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A Novel Role of Cannabinoids in Synaptogenesis

par
Sara Hamzeh

Faculté de Pharmacie

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présentée par :

Sara Hamzeh

a été évaluée par un jury composé des personnes suivantes :

Albert Adam, président-rapporteur

Jean-François Bouchard, directeur de recherche

Daniel Lamontagne, co-directeur

Maurice Ptito, membre du jury

Résumé

Les synapses jouent un rôle important dans la transmission de l'information au sein du système nerveux. Elles montrent une certaine plasticité tout au long de la vie et jouent un rôle essentiel lors du développement, de l'apprentissage et de la mémoire. Dans cette étude, nous avons exploré un nouveau rôle des cannabinoïdes endogènes ainsi que leur récepteur CB_1 dans la formation, le remodellage, et le maintien des synapses. Les cannabinoïdes endogènes et leurs récepteurs CB_1 sont surtout connus pour l'effet modulateur qu'ils excercent sur la libération de neurotransmetteurs au niveau de la synapse. Mais, ils sont également impliqués dans plusieurs événements lors du développement. Récemment, il a été rapporté que les cannabinoïdes endogènes diminuaient le nombre de synapses fonctionnelles entre des neurones pyramidaux en culture. Ici, nous démontrons que les cannabïnoides endogènes et les récepteurs CB_1 régulent la formation de filopodes axonaux et dendritiques (précurseurs de synapse) et sont impliqués lors de la synaptogénèse. La stimulation de neurones corticaux in vitro à l'aide d'un agoniste du récepteur CB_1, l'arachidonyl-2'-chloroethylamide (ACEA), produit une diminution significative du nombre de filopodes (précurseurs synaptiques) à DIV8, et par la suite une réduction de la densité synaptique à DIV10. D'autre part, antagoniser l'action des endocannabinoïdes et de leur récepteurs CB_1 à l'aide d'un agoniste inverse, le AM251, ou d'un antagoniste pur, le O2050, augmente la densité des filopodes à DIV8 et éleve le nombre de contacts synaptiques à DIV10. Aussi, nous avons constaté que ces augmentations étaient bloquées lorsque les cultures étaient préalablement traitées à l'aide du H89 ou du KT5720 (deux inhibiteurs de protéine kinase A(PKA)) ou d'un anticorps blocant la fonction du récepteur DCC (Deleted in Colorectal Cancer). Fait à noter, nous avons observé une diminution significative de la présence des récepteurs DCC à la surface membranaire des cellules traitées avec l'ACEA. D'autre part, nous avons remarqué une augmentation de la présence de DCC à la surface quand les récepteurs CB_1 étaient antagonisés à l'aide de l'AM251

ou de l'O2050. Cette augmentation a été bloquée lorsque les cellules étaient prétraitées avec le H89 ou le KT5720.Ceci a validé les observations précédentes démontrant que l'activation de la voie de l'adénylate cyclase et de la PKA produisait une augmentation de la formation de contacts synaptiques dépendant de la voie de la nétrine-1 et de son récepteur DCC. Afin de confirmer que l'effet produit par les endocannabinoïdes et leur récepteur CB_1 est médié par la nétrine-1 et son récepteur DCC, nous avons traité des neurones corticaux provenant d'embryons de souris où le gène codant pour le récepteur DCC a été délété à l'aide d'un agoniste inverse ou d'un antagoniste pur du récepteur CB_1. En l'absence du récepteur DCC, l'agoniste inverse AM251 et l'antagoniste O2050 n'ont montré aucune augmentation du nombre de filopodes axonaux et dendritiquse. Le nombre de contact synaptique demeura également inchangé, confirmant ainsi le lien existant entre les endocannabinoïdes, leur récepteur CB_1 et le récepteur DCC lors de la synaptogenèse. Nous proposons donc que les endocannabinoïdes agissant sur les récepteurs CB_1, diminuent la concentration cytosolique de l'AMPc, ce qui diminue l'activité de la PKA, bloquant ainsi le recrutement du récepteur de DCC à la surface membranaire, empêchant l'action de la nétrine-1, ce qui diminue la synaptogenèse. Dans cette étude, nous prouvons qu'une interrelation existe entre le système des endocannabinoïdes et celui de la nétrine-1, molécule habituellement impliquée dans le guidage axonal. Ce nouveau mécanisme participe à la formation de synapse au cours du développement neural. Ces résultats démontrent un rôle nouveau majeur des cannabinoïdes endogènes et représentent une percée importante dans l'identification des mécanismes impliqués dans la synaptogenèse.

Mots-clés: Synaptogenèse, récepteur aux cannabinoïdes CB_1, Deleted in Colorectal Cancer, nétrine-1, filopodes, protéine kinase A.

Abstract

Synapses play a major role in signalling transduction in the nervous system. They display extensive activity-driven plasticity during development, learning and memory. Here we have explored a new role of endogenous cannabinoids and their CB_1 receptors in synapse formation, remodelling, and maintenance.

Endogenous cannabinoids and their CB_1 receptors have been known to regulate neurotransmitter release at the level of the synapse and have also been implicated in several developmental events. Recently, it was reported that endogeneous cannabinoids decrease functional synapses in pyramidal neurons. We show here that endogenous cannabinoids and their CB_1 receptors regulate the dendritic and axonal filopodia formation (synapse precursors) and synaptogenesis obtained from embryonic mice cortical cultures. Stimulating the cortical cultures with the synthetic CB_1 receptor agonist, arachidonyl-2'-chloroethylamide (ACEA), produces a significant decrease in filopodia number at DIV8, and subsequently a lower synaptic contact density at DIV10 compared with the control group. On the other hand, inhibiting the action of endogenous cannabinoids and their CB_1 receptors by the inverse agonist AM251 or by the pure antagonist O2050 increases filopodia density at DIV8, and elevates synaptic density formation at DIV10. Furthermore, we found that this increase was reversed when cultures were pre-treated with H89, KT5720 (both inhibitors of Protein Kinase A (PKA)) or DCCfb antibody, (an antibody which blocks the function of Deleted in Colorectal Cancer Receptor). Interestingly, a decrease of DCC receptors present at the surface of the neurons was observed when treated with ACEA. Conversely, an externalisation of DCC was observed when CB_1 receptors were antagonised by AM251 or O2050 and this effect was prevented when neurons were pretreated using H89, KT5720. This confirms the previous observations showing that the activation of adenylate cyclase and PKA pathway produces a netrin-1-DCC dependent increase in synaptogenesis.

In order to verify the putative link between cannabinoid and netrin-1 systems, we performed in vitro experiments on primary cortical neurons obtained from dcc knockout mouse embryos. In the absence of the DCC receptor, the inverse agonist AM251 and the antagonist O2050 show no increase in axonal and dendritic filopodia, or synapse density confirming a connection between the two systems in the underlying mechanisms of synapse formation. We propose that endocannabinoids acting on their CB_1 receptors, decrease cytosolic cAMP concentration and inhibit PKA. This blocks the recruitment of the DCC receptor to the membrane surface and therefore, inhibits the action of netrin-1 regulating synaptogenesis.

In this study, we show that an interplay between the endogeneous cannabinoids and the DCC / netrin-1 pathways regulates synapse formation during neural development. These findings indicate a profound role of endogenous cannabinoids and a breakthrough in understanding the mechanisms implicated in synaptogenesis.

Keywords: Synaptogenesis, Cannabinoid Receptor 1, Deleted in Colateral Cancer Receptor, netrin-1, filopodia, Protein Kinase A.

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List of Abbreviations

A	Adult
AC	Adenylate Cyclase
ACEA	Arachidonyl-2'-chloroethylamide
2-AG	2-Arachidonylglycerol
AM251	N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide, a CB_1 receptor Inverse-Agonist
AMPA	α-Amino-3-hydroxy-5-Methyl-isoxasole Propionic Acid
ANOVA	Analysis of Variance
Arp2/3	Actin-Related Protein complex 2/3
AZ	Active Zone
BDNF	Brain-Derived Neurotrophic Factor
Ca^{2+}	Calcium
$CaCl_2$	Calcium Chloride
CAM	Cell Adhesion Molecule
CaMK	Calcium/calmodulin-dependent Kinase
cAMP	Adenosine 3', 5'-monophosphate
CapZ	Capping Protein
CASK	CaMK-,SH3- and guanylate-Kinase-domain containing protein
CB_1	Cannabinoid Receptor 1
CB_2	Cannabinoid Receptor 2
cc	Corpus callosum
Cdc42	Cell Division Cycle 42
cGMP	Guanosine 3',5'-monophosphate

CNS	Central Nervous system
CNR	Cadherin-related Neuronal Receptor
CRE	cAMP Response Element
CREB	cAMP Response Element Binding protein
DAGL	Diacylglycerol lipase, the 2-AG synthesizing enzyme
DB	DCC-Binding domain
DCC	Deleted in Colorectal Cancer
DCCfb	Deleted in Colorectal Cancer function-blocking antibody
DCCko	Deleted in Colorectal Cancer knockout
DIC	Differential Interference Contrast
DIV	Days *In Vitro*
DSI	Depolarization-induced Suppression of Inhibition
E	Embryonic
ECL	Enhanced Chemiluminescence
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
FAAH	Fatty acid amide hydrolase
F-actin	Filamentous actin
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
FM1-43	Fluorescent dye
FNIII	Fibronectin-type III
FSK	Forskolin
GABA	Gamma aminobutyric acid
GAP-43	Growth Associated Protein of 43 kDa
GFP	Green Fluorescent Protein
GluR1	Glutamate Receptor 1
GRIP	Glutamate Receptor Interacting Protein

GGP-HRP	Peroxidase AffiniPure Donkey Anti-Goat IgG (H+L)
GMP-HRP	Peroxidase AffiniPure Donkey Anti-Mouse IgG (H+L)
GRP-HRP	Peroxidase AffiniPure Donkey Anti-Rabbit IgG (H+L)
GRP55	G protein-coupled receptor 55
GTP	Guanosine Triphosphate
GTPase	Guanosine Triphosphatase
H89	Isoquinoline H89, inhibitor of PKA
HB-GAM	Heparin-Binding Growth-Associated Molecule
HEK	Human Embryonic Kidney cell
Hoe	Hoechst 33258 staining used to detect DNA content
ICAM	Intercellular Adhesion Molecule
Ig	Immunoglobulin
Ki	*In vitro* inhibition constant
KT5720	Hexylester derivative of K-252a and selective inhibitor of PKA
LTD	Long-Term Depression
LTM	Long-Term Memory
LTP	Long-Term Potentiation
MAG	Myelin-Associated Glycoprotein
MAP2	Microtubule Associated Protein 2
MAPK	Mitogen Activated Protein Kinase
mEPSC	miniature Excitatory Post-Synaptic Current
MGL	monoglyceride lipase, a 2-AG degrading enzyme
Mint1	Munc18-1 Interacting protein 1
mRNA	messenger Ribonucleic Acid
Munc	Mouse Unc homologs
N-Cadherin	Neural Cadherin
NCAM	Neural Cell Adhesion Molecule
NFM	Medium-sized Neurofilament

NGF	Nerve Growth Factor
N-GFP	Netrin-1: Green Fluorescent Protein chimera
NMDA	N-Methyl-D-Aspartate
NMJ	Neuromuscular Junction
NP-40	Tergitol-type NP-40, nonyl phenoxylpolyethoxylethanol
NT	Neurotrophin
N-WASP	Neuronal Wiskott - Aldrich syndrome Protein
O2050	Pure antagonist of CB_1 Receptor
P	Postnatal
Pak1	P21-activated kinase
PBS	Phosphate Buffered Saline
PDZ	PSD-95, Dlg and ZO-1
PFA	Paraformaldehyde
PI_3K	Phosphatidylinositol 3-Kinase
PKA	cAMP-dependent Protein Kinase
PKC	Calcium-dependent Protein Kinase
PLC	Phospholipase C
PSD	Post-Synaptic Density
RET	Rearranged During Transfection Protooncogene
Rac	Ras-related C3 botulinum toxin substrate
RCM	Repulsive Guidance Molecule
RFP	Red Fluorescent Protein
RIM	Rab3-Interacting Molecule
Rho	Ras Homolog
rNet	Recombinant Netrin-1 protein
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNAP-25	Synaptosomal-Associated Protein 25

SR141716A	Selective inverse agonist of the CB_1 receptor
SEM	Standard error of the mean
STM	Short-Term Memory
STP	Short-Term Potentiation
SV	Synaptic Vesicle
SV2	Synaptic Vesicle protein 2
SVP-38	Synaptic Vesicle Protein of 38 kDa, synaptophysin
SynCAM	Synaptic Cell-Adhesion Molecule
Δ^9-THC	Delta-9-tetrahydrocannabinol
Trk	Tropomyosin-Receptor Kinase
Tsp-1	Thrombospondin type 1
UNC	Uncoordinated
VAMP2	Vesicle-Associated Membrane Protein 2
ZO-1	Zona Occludens protein 1
ZU5	ZO-1 and Unc5-like domain

I would like to dedicate this Thesis to my family in appreciation of their continuous support of my dreams and their faith in me at all times.

