single CD1 mouse using the method of Harris and Allen (1985) was used for plasma membrane potential measurements. All fractionation procedures were carried out between 1 and 4 °C and the final synaptoneurosomal pellet was suspended in saline (1 ml) and held on ice. The membrane potential of synaptoneuroosomes was measured based on the fluorescence output of the voltage-sensitive indicator rhodamine 6G, as we have outlined in a previous report (Nicholson et al., 2003).

6.3.5 Release of [³H]-D-aspartic acid from superfused synaptosomes

Synaptosomes were incubated for 5 minutes at 32 °C in the presence of 5 µCi [³H]-D-aspartic acid (specific activity 23.9 Ci/mmol) in saline (NaCl 128 mM, KCl 5 mM, Na₂HPO₄·7H₂O 1 mM, CaCl₂·2H₂O 0.8 mM, MgCl₂·7H₂O 1.2 mM, glucose 14 mM and HEPES 20 mM buffered to pH 7.4 with Tris; total volume 1500 µl). Loading was terminated by transferring synaptosomes to ice. Pellets were resuspended in 800 µl of ice-cold saline and synaptosomes were transferred to each of ten Swinnex filtration units (Millipore) containing Whatman GF/B filters. Superfusions were carried out as we have described previously (Verdon et al., 2000). Individual filter units were constantly supplied with saline from a 30 ml syringe barrel. Saline was then routed to an LKB 2070 Ultrarac fraction collector adapted to collect 10 superfusates simultaneously. A constant 1 ml/minute flow rate was maintained using a 10 channel

peristaltic pump (Watson Marlow 503S) set up between the filtration units and the fraction collector. The effects of amino alcohols were examined after nerve preparations had been superfused with 40 ml control saline to establish consistent rates of release. From this point onwards, ten 3 ml fractions were collected from each filtration unit and total radioactivity in each tube measured by liquid scintillation counting. Amino alcohols were dissolved directly in saline. The depolarizing (35 mM KCl) treatment (with the [NaCl] reduced by an equivalent amount to maintain isoosmotic conditions) was routinely added at fraction 4. All superfusions were conducted between 22 and 25 °C.

6.3.6 Preparation of synaptic vesicles

Synaptic vesicles were prepared from bovine brain using procedures described in the literature (Shioi and Naito, 1989; Roz and Rehavi, 2003). A brain from a freshly killed bovine was obtained from a local abattoir and transported to the laboratory on ice. All the following fractionation procedures were performed at 1-4 °C. After removing as much white matter as possible, cerebral cortex tissue was chopped, homogenized (10% w/v) in 0.32 M sucrose (containing 1 mM NaHCO₃, 1 mM MgCl₂ and 0.5 mM CaCl₂) using a motor driven homogenizer (6 up and down strokes) and synaptosomes isolated according to Hajos (1975). The synaptosomal pellets were gently dispersed in the sucrose solution (0.3 ml) and then synaptosomes were osmotically disrupted by the addition of 10 ml of 5 mM Tris-HCl buffer solution followed by incubation on ice for 45 min. Suspensions were centrifuged at 20,000 x g for 20 min. and the supernatants then centrifuged at 62,000 x g for 40 minutes to yield

the synaptic vesicle pellets, which were suspended in 0.32 M sucrose, 1 mM NaHCO₃ at protein concentration of 7.5 mg/ml and stored at -80 °C.

6.3.7 Measurement of the effects of amino alcohols on proton levels within synaptic vesicles

The effects of ethanolamine, methylethanolamine, dimethylethanolamine and CCCP on ATP-dependent proton accumulation into synaptic vesicles were studied using the acridine orange method (Roz and Rehave, 2003). Recordings were carried out with a Perkin-Elmer LS-50 fluorescence spectrophotometer. The excitation wavelength was 493 nm excitation (slit width: 3 nm) and the emission signal was sampled at 530 nm (slit width: 3 nm). Synaptic vesicles (110 µg of protein) were first incubated in 2 ml buffer (150 mM KCl, 4 mM MgSO₄, 10 mM HEPES, pH 7.4) containing acridine orange (1.5 µM) for one minute at 30 °C. ATP (1 mM final concentration) was then added followed by the study compounds. Fluorescence changes were monitored for 340 seconds.

6.3.8 Measurement of amino alcohol-induced changes to the electrical (membrane) potential of synaptic vesicles

The effects of ethanolamine and other compounds on ATP-induced polarization of synaptic vesicles were monitored by recording fluorescence changes of the lipophilic anionic dye oxonol V as described by Tabb et al., 1992. These experiments were performed at 30 °C using the Perkin-Elmer LS-50 with the excitation wavelength set at 617 nm (slit width: 5 nm) and an emission wavelength of 643 nm (slit width:10 nm). Synaptic vesicles (210 µg protein) were allowed to equilibrate with oxonol V (0.65 µM final concentration) in 2 ml buffer (0.25 M sucrose, 4 mM KCl, 1 mM MgSO₄, 25 mM

Tris/maleate adjusted to pH 7.4) over 1 minute. ATP (1.5 mM final concentration) was then introduced which allowed synaptic vesicles to polarize. This was followed by addition of the amino alcohols followed by CCCP as required. Fluorescence recordings were run for a total of 300 seconds.

6.3.9 The effect of amino alcohols on the uptake of [³H]-D-aspartic acid by synaptic vesicles

Synaptic vesicles were equilibrated at 0 °C for at least an hour in a 20-fold excess of buffer (0.25 M sucrose, 1 mM MgSO₄, 4 mM KCl, 35 mM Tris/maleic acid adjusted to pH 7.4). Polarization of synaptic vesicles was achieved by warming to 30 °C, and incubating for 5 minutes with ATP (2 mM) with stirring. To 200 µl synaptic vesicles (approx. 70 µg protein) in buffer, D-aspartate (0.8 µCi [³H]-D-aspartate; final concentration 38 µM) was added with or without ethanolamine, methylethanolamine or dimethylethanolamine, as appropriate, and incubations continued for 5 min at 30 °C. Uptake was terminated by the addition of ice-cold buffer (2 ml) containing ATP and the appropriate amino alcohol and, after vortexing, the synaptic vesicles were quickly filtered under vacuum through a nylon filter (pore size < 0.45 µm). The synaptic vesicles retained on the filters were then washed twice with 2 ml of the same ice-cold same solution. The filters were then placed in 1 ml 10% sodium dodecyl sulfate to release radioactivity from synaptic vesicles. Radioactivity was quantitated using liquid scintillation counting. Uptake of [³H]-D-aspartic acid was temperature-sensitive and all uptake values at 30 °C were corrected by subtracting the radioactivity non-specifically associating with synaptic vesicles at 0 °C.

6.3.10 Protein assay

Protein levels were measured using the method of Lowry as adapted by Peterson (1977).

6.3.11 Analysis of results

Where appropriate results are expressed as mean \pm standard error (S.E.M.) with the level of replication stated. Fluorescence profiles were constructed and statistical analysis (Student's *t* test) performed using Prism 4 software (Graphpad, CA, USA).

6.4 Results

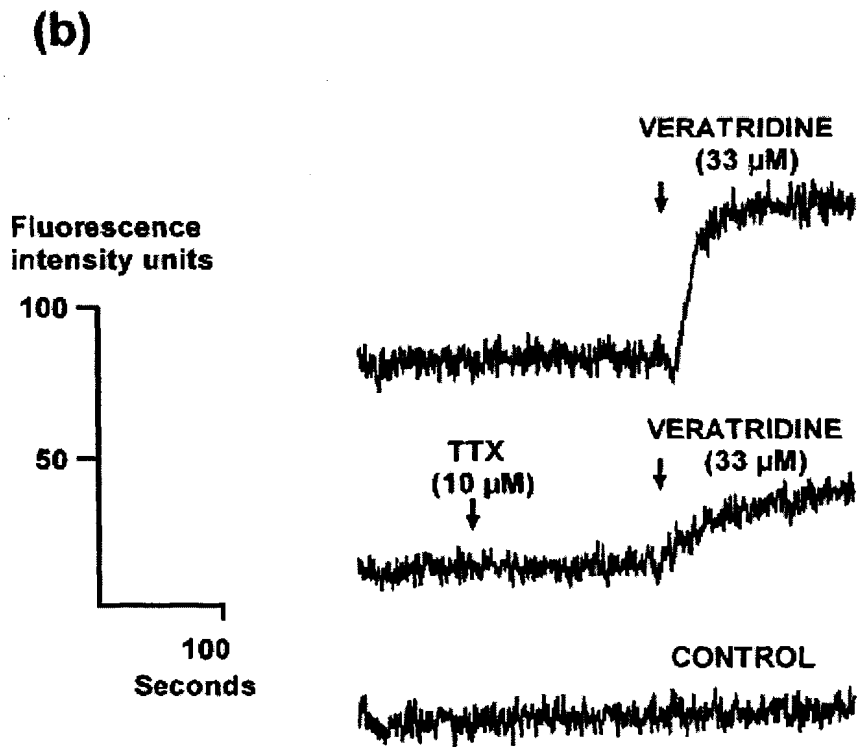
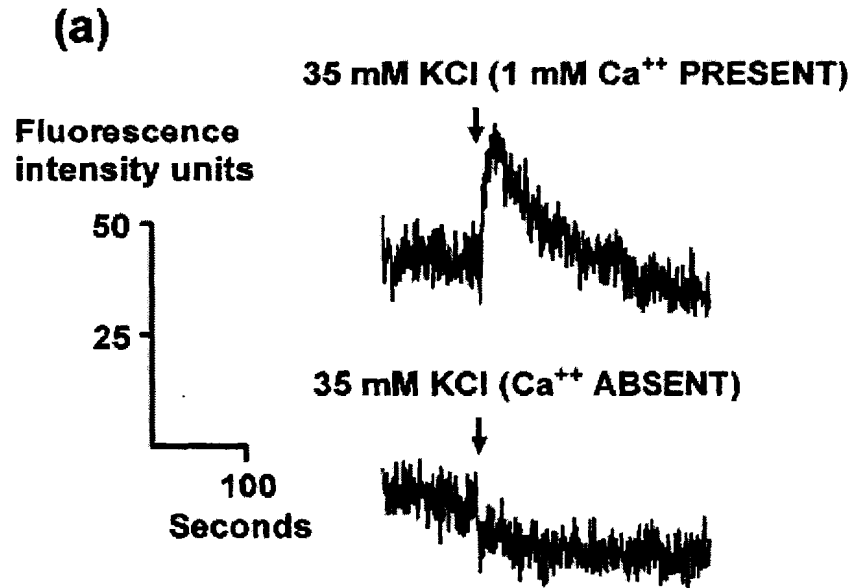
6.4.1 Effects of ethanolamine and other amino alcohols on acridine orange fluorescence in synaptosomes

In synaptosomal preparations 35 mM KCl produced a distinct rise in AO fluorescence above steady state control levels which only occurred in the presence of Ca^{++} (Fig. 28a) in accordance with previous reports (Zoccarto et al., 1999; Melnik et al., 2001). The sodium channel activator veratridine (33 μM) also enhanced AO fluorescence and this response was reduced substantially by 10 μM tetrodotoxin (Fig. 28b). Ethanolamine (5 mM) caused a marked and sustained increase in the intensity of AO fluorescence (Fig 29a). Responses to ethanolamine were concentration-related and threshold increases were detected at 0.31 mM. The increase in AO fluorescence produced by ethanolamine was independent of Ca^{++} in the saline and was unaffected by prior treatment of synaptosomes with 10 μM tetrodotoxin (Fig. 29b). Other amino alcohols methylethanolamine and dimethylethanolamine at 5 mM, caused similar rises in AO fluorescence, however choline was devoid of any effect (Fig. 30). In Fig. 31a, a typical profile for 5 mM ethanolamine is shown together with responses to 35 mM KCl,

the magnitude of which is unaffected by time of challenge. From the time at which the increase in AO fluorescence produced by 5 mM ethanolamine nears its peak and onwards 35 mM KCl becomes progressively less able to increase AO fluorescence (Fig. 31b).

Fig 28. Depolarization-induced exocytosis observed as an AO fluorescence increase arising from exposure of synaptosomes to 35 mM KCl and 33 μ M veratridine.

(a) KCl can't initiate exocytosis in the absence of Ca^{2+} (below) comparing Ca^{2+} presence (above). In (b) the release of AO occurring after challenge with 33 μ M veratridine (above), inhibition of veratridine's effect by TTX (middle) and a control profile (lower) are displayed. Traces are representative of results from 3-5 independent experiments. Ca^{2+} was present at 1 mM except where otherwise indicated.



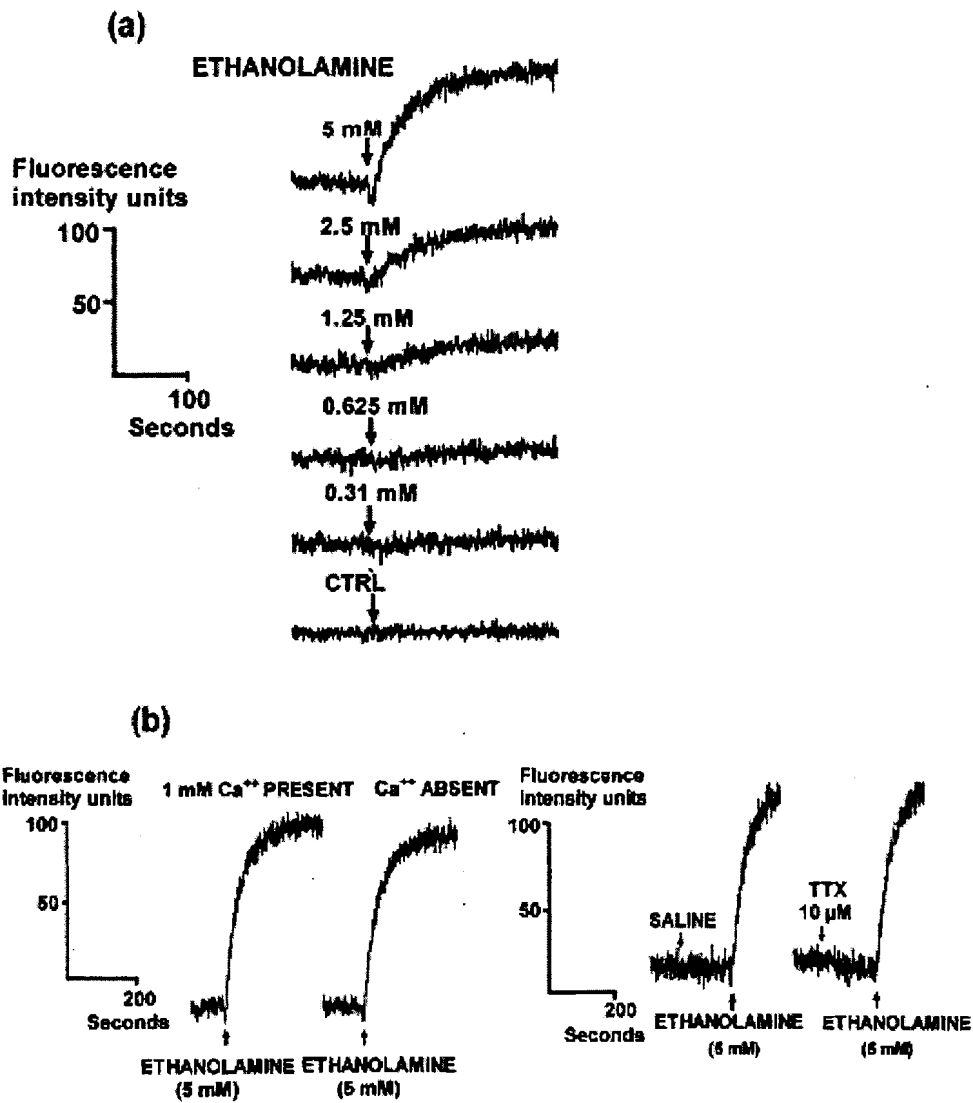


Fig 29. Showing (a) increases in AO fluorescence intensity in synaptosomal suspensions exposed to ethanolamine concentrations between 0.31 and 5 mM and (b) the lack of effect on ethanolamine's response when Ca²⁺ is absent from the saline (left) or when synaptosomes are pretreated with 10 μM tetrodotoxin (right). Profiles are typical of three experiments and Ca²⁺ was included in the saline at 1 mM except where indicated otherwise.

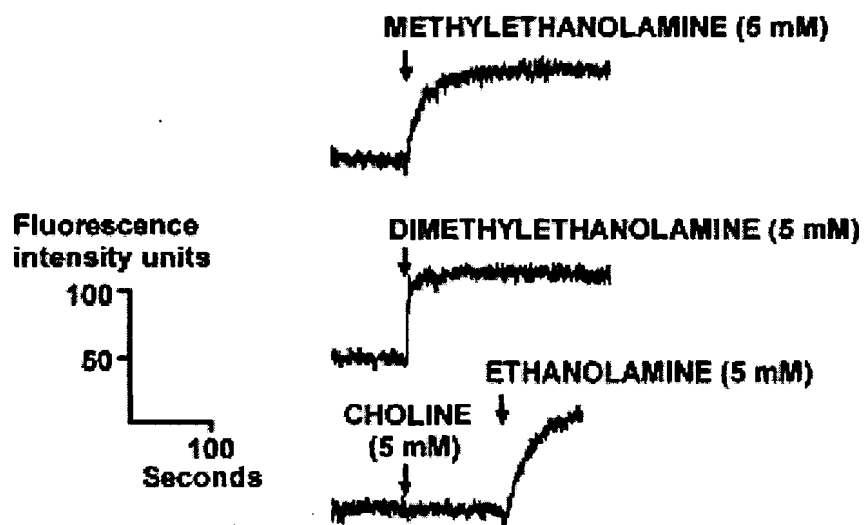


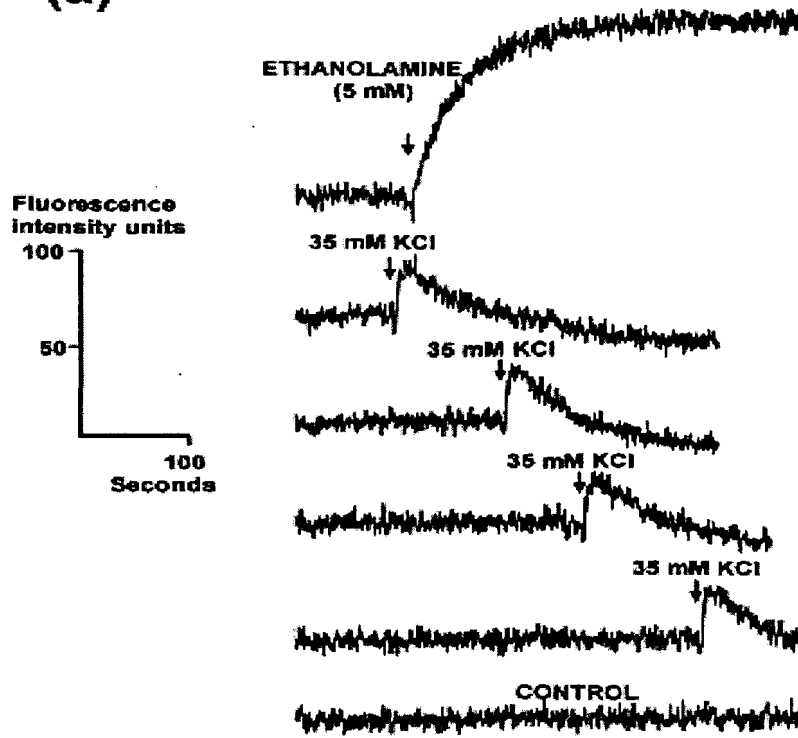
Fig 30. Typical recordings of changes in AO fluorescence in synaptosomal suspensions exposed to methylethanolamine and dimethylethanolamine.

Note the base choline produced no change in fluorescence, however following choline application, the preparation was still responsive to ethanolamine. Study compounds were added at 5 μ M and Ca^{2+} was present at 1 mM throughout. Results are representative of 3 experiments.

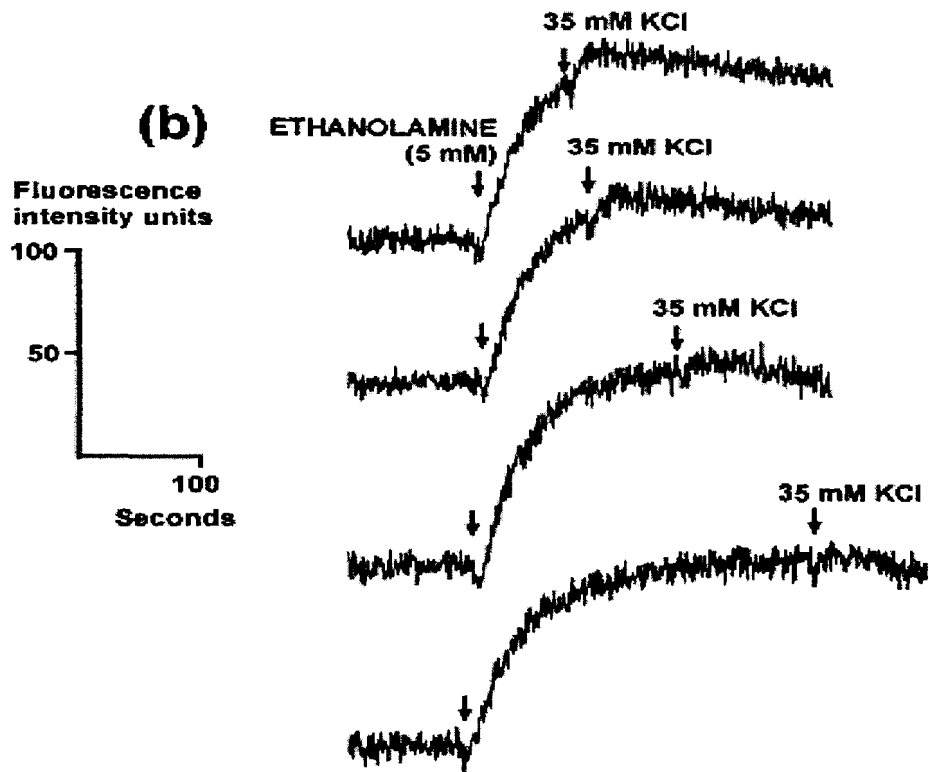
Fig 31. (a) Control responses to 5 mM ethanolamine and 35 mM KCl, the latter applied at various times after approximately 100 seconds. In (b) traces show the diminishing ability of 35 mM KCl to add to the increase in AO fluorescence induced by 5 mM ethanolamine over time.

Ca²⁺ (1 mM) was included throughout. All traces are typical of 3 experiments.

(a)



(b)



6.4.2 Inability of ethanolamine to influence the plasma membrane potential

Measurements of synaptoneurosomal plasma membrane potential using the fluorescent voltage-sensitive probe rhodamine 6G demonstrated that ethanolamine and its methyl and dimethyl forms do not depolarize or hyperpolarize the nerve membrane (Table 3). It is also apparent from our results that the neuronal plasma membrane can still be depolarized by KCl in the presence of each amino alcohol.

Table 3. Inability of ethanolamine, methylethanolamine and dimethylethanolamine (all at 5 mM) to modify the membrane potential of synaptoneurosomes.

Treatment	Membrane potentials (mV) 100 sec after exposing synaptoneurosomes to various treatments	Membrane potentials (mV) produced by 35 mM KCl after each amino alcohol treatment
Control (n = 4)	-78 ± 2	---
Ethanolamine (n = 6)	-82 ± 1 NS	-45 ± 5
Methylethanolamine (n = 5)	-79 ± 1 NS	-32 ± 1
Dimethylethanolamine (n = 5)	-79 ± 1 NS	-30 ± 2
KCl (n = 5)	-38 ± 4 *	---
Veratridine (n = 4)	-15 ± 8 *	---

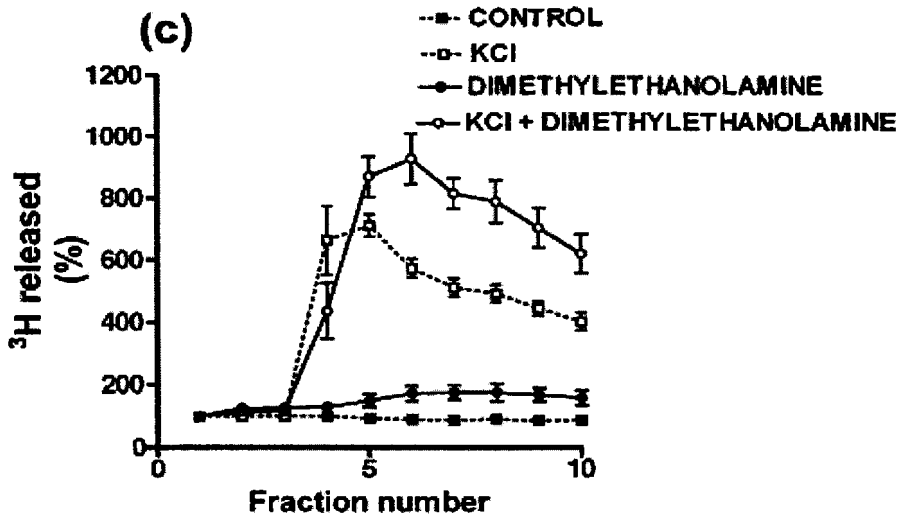
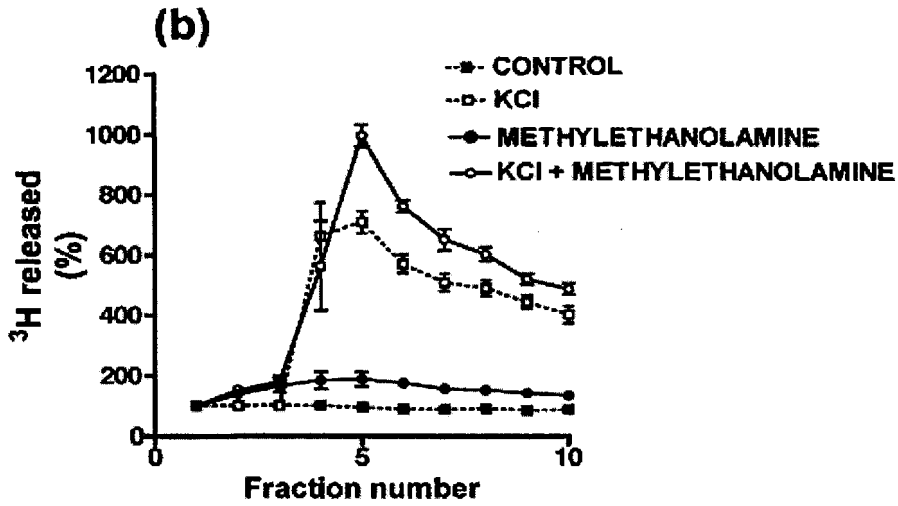
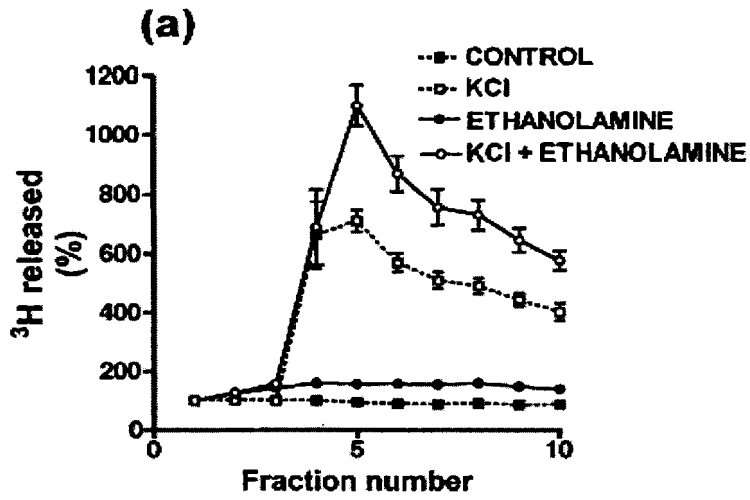
Membrane potentials were quantitated using the voltage-sensitive fluorescent dye rhodamine 6G as described in Experimental Procedures. Responses to standard depolarizing treatments (35 mM KCl and 33 μM veratridine) are provided for comparison. In addition the response to 35 mM KCl added after each amino alcohol challenge is displayed. Results are means ± S.E.M.; n values are given in brackets. In the middle column, asterisks signify instances where differences from controls are significant, (P < 0.01). NS = not significant, (P > 0.05).

6.4.3 Release of [³H]-D-aspartic acid from synaptosomes

The effects of amino alcohols (all at 5 mM) on the basal and 35 mM KCl-evoked release of [³H]-D-aspartic acid from superfused synaptosomes are displayed in Fig 32. Ethanolamine, methylethanolamine and dimethylethanolamine gradually increased basal release of [³H]-D-aspartic acid which overall amounted to increases of 65, 68 and 65% respectively. Amino alcohol-induced increases in the level of resting release were in the main sustained for the remainder of the exposure period. Ethanolamine, methylethanolamine and dimethylethanolamine also enhanced 35 mM KCl-evoked release of [³H]-D-aspartic acid from synaptosomes (by 52, 50, and 50% respectively) and these amino alcohol-enhanced [³H]-D-aspartic acid release profiles generally shadowed that of KCl alone.

Fig 32. The effect of (a) ethanolamine, (b) methylethanolamine and (c) dimethylethanolamine on the resting (basal) and KCl-evoked release of [^3H]-D-aspartic acid from mouse brain synaptosomes.

Values represent ^3H released as a percentage of ^3H present in fraction 1. Data points represent means and vertical bars the S.E.M. of 3-6 experiments. Amino alcohols (5 mM) were applied just after the start of sample collection and saline containing KCl (35 mM) was routinely introduced at fraction 4. The % increases given in the Results section were calculated by adding the % increases for each amino alcohol alone or with KCl and expressing these values as a % of the summed basal or KCl alone values respectively.



6.4.4 The effects of ethanolamine, methylethanolamine and dimethylethanolamine on the ATP-activated increase in intravesicular proton levels

Experiments to directly measure the effects amino alcohols on the H^+ -ATPase-activated increase in intravesicular proton concentration were conducted with a synaptic vesicle preparation from bovine cortex. ATP caused the typical end point decrease in acridine orange fluorescence (Roz and Rehave, 2003), consistent with increased pumping of protons into the interior of synaptic vesicles by the H^+ -ATPase (Fig 33). Subsequent exposure to ethanolamine, methylethanolamine and dimethylethanolamine (all 5 mM) caused an immediate drop in intravesicular proton concentration which reduced to a level lower than that observed before the ATP addition (Fig. 33 a-c). The protonophore CCCP also brought about a rapid decline in intravesicular acidification (Fig. 33d) and produced negligible (ethanolamine and methylethanolamine) or slight (dimethylethanolamine) enhancement of amino alcohol-induced proton depletions (Fig. 33 a-c).