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Introduction

A major issue of modern neurobiology is to understand how neurons extend their neurites to form a functional network. While a large amount of information is available on the mechanisms driving neuritogenesis and synaptogenesis, the study of the intricate molecular machinery underlying the intracellular mechanisms has only been recently addressed.

Formation of synapses during development is essential in the wiring of the brain. Synaptogenesis requires molecular recognition cues and guiding interaction cues to regulate the axons and dendrites toward their final target. The morphological development is initiated with the protrusion of filopodia transiently from dendritic shafts. Dendritic filopodia extend and initiate physical contacts with nearby axons or axonal filopodia which results in filopodial stabilization and the formation of functional presynaptic boutons. The increase in the number of boutons coincides with a decrease in the number of transient filopodia and an increase in the number of stable dendritic spines (Ziv NE and Smith SJ, 1996). After the initial contact, a stable synaptic adhesion site is established and the axonal and dendritic compartments differentiate into pre- and postsynaptic specializations respectively.

Synapses play a significant role in plasticity. Mechanisms of synaptic plasticity are regulated by the state of the postsynaptic cell, which is controlled by the interactions of synaptic inputs from multiple sources.

Recent reports on the effects of cannabis on cognitive processes have lead to investigations on their effect on synaptic plasticity. Due to their presence at the fetal and early postnatal periods, endocannabinoids and their CB_1 receptors have been implicated in playing a significant role in developmental events, such as proliferation and migration of neuronal cells, and synaptogenesis. Although cannabinoids have been reported before to have a retrograde regulatory role on the neurotransmitter release, they have not been clearly implicated in synapse formation. Recently, Kim and Thayer observed that endogenous cannabinoids by activating the CB_1 receptors decrease the number of synapses, however the underlying mechanisms are still to be uncovered.

Here we show that endogenous cannabinoids via the activation of CB_1 receptors with the agonist ACEA negatively modulate axonal and dendritic filopodia formation. The decrease in axonal and dendritic filopodia number reduces the contact probability between pre- and postsynaptic counterparts and therefore decreases the formation of synapses. This mechanism was found to be dependent of PKA activity and the action of netrin-1, a guidance molecule that acts on DCC receptors. Therefore, a novel role of CB_1 receptor in regulating synaptogenesis during development has been discovered.

Chapter 1: Literature Review

1. Synaptogenesis

Synaptogenesis is a process involving the formation of neurotransmitter release sites pre-synaptically and receptive fields post-synaptically in neurons. Progress towards understanding the molecular basis of synaptogenesis is still in its early stages.

Neurons acquire the ability to form synapses as part of a developmental maturation process. Synaptic specificity is determined by: (1) neuronal and glial cues that influence competence for synaptogenesis, (2) by long-range and local axon and dendrite guidance cues, (3) by cell-adhesion molecules that mediate contact, (4) and by local presentation of differentiation-inducing molecules. Moreover, the differentiation process is so intricate that neurons develop and maintain molecularly distinct synaptic specializations for excitatory and inhibitory actions, often only microns apart (Linhoff et al., 2006).

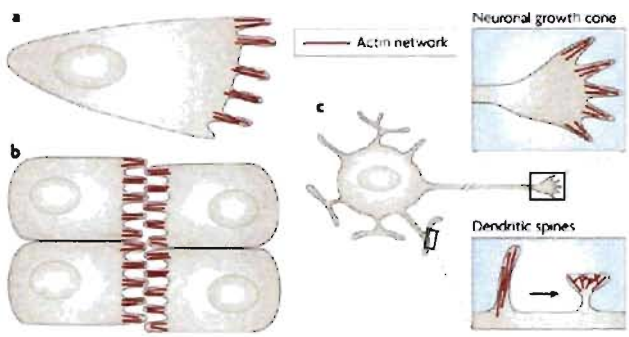
1.1 Filopodia and Spine formations

Filopodia formation is a key prerequisite for synaptogenesis in central nervous system neurons. The filopodia are slender cytoplasmic projections which extend from dendritic shafts (Mattila et al., 2008). They contain actin filaments (Figure 1a) cross-linked into bundles by actin-binding proteins (Hanein et al., 1997). Filopodia extend

to form adhesions with the neuronal axons and are attracted by guidance cues that regulate their migratory pathways, and then they differentiate into stable spines (Mattila and Lappalainen, 2008). A dendritic spine (Figure 1c) is a small membranous protrusion from the central stalk of a dendrite that is typically electrophysiologically active and synapses with a single axon. Typically, spines have a bulbous head and a thin neck that connects the head of the spine to the stalk of the dendrite. Spiny dendritic stalks host many spines, so because each individual spine typically synapses with a reciprocal axon, a spiny dendrite could receive a multitude of signals whereas a traditional dendrite would receive less (Nimchinsky et al., 2002).

Dendritic spines, sites of excitatory input in CNS neurons, can be highly dynamic, in later development as well as in the mature brain. Spine motility has been proposed to facilitate the formation of new synaptic contacts, and they continue to be dynamic even after bearing synaptic connections (Dailey et Smith, 1993).

Figure 1. Filopodia and Spines: Actin network in neurons



Nature Reviews | Molecular Cell Biology

Pieta K. Mattila & Pekka Lappalainen, Filopodia: molecular architecture and cellular functions, Nature Reviews Molecular Cell Biology 9, 446-454 (June 2008)

1.2 Axo-Dendritic Contact

A synaptic site develops when contact between axons and dendrites is established (Ziv and Smith, 1996; Fiala et al., 1998; Ahmari et al., 2000; Friedman et al.,2000; Alsina et al., 2001 ; Ziv and Garner, 2001). Factors enhancing the axo-dendritic contact therefore are significant role players in synaptogenesis. It was previously thought that axons extend and initiate contact with dendrites or other target cells while dendrites are more stationary. This was best explained by the neuromuscular junction model, which is mainly based on the well-characterized development of the neuromuscular junction (NMJ) (Sanes and Litchman, 1999; Burden, 2002), where target muscles are rather stationary, and axonal growth cones migrate toward the muscle and initiate contact forming a synapse.

Although axonal growth cones play a central role in wiring the nervous system and initiating axo-dendritic contacts, recent numerous studies have also attributed an important role for dendritic filopodia and spines in synaptogenesis (Ziv and Smith, 1996; Jontes et al., 2000; Luscher et al., 2000). The observation that the majority of excitatory synapses in the CNS are formed between spines and varicosities along axons (Gray, 1959; Vaughn, 1989; Harris and Kater, 1994), suggested that dendritic extensions, which are actin filaments, function to efficiently connect axons with a multitude of dendritic shafts, without the need for axons to run convoluted paths (Anderson and Martin, 2001). Dendritic filopodia are often present before the

formation of synaptic contacts and spines, and their number decreases as neurons mature, therefore they were believed to initiate axo-dendritic synaptogenesis and to be the precursors for dendritic spines and (Dailey and Smith, 1996; Ziv and Smith, 1996; Smith, 1999) important role players in synaptogenesis.

1.3 Synapse formation

Synapses must remain dynamic during development, allowing neurons to remodel their connections. Such forms of synaptic plasticity are influenced by the activity of synapses in neurons, and their variations are thought to underlie the adaptive responses of neural circuits, including the adaptive characteristics of learning and memory (Kandel, 2000). The mechanisms underlying plasticity stem from both the modulation of channel and receptor activity, and the physical movement of channels and receptors into and out of the synapse. The mechanisms that govern synapse formation and elimination are fundamental to our understanding of neural development and plasticity.

The wiring of neural circuitry requires that vast numbers of synapses be formed and disassembled. The subsequent refinement of neural circuitry involves the formation of additional synapses coincident with the disassembly of previously functional synapses. There is increasing evidence that activity-dependent plasticity also involves the formation and disassembly of synapses (Goda and Davis, 2003).

Complexity of synapse differentiation development arises from the presence of multiple neuron types in the CNS, each forming molecularly and functionally distinct synaptic specializations.

Differentiation of neurons into glutamatergic or GABAergic subtypes occurs early, before neurons extend axonal processes (Tozuka. and Hisatsune., 2005). Thus, the type of neurotransmitter released from a given presynaptic active zone is predetermined well before synapse formation (Hampson and Deadwyler, 1999). Consequently, the question arises of how a dendrite can specifically cluster glutamatergic and GABAergic postsynaptic specializations opposite glutamatergic and GABAergic presynaptic contacts, respectively. A likely possibility is that local signals from the axon at nascent contact sites direct the aggregation of appropriate postsynaptic proteins at these sites. Recent evidence suggests that b-neurexins could be good candidates for such signals. Exogenous focal application of neurexin-1b induces the clustering of glutamatergic and GABAergic postsynaptic receptors, scaffolding proteins and signalling proteins via neuroligins (Dean and Dresbach, 2005).The localization of neuroligin-1 to glutamatergic synapses and neuroligin-2 to GABAergic synapses, and the apparent linkage of neuroligins-1, -3, and -4 to proteins of glutamatergic postsynapses and of neuroligin-2 to proteins of GABAergic postsynapses, suggests that neurexins can influence postsynaptic differentiation by aggregating the proper neuroligin isoforms at nascent contact sites. Therefore, differential expression of neurexin isoforms by glutamatergic versus GABAergic neurons could contribute to local induction of glutamatergic versus GABAergic postsynaptic specializations. However, such a model of postsynaptic specification based solely on neurexins is probably too simplistic. Additional proteins that interact with neurexins and neuroligins and/or that act independently, such as NARP and ephrins, are probably needed to specify appropriate postsynaptic differentiation

(Craig and Linhoff, 2006). Interestingly, N-cadherin is found at hippocampal glutamatergic and GABAergic synapses early in development and then lost from GABAergic synapses (Benson and Tanaka, 1998). Thus, cadherin isoforms could also contribute to maintaining aspects of specificity. Further studies of synaptic molecules would give a better understanding of the basis for matching pre- and pro-synaptic compartments.

2. Synaptic Plasticity

Synaptic plasticity was first proposed as a mechanism for learning and memory on the basis of theoretical analysis (Hebb, 1949). The plasticity rule proposed by Hebb postulates that when one neuron drives the activity of another neuron, the connection between these neurons is potentiated. Theoretical analysis indicates that not only Hebbian like synaptic potentiation is necessary but also depression between two neurons that are not sufficiently coactive (Stent, 1973, Sejnowski 1977). Depression is necessary for several reasons, among them to prevent all synapses from saturating to their maximal values and thereby loosing their selectivity. It is also important to prevent a positive feedback loop between network activity and synaptic weights. The experimental correlates of these theoretically proposed forms of synaptic plasticity are called long-term potentiation (LTP) and long-term depression (LTD). LTP and LTD, the long-term potentiation and depression of excitatory synaptic transmission, are widespread phenomena expressed at possibly every excitatory synapse. It is now clear that (LTP) and (LTD) are not unitary phenomena. Their mechanisms vary

depending on the synapses and circuits in which they operate (Bear and Malenka, 1994).

Recent evidence suggests that induction of LTP may require, in addition to postsynaptic Ca^{2+} entry, activation of glutamate receptors and the generation of diffusible intercellular messengers. On the other hand, long-term depression (LTD) requires Ca^{2+} entry through the NMDA receptor (Yashiro and Philpot, 2008).

Two broad classes of models of synaptic plasticity can be described: 1) Phenomenological models: these are very simple models that are typically based on an input-output relationship between neuronal activity and synaptic plasticity. Phenomenological models are typically used in simulations to account for higher level phenomena such as the formation of memory, or the development of neuronal selectivity (Bliss and Lomo, 1973).

2) Biophysical models: These more detailed models incorporate more of the cellular and synaptic biophysics of neurons, and are typically used to account for controlled synaptic plasticity experiments. Substantial evidence indicates that the number and strength of synaptic connections can be changed by neuronal activity (Bailey and Kandel, 1993; Bliss and Collingridge, 1993; Malenka, 2003). These changes must be stabilized or consolidated in order for memory to persist. Temporary reversible changes are referred to as short-term memory (STM) and the persistent changes as long-term memory (LTM).