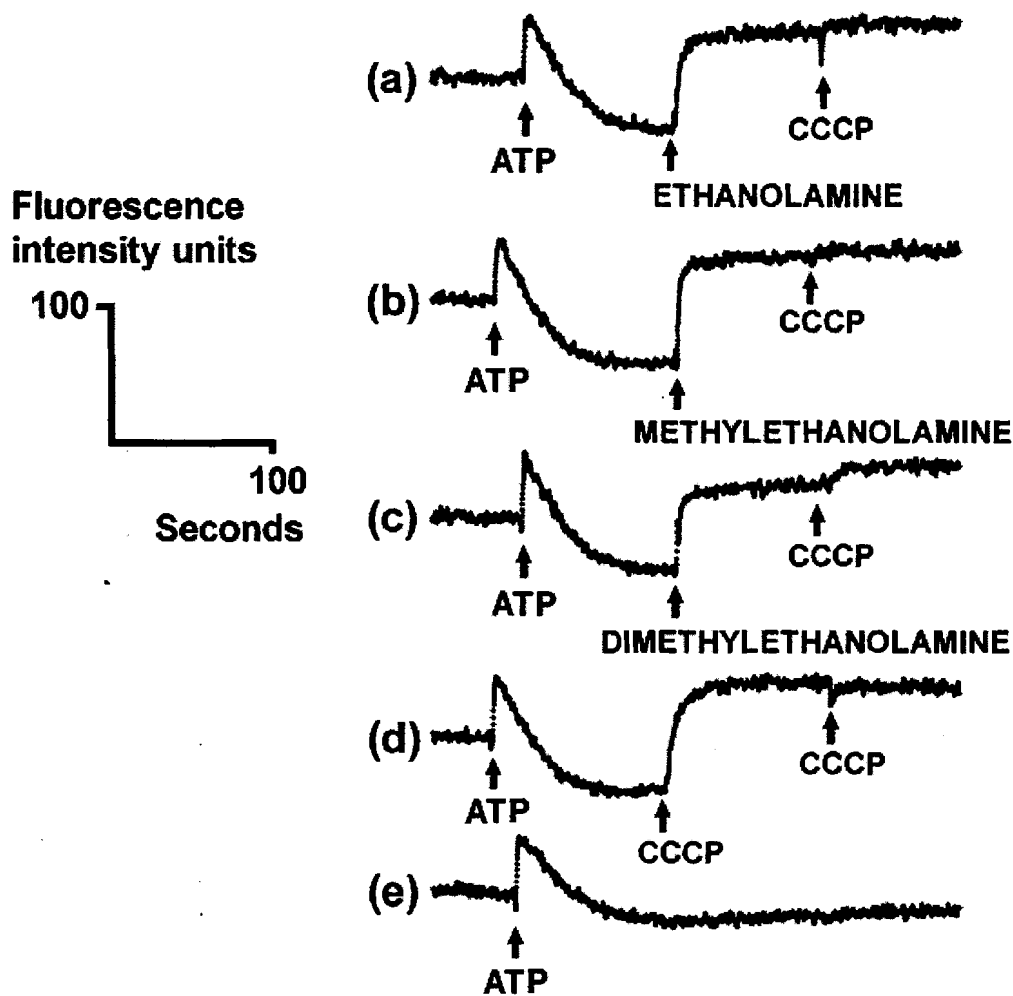


Fig 33. The effect of (a) ethanolamine, (b) methylethanolamine, (c) dimethylethanolamine and (d) CCCP (7.5 μ M) on ATP-dependent quenching of acridine orange fluorescence in synaptic vesicle preparations.

Recording (e) shows the baseline profile. Amino alcohols were added at 5 mM. Each trace is representative of at least 3 experiments. Note that the upward deflections produced by study compounds equate to a loss of intravesicular protons and that the ability of CCCP to reduce proton levels in the interior of synaptic vesicles beyond that of the amino alcohols was negligible or weak (traces a-c).

6.4.5 The effects of ethanolamine, methylethanolamine and dimethylethanolamine on the transmembrane potential of synaptic vesicles.

Changes to the electrical transmembrane potential of synaptic vesicles are conveniently measured using the anionic fluoroprobe oxonol V. Addition of ATP to synaptic vesicles led to rapid formation of the membrane potential, as evidenced by a decline in fluorescence (due to increased association of free oxonol with synaptic vesicles; Fig 34 a-d). Ethanolamine, methylethanolamine and dimethylethanolamine (at 5 mM) strongly increased the extent of synaptic vesicle polarization (Fig. 34 a-c). CCCP strongly depolarized synaptic vesicles in the presence of the amino alcohols (Fig. 34 a-c).

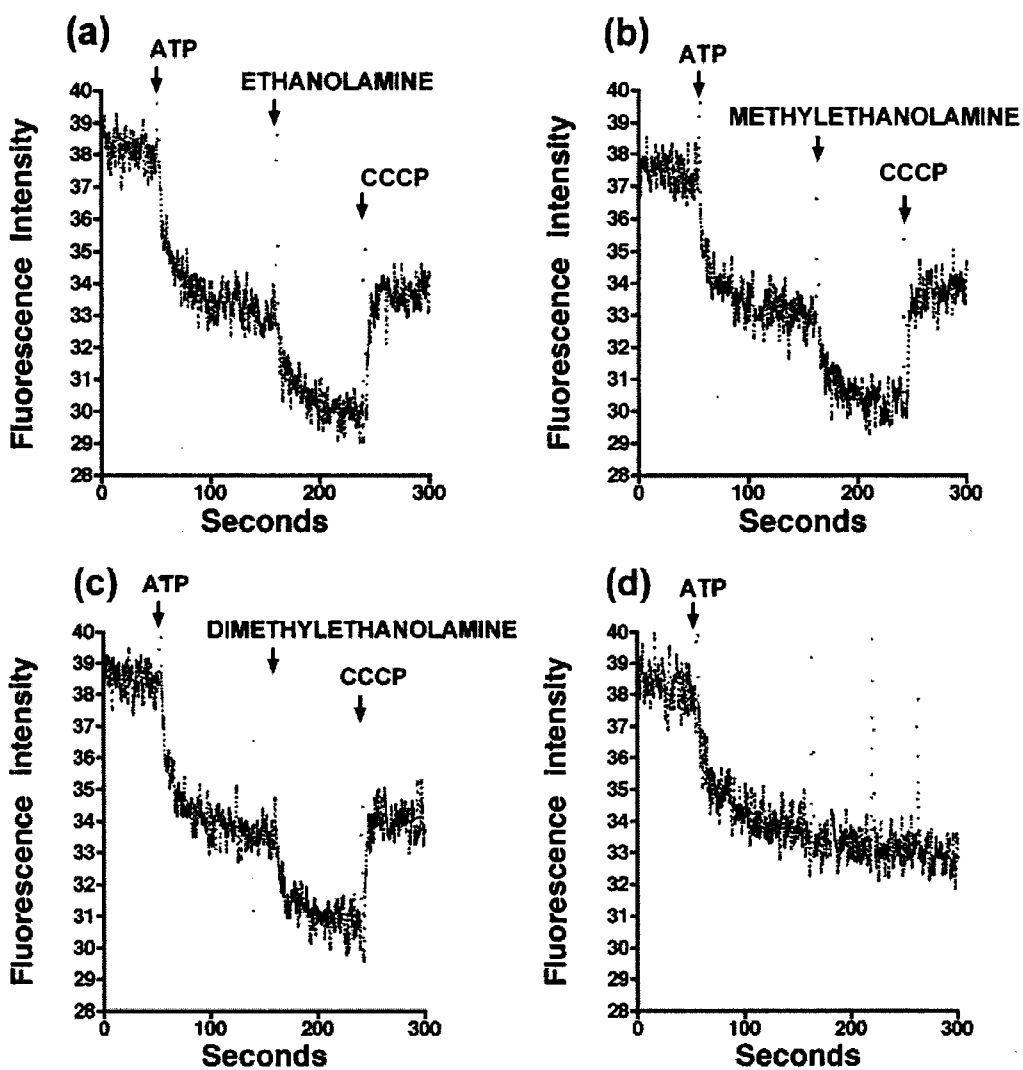


Fig 34. The effect of (a) ethanolamine, (b) methylethanolamine, (c) dimethylethanolamine on the ATP-supported (H^+ -ATPase-dependent) electrical potential of synaptic vesicles as determined using oxonol V.

Downward deflections represent polarization and upward deflections signify depolarization of the synaptic vesicular membrane. Amino alcohols were added at 5 mM. CCCP fully reverses the membrane polarizing influence of all amino alcohols and ATP combined (a-c). Recording (d) is the baseline (control). Each trace is typical of 3 or more experiments.

6.4.6 The effects of amino alcohols on the uptake of [³H]-D-aspartate into synaptic vesicles

Each amino alcohol was examined for their ability to influence the accumulation of [³H]-D-aspartic acid by synaptic vesicles in the presence of ATP (Table 4). The uptake of [³H]-D-aspartic acid by synaptic vesicles was significantly ($P < 0.05$) increased in the presence of ethanolamine, methylethanolamine and dimethylethanolamine by amounts which averaged 76, 62 and 145 % respectively.

Table 4. The effect of ethanolamine, methylethanolamine and dimethylethanolamine (all at 5 mM) on the uptake of [³H]-D-aspartic acid into synaptic vesicles prepared from bovine cortex.

Treatment	Uptake of [³ H]-D-aspartic acid into synaptic vesicles (nmol/mg protein/min)
Control	0.56 + 0.01
Ethanolamine	0.99 + 0.05 **
Methylethanolamine	0.91 + 0.08 *
Dimethylethanolamine	1.37 + 0.15**

Values represent means ± S.E.M. of 3-4 experiments. Asterisks signify a significant difference from control (* = P < 0.05; ** = P < 0.01)

6.5 Discussion

The pH-sensitive fluorescent indicator acridine orange (AO) has found particular utility in investigations of exocytotic function in synaptosomes. The quenching of AO fluorescence upon addition of this fluoroprobe to synaptosomal suspensions arises from its ability to become protonated and dimerize when sequestered within the acidic interior of synaptic vesicles, whereas high $[K^+]$ -induced increases in fluorescence above steady state are Ca^{2+} -dependent and reflect release of AO-rich synaptic vesicle contents by exocytosis (Zoccarto et al., 1999; Melnik et al., 2001). In support of the specificity of AO for synaptic vesicles, bafilomycin A_1 , which selectively blocks H^+ translocation by vacuolar H^+ -ATPases (Floor et al., 1990; Moriyama, 1990), eliminates both initial quenching of AO fluorescence by synaptosomes and markedly stimulates loss of AO from synaptosomes previously loaded with probe (Zoccarto et al., 1999). Inorganic weak bases also elicit similar responses. For instance, ammonium chloride prevents the loading of synaptic vesicles with AO and activates the efflux of AO from synaptic vesicles previously equilibrated with fluoroprobe by sequestration of protons (Melnik et al., 2001). The sodium channel-independent components of AO efflux observed with organic bases such as 4-aminopyridine (Zoccarto et al., 1999) and veratridine (this study), likely also occur through a similar mechanism. Ethanolamine and the other amino alcohols used in this investigation also possess weakly basic properties.

In the present experiments I observed profound stimulatory effects of ethanolamine, methylethanolamine and dimethylethanolamine on the fluorescence signal of synaptosomes pre-equilibrated with AO suggesting that these amino alcohols either

enhance the exocytosis of synaptic vesicles or reduce the level of protons in the synaptic vesicle interior. In parallel assays, the depolarizing agents KCl and veratridine produced calcium-dependent and tetrodotoxin-sensitive efflux of AO respectively. However, unlike KCl which directly depolarizes synaptosomes and opens voltage-gated calcium channels (Blaustein and Goldring, 1975; Nachsien and Blaustein, 1980), ethanolamine does not affect the synaptosomal plasma membrane potential and its AO fluorescence response is not affected when Ca^{2+} is absent from the external saline. These observations suggest that these amino alcohols do not act by opening voltage-gated calcium channels in the plasma membrane. My findings therefore invoke a different mechanism to that suggested by Bostwick et al. (1993), who proposed enhancement of calcium entry into nerve terminals after activation of L-type calcium channels. Voltage-gated sodium channel activation cannot be of relevance to the actions of ethanolamine either, since in addition to the failure of ethanolamine to depolarize the plasma membrane, ethanolamine-induced release of AO from synaptosomes was unaffected by TTX. Additionally, my results demonstrate that ethanolamine eliminates the ability of 35 mM K^+ to release AO from synaptosomes, clearly suggesting that amino alcohols also discharge the K^+ -sensitive synaptic vesicle pool labelled with AO. However, I found that ethanolamine, methylethanolamine and dimethylethanolamine enhance both basal and KCl-evoked release of [^3H]-D-aspartic acid from synaptosomes. Bostwick et al. (1993) reported that ethanolamine and other amino alcohols (R-alaninol and R-prolinol) enhance KCl-induced release of acetylcholine from hippocampal slices. While my observations on the release of [^3H]-D-aspartate from synaptosomes showed good agreement with those of Bostwick et al.,

my data also presented somewhat of a paradox since it has also been shown that bafilomycin A₁, which reduces the proton concentration of neurite vesicles (Cousin et al., 1997), also reduces KCl-evoked release of glutamic acid from synaptosomes (Pocock et al., 1995).

To explore this apparent anomaly in more depth I conducted investigations on synaptic vesicles isolated from bovine brain. Experiments with acridine orange provided direct confirmation that ethanolamine as well as the mono- and dimethyl amino alcohols dramatically reduce proton levels in the synaptic vesicle interior. In these assays, clear parallels with CCCP were evident. The pumping of protons from the cytoplasm into synaptic vesicles by V-H⁺-ATPases generates the main electrochemical gradient powering vesicular accumulation of neurotransmitters (Morel, 2003). Carbonylcyanidehalophenylhydrazones are known to dissipate proton gradients in synaptic vesicles (Roz and Rehavi, 2003). However, the oxonol V experiments also revealed that in marked contrast to CCCP, amino alcohols strongly increase the electrical transmembrane potential of synaptic vesicles and this latter observation correlates well their ability to enhance the uptake of [³H]-D-aspartic acid into synaptic vesicles. Taken together, my data strongly imply that the reduction in intravesicular proton levels seen with amino alcohols cannot be due to efflux of protons from synaptic vesicles. Given the basic (proton accepting) nature of amino alcohols, ethanolamine most likely acts by rapidly accessing the interior of synaptic vesicles where it immediately sequesters protons. Protonation can occur on the amine and possibly also on the hydroxyl oxygen yielding positively charged species which, as the oxonol V experiments predict, will contribute more to the overall electrochemical

potential than proton equivalents. Comparable hyperpolarizing actions of nigericin have been observed, although this substance facilitates K^+ entry into the intravesicular compartment coupled to H^+ removal (Tabb et al., 1992). Since the protonated forms of amino alcohols are unable to traverse membranes easily, any decline in the higher transvesicular membrane potential should be limited. Additionally, the oxonol V data suggest that by reversing amino alcohol-induced hyperpolarization, CCCP shifts the equilibrium in favor of proton dissociation from amino alcohols and allows proton efflux from the synaptic vesicle interior to proceed. In summary, therefore, I propose that enhanced filling of synaptic vesicles with [3H]-D-aspartate from the cytoplasmic compartment explains the increased basal and evoked amino acid release I observed with amino alcohols in the synaptosome experiments. Since [3H]-D-aspartic acid is not metabolized, the quantities originally taken up into synaptosomes should be sufficient to sustain the increased amino alcohol-dependent transfer to synaptic vesicles.