2.1 Elements inducing Synaptic Plasticity

Since its discovery by Bliss and Lomo (1973), LTP has been the object of intense investigation as it is believed to provide an understanding of the cellular and molecular mechanisms by which memories are formed and stored (Bliss and Collingridge, 1993). Calcium influx into the postsynaptic spine is crucial for the induction of many forms of bidirectional synaptic plasticity. Much of the calcium entering the postsynaptic spine comes through NMDA receptors (Garner and Ziv, 2002). Blocking NMDA receptors pharmacologically can eliminate both LTP and LTD, and a partial block of NMDA receptors can convert an LTP to LTD. Moreover, experimental results show that a strong postsynaptic calcium transient, in the absence of a presynaptic stimulus can produce LTP while a prolonged moderate calcium transient results in LTD (Yang et al., 1999). Many intracellular cascades are initiated after NMDA receptor activation leading to the development of synaptic plasticity. One particular signalling cascade is the one of CaMKII (Kawaguchi and Hirano, 2002). This kinase is unique in that after sufficient stimulation, auto phosphorylation occurs and transforms it from a Ca^{2+}-dependent to a Ca^{2+}-independent state. In this hyper phosphorylated state, CaMKII activity will continue independently of the NMDA receptor activation (Lisman et al., 2002). Moreover, acute CaMKII activation has been reported to enhance $GABA_A$ receptor-mediated transmission (Churn et al., 2002 ; Kawaguchi and Hirano, 2002). However, a preferential down regulation of $GABA_A$ receptors has been reported in the neocortex after withdrawal from periods of elevated $GABA_A$ receptor activation (S. S. Smith et al. 1998 ; Casasola et al., 2001).

Sustained CaMKII activity could result in a transient elevation of $GABA_A$ receptor function and may trigger a subsequent down regulation of $GABA_A$ receptor expression. During brain development, transmitter-gated receptors are operative before synapse formation, suggesting that their action is not restricted to synaptic transmission. GABA, which is the principal excitatory transmitter in the developing brain, acts as an epigenetic factor to control processes including cell proliferation, neuroblast migration and dendritic maturation. Neurons express functional GABA receptors before formation of functional synaptic contacts (Represa.and Ben-Ari, 2005).

2.2 Signal Transduction Mechanisms

In neurons, signal transduction mechanisms underlie the action potentials that travel along nerves. The influx of ions that occurs in response to ligand-gated ion channels often induce action potentials by depolarizing the membrane of the post-synaptic cells, which results in the wave-like opening of Na^+ voltage-gated ion channels (Huang and Kandel,1996). In addition, calcium ions are also commonly allowed into the cell during ligand-induced ion channel opening. This calcium can act as a classical second messenger, setting in motion signal transduction cascades and altering the cellular physiology of the responding cell. This may result in strengthening of the synapse between the pre- and post-synaptic neurons by remodeling the dendritic spines involved in the synapse (Kandel, 2000). Overwhelming evidence implicates Ca^{2+}-sensitive kinases such as PKC and calcium/calmodulin-dependent kinases (CaMK), as well as cAMP/PKA signalling, in

the enhancement of synaptic transmission (Lisman et al.,2002; Nguyen and Woo,2003). Conversely, Ca^{2+}-dependent phosphatases such as calcineurin and protein phosphatases 1/2A are shown to decrease synaptic transmission (Morishita et al., 2001; Winder and Sweatt, 2001). Ca^{2+} levels therefore bi-directionally control synaptic efficacy by influencing the balance between the activity of protein kinases and phosphatases.

3. Role of PKA and cAMP in CNS Synapse Formation and Plasticity

Adenylyl cyclases are a critically important family of multiply regulated signalling proteins. It is a transmembrane protein that passes through the plasma membrane twelve times. Some adenylate cyclases are stimulated by G_s proteins, and by forskolin. In neurons, a majority of adenylate cyclases are located next to calcium ion channels for faster reaction to Ca^{2+} influx; they are suspected of playing an important role in the learning processes. This is supported by the fact that adenylate cyclases are coincidence detectors, meaning that they are only activated by several different signals occurring together (Cooper, Mons, and Karpen, 1995). Protein kinase A, refers to a family of enzymes whose activity is dependent on the level of cyclic AMP (cAMP) in the cell. PKA is also known as cAMP-dependent protein kinase. Each PKA is a holoenzyme that consists of two regulatory and two catalytic subunits (Deadwyler et al., 2000). Under low levels of cAMP, the holoenzyme remains intact and is catalytically inactive. When the concentration of cAMP rises, cAMP binds to the two binding sites on the regulatory subunits, which then undergo a conformational change that releases the catalytic subunits. The cAMP signalling

cascade is central to certain types of learning and memory. Changing the strength of connections between neurons is thought to underlie memory formation and may result from the recruitment of new sites of synaptic transmission. New functional synapses can be induced between neurons in culture by elevating cAMP levels (Kim and Thayer, 2001).

3.1 Implication of cAMP in synaptogenesis and plasticity

It had long been believed that almost all synaptic effects of cAMP could be attributed to the direct binding of cAMP to cAMP-dependent protein kinase (PKA) (Evan and Morgan, 2003). PKA is a ubiquitous serine/threonine protein kinase that regulates many cellular functions. Cyclic AMP-GEFs bind cAMP and selectively activate the Ras superfamily guanine nucleotide binding protein (Rap1A) in a cAMP-dependent but PKA-independent manner. B-Raf, a member of the Raf family of Serine/threonine kinases, stimulated by Rap1 subsequently activates mitogen-activated protein kinase (MAPK) pathways (Vossler et al., 1997; York et al., 1998). The MAPK pathways (including at least three pathways: extracellular-signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK), and p38 MAPK) are involved in a widespread set of cellular functions including neuronal differentiation in a transcription-dependent manner (Hazzalin and Mahadevan, 2002), such as ion-channel conductivity, and synaptic release of neurotransmitters. Rapid modulation of synaptic transmission efficacy during the early phase of synaptic plasticity is likely mediated by both pre- and postsynaptic events. However, it remains unclear to what extent these changes contribute to the long-term maintenance of facilitated or depressed synaptic

transmission. It is currently thought that use-dependent modulation of gene expression and protein translation confers long-term plastic changes. In fact, modulation of cAMP-response element (CRE)-regulated gene expression by CRE-binding protein (CREB) appears to be a universal requirement for this process (Kandel, 2001; West et al., 2001).

3.2 Dual role of PKA and cAMP in neuritogenesis and synaptogenesis

To create precise neural circuits in the nervous system, neuritogenesis, axon guidance, and synaptogenesis are the critical cellular processes during neuronal differentiation. Recent studies have examined the role of cAMP in signalling pathways for regulating neuritogenesis and synaptogenesis. A rise in intracellular cAMP concentration by a membrane-permeable cAMP analog, dibutyryl cAMP (dbcAMP), is thought to increase the number of neurites and varicosities (Tojima,Kobayashi, and Ito, 2003). On the other hand, inhibition of cAMP-dependent protein kinase (PKA) activity by a PKA inhibitor (H89) although accelerates, neuritogenesis.neurite outgrowth rate; but it, however, decreases the number of varicosities and the frequency of postsynaptic miniature currents, resulting in suppression of synaptogenesis (Cesa, Scelfo, andStrata, 2007). PKA activity mediates phosphorylation of a gene transcription factor, CREB. On the other hand, inhibition of ERK pathway by PD98059 suppresses both neuritogenesis and neurite outgrowth without CREB phosphorylation. This strongly suggests that PKA simultaneously plays two different roles in neuronal differentiation: inhibition of neuritogenesis and

stimulation of synaptogenesis, via CREB-mediated gene expression. (Tojima et al., 2003)

4. Netrins

Netrins are a family of proteins that guide cell and axon migration during development. Three secreted netrins (netrin-1, -3/2-like and -4/B) have been identified in mammals, in addition to the two GPI-anchored membrane proteins, netrin-G1 and G2 (Mehlen and LIambi, 2005).

4.1 Netrin Structure and Function

The netrin family of guidance molecules is mainly known to be involved in axon guidance. Netrins are chemotropic; a growing axon will either move towards or away from a source of netrin (Stretaven et al., 1999). Though the detailed mechanism of axon guidance is not fully understood, it is known that netrin attraction is mediated through UNC-40/DCC cell surface receptors and repulsion is mediated through UNC-5 receptors. This protein gradient is bifunctional, attracting some axons to the midline and repelling others. Receptors for the secreted netrins include DCC (deleted in colorectal cancer) and the UNC5 homologues: UNC5 -1/A, 2/B, 3/C, 4/D in mammals. DCC mediates chemoattraction, while repulsion requires an UNC5 homologue and, in some cases, DCC (Stretaven et al., 1999). The netrin-G proteins bind NGLs (netrin G ligands), single pass transmembrane proteins unrelated to either DCC or the UNC5 homologues. Netrin function is not limited to the developing CNS

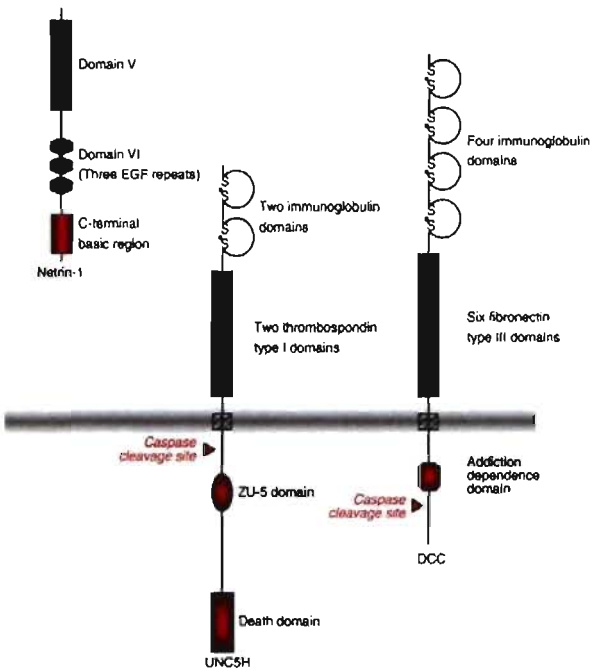
midline. Various netrins direct cell and axon migration throughout the embryonic CNS, and in some cases continue to be expressed in the mature nervous system (de Castro, 2003). Furthermore, although initially identified for their ability to guide axons, functional roles for netrins have now been identified outside the nervous system where they influence tissue morphogenesis by directing cell migration and regulating cell-cell and cell-matrix adhesion.

4.2 DCC, a netrin-1 receptor

Deleted in Colorectal Cancer, also known as DCC, is a human gene that has long been implicated in colorectal cancer. The protein coded by the *dcc* gene is a single transmembrane receptor also known as DCC. Since it was first discovered in a colorectal cancer study in 1990, DCC has been the focus of a significant amount of research (Fearon , Cho, Nigro et al, 1990). DCC held a controversial place as a tumour suppressor gene for many years, and is well known as an axon guidance receptor that responds to netrin-1(Hahn SA, Schutte M, Hoque AT, et al., 1996).

More recently DCC has been characterized as a dependence receptor, and theories have been put forward that have revived interest in DCC's candidacy as a tumour suppressor gene, as it may be a ligand-dependent suppressor that is frequently epigenetically silenced.

Figure 2. Structure of netrin-1 and netrin-1 dependence receptors



Mehlen P and Llambi F,Role of netrin-1 and netrin-1 dependence receptors in colorectal cancers, British Journal of Cancer (2005) 93, 1–6.

The *dcc* gene is located at 18q21.3, and has a total of 57 possible exons and 43 possible introns. This theoretically results in 13 correctly sliced, putatively functional proteins. The typical DCC protein has one signal peptide motif and eleven domains, including multiple immunoglobulin-like domains, a transmembrane domain, and several fibronectin type 3 domains (Michael A. Reale, Fearon et al., 1994).

DCC has extracellular binding sites for both netrin-1 and heparin. Heparin sulphate is believed to also be present during neural growth as a type of co-factor for axon

guidance. Intracellularly, DCC has been shown to have a caspase-3 proteolysis site at Asp 1290 (Mehlen et al., 2001).

DCC and neogenin, two of the netrin-1 receptors, have recently been shown to have sites for tyrosine phosphorylation (at Y1420 on DCC) and are likely interacting with Src family kinases in regulating responses to netrin-1(Xiu-Rong Ren et al., 2008).

4.3 Signaling Downstream of Netrin Receptors

Historically, cellular receptors have been thought to be activated when bound to their ligand, and are relatively inactive when no ligand is present. A number of receptors have been found that do not fit into this conceptual mould, and DCC is one of them. These receptors are active both with ligand bound and unbound, but the signals transmitted are different when the receptors are ligand bound (Mehlen et al., 2004). Collectively, this type of receptor is known as a dependence receptor because the unbound pathway is usually apoptotic, meaning that cell survival depends on ligand presence. Other receptors also show this functional profile, including $p75^{NTR}$, the androgen receptor, RET, several integrins and Patched (Mehlen et al., 2004).