In the synaptosomal assays I found that choline had no effect on AO fluorescence. Since choline contains a quaternary nitrogen it is unable to traverse membranes and is also considerably less susceptible to protonation. Moreover, the ability of choline to access synaptic vesicles will be very limited because although choline would be expected to be transported efficiently into the cytoplasmic compartment of cholinergic nerve endings, it must undergo metabolism to acetylcholine which is then pumped into synaptic vesicles (Sha et al., 2004).

The physiological relevance of these findings needs placing in proper perspective. My concentration-response data for ethanolamine demonstrate threshold

effects on AO fluorescence close to 0.31 mM (18.9 $\mu\text{g/ml}$), while well-defined increases in fluorescence occur up to 5 mM (305.4 $\mu\text{g/ml}$). The concentrations of ethanolamine required to influence AO fluorescence align well with a report (Ellison et al., 1987), which established that resting levels of this amino alcohol in various regions of mammalian brain lie between between 197 - 870 nmol/g wet wt. (12.03 - 53.1 $\mu\text{g/ml}$ equivalent). In other tissues, for example liver, a similar resting concentration of 0.54 mM (32.9 $\mu\text{g/ml}$) ethanolamine has been reported (Houweling et al., 1992). Therefore the concentrations of ethanolamine I employed in this research are close to physiological. A report by Andriamampandry et al. (1989) indicates that the steady state levels of methyl- and dimethyl ethanolamine may be lower than that of ethanolamine. Although it is generally assumed that ethanolamine exists mostly in the protonated form in tissues, it remains to be established whether intracellular enzymes for example, serine decarboxylase or those involved in base exchange reactions, initially release the unprotonated species and, indeed, if such reactions can occur in synaptic vesicles to the extent that might permit amino alcohol-dependent regulation of synaptic vesicle filling. The mechanism I outline in this report may also have relevance to the development of certain neuropathophysiological states where ethanolamine concentrations are known to change. For example, ethanolamine levels in brain tissues from epilepsy patients are elevated five-fold compared to non-epileptogenic samples and considerable intracellular buildup of ethanolamine has been reported in epileptiform brain (Hamberger et al., 1991; Hamberger et al., 1993). In addition, ischemic episodes are thought to trigger the release of ethanolamine by activation of base exchange reactions in the brain (Buratta et al., 1998). In both disease

states, increased release of L-glutamate (for which [³H]-D-aspartate acts as a surrogate) has been implicated (Meldrum, 1994; Choi and Rothman, 1990).

In closing, the central finding of this investigation is that at concentrations close to those found in normal brain tissue, ethanolamine and closely related amino alcohols have the ability to positively modulate the vesicular membrane potential, enhancing the capacity of both synaptic vesicles to take up [³H]-D-aspartate and synaptosomes to release this amino acid. We are now considering the relevance of this mechanism to other neurotransmitters and how changes in neuronal activity might influence the concentrations of amino alcohols such as ethanolamine within the nerve ending.

Acknowledgment

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CHAPTER 7

SUMMARY AND PHYSIOLOGICAL SIGNIFICANCE OF MY FINDINGS

Endocannabinoids are widely distributed in higher animals and exert important physiological effects by binding to various molecular targets, such as CB1 receptors, CB2 receptors, vanilloid receptors, 5-HT receptors, potassium channels, and calcium channels. My data in Chapter 2 provides strong evidence that VSSCs are also critical targets of endocannabinoids and synthetic cannabinoids. In support of this I found that the endocannabinoid anandamide and the cannabinimetic drugs AM 404 and WIN 55, 212-2 inhibit the binding of [³H]BTX-B to VSSCs suggesting they bind directly to this complex. Experiments using synaptosomes demonstrated that anandamide and the synthetic drugs selectively inhibit sodium channel-dependent release of amino acid neurotransmitters (L-glutamic acid and GABA). As the cannabinoid antagonist AM 251 failed to reduce the inhibitory effect of anandamide and cannabinimetics in the radioligand binding and transmitter release assays, it is reasonable to conclude that this inhibition of VSSCs by the study compounds is totally independent of CB1 receptor activation.

In support of my conclusions our collaborator Dr. George Lees found that anandamide reversibly inhibits TTX-suppressible sustained repetitive firing in cortical neurons. This inhibition is also AM 251-insensitive. Laurence David in our lab confirmed that anandamide, AM 404 and WIN 55, 212-2 inhibits VSSCs dependent

depolarization in synaptoneurosomes and more recently Daniel Duan in our lab has found that other endocannabinoids (2-AG, AGE, NADA, A-GABA and A-GLY) as well as the highly potent CB-1 receptor agonist CP 55,940 also inhibit VSSCs. Further extending this idea, I demonstrate in Chapter 3 that AM251 binds to VSSCs and inhibit sodium channel independent transmitter release although the concentration for inhibition of VSSCs by AM 251 is higher than those reported in the literature for blocking CB1 receptors.

In Chapter 4 I present evidence that anandamide can be synthesized in synaptic preparations. However, my results did not support the prevailing belief that depolarization of the soma portion of the neuron increases the biosynthesis of anandamide. Nor did depolarization enhance release of anandamide from synaptoneurosomes preloaded with this substance, although a substantial amount of anandamide was retained in synaptoneurosomes after extended superfusion.

Chapter 5 focuses on novel actions of the anandamide metabolite ethanolamine in synaptic preparations. These experiments revealed that synaptosomes and synaptoneurosomes preloaded with [³H]ethanolamine release this substance in response to various forms of depolarization (KCl, VTD, and 4-aminopyridine). Depolarization-induced release of [³H]ethanolamine is partially calcium-dependent, indicating that a significant component of the release may be via classical exocytosis. Base exchange was not involved in the release of ethanolamine supporting the idea that this substance is stored intracellularly in free form. The significance of these findings lie in the possibility that ethanolamine may play a role in signaling across the synapse in the brain.

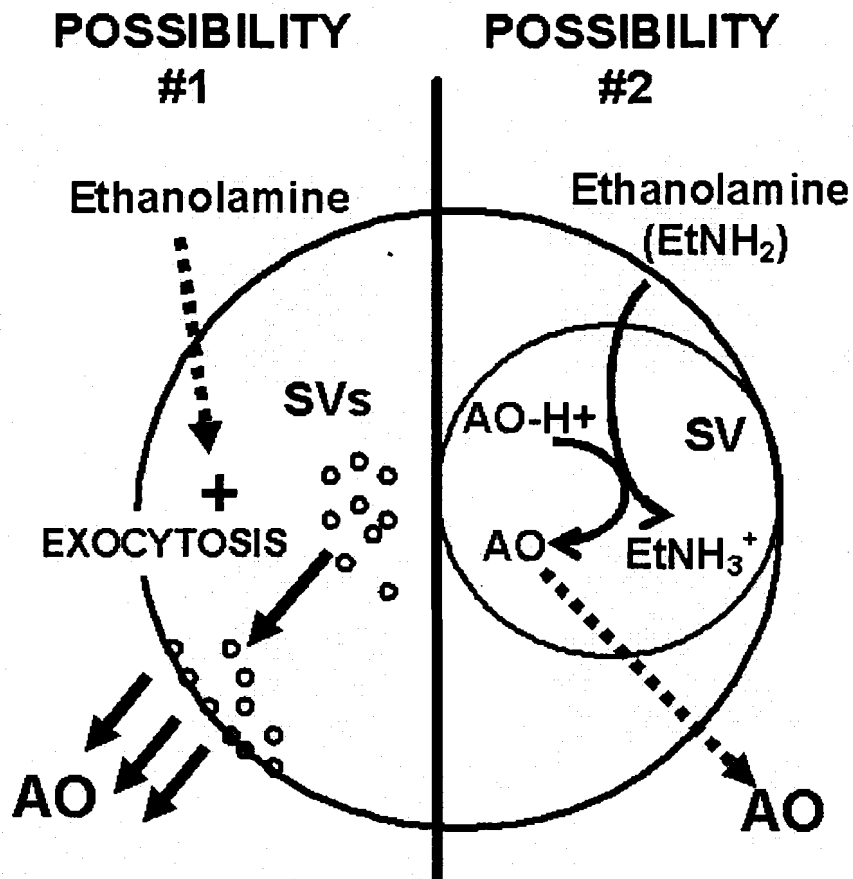
In Chapter 6 I review the experimental evidence supporting my hypothesis that ethanolamine and other endogenous amino alcohols also have an important presynaptic regulatory potential. Ethanolamine and related amino alcohols are taken up into synaptic vesicles where they rapidly sequester protons (as indicated by AO release). Proton sequestration by ethanolamine generates membrane impermeant positively charged (quaternary nitrogen) species which, in tandem with the pumping of protons into the synaptic vesicle interior, increase the transvesicular membrane potential (as indicated by oxonol V fluorescence decreases). This in turn enhances [³H]D-aspartic acid uptake into the synaptic vesicle. The effects of ethanolamine and related amino alcohols on synaptic vesicle function explain my original observations on how these amino alcohols affect the dynamics of acridine orange and transmitter release from synaptosomes. As ethanolamine sequesters protons in synaptic vesicles, acridine orange becomes deprotonated and undergoes rapid efflux from the synaptosomal compartment, therefore KCl is incapable of stimulating AO release. Lastly, the boosting of both the synaptic vesicle membrane potential and enhanced loading of synaptic vesicles with [³H]D-aspartic acid in the presence of amino alcohols is the reason why I found that these compounds enhance the basal and evoked release of [³H]-D-aspartate release from synaptosomes. These results show that ethanolamine and related amino alcohols have the ability to regulate synaptic transmission by affecting synaptic vesicle filling with [³H]-D-aspartic acid. The relative importance to synaptic vesicle filling of the transvesicular proton gradient versus electrical potential gradient is known to be different for different transmitters. In this context, the vesicular uptake of L-glutamic acid and GABA is more reliant on the vesicular transmembrane potential

than the proton gradient (Wolosker et al., 1996; Tabb et al., 1992), whereas the converse is true for acetylcholine and monoamines (Reimer et al., 1998). Thus ethanolamine, which completely collapses the transmembrane proton gradient but strengthens the transmembrane potential, should be more important in the regulation of filling of synaptic vesicles with amino acid neurotransmitters. Further, my results suggest that in addition to the amino alcohols, other endogenous bases such as ammonia and methylamine warrant future investigation.

Schemes illustrating potential mechanisms by which ethanolamine reduces KCl-induced release of AO and increases the polarization of synaptic vesicles are given in Appendices 1 and 2.

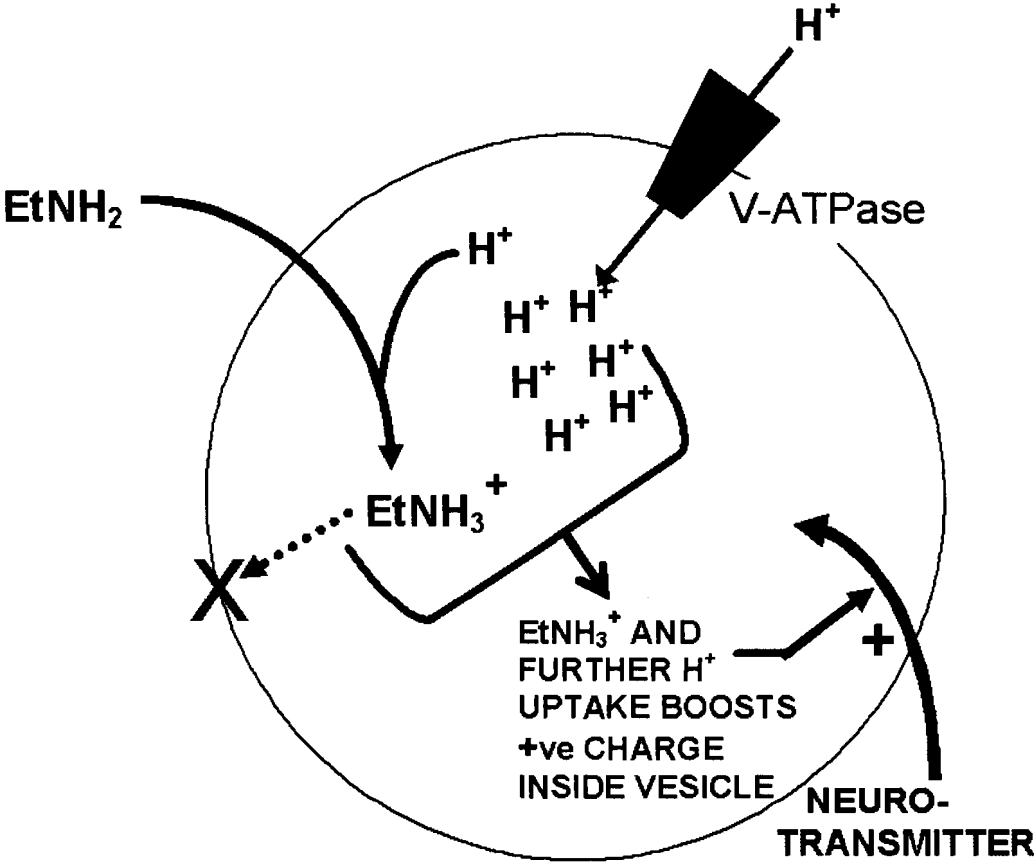
APPENDICES

Appendix 1



Possible mechanisms by which ethanolamine prevents KCl from release AO from synaptosomes

Appendix 2



Proposed mechanism underlying the increase in synaptic vesicle polarization by ethanolamine

REFERENCES

- Adams, I. B., Ryan, W., Singer, M., Thomas, B. F., Compton, D. R., Razdan R. K., Martin, B. R. (1995) Evaluation of cannabinoid receptor binding and in vivo activities for anandamide analogs. *J Pharmacol Exp Therap*, 273, 1172–1181.
- Alberghina, M., Giacchetto, A., Cavallaro, N. (1993) Levels of ethanolamine intermediates in the human and rat visual system structures: Comparison with neural tissues of a lower vertebrate (*Mustelus canis*) and an invertebrate (*Loligo pealei*). *Neurochem Int*, 22, 45-51.
- Alger, B. E. (2002) Retrograde signaling in the regulation of synaptic transmission: focus on endocannabinoids. *Prog Neurobiol*, 68, 247-286.
- Alger, B. E., Pitler, T. A., Wagner, J. J., Martin, L. A., Morishita, W., Kirov, S. A., Lenz, R.A. (1996) Retrograde signalling in depolarization-induced suppression of inhibition in rat hippocampal CA1 cells. *J Physiol*, 496, 197-209.
- Andriamampandry, C., Freysz, L., Kanfer, J.N., Dreyfus, H., Massarelli, R. (1989) Conversion of ethanolamine, monomethylethanolamine and dimethylethanolamine to choline-containing compounds by neurons in culture and by the rat brain. *Biochem J*, 264, 555–562.
- Arai, Y., Fukushima, T., Shiraz, M., Yang, X., Imai, K. (2000) Sensitive determination of anandamide in rat brain utilizing a coupled-column HPLC with fluorimetric detection. *Biomed Chromatogr*, 14, 118-124.
- Arnoult, C., Cardullo, R. A., Lemos, J. R., Florman, H. M. (1996) Activation of mouse sperm T-type Ca^{2+} channels by adhesion to the egg zona pellucida. *Proc Natl Acad Sci USA*, 93, 13004–13009.
- Axelrod, J., Felder, C. C. (1998) Cannabinoid receptors and their endogenous agonist, anandamide. *Neurochem Res*, 23, 575-81.
- Baba, S., Watanabe, Y., Gejyo, F., Arakawa, M. (1984) High-performance liquid chromatographic determination of serum aliphatic amines in chronic renal failure. *Clin Chim Acta*, 136, 49–56.
- Barann, M., Molderings, G., Bruss, M., Bonisch, H., Urban, B. W., Gothert, M. (2002) Direct inhibition by cannabinoids of human 5-HT_{3A} receptors: probable involvement of an allosteric modulatory site, *Br J Pharmacol*, 137, 589–596.
- Begg, M., Pacher, P., Batkai, S., Osei-Hyiaman, D., Offertaler, L., Mo, F.M., Liu, J., Kunos, G. (2005) Evidence for novel cannabinoid receptors. *Pharmacol Ther*, 106, 133-145.

- Bell, R. L., Kennerly, D. A., Stanford, N., Majerus, P. W. (1979) Diglyceride lipase: A pathway for arachidonate release from human platelets. *Proc Natl Acad Sci USA*, 76, 3238–3241.
- Beltramo, M. Stella, N. Calignano, A. Lin, S. Y. Makriyannis, A., Poimelli, D. (1997) Functional role of high affinity anandamide transport, as revealed by selective inhibition. *Science*, 277, 1094–1097.
- Bhave, G., Hu, H. J., Glauner, K. S., (2003) Protein kinase C phosphorylation sensitizes but does not activate the capsaicin receptor transient receptor potential vanilloid 1 (TRPV1). *Proc Natl Acad Sci USA*, 100, 12480-12485.
- Bhave, G., Zhu, W., Wang, H., Brasier, D. J., Oxford, G. S., Robert, W., Gereau, IV. (2002) cAMP-dependent protein kinase regulates desensitization of the capsaicin receptor (VR1) by direct phosphorylation. *Neuron*, 35, 721-731.
- Bisogno, T., Berrendero, F., Ambrosino, G., Cebeira, M., Ramos, J. A., Fernandez-Ruiz, J. J., Di Marzo, V. (1999) Brain regional distribution of endocannabinoids: implications for their biosynthesis and biological function. *Biochem Biophys Res Commun*, 256, 377-380.
- Bisogno, T., Ligresti, A., Di Marzo, V. (2005) The endocannabinoid signalling system: biochemical aspects. *Pharmacol Biochem Behav*, 81, 224-238.
- Blaustein, P., Goldring, J. M., (1975) Membrane potentials in pinched-off presynaptic nerve terminals monitored with a fluorescent probe: evidence that synaptosomes have potassium diffusion potentials. *J Physiol (Lond.)* 247, 589-615.
- Bligh, E. G., Dyer, W. J. (1959) A rapid method for total lipid extraction and purification. *Can J Biochem Physiol*, 37, 911–917.
- Boger, D. L., Sato, H., Lerner, A. E., Austin, B. J., Patterson, J. E., Patricelli, M. P., Cravatt, B. F. (1999) Trifluoromethylketone inhibitors of fatty acid amide hydrolase: a probe of structural and conformational features contributing to inhibition. *Bioorg Med Chem Lett*, 9, 265–270.
- Bojesen, I. N., Hansen, H. S. (2003) Binding of anandamide to bovin serum albumin. *J Lipid Res*, 44, 1790-1794.
- Bojesen, I. N., Hansen, H. S. (2006) Effect of an unstirred layer on the membrane permeability of anandamide. *J Lipid Res*, 47, 561-570.
- Bojesen, I. N., Hansen, H. S. (2005) Membrane transport of anandamide through resealed human red blood cell membranes. *J Lipid Res*, 46, 1652-169.
- Bonanno, G., Pittaluga, A., Fedele, E., Fontana, G., Raiteri, M. (1993) Glutamic acid and gamma-aminobutyric acid modulate each others release through heterocarriers sited on the axon terminals of rat brain. *J Neurochem*, 61, 222–230.

- Bostwick, J. R., Abbe, R., Appel, S. H. (1993) Modulation of acetylcholine release in hippocampus by amino alcohols and Bay K 8644. *Brain Res*, 629, 79-87.
- Bostwick, J. R., Abbe, R., Sun, J., Appel, S. H. (1992) Amino alcohol modulation of hippocampal acetylcholine release. *NeuroReport*, 3, 425-428.
- Bostwick, J. R., Landers, D. W., Crawford, G., Lau, K., Appel, S. H. (1989) Purification and characterization of a central cholinergic enhancing factor from rat brain its identity as phosphoethanolamine. *J Neurochem*, 53, 448-458.
- Brady, R. O., Carbone, E. (1973) Comparison of the effects of 9-tetrahydrocannabinol, 11-hydroxy-9-tetrahydrocannabinol, and ethanol on the electrophysiological activity of the giant axon of the squid. *Neuropharmacology*, 12, 601-605.
- Breivogel, C. S., Griffin, G., Di Marzo, V. Martin, B.R. (2001) Evidence for a new G protein-coupled cannabinoid receptor in mouse brain. *Mol Pharmacol*, 60, 155-63.
- Brenowitz, S. D., Regehr, W. G. (2003) Calcium dependence of retrograde inhibition by endocannabinoids at synapses onto purkinje cells. *J Neurosci*, 23, 6373-6384.
- Buckley, N. E., Hansson, S., Harta, G., Mezey, E. (1998) Expression of the CB1 and CB2 receptor messenger RNAs during embryonic development in the rat. *Neuroscience*, 82, 1131-1149.
- Buratta, S., Hamberger, A., Ryberg, H., Nystrom, B., Sandberg, M., Mozzi, M. (1998) Effect of serine and ethanolamine administration on phospholipid-related compounds and neurotransmitter amino acids in the rabbit hippocampus. *J Neurochem*, 71, 2145-2150.
- Cabot, M. C., Gatt, S. (1977) Hydrolysis of endogenous diacylglycerol and monoacylglycerol by lipases in rat brain microsomes. *Biochemistry*, 16, 2330-2334.
- Cadogan, A. K., Alexander, S. P. H., Boyd, E. A., Kendall, D. A. (1997) Influence of cannabinoids on electrically evoked dopamine release and cyclic AMP generation in the rat striatum. *J Neurochem*, 69, 1131-1137.
- Cantrell, A. R., Scheuer, T., Catterall, W. A. (1999) Voltage-dependent neuromodulation of Na⁺ channels by D1-like dopamine receptors in rat hippocampal neurons. *J Neurosci*, 19, 5301-5310.
- Catterall, W. A. (1980) Neurotoxins that act of voltage-sensitive sodium channels in excitable membranes. *Ann Rev Pharmacol Toxicol*, 20, 15-43.
- Catterall, W. A. (1981) Inhibition of voltage-sensitive sodium channels in neuroblastoma cells by antiarrhythmic drugs. *Mol Pharmacol*, 20, 356-362.
- Catterall, W. A., Morrow, C. S., Daly, J. W., Brown, G. B. (1981) Binding of batrachotoxinin A 20- α -benzoate to a receptor site associated with sodium channels in synaptic nerve ending particles. *J Biol Chem*, 256, 8922-8927.

- Chemin, J., Monteil, A., Peres-Reyes, E., Nargeot, J., Lory, P. (2001) Direct inhibition of T-type calcium channels by the endogenous cannabinoid anandamide. *EMBO J*, 20, 7033–7040.
- Chen, T., Law, B., Kondratyuk, T., Rossie, S. (1995) Identification of soluble protein phosphatases that dephosphorylate voltage-sensitive sodium channels in rat brain. *J Biol Chem*, 270, 7750–7756.
- Chevaleyre, V., Castillo, P. E. (2003) Heterosynaptic LTD of hippocampal GABAergic synapses. A novel role of endocannabinoids in regulating excitability. *Neuron*, 38, 461-472.
- Childers, S., Sexton, T., Roy, M. (1994) Effects of anandamide on cannabinoid receptors in rat brain membranes. *Biochem Pharmacol*, 47, 711–715.
- Choi, D. W., Rothman, S. M. (1990) The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death. *Ann Rev Neurosci*, 13, 171-182.
- Corazzi, L., Porcellati, G., Freysz, L., Binaglia, L., Roberti, R., Arienti, G. (1986) Ethanolamine base-exchange reactions in rat brain microsomal subfractions. *J Neurochem*, 46, 202-207.
- Cousin, M. A., Nicholls, D. G. (1997) Synaptic vesicle recycling in cultured cerebellar granule cells: role of vesicular acidification and refilling. *J Neurochem*, 69, 1927-1935.
- Cravatt, B. F., Demarest, K., Patricelli, M. P., Bracey, M. H., Giang, D. K., Martin, B. R., Lichtman, A. H. (2001) Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. *Proc Natl Acad Sci USA*, 98, 9371–9376.
- Crawley, J. N., Corwin, R. L., Robinson, J. K., Felder, C. C., Devane, W. A., Axelrod, J. (1993) *Pharmacol Biochem Behav*, 46, 967-972.
- Crevling, C. R., Bell, M. E., Burke, T. R., Chang, Jr., E., Lewendowski-Lovenberg, G. A., Kim, C.-H., Rice, K. C., Daly, J. W. (1990) Procaine isothiocyanate: an irreversible inhibitor of the specific binding of [³H]batrachotoxinin A benzoate to sodium channels. *Neurochem Res*, 15, 441–448.
- Crevling, C. R., McNeal, E. T., Daly, J. W., Brown, G. B. (1983) Batrachotoxin-induced depolarization and [³H]batrachotoxin-A 20-alpha-benzoate binding in a vesicular preparation from guinea pig cerebral cortex: Inhibition by local anesthetics. *Mol Pharmacol*, 25, 350–358.
- Daniel, H. and Crepel, F. (2001) Control of Ca²⁺ influx by cannabinoid and metabotropic glutamate receptors in rat cerebellar cortex requires K⁺ channels. *J Physiol*, 537, 793-800.
- Daniel, H., Rancillac, A., Crepel, F. (2004) Mechanisms underlying cannabinoid inhibition of presynaptic Ca²⁺ influx at parallel fibre synapses of the rat cerebellum. *J Physiol*, 557, 159-174.

- Day, T. A., Rakhshan, F., Deutsch, D. G., Barker, E. L. (2001) Role of Fatty Acid Amide Hydrolase in the Transport of the Endogenous Cannabinoid Anandamide. *Mol Pharmacol*, 59, 1369-1375.
- Deecher, D. C., Payne, G. T., Soderlund, D. M. (1991) Inhibition of [³H]batrachotoxinin A 20- α -benzoate binding to mouse brain sodium channels by the dihydropyrazole insecticide RH 3421. *Pestic Biochem Physiol*, 41, 265–273.
- Derkinderen, P., Toutant, M., Burgaya, F., Le Bert, M., Siciliano, J. C., de Franciscis, V., Gelman, M., Girault, J. A. (1996) Regulation of a neuronal form of focal adhesion kinase by anandamide. *Science*, 273, 1719-1722.
- Deutsch, D. G., Ueda, N., Yamamoto, S. (2002) The fatty acid amide hydrolase (FAAH). *Prostaglandins Leukot Essent Fatty Acids*, 66, 201–210.
- Devane, W. A., Dysarz, F. A., Johnson, M. R., Melvin, L. S., Howlett, A. (1988) Determination and characterization of a cannabinoid receptor in rat brain. *Mol Pharmacol*, 34, 605-613.
- Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A., Mechoulam, R. (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science*, 258, 1946-1949.
- Di Marzo, V., Breivogel, C. S., Tao, Q., Bridgen, D. T., Razdan, R. K., Zimmer, A. M., Zimmer, A., Martin, B. R. (2000) Levels, metabolism and pharmacological activity of anandamide in CB1 receptor knockout mice: Evidence for non-CB1, non-CB2 receptor mediated actions in mouse brain. *J Neurochem*, 75, 2434–2444.
- Di Marzo, V., Fontana, A., Cadas, H., Schinelli, S., Cimino, G., Schwartz, J. C., Piomelli, D. (1994) Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature*, 372, 686–691.
- Di Marzo, V., Melck, D., Bisogno, T., Petrocellis, L. (1998) Endocannabinoids: endogenous cannabinoid receptor ligands with neuromodulatory action. *Trends Neurosci*, 21, 521–528.
- Drescher, M. J., Drescher, D. G., Hatfield, J. S. (1987) Potassium-evoked release of endogenous primary amine-containing compounds from the trout *sacculus macula* and sacculus nerve *in vitro*. *Brain Res*, 417, 39–50.
- Dunkley, P. R., Jarvie, P. E., Heath, G. W., Kidd, G. J., Rostas, J. A. P. (1986) A rapid method for isolation of synaptosomes on percoll gradients. *Brain Res*, 372, 115–129.
- Eells, J. T., Bandettini, P. A., Holman, P. A., Propp, J. M. (1992) Pyrethroid insecticide-induced alterations in mammalian synaptic membrane potential. *J Pharmacol Exp Therap*, 262, 1173–1181.

- Ellison, D. W., Beal, M. F., Martin, J. B. (1987) Amino acid neurotransmitters in postmortem human brain analyzed by high performance liquid chromatography with electrochemical detection. *J Neurosci Methods*, 19, 305-315.
- Ellison, D. W., Beal, M. F., Martin, J. B. (1987) Phosphoethanolamine and ethanolamine are decreased in Alzheimer's disease and Huntingdon's disease. *Brain Res*, 412, 389-392.
- Fan, P. (1995) Cannabinoid agonists inhibit the activation of 5-HT₃ receptors in rat nodose ganglion neurons. *J Neurophysiol*, 73, 907-910.
- Fassio, A., Bonanno, G., Fontana, G., Usai, C., Marchi, M., Raiteri, M. (1996) Role of external and internal calcium on heterocarriger-mediated transmitter release. *J Neurochem*, 66, 1468-1474.
- Fegley, D., Kathuria, S., Mercier, R., Li, C., Goutopoulos, A., Makriyannis, A., Piomelli, D. (2004) Anandamide transport is independent of fatty-acid amide hydrolase activity and is blocked by the hydrolysis-resistant inhibitor AM1172. *Proc Natl Acad Sci U S A*, 101, 8756-8761.
- Felder, C. C., Briley, E. M., Axelrod, J., Simpson, J. T., Mackie, K., Devane, W. A. (1993) Anandamide, an endogenous cannabimimetic eicosanoid, binds to the cloned human cannabinoid receptor and stimulates receptor-mediated signal transduction. *Proc Natl Acad Sci USA*, 90, 7656-7660.
- Felder, C. C., Joyce, K. E., Briley, E. M., Mansouri, J., Mackie, K., Blond, O., Lai, Y., Ma, A. L., Mitchell, R. L. (1995) Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors. *Mol Pharmacol*, 48, 443-450.
- Fezza, F., Bisogno, T., Minassi, A., Appendino, G., Mechoulam, R., Di Marzo, V. (2002) Noladin ether, a putative novel endocannabinoid: inactivation mechanisms and a sensitive method for its quantification in rat tissues. *FEBS Lett*, 513, 294-298.
- Floor, E., Leventhal, P. S., Schaeffer, S. F. (1990) Partial purification and characterization of the vacuolar H⁺-ATPase of mammalian synaptic vesicles. *J Neurochem*, 55, 1663-1670.
- Freund, T. F., Katona, I., Piomelli, D. (2003) Role of endogenous cannabinoids in synaptic signaling. *Physiol Rev*, 83, 1017-1066.
- Fride, E. and Mechoulam, R. (1993) Pharmacological activity of the cannabinoid receptor agonist, anandamide, a brain constituent. *Eur J Pharmacol*, 231, 313-314.
- Galiègue, S., Mary, S., Marchand, J., Dussossoy, D., Carrière, D., Carayon, P., Bouaboula, M., Shire, D., Le Fur, G., Casellas, P. (1995) Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur J Biochem*, 232, 54-61.