When DCC is present on the membrane and bound to netrin-1, signals are conveyed that can lead to proliferation and cell migration (Zänker et al, 2007). Only in the absence of the DCC receptor is there an absence of downstream signaling. There are therefore three possible signaling states for dependence receptors: on (ligand-bound, migration and proliferation), off (ligand-unbound, apoptosis inducing) and absent (lack of signal).

4.4 Developmental and Neurological Roles

DCC's role in commissural axon guidance is perhaps it's most characterized. In the developing spinal cord, commissural neurons located dorsally extend axons ventrally using a mechanism dependent on a ventral midline structure, the floor plate (Imondi and Kaprielian, 2001). A gradient of netrin-1 is produced from the floor plate, which allows orientation of the extending axons, aiding the development of the dorsal-ventral axis of the brain and spinal cord. A variety of receptors are present on the axon surface which either repel or attract axons to the midline. When membrane DCC is stimulated by netrin-1, it promotes axon progression towards the midline (Kaprielian,, Runko., and Imondi, 2001).

Several other molecules are also involved in the guidance of axons to and across the midline. The slit proteins have repulsive functions, as opposed to netrins, and are mediated by the transmembrane protein Robo (Simpson et al., 2000). Axonal growth cones that are attracted to the midline by netrin/DCC signaling eventually cross the floor plate. When this occurs they lose responsiveness to netrin and become repulsed by slit/Robo signaling. This is accomplished by the formation of a DCC-Robo complex, which inhibits attractive netrin/DCC signals while allowing slit/Robo signals (Farmer et al., 2008). Netrin also has other receptors, the UNC5 family. The UNC5 receptors have repellant migratory responses to netrin binding, and have similar effects to the slit/Robo system (Farmer et al., 2008).

The intracellular signaling responses to netrin-1 are not fully understood. Several phosphorylation events have been established, as have the involvement of several src

family kinases and small GTPases, but the sequence of events has not yet been determined (Zheng et al., 2003). DCC is also required to be recruited to lipid rafts for axon outgrowth and apoptotic signaling. Activating PKA increased the distance over which axons turned toward a source of netrin-1, whereas PKA inhibition reduced this distance (Bartoe et al., 2006). However, in contrast to the cyclic nucleotide switch model, inhibiting PKA does not cause these axons to be repelled by netrin-1 (Bouchard et al., 2004; Moore, and Kennedy, 2006). Thus the mechanisms underlying chemoattraction to netrin-1 are independent of mechanisms required for cyclic nucleotide-dependent switching. PKA regulates the sensitivity of spinal commissural axon chemoattraction to netrin-1 and mobilizes DCC from an intracellular vesicular pool to the growth cone plasma membrane (Bouchard et al., 2004; Moore and Kennedy, 2006).

Axon growth and pathfinding are crucial events involved in the establishment of the correct neuronal circuitry during the development of the nervous system. This is achieved by the complex integration of intracellular mechanisms mediated by several highly conserved families of guidance cues, including the netrins (Huber et al., 2003). Netrins are bifunctional ligands attracting or repelling different classes of neurons, depending on the expression of the receptors at the cell surface of the growth cone (Round and Stein, 2007).

Remodelling of the actin cytoskeleton within the neuronal growth cone is an important step in the response of an axon to attractive and repulsive cues leading the axon to advance, retract, or turn in the appropriate direction. Rho-family GTPases, in

particular Rac1, Cdc42 and RhoA are important regulators of cytoskeletal dynamics in neuronal and non-neuronal cells and act downstream of most guidance cue receptors (Hall, 1998; Luo, 2000; Dickson, 2001; Govek et al., 2005). Rho GTPase activities regulate growth cone extension and axon outgrowth (Mueller, 1999; Dickson, 2001). Rac1 promotes the formation of actin-based lamellipodia, which provides tension necessary for neurite elongation, while Cdc42 is responsible for the formation of filopodia that serves to orient the growth cone by sensing the extracellular environment (Waterman-Storer et al., 1999; Suzukia and Takahashi, 2008).

RhoA activity also affects the growth cone morphology by decreasing actin polymerization, regulating actomyosin contraction and inducing growth cone collapse (Tashiro et al., 2000). A widely accepted model in neuronal morphogenesis suggests that attractive cues promote actin polymerization and neurite outgrowth via activation of Rac1 and Cdc42 whereas repulsive cues induce neurite collapse via RhoA activation (Hall, 1998; Mueller, 1999). However, in some cases, their roles in neurite outgrowth seem to be interchanged (Meyer and Feldman, 2002). For instance, Sema3D-induced chick dorsal root ganglia (DRG) growth cone collapse is mediated by Rac1 (Jin and Strittmatter, 1997). Similarly, Rac1 activity is required for Ephrin-A2-mediated growth cone collapse in chick retinal cells and sensory neurons (Jurney et al., 2002). Conversely, RhoA activity has been implicated in mediating neurite outgrowth (Sebok et al., 1999; Arakawa et al., 2003) and axon guidance (Bashaw et al., 2001; Yuan et al., 2003). Indeed, low concentration of stromal cell-derived factor (SDF-1α) promotes axon elongation by activation of the RhoA/mDia pathway in

cultured cerebellar granule neurons (Arakawa et al., 2003). Similarly, a Dbl family RhoGEF was found to promote axon attraction in Drosophila, in a Rho but not Rac or Cdc42-dependent manner (Bashaw et al., 2001). Rac1 and Cdc42 are important downstream signaling components of netrin-1 receptor DCC signaling (Li et al., 2002b; Shekarabi and Kennedy, 2002; Shekarabi et al.,2005). Rho GTPases are implicated in the signalling mechanisms induced by the netrin-1 receptor UNC5 (Briançon-Marjollet and Lamarche-Vane, 2008). Rac1, Cdc42 and RhoA have been shown to play a role in UNC5a signalling and are implicated in the regulation of neurite outgrowth (Sebok et al., 1999; Arakawa et al., 2003).Also recent studies showed that UNC5a highly activate RhoA and to a lower extent Rac1 and Cdc42 in response to netrin-1 in fibroblast cells (Cirulli andYebra, 2007). Interestingly, UNC5a has been shown to induce neurite outgrowth but not growth cone collapse in N1E-115 neuroblastoma cells in a netrin-1-dependent manner (Huber et al., 2003). UNC5a was shown to strongly activate RhoA and thus RhoA activity is thus required for UNC5a-mediated neurite outgrowth (Cirulli and Yebra, 2007).

DCC is developmentally regulated, being present in most fetal tissues of the body at higher levels than what is found in adult tissues (Jiang et al., 2003). DCC and netrin have been found to be specifically involved in the secondary migration of neural crest cells into the pancreas and developing gut structures, and may prove to be vital to other areas during fetal growth (Jiang et al., 2003).

4.5 Novel Functions for Netrins

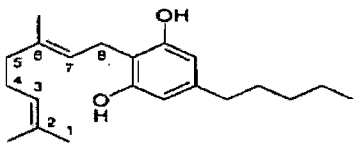
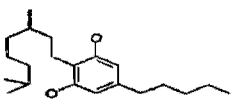
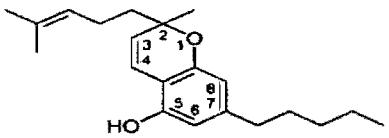
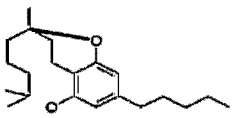
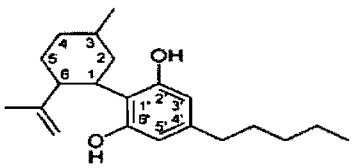
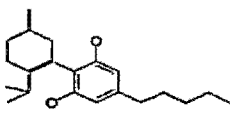
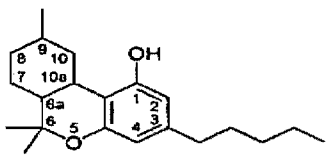
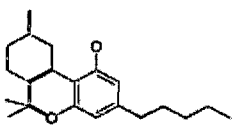
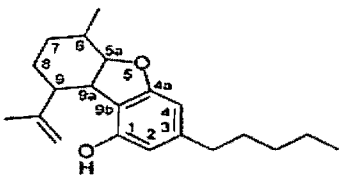
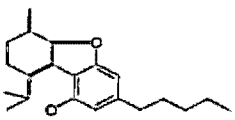
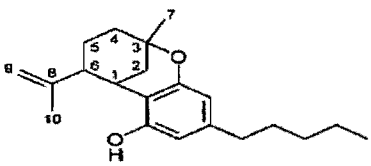
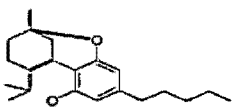
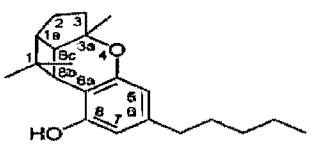
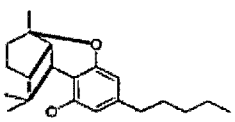
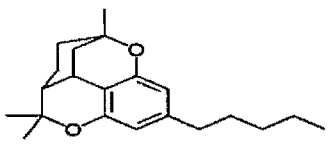
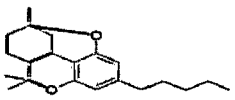
Netrin-1 and DCC play a role in the development of the embryonic nervous system, specifically in the formation of axonal and dendritic filopodia, and have a possible role in initiating synaptic contacts and modulating synaptic transmission. Recently, Bouchard et al. showed that activation of PKA enhances the presence of DCC at the cell surface and increases axon extension in response to netrin-1. While inhibiting adenylate cyclase, PKA, or exocytosis blocks DCC translocation decreasing axon extension (Bouchard et al., 2004; Bouchard et al. 2008).

5. Cannabinoids

The marijuana plant contains more than 60 bioactive ingredients, of which delta9-tetrahydrocannabinol (Δ^9-THC) is mainly responsible for its psychoactive properties. Cannabinoids are a group of substances that are structurally related to Δ^9-THC or that bind to cannabinoid receptors. Currently, there are three general types of cannabinoids: herbal cannabinoids occur uniquely in the cannabis plant; endogenous cannabinoids are produced in the bodies of humans and other animals; and synthetic cannabinoids which are produced in a laboratory (Lambert and Fowler, 2005). Phytocannabinoids also called natural cannabinoids, herbal cannabinoids, and classical cannabinoids, are only known to occur naturally in significant quantity in the cannabis plant, and are concentrated in a viscous resin that is produced in glandular structures known as trichomes (Lambert and Fowler, 2005). In addition to cannabinoids, the resin is rich in terpenes, which are largely responsible for the odour

of the cannabis plant (Lambert and Fowler, 2005). Phytocannabinoids are nearly insoluble in water but are soluble in lipids, alcohols, and other non-polar organic solvents (Taura et al., 2007). However, as phenols they form more water-soluble phenolate salts under strongly alkaline conditions (Taura et al., 2007). All natural cannabinoids are derived from their respective 2-carboxylic acids (2-COOH) by decarboxylation (Thakur et al., 2005). Many cannabinoids have been isolated from the cannabis plant. All classes derive from cannabigerol-type compounds and differ mainly in the way this precursor is cyclised (Burns and Ineck, 2006).

Figure 3. Natural cannabinoids

Type	Skeleton	Cyclization
Cannabigerol-type **CBG**		
Cannabichromene-type **CBC**		
Cannabidiol-type **CBD**		
Tetrahydrocannabinol- and Cannabinol-type **THC, CBN**		
Cannabielsoin-type **CBE**		
iso-Tetrahydrocannabinol-type ***iso*-THC**		
Cannabicyclol-type **CBL**		
Cannabicitran-type **CBT**		

http://en.wikipedia.org/wiki/Cannabinoids

5.1 Cannabinoid Structure and Function

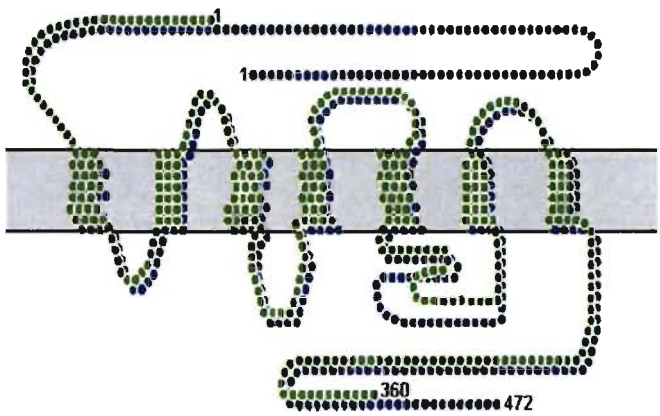
In the 1970's, it was believed that Δ^9-THC produced its effects by perturbing neuronal membranes due to its lipid-soluble, hydrophobic nature. It was subsequently demonstrated that saturable, stereo selective, high-affinity membrane binding sites for cannabinoids are present in the mammalian brain (Thakur et al., 2005). There are currently two known subtypes, CB_1 which is expressed mainly in the brain, but also in the lungs, liver and kidneys and CB_2 which is mainly expressed in the immune system and in hematopoietic cells (Begg et al., 2005). Mounting evidence suggests that there are novel cannabinoid receptors that is, non-CB_1 and non-CB_2, which are expressed in endothelial cells and CNS. One of these is the GPR55, which was identified and cloned for the first time in 1999 (Sawzdargo et al., 1999). Later it was identified by an in silico screen as a putative cannabinoid receptor because of a similar amino acid sequence in the binding region (Baker et al., 2006). GPR55 is only 13.5% identical to CB_1 and 14.4% identical to CB_2, and its mRNA is present in the brain and periphery (Ryberg et al., 2007). The physiological role of GPR55 is unclear. Mice with a target deletion of the GPR55 gene show no specific phenotype (Johns et al., 2007). GPR55 is widely expressed in the brain, especially in the cerebellum. GPR55 is not expressed in the periphery, except for the jejunum and ileum (Ryberg et al., 2007). This profile as a distinct non-CB_1/CB_2 receptor which responds to a variety of both endogenous and exogenous cannabinoid ligands, has led some groups to suggest GPR55 should be categorised as the CB_3 receptor, and this re-classification may follow in time (Overton et al., 2003). However this is

complicated by the fact that another possible CB_3 receptor has been discovered in the hippocampus, although its gene has not yet been cloned, (De Fonseca and Schneider, 2008) suggesting that there may be at least four cannabinoid receptors which will eventually be characterised.