- Gaoni, Y., Mechoulam, R. (1964) Isolation, structure, and partial synthesis of an active constituent of hashish. *J Amer Chem Soc*, 86, 1646-1647.
- Gerard, C. M., Mollereau, C., Vassart, G., Parmentier, M. (1991) Molecular cloning of a human cannabinoid receptor which is also expressed in testis. *Biochem J*, 279, 129-134.
- Gerdeman, G. L., Ronesi, J., Lovinger, D. M. (2002) Postsynaptic endocannabinoid release is critical to long-term depression in the striatum. *Nat Neurosci*, 5, 446-451.
- Giang, D. K., Cravatt, B. F. (1997). Molecular characterization of human and mouse fatty acid amide hydrolases. *Proc Natl Acad Sci USA*, 94, 2238-2242.
- Gifford, A. N., Ashby Jr, C. R. (1996) Electrically evoked acetylcholine release from hippocampal slices is inhibited by the cannabinoid receptor agonist WIN 55212-2, and is potentiated by the cannabinoid antagonist, SR 141716A. *J Pharmacol Exp Therap*, 277, 1431-1436.
- Giuffrida, A., Piomelli, D. (1998) Isotope dilution GC/MS determination of anandamide and other fatty acylethanolamides in rat blood plasma. *FEBS Lett*, 422, 373-376.
- Giuffrida, A., Rodriguez de Fonseca, F., Nava, F., Loubet-Lescoulié, P., Piomelli, D. (2000) Elevated circulating levels of anandamide after administration of the transport inhibitor, AM404. *Eur J Pharmacol*, 408, 161-168.
- Glaser, S. T., Abumrad, N. A., Fatade, F., Kaczocha, M., Studholme, K. M., Deutsch, D. G. (2003) Evidence against the presence of an anandamide transporter. *Proc Natl Acad Sci USA*, 100, 4269-4274.
- Glaser, S. T., Kaczocha, M., Deutsch, D. G. (2005) Anandamide transport: a critical review. *Life Sci*, 77, 1584-1604.
- Glitsch, M., Parra, P., Llano, I. (2000) The retrograde inhibition of IPSCs in rat cerebellar Purkinje cells is highly sensitive to intracellular Ca^{2+} . *Eur J Neurosci*, 12, 987-993.
- Godlewski, G., Gothert, M., Malinowska, B. (2003) Cannabinoid receptor-independent inhibition by cannabinoid agonists of the peripheral 5-HT(3) receptor-mediated von Bezold-Jarisch reflex. *Br J Pharmacol*, 138, 767-774.
- Gong, J. P., Onaivi, E. S., Ishiguro, H., Liu, Q., Tagliaferro, P. A., Bruscoe, A., Uhl, G. R. (2006) Cannabinoid CB2 receptors: Immunohistochemical localization in rat brain. *Brain Res*, 1071, 10-23.
- Goparaju, S. K., Ueda, N., Taniguchi, K., Yamamoto, S. (1999) Enzymes of porcine brain hydrolyzing 2-arachidonoylglycerol, an endogenous ligand of cannabinoid receptors. *Biochemical Pharmacology*, 57, 417-423.

- Goparaju, S. K., Ueda, N., Yamaguchi, H., Yamamoto, S. (1998) Anandamide amidohydrolase reacting with 2-arachidonoylglycerol, another cannabinoid receptor ligand. *FEBS Lett*, 422, 69–73.
- Grassl, S. M. (2001) Ethanolamine Transport in Human Placental Brush-Border Membrane Vesicles. *J Pharmacol Exp Ther*, 298, 695-702.
- Greenshaw, A. J. (1993) Behavioural pharmacology of 5-HT₃ receptor antagonists: a critical update on therapeutic potential. *Trend Pharmacol Sci*, 14, 265-270,
- Hagiwara, N., Irisawa, H. and Kameyama, M. (1988) Contribution of two types of calcium currents to the pacemaker potentials of rabbit sino-atrial node cells. *J Physiol*, 395, 233–253.
- Hajos, F. (1975) An improved method for the preparation of synaptosomal fractions in high purity. *Brain Res*, 93, 485-489.
- Hajos, N., Ledent, C., Freund, T. F. (2001) Novel cannabinoid-sensitive receptor mediates inhibition of glutamatergic synaptic transmission in the hippocampus. *Neuroscience*, 106, 1–4.
- Hamberger, A., Haglid, K., Nystrom, B., Silfvenius, H. (1993) Co-variation of free amino acids in human epileptogenic cortex. *Neurochem Res*, 18, 519-525.
- Hamberger, A., Nystrom, B., Larsson, S., Silfvenius, H., Nordberg, C. (1991) Amino acids in the neural microenvironment of focal epileptic lesions. *Epilepsy Res*, 9, 32-43.
- Hanoune, J., Defer, N. (2001) Regulation and role of adenylyl cyclase isoforms. *Ann Rev Pharmacol Toxicol*, 41, 145-174.
- Harris, R. A., Allen, A. M. (1985) Functional coupling of γ -aminobutyric acid receptors to chloride channels in brain membranes. *Science*, 228, 1108–1110.
- Herkenham, M. (1995) Localization of cannabinoid receptors in brain and periphery. In: Pertwee RG, ed. *Cannabinoid Receptors*. New York: Academic Press, 145-166.
- Hilborn, M. D., Vaillancourt, R. R., Rane, S. G. (1998) Growth factor receptor tyrosine kinases acutely regulate neuronal sodium channels through the src signaling pathway. *J Neurosci*, 18, 590–600.
- Hill, R. J., Duff, H. J., Sheldon, R. S. (1989) Class I Antiarrhythmic drug receptor: biochemical evidence for state-dependent interaction with quinidine and lidocaine. *Mol Pharmacol*, 36, 150–159.
- Hillard, C. J., Edgmond, W. S., Jarrahian, A., Campbell, W. B. (1997) Accumulation of N-arachidonylethanolamine (anandamide) into cerebellar granule cells occurs via facilitated diffusion. *J Neurochem*, 69, 631–638.

- Hillard, C. J., Jarrahian, A. (2005) Cellular accumulation of anandamide: consensus and controversy. *Br J Pharmacol*, 140, 802-808.
- Hillard, C. J., Jarrahian, A. (2000) The movement of N arachidonylethanolamine (anandamide) across cellular membranes. *Chem Phys Lipids*, 108, 123-34.
- Holbrook, P. G., Wurtman, R. J. (1988) Presence of base-exchange activity in rat brain nerve endings: dependence on soluble substrate concentrations and effect of cations. *J Neurochem*, 50, 156– 162.
- Houweling, M., Tijburg, L. B. M., Vaartjes, W. J., Van Golde, L. M. G. (1992) Phosphatidylethanolamine metabolism in rat liver after partial hepatectomy. Control of biosynthesis of phosphatidylethanolamine by the availability of ethanolamine. *Biochem J*, 283, 55–61.
- Houweling, M., Tijburgh, L. B. M., Vaartjes, W. J., Van Golde, L. M. G., 1992. Phosphatidylethanolamine metabolism in rat liver after partial hepatectomy. *Biochem J*, 283, 55-61.
- Howlett, A. C., Fleming, R. M. (1984) Cannabinoid inhibition of adenylate cyclase; pharmacology of the response in neuroblastoma cell membranes. *Mol Pharmacol*, 26, 532-538.
- Huang, C., Lo, S., Hsu, K. (2001) Presynaptic mechanisms underlying cannabinoid inhibition of excitatory synaptic transmission in rat striatal neurons, *J Physiol*, 532, 731-748.
- Huguenard, J. R. (1996) Low-threshold calcium currents in central nervous system neurons. *Annu Rev Physiol*, 58, 329–348.
- Jarai, Z., Wagner, J. A., Varga, K., Lake, K. D., Compton, D. R., Martin, B. R., Zimmer, A. M., Bonner, T. I., Buckley, N. E., Mezey, E., Razdan, R. K., Zimmer, A., Kunos, G. (1999) Cannabinoid-induced mesenteric vasodilation through an endothelial site distinct from CB1 or CB2 receptors. *Proc Natl Acad Sci U S A*. 96, 14136-41.
- Jia, Y., McLeod, R. L., Hey, J. A. (2005) TRPV1 receptor: a target for the treatment of pain, cough, airway disease and urinary incontinence. *Drug News Perspect*, 18, 165-71.
- Joy, J. E., Watson, S. J., Benson, J. A., eds. (1999) *Marijuana and Medicine: Assessing the Science Base*. Washington, DC: National Academy Press.
- Kaplan, B. L., Ouyang, Y., Rockwell, C. E., Rao, G. K., Kaminski, N. E. (2005) 2-Arachidonoyl-glycerol suppresses interferon-gamma production in phorbol ester/ionomycin-activated mouse splenocytes independent of CB1 or CB2. *J Leukoc Biol*, 77, 966-74.
- Kapoor, R., Li, Y.-G., Smith, K. J. (1997) Slow sodium-dependent potential oscillations contribute to ectopic firing on mammalian demyelinated axons. *Brain*, 120, 647–652.

- Katona, I., Sperlagh, B., Sik, A., Kafalvi, A., Vizi, E. S., Mackie, K., Freund, T. F., (1999) Presynaptically located CB1 cannabinoid receptors regulate GABA release from axon terminals of specific hippocampal interneurons. *J Neurosci*, 19, 4544–4558.
- Kelley. B. G., Thayer, S. A. (2004) Anandamide transport inhibitor AM404 and structurally related compounds inhibit synaptic transmission between rat hippocampal neurons in culture independent of cannabinoid CB1 receptors. *Eur J Pharmacol*, 496, 33–39.
- Kempe, K., Hsu, F. F., Bohrer, A., Turk, J. (1996) Isotope dilution mass spectrometric measurements indicate that arachidonylethanolamide, the proposed endogenous ligand of the cannabinoid receptor, accumulates in rat brain tissue post mortem but is contained at low levels in or is absent from fresh tissue. *J Biol Chem*, 271, 17287–17295.
- Kilpatrick, I. C. (1991) Rapid, automated HPLC analysis of neuroactive and other amino acids in microdissected brain regions and brain slice superfusates using fluorimetric detection. In: B. Greenstein, Editor, *Neuroendocrine Research Methods Vol. 2*, Harwood Academic Publishers, Chur, Switzerland, 555–578.
- Kim, H. I., Kim, T. H., Shin, Y. K., Lee, C. S., Park, M. and Song, J. H. (2005) Anandamide suppression of Na⁺ currents in rat dorsal root ganglion neurons. *Brain Res*, 1062, 39–47.
- Kim, J., Isokawa, M., Ledent, C., Alger, B. E. (2002) Activation of muscarinic acetylcholine receptors enhances the release of endogenous cannabinoids in the hippocampus. *J Neurosci*, 22, 10182–10191.
- Kim, J., Wei, D. S., Hoffman, D. A. (2005) Kv4 potassium channel subunits control action potential repolarization and frequency-dependent broadening in rat hippocampal CA1 pyramidal neurones. *J Physiol*, 569, 41–57.
- Kiss, Z. (1999) Regulation of Mitogenesis by Water-Soluble Phospholipid Intermediates. *Cell Signal*, 11, 149–157.
- Kobayashi, Y., Arai, S., Waku, K., Sugiura, T., (2001) Activation by 2-arachidonylglycerol, an endogenous cannabinoid receptor ligand, of p42/44mitogen-activated protein kinase in HL-60 cells. *J of Biochem*, 129, 665–669.
- Kofalvi, A., Rodrigues, R. J., Ledent, C., Mackie, K., Vizi, E. S., Cunha, R. A., Sperlagh, B. (2005) Involvement of cannabinoid receptors in the regulation of neurotransmitter release in the rodent striatum: A combined immunochemical and pharmacological analysis. *J Neurosci*, 25, 2874–2884.
- Kofalvi, A., Vizi, E. S., Ledent, C., Sperlagh, B. (2003) Cannabinoids inhibit the release of [³H]glutamate from rodent hippocampal synaptosomes via a novel CB1 receptor-independent action. *European J of Neurosci*, 18, 1973–1978.

- Koga, D., Santa, T., Fukushima, T., Homma, H., Imai, K. (1997) Liquid chromatographic-atmospheric pressure chemical ionization mass spectrometric determination of anandamide and its analogs in rat brain and peripheral tissues. *J Chromatogr B Biomed Sci Appl*, 690, 7-13.
- Korf, J., Venema, K. (1985) Amino acids in rat striatal dialysates: Methodological aspects and changes after electroconvulsive shock. *J Neurochem*, 45, 1341-1348.
- Kozak, K. R., Marnett, L. J. (2002) Oxidative metabolism of endocannabinoids, Prostaglandins Leukot Essent Fatty Acids, 66, 211–220.
- Kreitzer, A. C., Regehr, W. G. (2001) Retrograde inhibition of presynaptic calcium influx by endogenous cannabinoids at excitatory synapses onto Purkinje cells. *Neuron*, 29, 717–727.
- Kreitzer, A. C., Carter, A. G., Regehr, W. G. (2002) Inhibition of interneuron firing extends the spread of endocannabinoid signaling in the cerebellum. *Neuron*, 34, 787–796.
- Lambert, D. M., Fowler, C. J. (2005) The endocannabinoid system: Drug targets, lead compounds, and potential therapeutic applications. *J Med Chem*, 48, 5059-5087.
- Lan, R, Liu, Q., Fan, P., Lin, S., Fernando, S. R., McCallion, D., Pertwee, R., Makriyannis, A. (1999) Structure-activity relationships of pyrazole derivatives as cannabinoid receptor antagonists. *J Med Chem*, 42, 769–776.
- Ledent, C., Mody, I., Hajos, N., Katona, I., Naiem, S. S., Mackie, K., Freund, T. F. (2000) Cannabinoids inhibit hippocampal GABAergic transmission and network oscillations. *Eur J Neurosci*, 12, 3239–3249.
- Ledent, C., Valverde. O., Cossu. G., Petitet. F., Aubert, J. F., Beslot. F., Bohme. G. A., Imperato. A., Pedrazzini. T., Roques. B. P., Vassart. G., Fratta. W., Parmentier, M. (1999) Unresponsiveness to cannabinoids and reduced addictive effects of opiates in CB1 receptor knockout mice. *Science*, 283, 401-404.
- Lenz, R. A., Wagner, J. J., Alger, B. E. (1998) N- and L-type calcium channel involvement in depolarization-induced suppression of inhibition in rat hippocampal CA1 cells. *J Physiol (London)*, 512, 61–73.
- Levenes, C., Daniel, H., Soubrie, P., Crepel, F. (1998) Cannabinoids decrease excitatory synaptic transmission and impair long term depression in rat cerebellar Purkinje cells. *J Physiol*, 510, 867–879.
- Li, M., West, J. W., Lai, Y., Scheuer, T., Catterall, W. A. (1992) Functional modulation of brain sodium channels by cAMP-dependent phosphorylation. *Neuron*, 8, 1151–1159.
- Liao, C., Nicholson, R. A. (2005) Depolarization-induced release of ethanolamine from brain synaptic preparations *in vitro*. *Brain Res*, 1060, 170-178.