The currently recognized cannabinoid receptors, CB_1 and CB_2, are about 45% similar in their protein sequences (Munro et al., 1993). The existence of specific membrane receptors for plant-derived substances in mammalian neurons triggered a search for an endogenous ligand. In 1992, this search culminated in the identification of arachidonyl ethanolamide, named anandamide, a brain-derived lipid that binds to cannabinoid receptors and mimics the biological effects of Δ^9-THC (Mechoulam et al., 1998). Three years later, a second endogenous cannabinoid, 2-arachidonoylglycerol (2-AG) was isolated (Mechoulam et al., 1998). In the ensuing years, several other related lipids with endocannabinoid properties were identified, but have been characterized less extensively (Pagotto et al., 2007). CB_1 receptor is a 7 pass transmembrane super family member. It couples to inhibitory G-proteins (Gi/o) and therefore, cannabinoid agonists inhibit adenylate cyclase and decrease the activity of protein kinase A (Collins et al., 1994). Stimulation of adenylyl cyclase has been reported in pertussis toxin-treated cell, suggesting that in the absence of functional Gi/o coupling, the CB_1 receptor can activate Gs (Olianas and Onali, 2006). Activation of CB_1 receptors also inhibits synaptic transmission, probably via inhibition of voltage-gated Ca^{2+} channels and activation of K^+ channels (Deadwyler

et al., 1993). The cannabinoid CB_1 receptor is one of the most abundant G-protein-coupled receptors in the brain (Begg et al., 2005).

Figure 4. CB_1 and CB_2 receptors Structures



Straiker A, J Neurphysiol, 2003; Howlett AC, Pharmacol Rev, 2002; Howlett AC, Handb Exp Pharmacol, 2005

5.2 CB_1 receptor: Agonist, Antagonist and Inverse Agonist

Historically, laboratory synthesis of cannabinoids was often based on the structure of herbal cannabinoids and a large number of analogs have been produced and tested. Later novel compounds were not related to natural cannabinoids but actually based on the structure of the endogenous cannabinoids. Synthetic cannabinoids are particularly useful in experiments to determine the relationship between the structure and activity of cannabinoid compounds and for studying cannabinoid receptor mechanisms. Some of these are described below:

Arachidonyl-2'-chloroethlamide (ACEA) is a potent and highly selective CB_1 receptor agonist (Ki = 1.4 nM). It displays a greater than 1400-fold selectivity over CB_2 receptor active *in* vivo (Hillard et al., 1999).

AM251 is a CB_1 cannabinoid receptor inverse agonist. AM251 is structurally very close to SR141716A (rimonabant), which both are biarylpyrazole cannabinoid receptor inverse agonists. In AM251, the p-chloro group attached to the phenyl substituent at C-5 of the pyrazole ring is replaced with a p-iodo group. The resulting compound exhibits slightly better binding affinity for the CB_1 receptor with a Ki value of 7.5 nM compared to SR141716A, which has a Ki value of 11.5 nM (Hillard et al, 1999; Pertwee, 2005). However AM251 is about two-fold more selective for the CB_1 receptor when compared to SR 141716A. AM 251 displays a K_i value at CB_1 receptors which is 306-fold selective over CB_2 receptors. In comparison, SR141716A displays a K_i value which is 143-fold selective over CB_2 receptors (Hillard et al, 1999; Pertwee, 2005).

O2050 is a high affinity cannabinoid CB_1 receptor pure antagonist (K_i = 2.5 nM); devoid of agonist effects (Hillard et al, 1999;Pertwee, 2005).

5.3 CB_1 cannabinoid receptor and their behavioural effects

Cannabis derivatives are among the most ancient and frequently consumed drugs. Cannabinoid dependence and self-administration have been verified in animal tests (Tsou et al., 1995; de Fonseca et al., 1997; Martellotta et al., 1998; Tanda et al. 2000), further confirming that cannabinoids hold a considerable abuse potential (Abood and Martin, 1992). It is generally considered that the recreational use of cannabinoids is related to their positive modulatory effects on brain-rewarding processes along with their ability to positively influence emotional states and remove stress responses to environmental stimuli (De Fonseca et al., 1997). Indeed, recent studies have shown that dopamine release is significantly increased in the nucleus accumbens after cannabinoid treatment presumably because of increased activity of dopaminergic neurons in the ventral tegmental area (Chen et al., 1990; Tanda et al., 1997). In addition, cannabinoid exposure decreases corticotropin-releasing hormone level in the amygdala, which may account for the reduced stress responses (De Fonseca et al., 1997).

The neuronal cannabinoid receptor CB_1 has been shown to be responsible for most behavioral effects of cannabinoids (Ledent et al., 1999; Zimmer et al., 1999). Accordingly, CB_1 knock-out animals do not develop cannabinoid dependence or self-administration (Ledent et al., 1999). CB_1 receptors are widely distributed in the brain (Tsou et al., 1998), suggesting that several brain areas may be affected by cannabinoids and contribute to their behavioral effects and abuse potential.

Thus, to understand how cannabinoids modulate emotional states, one should consider that other brain regions may also play important roles in different aspects of these phenomena and elucidate the role of CB_1 receptors at the synaptic, cellular, and network levels in these regions.

5.4 DSI and DSE: a role of endocannabinoids in short-term plasticity.

In the hippocampus, depolarization of pyramidal neurons induces a short-term suppression of GABAergic IPSCs. This phenomenon termed Depolarization-induced Suppression of Inhibition (DSI) is blocked by perfusion of CB_1 receptor inverse agonists (SR141617A and AM251), and it is absent in *cnr1*-/- mice (*cnr1:* gene coding for CB_1 receptors). DSI expressed in the hippocampus is transient (<30s) and suppression of the resulting inhibition could not account for long-term plasticity of synapses. However, more recent *in vivo* studies fail to confirm DSI in response to a variety of behaviourally relevant neuronal activation patterns. Furthermore, recent studies demonstrate that not all neuronal phenotypes in the hippocampus exhibit the capacity for endocannabinoid-mediated DSI. In the cerebellum, researchers obtain similar results. Moreover, Kreitzer and Regehr found that both climbing fiber and parallel fiber excitatory inputs to Purkinje neurons could also be transiently suppressed following Purkinje cell depolarization, the Depolarization-induced Suppression of Excitation (DSE) phenomenon. They showed also that DSE could be blocked by AM251. This suggests that endocannabinoids have a fundamental role in DSI/DSE and synaptic plasticity.

5.5 Long-Term Potentiation (LTP) and Long-Term Depression (LTD): a role of endocannabinoids in long-term plasticity

The first investigation on the role of cannabinoids on LTP shows that Δ^9-THC could either inhibit or potentiate high-frequency stimulation-induced LTP, depending of the concentration used (Bear et al., 1994). A few years later, Collins et al demonstrated that cannabinoids are stereo-selective inhibitors of LTP (Collins et al., 1995). This effect has been confirmed for another cannabinoid, where perfusion of WIN55, 212-2, blocks LTP (Carlson et al, 2002). Inverse agonist of CB_1 receptors, SR141716A prevents the blockade of LTP induced by HU-210, WIN55, 212-2, 2-arachidonylglycerol, or anandamide (Davies et al., 2002). Furthermore, high frequency stimulation induces significantly larger LTP in hippocampal slices prepared from *cnr*$^{-/-}$ mice compared with wild-type controls (Alger et al., 2002). These results contrast with the study published by Alger's group showing that LTP at hippocampal excitatory synapses can be facilitated by endocannabinoid release (Alger et al., 2002). Thus when weak stimulus trains that not alone produce LTP are preceded by DSI-inducing depolarizing step, LTP is induced (Carlson et al, 2002). This form of DSI-paired LTP is blocked by AM251 suggesting that the postsynaptic depolarization produces an endogenous cannabinoids-dependent inhibition of GABA release that is significant to facilitate glutamatergic transmission. However, because recordings from neuron pairs demonstrate that DSI is spatially restricted to $< 20\mu m$ in the hippocampus, it can be assumed that endocannabinoids released by post-synaptic

depolarization will only facilitate LTP within a circumscribed region surrounding the depolarized cell. In contrast, a reduction in LTP will tend to occur during periods of global CB_1 receptor activation. In the striatum, another type of long-term plasticity, LTD, is dependent upon endocannabinoid release. In the prefrontal cortex, WIN55.212-2 increases the proportion of cells exhibiting LTD and decreases the proportion exhibiting LTP, whereas SR141716A increases the proportion of neurons exhibiting LTP and decreases the proportion exhibiting LTD (Pertwee, 2005).

5.6 Putative roles of endocannabinoids and their CB_1 receptors during development and early postnatal period.

Due to their presence at the fetal and early postnatal periods, endocannabinoids and their CB_1 receptor might be involved in several developmental events, such as proliferation and migration of neuronal cells, or during synaptogenesis (Fernandez-Ruiz et al., 2000). One of the evident roles of endogeneous cannabinoids is modulation of retrograde signalling at the synapse. Postsynaptic neurons in the nervous system release the endogenous cannabinoids anandamide and 2-arachidonylglycerol, which act retrogradely at presynaptic neurons to block neurotransmitter release (Freund et al., 2003). In the developing brain, CB_1 cannabinoid receptors are expressed in neural precursors and growing axons, and CB_1 receptor expression increases during synaptogenesis period (Piomelli, 2003).

CB_1 receptors are localized to the developing axons of cortical and hippocampal pyramidal cells in mice (Hoffman et al., 2003). Similarly, diacylglycerol lipases,

DAGL, which are necessary for 2-arachidonylglycerol production, are present in elongating axons, suggesting that endocannabinoid production and signalling may occur in the same neurons during axon outgrowth (Leterrier et al., 2004). Also, CB_1 receptors localized to the axons and growth cones of GABAergic interneurons (Katona et al., 2006). In contrast, DAGL localized to glutamatergic pyramidal cell dendrites, suggesting that CB_1 receptors and endocannabinoids are positioned on opposite sides of pyramidal cell synapses during synaptogenesis (Leterrier et al., 2004). In cultures of GABAergic interneurons, CB_1 receptors localized to the F-actin–rich filopodial tips of growth cones (Katona et al., 2006). Anandamide treatment shifted CB_1 receptors into the center of the growth cone (Terranova et al., 1995). In cultured hippocampal neurons, anandamide treatment induced the retrograde transport of CB_1 receptors through the axon, away from the growth cone (Terranova et al., 1995). Endocannabinoids are chemorepulsive; the CB_1 receptor agonist WIN55, 212-2 induced growth cones to turn away from the drug application site and collapse (Maneuf et al., 1997). During growth cone repulsion, RhoA GTPase activates the serine-threonine Rho kinase (ROCK). In contrast to the WIN55, 212-2 treatment alone, co application of WIN55, 212-2 with the ROCK inhibitor Y-27632 was chemo attractive; inducing growth cones to turn toward the drug application site, suggesting that CB_1 receptor is coupled to RhoA (Carlson et al., 2002). GABAergic interneurons express the homeobox genes Dlx5 and Dlx6 during embryonic development and transporters for vesicular GABA (VGAT) and vesicular glutamate 3 (VGLUT3) in adulthood (Mu and Deadwyler et al.,2000). In one study, mated mice were mated with the CB_1 receptor gene surrounded by loxP recombination sites to

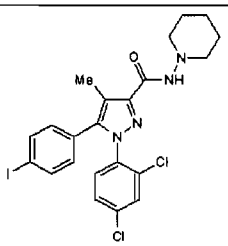
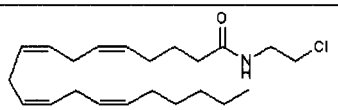
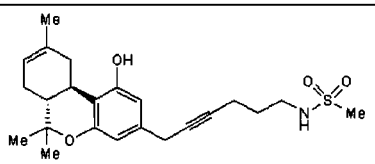
mice expressing Cre recombinase in Dlx5- and Dlx6-expressing cells (Monory et al., 2006). Pyramidal cells in the resulting progeny had more VGAT- and VGLUT3-positive synaptic inputs relative to wild-type mice, suggesting that the absence of CB_1 receptors impairs axon target selection (Berghuis et al, 2007). Therefore, marijuana exposure might cause premature growth cone collapse and impaired synaptogenesis in the fetal brain (Robbe et al., 2006). In adults, cannabinoids localize to dendritic spines in the hippocampus, so marijuana exposure may inhibit synaptic plasticity. Because RhoA inhibits peripheral axon regeneration, the cannabinoid receptor inhibitor AM251 might induce the regrowth of axons following injury (Piomelli et al., 2003).