- Liao, C., Zhen, J., David, L. S., Nicholson, R. A. (2004) Inhibition of voltage-sensitive sodium channels by the cannabinoid 1 receptor antagonist AM251 in mammalian brain. *Basic Clin Pharmacol Toxicol*, 94, 73–78.
- Ligresti, A., Morera, E., Van Der Stelt, M., Monory, K., Lutz, B., Di Marzo, V. (2004) Further evidence for the existence of a specific process for the membrane transport of anandamide. *Biochem J*, 380, 265-272.
- Ligresti, A., Cascio, M. G., Pryce, G., Kulasegram, S., Beletskaya, I., De Petrocellis, L., Saha, B., Mahadevan, A., Visintin, C., Wiley, J. L., Baker, D., Martin, B. R., Razdan, R. K., Di Marzo, V. (2006) New potent and selective inhibitors of anandamide reuptake with antispastic activity in a mouse model of multiple sclerosis. *Br J Pharmacol*, 147, 83-91.
- Lin, S., Khanolkar, A. D., Fan, P., Goutopoulos, A., Qin, C., Papahadjis, D., Makriyannis, A. (1998) Novel analogues of arachidonylethanolamide (anandamide): affinities for the CB1 and CB2 cannabinoid receptors and metabolic stability. *J Med Chem*, 41, 5353-5361.
- Lindstrom, J. (1996) Neuronal nicotinic acetylcholine receptors. In: T. Narahashi, Editor, *Ion Channels* vol. 4, Plenum, New York, pp. 377–390.
- Lipton, B. A., Davidson, E.P., Ginsberg, B.H., Yorek, M. A. (1990) Ethanolamine metabolism in cultured bovine aortic endothelial cells. *J Biol Chem*, 265, 7195-7201.
- Liu, Q., Tonai, T., Ueda, N. (2002) Activation of N-acylethanolamine-releasing phospholipase D by polyamines. *Chem Phys Lipids*, 115, 77– 84.
- Llano, I., Leresche, N., Marty, A. (1991) Calcium entry increases the sensitivity of cerebellar Purkinje cells to applied GABA and decreases inhibitory synaptic currents. *Neuron*, 6, 565–574.
- Lopez-Rodriguez, M. L., Viso, A., Ortega-Gutierrez, S., Fowler, C. J., Tiger, G., de Lago, E., Fernandez-Ruiz, J., Ramos, J. A. (2003) Design, synthesis, and biological evaluation of new inhibitors of the endocannabinoid uptake: comparison with effects on fatty acid amidohydrolase. *J Med Chem*, 46, 1512–1522.
- Loscher, W. (1983) Effect of 2-aminoethanol on the synthesis, binding, uptake and metabolism of GABA. *Neurosci Lett*, 42, 293–297.
- Lu, Y., Grady, S., Marks, M. J., Picciotto, M., Changeux, J. P., Collins, A. C. (1998) Pharmacological characterization of nicotinic receptor-stimulated GABA release from mouse brain synaptosomes. *J Pharmacol Exp Ther*, 287, 648–657.
- Luo, C., Kumamoto, E., Furue, H., Chen, J., Yoshimura, M. (2002) Anandamide inhibits excitatory transmission to rat substantia gelatinosa neurones in a manner different from that of capsaicin. *Neurosci Lett*, 321, 17–20.

- Maccarrone, M., De Felici, M., Bari, M., Klinger, F., Siracusa, G., Finazzi-Agrò, A. (2000) Down-regulation of anandamide hydrolase in mouse uterus by sex hormones. *Eur J Biochem*, 267, 2991–2997.
- Maccarrone, M., Finazzi-Agro, A. (2003) The endocannabinoid system, anandamide and the regulation of mammalian cell apoptosis. *Cell Death Differ*, 10, 946-955.
- Maccarrone, M., van der Stelt, M., Rossi, A., Veldink, G. A., Vliegthart, J. F. G., Finazzi-Agrò, A. (1998) Anandamide hydrolysis by human cells in culture and brain. *J Biol Chem*, 273, 32332–32339.
- Mackie, K., Lai, Y., Westenbroek, R., Mitchell, R. (1995) Cannabinoids activate an inwardly rectifying potassium conductance and inhibit Q-type calcium currents in AtT20 cells transfected with rat brain cannabinoid receptor. *J Neurosci*, 15, 6552–6561.
- MacKinnon, A. C., Wyatt, K. M., McGivern, J. G., Sheridan, R. D., Brown, C. M. (1996) [³H]Lifarizine, a high affinity probe for inactivated sodium channels. *Br J Pharmacol*, 118, 162–166.
- Maejima, T., Hasimoto, K., Yoshida, T., Aiba, A., Kano, M., (2001). Presynaptic inhibition caused by retrograde signals from metabotropic glutamate to cannabinoid receptors. *Neuron*, 31, 463–475.
- Maingret, F., Patel, A. J., Lazdunski, M., Honore, E. (2001) The endocannabinoid anandamide is a direct and selective blocker of the background K⁺ channel TASK-1. *EMBO J*, 20, 47–54.
- Mandala, M., Serck-Hanssen, G., Martino, G., Helle, K. B. (1999) The fluorescent cationic dye rhodamine 6G as a probe for membrane potential in bovine aortic endothelial cells. *Analyt Biochem*, 274, 1-6.
- Marinelli, S., Vaughan, C. W., Christie, M. J., Connor, M. (2002) Capsaicin activation of glutamatergic synaptic transmission in the rat locus coeruleus *in vitro*. *J Physiol*, 543, 531–540.
- Martin, B. R., Mechoulam, R., Razdan, R. K. (1999) Discovery and characterization of endogenous cannabinoids. *Life Sci*, 65, 573-736.
- Massarelli, A. C., Dainous, F., Hoffmann, D., Mykita, S., Freysz, L., Dreyfus, H., Massarelli, R. (1986) Uptake of ethanolamine in neuronal and glial culture. *Neurochem Res*, 11, 29–36.
- Massarelli, R., Dainous, F., Freysz, L., Dreyfus, H., Mozzi, R., Floridi, A., Siepi, D., Porcellati, G. (1982) The role of choline phospholipids and choline neosynthesis in the regulation of acetylcholine metabolism. In: Giuffrida-Stella, A.M., Gombos, G., Benzi, G. and Bachelard, H.S. Editors, *Basic and Clinical Aspects of Neurobiology*, Found Internat Menarini, Milan, 147–155.

- Matsuda, L. A., Lolait, S. J., Brownstein, M., Young, A., Bonner, T. I. (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature*, 346, 561-564.
- Matyas, F., Yanovsky, Y., MacKie, K., Kelsch, W., Misgeld, U., Freund, T. F. (2006) Subcellular localization of type 1 cannabinoid receptors in the rat basal ganglia. *Neurosci*, 137, 337-361
- McFarland, M. J., Porter, A. C., Rakhshan, F. R., Rawat, D. S., Gibbs, R. A., Barker, E. L. (2004) A Role for Caveolae/Lipid Rafts in the Uptake and Recycling of the Endogenous Cannabinoid Anandamide. *J Biol Chem*, 279, 41991 – 41997.
- McPartland, J., Di Marzo, V., De Petrocellis, L., Mercer, A., Glass, M. (2001) Cannabinoid receptors are absent in insects. *J Comp Neuro*, 436, 423-429.
- Mechoulam, R., Ben-Shabat, S., Hanus, L., Ligumsky, M., Kaminski, N. E., Schatz, A. R., Gopher, A., Almog, S., Martin, B. R., Compton, D. R., Pertwee, R. G., Griffin, G., Bayewitch, M., Barg, J., Vogel, Z. (1995) Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem Pharmacol*, 50, 83- 90.
- Mechoulam, R., Deutsch, D. G. (2005) Toward an anandamide transporter. *Proc Natl Acad Sci U S A*, 102, 17541-1742.
- Meldrum, B. S. (1994) The role of glutamate in epilepsy and other CNS disorders. *Neurology*, 44, 14-23.
- Melnik, V. I., Bikbulatova, L. S., Gulyaeva, N. V., Basyan, A. S. (2001) Synaptic vesicle acidification and exocytosis studied with acridine orange fluorescence in rat brain synaptosomes. *Neurochem Res*, 26, 549-554.
- Melvin, L. S., Johnson M. R. (1987) Structure-activity relationships of tricyclic and nonclassical bicyclic cannabinoids. *NIDA Res Monogr*, 79, 31-47.
- Mendelson, W. B., Basile, A. S. (1999) The hypnotic actions of oleamide are blocked by a cannabinoid antagonist. *Neuroreport*, 10, 3237–3239.
- Moore, S. A., Nomikos, G. G., Dickason-Chesterfield, A. K., Schober, D. A., Schaus, J. M., Ying, B.-P., Xu, Y.-C., Phebus, L., Simmons, R. M. A., Li, D., Iyengar, S., Felder, C. C. (2005) Identification of a high-affinity binding site involved in the transport of endocannabinoids. *Proc Natl Acad Sci U S A*, 102, 17852-17857.
- Morel, N. (2003) Neurotransmitter release: the dark side of the vacuolar-H⁺-ATPase. *Biol Cell*, 95, 453-457.
- Morisset, V., Urban, L. (2001) Cannabinoid-induced presynaptic inhibition of glutamatergic EPSCs in substantia gelatinosa neurons of the rat spinal cord. *J Neurophysiol*, 86, 40–48.

- Moriyama, Y., Futai, M. (1990) H⁺-ATPase, a primary pump for accumulation of neurotransmitters, is a major constituent of brain synaptic vesicles. *Biochem Biophys Res Commun*, 173, 443-448.
- Mukhopadhyay, S., Shim, J. Y., Assi, A. A., Norford, D., Howlett, A. C. (2002) CB1 cannabinoid receptor-G protein association: a possible mechanism for differential signalling. *Chem Phys Lipids*, 121, 91-109.
- Munro, S., Thomas, K. L., Abu-Shaar, M. (1993) Molecular characterization of a peripheral receptor for cannabinoids. *Nature*, 365, 61-65.
- Nachshen D. A., Blaustein, M. P. (1980) Some properties of potassium stimulated calcium influx in presynaptic nerve endings. *J Gen Physiol*, 76, 709-728.
- Nemeth, J., Helyes, Z., Than, M., Jakab, B., Pinter, E., Szolcsanyi, J. (2003) Concentration-dependent dual effect of anandamide on sensory neuropeptide release from isolated rat tracheae. *Neurosci Lett*, 336, 89-92.
- Nicholson, R. A. (1992) Insecticidal dihydropyrazoles antagonize the depolarizing action of veratridine in mammalian synaptosomes as measured with a voltage-sensitive dye. *Pestic Biochem Physiol*, 42, 197-202.
- Nicholson, R. A. and Merletti, E. L. (1990) The effect of dihydropyrazoles on release of [³H]GABA from nerve terminals isolated from mammalian cerebral cortex. *Pestic Biochem Physiol*, 37, 30-40.
- Nicholson, R. A., Liao, C., Zheng, J., David, L. S., Coyne, L., Errington, A. C., Singh, G., Lees, G. (2003) Sodium channel inhibition by anandamide and synthetic cannabimimetics in mammalian brain. *Brain Res*, 978, 194-204.
- Nicholson, R. A., Zheng, J., Ganellin, R., Verdon, B., Lees, G. (2001) Anesthetic-like interaction of the sleep-inducing lipid oleamide with voltage-gated sodium channels in mammalian brain. *Anesthesiology*, 94, 120-128.
- Noguron, M. I., Porgilsson, B., Schneider, W. E., Stucky, C. L., Hillard, C. J. (2001) Cannabinoid receptor agonists inhibit depolarization-induced calcium influx in cerebellar granule neurons. *J Neurochem*, 79, 371-381.
- Numann, R., Catterall, W. A. Scheuer, T. (1991) Functional modulation of brain sodium channels by protein kinase C phosphorylation. *Science*, 254, 115-118.
- Numazaki, M., Tominaga, T., Toyooka, H., Tominaga, M. (2002) Direct phosphorylation of capsaicin receptor VR1 by protein kinase C epsilon and identification of two target serine residues. *J Biol Chem*, 277, 13375-13378.
- Ohno-Shosaku, T., Maejima, T., Kano, M. (2001) Endogenous cannabinoids mediate retrograde signals from depolarized postsynaptic neurons to presynaptic terminals. *Neuron*, 29, 729-738.

- Ohno-Shosaku, T., Tsubokawa, H., Mizushima, I., Yoneda, N., Zimmer, A., Kano, M. (2002) Presynaptic cannabinoid sensitivity is a major determinant of depolarization-induced retrograde suppression at hippocampal synapses. *J Neurosci*, 22, 3864-3872.
- Ohta, M., Narahashi, T., Keeler, R. F. (1973) Effects of veratrum alkaloids on membrane potential and conductance of squid and crayfish axons. *J Pharmacol Exp Ther*, 184, 143-154.
- Oliver, D., Lien, C., Soom, M., Baukowitz, T., Jonas, P., Fakler, B. (2004) Functional conversion between A-type and delayed rectifier K⁺ channels by membrane lipids. *Science*, 304, 265-270.
- Onaivi, E. S., Leonard, C. M., Ishiguro, H., Zhang, P. W., Lin, Z., Akinshola B. E., Uhl, G. R. (2002) Endocannabinoids and cannabinoid receptor genetics. *Prog Neurobiol*, 66, 307-344.
- Ortar, G., Ligresti, A., De Petrocellis, L., Morera, E., Di Marzo, V. (2003) Novel selective and metabolically stable inhibitors of anandamide cellular uptake. *Biochem Pharmacol*, 65, 1473-1481.
- Oz, M. (2006) Receptor-independent actions of cannabinoids on cell membranes: Focus on endocannabinoids. *Pharmacol Ther*, 111, 114-144
- Oz, M., Zhang, L., Ravindran, A., Morales, M., Lupica, C. R. (2004) Differential effects of endogenous and synthetic cannabinoids on $\alpha 7$ -nicotinic acetylcholine receptor-mediated responses in *Xenopus* oocytes. *J Pharmacol Exp Ther*, 310, 1152-1160.
- Oz, M., Ravindran, A., Diaz-Ruiz, O., Zhang, L., Morales, M. (2003) The endogenous cannabinoid anandamide inhibits $\alpha 7$ -nicotinic acetylcholine receptor-mediated responses in *Xenopus* oocytes. *J Pharmacol Exp Ther*, 306, 1003-1010.
- Pagotto, U., Marsicano, G., Cota, D., Lutz, B., Pasquali, R. (2006) The emerging role of the endocannabinoid system in endocrine regulation and energy balance. *Endoc Rev*, 27, 73-100.
- Paria, B. C., Deutsch, D. D., Dey, S. K. (1996) The uterus is a potential site for anandamide synthesis and hydrolysis: differential profiles of anandamide synthase and hydrolase activities in the mouse uterus during the periimplantation period. *Mol Reprod Dev*, 45, 183-192.
- Patel, S., Carrier, E. J., Ho, W. S., Rademacher, D. J., Cunningham, S., Reddy, D. S., Falck, J. R., Cravatt, B. F., Hillard, C. J. (2004) The postmortal accumulation of brain N-arachidonylethanolamine (anandamide) is dependent upon fatty acid amide hydrolase activity. *J Lipid Res*, 46, 342-349.
- Perschak, H., Wolfensberger, M., Do, K. Q., Dunant, Y., Cuenod, M. (1986) Release of ethanolamine, but not serine or choline, in rat pontine nuclei on stimulation of afferents from the cortex, *in vivo*. *J Neurochem*, 46, 1338-1343.

- Pertwee, R. G. (1997) Pharmacology of cannabinoid CB1 and CB2 receptors. *Pharmacol Ther*, 74, 129-180.
- Peterson, G. L. (1977) A simplification of the method of Lowry et al. which is more generally applicable. *Anal Biochem*, 83, 346-356.
- Piomelli, D. (2003) The molecular logic of endocannabinoid signalling. *Nat Rev Neurosci*, 4, 873-884
- Piomelli, D., Beltramo, M., Giuffrida, A., Stella, N. (1998) Endogenous cannabinoid signaling. *Neurobiol Dis*, 5, 462-473.
- Pitler, T. A., Alger, B. E. (1992) Postsynaptic spike firing reduces synaptic GABA_A responses in hippocampal pyramidal cells. *J Neurosci*, 12, 4122-4132.
- Pitler, T. A., Alger, B. E., (1994) Depolarization-induced suppression of GABAergic inhibition in rat hippocampal pyramidal cells: G-protein involvement in a presynaptic mechanism. *Neuron*, 13, 1447-1455.
- Pocock, J. M., Cousin, M. A., Parkin, J., Nicholls, D. G. (1995) Glutamate exocytosis from cerebellar granule cells: the mechanism of a transition to an L-type Ca²⁺ channel coupling. *Neuroscience*, 67, 595-607.
- Porcellati, G., Arienti, G., Pirrotta, A. and Giogini, D. (1971) Base-exchange reactions for the synthesis of phospholipids in nervous tissue: the incorporation of serine and ethanolamine into phospholipids of isolated brain microsomes. *J Neurochem*, 18, 1395-1417.
- Porter, A. C., Sauer, J. M., Knierman, M. D., Becker, G. W., Berna, M. J., Bao, J., Nomikos, G. G., Carter, P., Bymaster, F. P., Leese, A. B., Felder, C. C. (2002) Characterization of a novel endocannabinoid, virodhamine, with antagonist activity at the CB1 receptor. *J. Pharmacol Exp Ther*, 301, 1020-1024.
- Postma, S. W., Catterall, W. A. (1984) Inhibition of binding of [³H]batrachotoxin A 20- α -benzoate to sodium channels by local anesthetics. *Mol Pharmacol*, 25, 219-227.
- Pu, G. A., Anderson, R. E. (1984) Ethanolamine accumulation by photoreceptor cells of the rabbit retina. *J. Neurochem*, 42, 185-191.
- Raiteri, L., Stigliani, S., Zedda, L., Raiteri, M., Bonanno, G. (2002) Multiple mechanisms of transmitter release evoked by "pathologically" elevated extracellular [K⁺]: involvement of transporter reversal and mitochondrial calcium. *J Neurochem*, 80, 706-714.
- Ratcliffe, C. F., Qu, Y., McCormick, K. A., Tibbs, V. C., Dixon, J. E., Scheuer, T., Catterall, W.A. (2000) Modulation of brain sodium channels by associated receptor protein tyrosine phosphatase beta. *Nat Neurosci*, 3, 437-444.

- Ratnakumari, L., Hemmings, H. C. (1996) Inhibition by propofol of [³H]batrachotoxinin-A 20- α -benzoate binding to voltage-dependent sodium channels in rat cortical synaptosomes. *Br J Pharmacol*, 119, 1498–1504.
- Reimer, R. J., Fon, E. A., Edwards, R. H. (1998) Vesicular neurotransmitter transport and the presynaptic regulation of quantal size. *Curr Opin Neurobiol*, 8, 405-412.
- Rhee, M. H., Bayewitch, M., Avidor-Reiss, T., Levy, R., Vogel, Z., (1998). Cannabinoid receptor activation differentially regulates the various adenylyl cyclase isozymes. *J Neurochem*, 71, 1525-1534.
- Richardson, J. D., Kilo, S., Hargreaves, K. M., (1998) Cannabinoids reduce hyperalgesia and inflammation via interaction with peripheral CB1 receptors. *Pain*, 75, 111–119.
- Rinaldi-Carmona, M., Barth, F., Heaulme, M., Shire, D., Calandra, B., Congy, C., Martinez, S., Marauni, J., Neliat, G., Caput, D., Ferrara, P., Soubrie, P., Breliere, J., Le Fur, G. (1994) SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. *FEBS Lett*, 350, 240–244..
- Ronesi, J., Gerdeman, G. L., Lovingen, D. M. (2004) Disruption of endocannabinoid release and striatal long-term depression by postsynaptic blockade of endocannabinoid membrane transport. *J Neurosci*, 24, 1673–1679.
- Rossier, M. F., Burnay, M. M., Vallotton, M. B., Capponi, A. M. (1996) Distinct functions of T- and L-type calcium channels during activation of bovine adrenal glomerulosa cells. *Endocrinology*, 137, 4817–4826.
- Roz, N., Rehavi, M. (2003) Hyperforin inhibits vesicular uptake of monoamines by dissipating pH gradient across synaptic vesicle membrane. *Life Sci*, 73, 461-470.
- Salzet, M. and Stefano, G. B. (2002) The endocannabinoid system in invertebrates. *Prostaglandins Leukot Essent Fatty Acids*, 66, 353-361.
- Sanchez-Prieto, J., Sihra, T.S., Nicholls, D. G. (1987) Characterization of the exocytotic release of glutamate from guinea-pig cerebrocortical synaptosomes. *J Neurochem*, 49, 58–64.
- Schmid, P. C., Paria, B. C., Krebsbachm, R. J., Schmid, H. H., Dey, S. K. (1997) Changes in anandamide levels in mouse uterus are associated with uterine receptivity for embryo implantation. *Proc Natl Acad Sci USA*, 94, 4188-4192.
- Sha, D., Jin, H., Kopke, R. D., Wu, J-Y. (2004) Choline acetyltransferase: regulation and coupling with protein kinase and vesicular acetylcholine transporter on synaptic vesicles. *Neurochem Res*, 29, 199-207.
- Sheldon, R. S., Duff, H. J., Thakore, E., Hill, R. J. (1994) Class I Antiarrhythmic drugs: allosteric inhibitors of [³H]batrachotoxinin binding to rat cardiac sodium channels. *J Pharmacol Exp Ther*, 268, 187–194.

- Sheldon, R. S., Hill, R. J., Cannon, N. J., Duff, H. J. (1989) Amiodarone: Biochemical evidence for binding to a receptor for Class I drugs associated with the rat cardiac sodium channel. *Circ Res*, 65, 477–482.
- Shen, M., Piser, T. M., Seybold, V. S., Thayer, S. A. (1996) Cannabinoid receptor agonists inhibit glutamatergic synaptic transmission in rat hippocampal cultures. *J Neurosci*, 16, 4322–4334.
- Shioi, J., Naito, S., Ueda, T. (1989) Glutamate uptake into synaptic vesicles of bovine cerebral cortex and electrochemical potential difference of proton across the membrane. *Biochem J*, 258, 499-504.
- Smith, P. B., Compton, D. R., Welch, S. P., Razdan, R. K., Mechoulam, R., Martin, B. R. (1994) The pharmacological activity of anandamide, a putative endogenous cannabinoid, in mice. *J Pharmacol Exp Ther*, 270, 219-227.
- Souilhac, J., Poncelet, M., Rinaldi-Carmona, M., Le Fur, G., Soubrie, P. (1995) Intrastratial injection of cannabinoid receptor agonists induced turning behavior in mice. *Pharmacol Biochem Behav*, 51, 3-7.
- Spanner, S., Ansell, G. B. (1978) The determination of free ethanolamine in brain tissue and its release on incubation. *J. Neurochem*, 30, 497–498.
- Steffens, M., Feuerstein, T. J., van Velthoven, V., Schnierle, P., Knörle, R. (2003) Quantitative measurement of depolarization-induced anandamide release in human and rat neocortex. *Naunyn Schmiedebergs Arch Pharmacol*, 368, 432-436.
- Stella, N., Schweitzer, P., Piomelli, D. (1997) A second endogenous cannabinoid that modulates long-term potentiation. *Nature*, 388, 773–778.
- Stremmel, W., Pohl, L., Ring, A., Herrmann, T. (2001) A new concept of cellular uptake and intracellular trafficking of long-chain fatty acids. *Lipids*, 36, 981-989.
- Strichartz, G., Rando, T., Wang, G. K. (1987) An integrated view of the molecular toxinology of sodium channel gating in excitable cells. *Annu Rev Neurosci*, 10, 237–267.
- Sugiura, T., Kondo, S., Sukagawa, A., Nakane, S., Shinoda, A., Itoh, K., Yamashita, A., Waku, K. (1995) 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. *Biochem Biophys Res Commun*, 215, 89-97.
- Sun, Y. X., Tsuboi, K., Okamoto, Y., Tonai, T., Murakami, M., Kudo, I., Ueda, N. (2004) Biosynthesis of anandamide and N-palmitoylethanolamine by sequential actions of phospholipase A 2 and lysophospholipase D. *Biochem J*, 380, 749–56.
- Szallasi, A., Blumberg, P. M., Szallasi, A., Blumberg, P. M. (1999) Vanilloid (capsaicin) receptors and mechanisms, *Pharmacol Rev*, 51, 159–211.

- Tabb, J. S., Kish, P. E., Van Dyke, R., Ueda, T. (1992) Glutamate transport into synaptic vesicles. *J Biol Chem*, 267, 15412-15418.
- Takahashi, K. A., Linden, D. J. (2000) Cannabinoid receptor modulation of synapses received by cerebellar Purkinje cells. *J Neurophysiol*, 83, 1167-1180.
- Talley, E. M., Lei, Q., Sirois, J. E., Bayliss, D. A. (2000) TASK-1, a two-pore domain K^+ channel, is modulated by multiple neurotransmitters in motoneurons. *Neuron*, 25, 399-410.
- Thomas, B. F., Gilliam, A. F., Burch, D. F., Roche, M. J., Seltzman, H. H. (1998) Comparative receptor binding analyses of cannabinoid agonists and antagonists. *J Pharmacol Exp Therap*, 285, 285-292.
- Tibbs, G. R., Barrie, A. P., Van Mieghem, F. J. E., McMahon, H. T., Nicholls, D. G. (1989) Repetitive action potentials in isolated nerve terminals in the presence of 4-aminopyridine: effects on cytosolic free Ca^{2+} and glutamate release. *J Neurochem*, 53, 1693-1699.
- Tisdale, E. J., Tartakoff, A. M. (1988) Extensive labeling with éthanolamine of a hydrophilic protein of animal cells. *J Biol Chem*, 263, 8244-8252.
- Townsend, D. 4th., Thayer, S. A., Brown, D. R. (2002) Cannabinoids throw up a conundrum. *Br J Pharmacol*, 137, 575-7.
- Turkanis, S. A., Partlow, L. M., Karler, R. (1991) Delta-9 tetrahydrocannabinol depresses inward sodium current in mouse neuroblastoma cells. *Neuropharmacology*, 30, 73-77.
- Turkanis, S. A., Karler, R., Partlow, L. M. (1991) Differential effects of delta-9-tetrahydrocannabinol and its 11-hydroxy metabolite on sodium current in neuroblastoma cells. *Brain Res*, 560, 245-250.
- Twitchell, W., Brown, S., Mackie, K. (1997) Cannabinoids inhibit N- and P/Q-type calcium channels in cultured hippocampal neurons. *J Neurophysiol*, 78, 43-50.
- Van Der Heyden, J. A. M., Venema, K., Korf, J. (1979) *In vivo* release of endogenous GABA from rat substantia nigra measured by a novel method. *J Neurochem*, 32, 469-476.
- Van der Stelt, M., Di Marzo, V. (2005) Anandamide as an intracellular messenger regulating ion channel activity. *Prostaglandins Other Lipid Mediat*, 77, 111-122.
- Van der Stelt, M., Trevisani, M., Vellani, V., De Petrocellis, L., Schiano, M. A., Campi, B., McNaughton, P. A., Geppetti, P., Di Marzo, V. (2005) Anandamide acts as an intracellular messenger amplifying Ca^{2+} influx via TRPV1 channels. *EMBO Journal*, 24, 3026-3037.
- Vandevoorde, S., Fowler, C. J. (2005) Inhibition of fatty acid amide hydrolase and monoacylglycerol lipase by the anandamide uptake inhibitor VDM11: evidence that VDM11 acts as an FAAH substrate. *Br J Pharmacol*, 145, 885-93.

- Varga, K., Wagner, J. A., Bridgen, T., Kunos, G. (1998) Platelet and macrophage-derived endogenous cannabinoids are involved in endotoxin-induced hypotension. *FASEB J*, 12, 1035–1044.
- Varma, N., Carlson, G. C., Ledent, C., Alger, B. E. (2001) Metabotropic glutamate receptors drive the endocannabinoid system in hippocampus. *J Neurosci*, 21, 1–5.
- Verdon, B., Zheng, J., Nicholson, R. A., Ganellin, C., Lees, G. (2000) Stereoselective barbiturate-like actions of oleamide on GABA_A receptors and voltage-gated Na⁺ channels *in vitro*: a putative endogenous ligand for depressant drug sites in CNS. *Brit J Pharmacol*, 129, 283–290.
- Vincent, P., Armstrong, C. M., Marty, A. (1992) Inhibitory synaptic currents in rat cerebellar Purkinje cells: modulation by postsynaptic depolarization. *J Physiol (London)*, 456, 453–471.
- Wang, J., Zucker, R. S. (2001) Photolysis-induced suppression of inhibition in rat hippocampal CA1 pyramidal neurons. *J Physiol*, 533, 757–763.
- Willow, M., Catterall, W. A. (1982) Inhibition of binding of [³H]batrachotoxin A 20- α -benzoate to sodium channels by the anticonvulsant drugs diphenylhydantoin and carbamazepine. *Mol Pharmacol*, 22, 627–635.
- Wilson, R. I., Nicoll, R. A. (2001) Endogenous cannabinoids mediate retrograde signalling at hippocampal synapses. *Nature*, 410, 588–592.
- Wilson, R. I., Nicoll, R. A. (2002) Endocannabinoid signaling in the brain. *Science*, 296, 678–682.
- Wolfensberger, M., Felix, D., Cuenod, M. (1982) 2-Aminoethanol as a possible neuromodulator in the pigeon optic tectum. *Neurosci Lett*, 32, 53–58.
- Wolosker, H., de Souza, D. O., de Meis, L. (1996) Regulation of glutamate transport into synaptic vesicles by chloride and proton gradient. *J Biol Chem*, 271, 11726–11731
- Yagen, B., Burstein, S. (2000) Novel and sensitive method for the detection of anandamide by the use of its dansyl derivative. *J Chromatogr B Biomed Sci Appl*, 740, 93–99.
- Zhang, A., Towner, P., Nicholson, R. A. (1996) Dihydropyrazole insecticides: Interference with depolarization-dependent phosphorylation of synapsin I and evoked release of L-glutamate in nerve terminal preparations from mammalian brain. *Pestic Biochem Physiol*, 54, 24–30.
- Zhang, C. Y., Baffy, G., Perret, P., Krauss, S., Peroni, O., Grujic, D., Hagen, T., Vidal-Puig, A. J., Boss, O., Kim, Y. B., Zheng X. X., Wheeler, M. B., Shulman, G. I., Chan, C. B., Lowell, B. B. (2001) Uncoupling protein-2 negatively regulates insulin secretion and is a major link between obesity, beta cell dysfunction, and type 2 diabetes. *Cell*, 105, 745–755.

- Zimmer, A., Zimmer, A. M., Hohmann, A. G., Herkenham, M., Bonner, T. I. (1999) Increased mortality, hypoactivity, and hypoalgesia in cannabinoid CB1 receptor knockout mice. *Proc Natl Acad Sci USA*, 96, 5780-5785.
- Zoccarato, F., Cavallini, L., Alexandre, A. (1999) The pH-sensitive dye acridine orange as a tool to monitor exocytosis/endocytosis in synaptosomes. *J Neurochem*, 72, 625-633.
- Zygmunt, P. M., Chuang, H., Movahed, P., Julius, D., Hogestatt, D. E. D. (2000) The anandamide transport inhibitor AM404 activates vanilloid receptors. *Eur J Pharmacol*, 396, 39-42.
- Zygmunt, P. M., Petersson, J., Andersson, D. A., Chuang, H. H., Sorgard, M., Di Marzo, V., Julius, D., Hogestatt, E. D. (1999) Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature*, 400, 452-457.