Recent physiological, pharmacological, and high-resolution anatomical studies provided evidence that the major physiological effect of cannabinoids is the regulation of neurotransmitter release via activation of presynaptic CB_1 receptors located on distinct types of axon terminals throughout the brain. Subsequent discoveries shed light on the functional consequences of this localization by demonstrating the involvement of endocannabinoids in retrograde signalling at GABAergic and glutamatergic synapses.

Under certain conditions, cannabinoids via their presynaptic CB_1 receptors decrease glutamate release; blocking the induction of LTP. Because LTP, via a positive modulation of glutamate release, increases pre and post-synaptic contacts, a decrease of glutamate release induced via CB_1 receptor activation perhaps could decrease the number of synaptic contacts (Carlson et al., 2002).

Here we show that endogenous cannabinoids via the activation of CB_1 receptors with ACEA agonist negatively modulate axonal and dendritic filopodia formation and synape formation dependant of PKA activity and the action of netrin-1 on DCC receptor.

Figure 5. Inverse agonist, agonist, and antagonist of CB_1 receptors

Type	Skeleton
AM251	
ACEA	
O 2050	

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Chapter 2: A Novel Role for Cannabinoids in Synaptogenesis

Introduction

Although, the endogenous cannabinoid signalling system has been studied for a decade, many of its physiological roles have yet to be revealed. Early studies have shown that cannabinoids inhibit adenylate cyclase (AC) in the nervous system (Howlett et al., 1984). Furthermore, AC inhibition was observed only in certain cell types, ruling out a direct inhibitory effect of cannabinoids on the enzyme itself, or a non-specific effect through changes in membrane fluidity (Howlett et al., 1988).

Before the 1970's, it was often speculated that the cannabinoid lipid soluble hydrophobic molecules produced their physiological and behavioral effects via nonspecific interaction with cell membranes, instead of interacting with specific membrane-bound receptors (Abel EL., 1970). In the late 1980's, specific binding sites for cannabinoids were described in a rat brain membrane preparation (Deadwyler et al., 1993). After that, a cannabinoid receptor was isolated and cloned from a rat cerebral cortex cDNA library. This new receptor in the presence of Δ^9-THC could inhibit forskolin-induced cyclic AMP accumulation (Matsuda et al., 1990). This discovery of the first cannabinoid receptor, namely CB_1 receptor, was followed by the characterization of a second cannabinoid receptor, CB_2, in immune cells (Devane et al., 1992).

CB_1 and CB_2 receptors are the two major cannabinoid receptors but some pharmacological data suggest the existence of other receptors (Munro, et al., 1993) such as GPR55 that has also been proposed to be a novel cannabinoid receptor (Begg et al., 2005).

Recent studies provided evidence that the major physiological effect of cannabinoids is the regulation of neurotransmitter release via activation of presynaptic CB_1 receptors located on distinct types of axon terminals throughout the brain (Caboche et al., 2003). Subsequent studies shed light on the functional effects of this localization by demonstrating the involvement of endocannabinoids in retrograde signalling at GABAergic and glutamatergic synapses (Piomelli et al., 2003).

Recently, using FM1-43 technique, endocannabinoids were reported to block the increase of functional synapses induced by forskolin (FSK), an activator of adenylate cyclase, in primary hippocampal neuron culture (Kim and Thayer, 2001; Kim and Thayer, 2008). Albeit these results are very preliminary, they suggest that the role of endocannabinoids is not only restricted to the modulation of synaptic transmission, but that they could possibly play a role in synapse formation and maintenance. The generation of a functional neuronal network requires the elaboration of precise synaptic connections between neurons and their targets. This connectivity is established during the development of the nervous system in a series of continuous events that can be divided into three stages: (1) neurite guidance, (2) target recognition, and (3) synapse formation. There is evidence that the initial contact

between axons and dendrites is sufficient to establish a functional synaptic site (Ziv and Smith, 1996; Fiala et al., 1998; Ziv and Garner 2001), and that dynamic actin-based filopodial extensions from both axonal and dendritic compartments are important in initiating this contact (Chang and De Camilli, 2001; Jontes and Smith, 2000). Moreover, activity-dependent actin reorganization was shown to contribute to synaptic plasticity (Krucker et al., 2000; Lendvai et al., 2000; Trachtenberg et al., 2002; Fukazawa et al., 2003). The identification of signals that influence actin dynamics and the formation of axonal and dendritic filopodia are therefore essential to understand the molecular mechanisms of synapse formation and synaptic plasticity.

Netrins are a family of secreted molecules involved in cellular, axonal and dendritic guidance during the development of the central nervous system (CNS) (Manitt and Kennedy, 2002; Kim and Chiba, 2004). Netrin-1 signals through Deleted in Colorectal Cancer (DCC) receptors (KeinoMasu et al., 1996) modulating actin cytoskeleton dynamics, enhancing the formation and motility of filopodia (Li et al., 2002b; Shekrabi and Kennedy, 2002; Dent, et al., 2004). Netrin-1 and DCC are involved in the formation of axonal and dendritic filopodia, and have a possible role in initiating synaptic contacts and modulating synaptic transmission (Kennedy et al., 2002; Bouchard et al. unpublished data). We propose that cannabinoids acting at CB_1 receptors regulate filopodia formation, and synaptogenesis. Activating the CB_1 receptors causes a decrease in cytosolic cAMP concentration, inhibiting PKA. This blocks the recruitment of DCC receptor to the membrane surface and therefore,

inhibits the action of netrin-1 regulating filopodia formation and synaptogenesis. In this study, we show that an interplay between the CB_1/ endogenous cannabinoids and the DCC / netrin-1 pathways coordinates and maintains synapse formation during neural development.

Materials and Methods

Reagents

All cell culture media and supplements were purchased from Invitrogen Canada (Burlington, ON). Monoclonal DCC antibody directed against intracellular epitope (G97-449) was obtained from PharMingen (Mississauga, ON) and the function blocking DCC monoclonal antibody, DCC_{FB} (DCC, clone AF5) from Calbiochem (LaJolla, CA). Polyclonal antibodies against GAP-43 (AB5220), NCAM (AB5032), GluR1 (AB1504), GluR2/3 (AB1506) and NFM (AB1987) were obtained from Chemicon (Temecula, CA). Anti-MAP2, anti-synaptophysin, and anti-actin (A5316) monoclonal antibodies were purchased from Sigma-Aldrich (Oakville, ON). Anti-CB_1 (209550) was obtained from Calbiochem (LaJolla, CA). Anti-CB_2 (101550), anti-FAAH (101600), and anti-MGL (100035) were obtained from Cayman Chemical (Ann Arbor, MI). Anti-CB_1 (sc-10066) and Anti-CB_2 (sc-10076) were purchased from Santa Cruz Biotechnology (Santa Cruz,CA). Anti-N-Cadherin (610920) was obtained from BD Pharmingen (San Diego,CA). Polyclonal anti-NR1 was purchased from Upstate (Lake Placid, NY).

Polyclonal anti-GFP (A-6455), the FM1-43 dye, the secondary antibodies Alexa 488 (A11001& A11008), and Alexa 546 (A11003 & A11010) were obtained from Molecular Probes (Eugene, OR). The secondary antibodies horseradish peroxidase-conjugated goat directed against rabbit,-mouse or goat IgG (GRP-HRP (711-035-152), GMP-HRP (715-035-150) and GGP-HRP (705-035-003)) were purchased from Jackson Immunoresearch (West Grove,PA). The inverse agonist AM251 (1117), the pure antagonist O2050 (1655), and the agonist ACEA (1319) were obtained from Tocris Bioscience (Ellisville, MO). FSK (F6886), KT5720 (K3761), H89 (B1427) and Hoe 33258 (Bis-benzamide) were purchased from Sigma-Aldrich (Mississauga, ON).

Cortical Neuron Cultures

All procedures with animals were performed in accordance with the Canadian Council on Animal Care guidelines for the use of animals in research. Staged pregnant CD1 mice were obtained from Charles River Laboratories (St-Constant, QC). *dcc* KO mice and their wild-type littermate were kindly provided by Dr. Timothy Kennedy (Montreal Neurological Institute, QC).

The cortices of embryonic day 14 mice were isolated by micro-dissection and dissociated to produce a suspension of single cells as previously described (Brewer et al., 1993; Banker and Goslin, 1998). Briefly, cortices were dissected in ice cold Hank-Balanced Salt Solution, diced and incubated for 15 min at 37°C in Hepes-buffered Minimum Essential Medium containing 0.25% Trypsin and 0.001% DNase I. The tissue was then washed once in cold Neurobasal supplemented with 10% heat-

inactivated fetal bovine serum (FBS) and gently triturated with a flamed Pasteur pipette to yield a suspension of single cells. Dissociated cells were plated at low density (15,000 cells/cm^2) for analyses of branching, filopodia and synapse number, at high density (40,000 cells/cm^2) for biochemical analyses, and for transfection. Cultures were maintained for 2 to 15 DIV in Neurobasal medium containing 1% B27, 0.5% N2, 2 mM glutamine, 1 unit/ml penicillin, and 1 μg/ml streptomycin at 37°C in a humidified 5% CO_2 incubator. Penicillin, streptomycin and glutamine were omitted from the culture medium after one week in culture. Under these conditions, cortical neurons formed large networks and developed functional synapses, while keeping glial cell contamination low. Cortical neuron cultures were stimulated by adding 300 nM AM251, 50nM ACEA, 1μM O2050, 10 μM forskolin (FSK), 200 nM KT5720, 200 nM H89 or anti-DCCfb antibody 10μg/ml directly to the culture media.

Immunocytochemistry

Dissociated neurons were plated and then cultured for either 8 days (dendritic and axonal filipodia analysis) or 10 days (synaptic punctae analysis) in 24-well plates (Sarstedt, St-Leonard, QC). Neurons were grown in the wells on 12 mm round glass coverslips (No.0 Deckgläser, Carolina Biological, NC) that had been coated with poly-D-lysine (PDL) (70-150 kDa, 20μg/ml). After a 1 hour (filopodia) or 24 hour (synaptic punctae) treatment with 1μM O2050, 300 nM AM251, or 10 μM forskolin respectively, cultures were fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS), pH 7.4, and blocked with 2% FBS/2% bovine

serum albumine/0.1 % Tween-20 in 0.1 M PBS, pH 7.4, for 1 hr at room temperature. Antibodies were added in blocking solution and left overnight at 4°C at the following dilutions: anti-DCC (1:500), anti-GAP-43 (1:1000), anti-GFP (1:1000), anti-GluR1 (1:500), anti-MAP2 (1:1000), anti-NFM (1:1000) and anti-synaptophysin (1:500). After three washes in PBS, cells were labelled with either Alexa 488 or Alexa 546 secondary antibodies for 2 hr at room temperature in blocking buffer. Then nuclei were stained using Hoe 33258 (Bis-benzamide). Photomicrographs were taken with an inverted Olympus IX71 microscope (Olympus Canada, Markham, ON) and an Evolution VF camera (Media Cybernetics, Bethesda, MD). Images were analyzed with Image Pro Plus 5.1 image analysis software (Media Cybernetics).

FM1-43:

Primary pyramidal neurons were cultured on cover slips for 10 DIV, and then cell culture media was exchanged for a saline solution consisting of (in mM) 128 NaCl, 5 KCl, 2.7 $CaCl_2$, 1 $MgCl_2$, 10 dextrose, and 20 HEPES. Synaptic terminals were loaded in the presence of 15 μM FM1-43 and 80 mM KCl. After loading, a field containing labeled punctae was imaged with a cooled charged-coupled device (CCD) camera (Evolution VF camera (Media Cybernetics, Bethesda, MD)) through a 100X objective mounted on an Olympus IX71 microscope and Image Pro Plus 5.1 image analysis software (Media Cybernetics). The FM1-43 was unloaded with a 90-s stimulation using 80 mM KCl in FM1-43 free saline solution. Then, another photomicrograph was taken.

Surface Receptor Biotinylation

Eight DIV and ten DIV cortical neurons were washed three times with ice-cold PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS++). Surface biotinylation was performed by adding 0.5 mg/ml EZ-Link Sulfo-NHS-LC-biotin (Pierce,Rockford, IL) in PBS++ for 30 min at 4°C. Excess biotin was quenched by washing the cells twice with 10 mM glycine in PBS++ at 4°C and twice with ice-cold PBS++. Cells were lysed in 1 ml RIPA buffer (150 mM NaCl, 20 mM Tris pH 8.0, 1 mM EDTA, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS) for 15 min on ice. Biotinylated proteins were precipitated with streptavidin-agarose beads (Pierce, Rockford, IL) for 1 hr at 4°C and analyzed by western blot.

Western Blot

Proteins were extracted by cell lysis and were run on an SDS-PAGE gel (8%) and then transferred to a nitrocellulose membrane. The membranes were exposed overnight to a primary antibody and then washed. A secondary antibody was added for two hours (GRP-HRP, GMP-HRP, or GGP-HRP).The immunoreactive bands were detected by enhanced chemiluminescence (ECL).

Data Analysis and Quantification

Dendritic filopodia were defined as GAP-43 positive protrusions from primary dendrites. Synapses were defined as synaptophysin immunoreactive punctae closely apposed to the GluR1 immunoreactive dendrites. The density of filopodia and synapses were calculated over the area that corresponds to a 30 μm length of the

neuronal process. Statistics were performed using the *Systat* Software package (Chicago, IL). The results were expressed as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni post hoc test. $p < 0.05$ was used as the criteria for statistical significance unless otherwise indicated.

Results

CB_1 receptor distribution in embryonic cortical neurons

First, we examined the distribution of CB_1 receptors using cultures of embryonic day 15 (E15) mouse cortical neurons. These cultures were maintained in serum-free medium to prevent the proliferation of glia (Brewer et al., 1993). Under these conditions, cortical neurons display typical features of mature pyramidal neurons such as well differentiated axonal and dendritic arbors, and functional synaptic connections (Evans et al., 1998).Western blot analysis of primary cortical culture lysates harvested after 1 to 21 days *in vitro* (DIV) revealed that these cultures express CB_1 receptor, synaptophysin (SVP38), AMPA receptor subunit GluR1, and NCAM (Figure 6A). Interestingly, there was an increase of CB_1 receptor protein levels around DIV 13 (Figure 6A).

We next analyzed by immunofluorescence microscopy the localization of CB_1 receptors in cortical neurons following fixation. Neurons were plated at low density to allow resolution of individual processes and cellular distribution of CB_1 receptors at 3DIV (non-polarized neurons) and 15DIV (polarized neurons). Examination of

neurons shortly after plating, before the development of polarity (3 DIV), showed CB_1 receptors present in all neurites (GluR1 or NFM positive) and their growth cones (Fig. 6B). At 15 DIV, CB_1 immunoreactivity was detected in neuronal processes labeled with either MAP2, a cytoskeletal protein restricted to dendrites, as well as in MAP2-negative axons (Fig. 6C; arrowhead), suggesting that CB_1 receptors distribute to both axonal and dendritic compartments in mature neurons. This observation was confirmed by the presence of CB_1 receptors in both NFM-positive axons and NFM-negative dendrites (Fig. 6C).

Endogenous cannabinoids decrease the number of synaptic contacts

Here, we investigated whether endocanabinoids could modulate the formation of synaptic connections. Cortical neurons cultured for 10 DIV were stimulated by bath application of 50nM ACEA, 300nM AM251, or 10μM FSK for 24 hr, and then they were fixed and processed for synapse visualization by immunocytochemistry. Synapses were identified as synaptophysin immunoreactive puncta (presynaptic) closely apposed to GluR1 (postsynaptic) immunoreactive dendrites (Fig. 7) (O' Brien et al., 1997; Rao et al., 1998). Quantitative immunocytochemical analysis revealed a FSK induced increase in the density of paired GluR1 (postsynaptic) and synaptophysin (presynaptic) immunoreactive punctae along the proximal 30 μm of primary dendrites. Addition of 50nM ACEA, a potent and highly selective CB_1 receptor agonist, to the cultures produced a decrease in synaptic contacts (Fig. 7C). The addition of a potent and selective CB_1 receptor inverse agonist, 300nM AM 251,

to neurons increased the colocalization of pre and postsynaptic punctae. Furthermore, treating the culture with 1μM O2050, a pure antagonist of CB_1 receptors also increased the synaptic punctae formation.

The dendritic thickness measured as the ratio of dendritic surface area to dendritic length was compared within the different treatments and was found not to be affected by ACEA, AM 251, O 2050, nor FSK stimulation (Fig. 7D).

Also, western blot analysis of lysates obtained from cultured cortical neurons treated with agonist, inverse agonist or antagonist of CB_1 receptors or FSK for 24 hrs did not reveal any significant changes in the protein expression levels of major synaptic proteins. This indicated that the observed modulation in synaptic density is due to the reorganization of synaptic components (Fig. 7E).

Endogenous cannabinoids decrease functional synapses

In order to directly measure presynaptic function without using post synaptic responsiveness as an indicator, we next examined the ability of primary cortical pyramidal neurons to take up the fluorescent stryryl dye FM1-43 into synaptic vesicles during activity (Betz et al., 1992; Betz and Bewick, 1992; Ryan et al., 1993; Cochilla et al., 1999). Primary cortical neurons that had been cultured in the presence of 300nM AM251, 1μM O2050, or 10 μM FSK for 24 hrs had significantly more FM1-43 positive synaptic terminals compared to the control condition (Fig 7J). Treatment with 50nM ACEA significantly decrease the number of FM1-43 positive punctae (Fig 7J). In all cases, FM1-43 was observed in synaptic puncta like spots

along dendrites and somas (Fig 7F & G), and these punctae destained with similar kinetics (data not shown). This finding indicates that cannabinoids strongly decrease the density of active presynaptic sites.

Cannabinoids modulates the number of synaptic punctae *in vitro*: link between cannabinoids, PKA, and synaptogenesis

Here, we investigated whether the endocanabinoids modulation of synaptogenesis was linked to the activation of cAMP/PKA pathway.

Immunocytochemical analysis of 10 DIV cortical neurons shows that 24 hr application of 300 nM AM 251 and 1μM O 2050, respectively a cannabinoid CB_1 receptor inverse agonist and antagonist, significantly increases the number of synaptophysin immunoreactive punctae closely apposed to GluR1 immunoreactive dendrites. This effect however was reversed when neurons were pre-treated with KT5720 of H89 both inhibitors of PKA (Results not shown).

Quantification of 10 DIV cortical neurons shows that 24 hrs application of 300 nM AM 251, and 1μM O 2050 significantly increases the number of synaptophysin immunoreactive punctae closely apposed to GluR1 immunoreactive dendrites when compared to control and that this effect was reversed when neurons were pretreated with KT5720 and H89, two inhibitors of PKA (Fig. 8A).

Activation of adenylate cyclase and PKA using 10μM forskolin (FSK) induced an increase in the number of presynaptic punctae closely apposed to dendrites. This response was blocked by 50 nM ACEA, an agonist of CB_1 receptors and by KT5720

and H89 (Fig. 8A). Mean dendritic thickness, measured as the ratio of dendrite surface area to dendrite length and plotted as % control, was not appreciably affected by AM 251, O 2050, H89, KT5720 or FSK stimulation (Fig. 8B).

Cannabinoids modulates the presence of DCC at the cell surface via the cAMP/PKA pathway

Our observations suggest that cannabinoids act as modulators in the creation and function of synaptic contacts.

Recent reports showed that activation of cAMP/PKA pathway increases the cell surface expression of DCC in commissural and cortical neurons (Bouchard et al., 2004; and Bouchard et al., 2008). In order to understand how cannabinoids might affect DCC-expressing neuronal processes, we tested whether they modulate the surface presentation of DCC in cortical neurons. Mature cortical neuron cultures were stimulated with ACEA, AM251, O2050, FSK, or AM251+KT5720 for 15 min, and processed for cell surface protein expression using biotinylation, streptavidin pull-downs and western blot analysis. DCC surface expression was significantly decreased after 15 min in the presence of ACEA (Fig. 8C). Interestingly, addition of AM251, O2050, or FSK increase DCC surface expression and this effect was blocked by a pretreatment with KT5750 (Fig. 8C).These results suggest that cannabinoids in cortical neurons modulates the relative amount of DCC at the plasma membrane via a cAMP/PKA dependent mechanism.

CB_1 receptors modulate synapse formation in vitro and this effect is dependent of DCC receptor

Furthermore, addition of DCCfb antibody prior the AM251, O2050, or FSK treatments completely abolished the increase in filopodia formation induced by these pharmacological agents (Fig. 9A). Immunocytochemical analysis of 10 DIV cortical neurons shows that a pre-treatment of neurons with DCCfb antibody blocks the effect of 300nM AM 251 or 1μM O 2050 on the number of synaptophysin immunoreactive punctae closely apposed to GluR1 immunoreactive dendrites.

Stimulating with 300 nM AM 251 and 1μM O 2050, a pure antagonist of CB_1 receptor, produced a significant increase of synaptic punctae. This effect was antagonized by an antibody blocking DCC function, DCCfb (Fig. 9A).

Mean dendritic thickness, measured as the ratio of dendrite surface area to dendrite length and plotted as % control, was not appreciably affected by ACEA, AM 251, AM 251+DCCfb, O 2050, O 2050 + DCCfb or FSK stimulation.

DCC is essential for the cannabinoid induced modulation of synaptic contacts

In order to confirm the link between cannabinoid and netrin-1/DCC systems, we performed *in vitro* experiments on the effects of the CB_1 inverse agonist/antagonist in primary cortical neuron cultures obtained from embryonic *dcc* knockout mice. In the absence of the DCC receptor, the inverse agonist AM 251 and the antagonist O 2050 showed no increase in synapse density (Fig. 9D). Activation of adenylate cyclase and PKA using 10μM forskolin (FSK) also induced no increase in the number of

presynaptic punctae closely apposed to dendrites in the embryonic *dcc* knockout mice. Treating with agonist ACEA also showed no decrease of synapses compared to control cortical neurons (Results not shown). These results confirmed a connection between the two systems in the underlying mechanisms of synapse formation.

Endogenous cannabinoids decrease filopodia density

Initial contact between axons and dendrites is sufficient to initiate assembly of a functional synaptic site (Ziv and Smith, 1996; Fiala et al., 1998). Factors that modulate the dynamics of branches and filopodia are therefore likely to influence synapse formation, simply by altering the probability of chance encounters between axons and dendrites. We recently reported that DCC influences cytoskeletal dynamics and promotes the formation of filopodia in commissural (Sherkarabi et al., 2005) and cortical neurons (unpublished data). To determine whether cannabinoids similarly influences the formation of initial contacts between axons and dendrites, dissociated cortical neurons cultured for 6 to 8 DIV were stimulated for 1 hour by bath application of cannabinoid pathway modulators. Then they were fixed and stained for GAP-43, a membrane protein associated with the cytoplasmic surface of growth cones and filopodia. GAP-43 staining is detected in both axons and dendrites of neurons cultured for up to 8 DIV, but is restricted to axons at later developmental stages, consistent with previous reports (Van Lookeren et al., 1992). Addition of 50 nM ACEA to the cultures produced a decrease in the density of axonal and dendritic filopodia formation (Fig. 10E). The addition of 300nM AM251, 1μM O2050, or 10

μM FSK to neurons increased it (Fig. 10E). To investigate the mechanism regulating the increase in filopodia formation, cultures were exposed to different enzyme inhibitors 15 min before the addition of AM251, O2050, or FSK to the media. To confirm that these drugs were acting by increasing through PKA, cortical neurons were pretreated with 200 nM KT5720 or 200 nM H89, specific inhibitors of PKA (Kaseet al., 1987, Lochner et al., 2006). KT5720 and H89 both blocked the increase in axonal and dendritic filopodia induced by AM251, O2050 and FSK (Fig. 10 E). These observations suggest that cannabinoids inhibit axonal and dendritic filopodia formation, well known precursors of synaptogenesis and this effect is related to the PKA activity and the DCC pathway (Figure 10 A-E).

To confirm and strengthen the link between CB_1 receptor, PKA and DCC, supplemental experiments were performed using *dcc* KO mice. In the wildtype group, ACEA decreased the number of filopodia density. Conversely AM 251, O 2050, and FSK increased that number (Fig. 10F). On the other hand, in their KO littermates, these pharmacological agents did not produce any effects (Figure 10(F-G)).

Discussion

Cannabinoids have been known to interfere with learning and memory in humans (Abel, 1970; Tart, 1970; Chaperon and Thiebot, 1999) and are thought to impair synaptic plasticity (Lévénès et al, 1998). LTP, a well studied form of synaptic plasticity, is associated with changes in synapse number and structure. Several reports demonstrate that activation of cannabinoid receptors will inhibit LTP (Stella et al.,

1997; Misner and Sullivan, 1999; Bohme et al., 2000). Interestingly, the late stage of long-term potentiation (L-LTP) is critically dependent on the activation of PKA. L-LTP can be induced by PKA activation, and electrically induced L-LTP is blocked by PKA inhibition. L-LTP involves a PKA induced increase in the number of quanta released by a single action potential and an increase in the number of functional presynaptic terminals between hippocampal pyramidal neurons (Bohme et al., 2000)..

The cAMP signalling cascade is central to certain types of learning and memory (Impey et al., 1998). Changing the strength of connections between neurons is thought to underlie memory formation and may result from the recruitment of new sites of synaptic transmission (Bolshakov et al., 1997). New functional synapses between hippocampal neurons in culture can be induced by an elevation in cAMP (Kavalali et al., 1999; Ma et al., 1999). cAMP-induced changes in synaptic plasticity contribute to memory formation and moreover cannabimimetic drugs are known to inhibit adenylyl cyclase and impair memory. CB_1 receptors can modulate cAMP-dependent synaptic plasticity (Impey et al., 1998).

Kim and Thayer, using a FM1-43-based assay to identify functional synaptic boutons in rat hippocampal cultures, found that cannabimimetic drugs prevent the recruitment of new synapses by inhibiting the formation of cAMP (Kim and Thayer, 2001).

Forskolin increased the number of FM1-43-labeled functional synapses between cultured hippocampal neurons (Kim et Thayer, 2001). The formation of new synaptic sites was dependent on activation of protein kinase A. The forskolin-induced increase in the number of functional boutons was compared to the increase in synaptic puncta

induced by Sp-cAMPS in hippocampal slices (Bozdagi et al., 2000). Cannabimimetic drugs inhibited recruitment of new synapses by activation of CB_1 receptors as indicated by antagonism with SR141716 (Collins et al, 1995). Δ^9-THC inhibited new synapse formation consistent with the partial agonist properties of this drug (Sim et al., 1996a; Shen and Thayer, 1999). Win55212-2 blocked the formation of new synaptic boutons induced by forskolin but not those induced by Sp-cAMPS, suggesting that cannabimimetic drugs block new synapse formation by inhibiting the synthesis, not the actions of cAMP (Shen and Thayer, 1999). Cannabinoid modulation of both cAMP signalling and neurotransmission contribute to changes in synaptic plasticity *in vivo*. Other receptors that couple to adenylyl cyclase may regulate synaptic plasticity by modulating cAMP levels. This is clearly true for G_s-coupled receptors such as the β adrenergic receptor, activation of which lowers the threshold for eliciting both the early and the late phase of mossy fiber LTP (Huang and Kandel, 1996). Activation of adenosine A_1 receptors inhibits LTP, which could theoretically be mediated via inhibition of adenylyl cyclase, but the strong inhibition of neurotransmitter release by adenosine appears to predominate (de Mendonca and Ribeiro, 1997).

In this article, we show that cannabinoids decrease axonal and dendritic filopodia and reduce the number of synaptic connections in a PKA/DCC-dependent manner *in vitro*. Our data suggest a novel function for cannabinoids in modulating synapse plasticity.

Effect of cannabinoids on synapse density

The effect of cannabinoids on changes in synapse number was studied by visualizing a colocalization punctae of synaptophysin and GluR1 proteins. Stimulation of CB_1 receptor agonist significantly decreased the number of synaptophysin immunoreactive punctae closely apposed to GluR1 immunoreactive dendrites when compared to control. Treatment with an inverse agonist or a pure antagonist of the CB_1 receptor produces a significant increase in synaptic punctae. The functionality of these synaptic contacts was confirmed using FM1-43. ACEA decreased the number of functional synapses. On the other hand, blocking CB_1 receptors increased the number of functional synapses.

Cannabinoids modulate synapse number via PKA

Long-term potentiation (L-LTP) of synaptic plasticity is critically dependent on the activation of PKA (Bohme et al., 2000). Cannabinoids via their CB_1 receptors could decrease cAMP levels and PKA activity as confirmed by the increase in the positive FM143 punctae in FSK treated neurons compared to the control. This effect was antagonized by PKA inhibitors (Figure 8).

Cannabinoids modulate synapse number through a connection between the netrin-1/DCC and cAMP/PKA pathways

Immunocytochemical analysis of 10 DIV cortical neurons shows that a pre-treatment of neurons with DCCfb antibody and an application of AM 251 or O 2050 for 24 hrs,

did not increase the number of synaptophysin immunoreactive punctae closely apposed to GluR1 immunoreactive dendrites when compared with control and significantly decreased the number of synaptophysin immunoreactive punctae compared to neurons treated with AM 251 or O 2050 only (Figure 9).

Interestingly, a decrease of DCC receptors present at the surface of the neuron membrane was observed when treated with ACEA. An increase of DCC was observed when CB_1 receptors were antagonized and this was prevented when neurons were pretreated with inhibitors of PKA (Figure 9). These observations implicated a possible connection between the DCC receptor, the cyclic AMP/PKA pathway and the effect of CB_1 on synaptic density. To further confirm this link we repeated the same experiments in *dcc* -/- cultures and interestingly, in this experimental group cannabimimetic drugs had no effect on synaptic density (Figure 8). When we compared the treated 10 DIV cortical neurons derived from DCC receptor knockout (-/-) compared to their wild type (+/+) littermates, no increase in synaptic density, revealed by the close apposition between GluR1-positive dendrites and synaptophysin-positive presynaptic boutons was observed when treated with CB_1 inverse agonist, antagonist or FSK for 24 hrs. Thus, it seems that the action of endocannabinoids at CB_1 in modulating synaptic density is interconnected with the DCC receptor and the netrin-1, neuronal guidance cue.

Modulation of synapse precursors by cannabinoids

It is well established that initial contact between axons and dendrites is sufficient to establish a functional synaptic site (Ziv et Smith,1996; Fiala et al.,1998), and that

dynamic actin-based filopodia extensions from both axonal and dendritic compartments are important in initiating this contact (Jontes et Smith, 2000; Chang et De Camilli, 2001). Neuronal signals that influence cytoskeletal dynamics are therefore likely to affect synapse formation.

Netrin-1 mediates its guidance function during development by inducing actin reorganization and increasing filopodia number in neuronal growth cones, through the activation of the Rho GTPases Rac1 and Cdc42 (Li et al.,2002; Shekarabi et Kennedy, 2002; Shekarabi et al.,2005). Accordingly, we find that netrin-1 increases the number of filopodia and branches in both axonal and dendritic compartments of cortical neurons in a DCC-dependent manner, suggesting that netrin-1 similarly affects Rho GTPases in cortical neurons (unpublished observations). This effect is consistent with previous reports that demonstrated a role for netrin-1 in axonal branch formation *in vitro* and *in vivo* (Wadsworth et al., 1996; Dent et al., 2004).

In agreement with the hypothesis that increased axo-dendritic contact favors synaptogenesis, we detect a decrease in the density of axonal and dendritic filopodia and synaptic contacts in cortical neuron cultures 24 hr after CB_1 agonist stimulation. Conversely, blockade of the CB_1 receptor using inverse agonist or antagonist increase the number of filopodia and synaptic contacts. This effect was reversed by PKA inhibitors or by an antibody which blocks DCC function. Furthermore, in the *dcc* -/- mouse, stimulation or blockade of CB_1 receptors had no effect on filopodia and synaptic density Taken together; these observations confirm that cannabinoids modulation requires signaling through PKA and DCC. We propose a modulatory role

for cannabinoids in decreasing the likelihood of axo-dendritic contact and synaptogenesis.

Link between the cannabinoids, PKA activity, and DCC during synaptogenesis

Long-term potentiation is associated with changes in synapse number and structure. This is dependent on the activation of PKA. It has been shown that FSK stimulation caused DCC externalization into the synaptic plasma membrane and increases the presence of DCC at the synapse. (Bouchard et al., 2004) In a few words, our hypothetical model could be summarized as follow. Endogenous cannabinoids acting via their CB_1 receptors decrease adenylate cyclase and PKA activities. This reduces the recruitment of DCC at the cell surface, diminishing axonal and dendritic filiopodia contacts, and thereby decreasing synapse density (figure. 11).

Conclusion

We have shown that CB_1 stimulation by endogenous cannabinoids causes a decrease in filopodia and synapse density. Meanwhile, the inhibition of CB_1 function with inverse agonist or antagonist induces an increase in filopodia and synapse number. Our results also showed that CB_1 regulatory action is dependant upon the DCC pathway. We have confirmed the role for CB_1 receptor as a major player in regulating synaptogenesis at the developmental stages. The identification of a fundamental molecular mechanism regulating the connections between neurons will provide important insight into normal brain function, learning process and will permit the

development of innovative therapeutic strategies aiming at treating congenital or neurodegenerative diseases and trauma injuries.

The implications of these findings open door to more research and a better understanding of synaptogenesis and its complexities. Identifying novel roles of receptors in synaptogenesis regulation brings us a step closer to uncovering neuron wiring in brain development. In the next chapter, examples of future investigations in this field are discussed.

Chapter 3: General Discussion

CB_1 has been linked to an endless list of research fields such as neurogenesis, fear extinction, neural cell development, plasticity in the CNS, obesity, learning and memory, addiction, pain suppression, control of motor function, Parkinson's disease and the literature is still expanding.

CB_1 and its ligands, the cannabinoids, have been shown to have a plethora of effects on nerve cells and brain circuits and our results show an outstanding new link that explain the role of CB_1 in modulating synaptogenesis.

Synapses play an important role in wiring the brain circuitry. The wiring of the central nervous system represents the biological basis of how we understand the world, how we represent knowledge, process information and store memories. For humans to have meaningful behavior synapses need to be wired up properly.

Synapse formation and stabilization in the central nervous system is a dynamic process, requiring contact between pre- and postsynaptic sites. Numerous mechanisms coordinate where and when synapses are made in the developing brain.

Implications of our findings

This new role of CB_1 in synapse formation brings us a step closer in understanding how signaling mechanisms in the brain develops. These findings will be important for understanding both developmental disorders and regeneration after disease or injury.

The discussion of our findings on the functional contribution of cannabinoids and their receptor CB_1 to synapse formation and their molecular mechanism can be divided into two points:

I. Role in synapse formation and synaptic remodeling.
II. Identifying the underlying mechanisms by which they modulate synaptogenesis and plasticity.

Western blot analysis of primary cortical culture lysates harvested after 1 to 21 days *in vitro* (DIV) revealed that these cultures express CB_1 receptor, synaptophysin (SVP38), AMPA receptor subunit GluR1, and NCAM (Figure 6A). Interestingly, there was an increase of CB_1 receptor protein levels around DIV 13 (Figure 6A).

We next analyzed by immunofluorescence microscopy the localization of CB_1 receptors in cortical neurons following fixation. In our study, we found that the distribution of CB_1 receptors in immature non-polarized neurons (DIV3) and mature cortical neurons (DIV15) were expressed in all neurites, dendrites and axons (Figure

6). CB_1 proteins were distributed on dendrites in mature cortical neurons as indicated by the colocalization of CB_1 with MAP2. CB_1 receptor also localizes to MAP2-negative axons. It is confirmed by its colocalization with NFM positive axons and NFM negative dendrite (Figure 6C).

CB_1 receptors played a role in regulating the formation, extension and stability of dendritic filopodia (Figure 10).

Like growth cones, developing dendritic shafts are decorated with filopodia. Recent observations suggest that many synaptic contacts are initiated by dendritic filopodia that actively seek out an axonal contact. Furthermore, dendritic filopodia have been proposed to differentiate into spines following their contact with an axon.

The emerging model is that both dendrites and axons seek for synaptic partners (Mattila et al., 2008). Following the initial contact, a stable synaptic adhesion site is established and the axonal and dendritic compartments differentiate into pre- and postsynaptic specializations. The molecular mechanisms regulating the formation of dendritic filipodia are not perfectly understood, but the observation that cytchalasin-D, a drug that interferes with actin polymerization; blocks the motility of dendritic protrusions suggests a key role for actin (Fischer et al., 1998; Zhang and Benson, 2002).

We show here that cannabinoids via their receptors CB_1 regulate the formation, extension and stability of dendritic filopodia. The treatment of dendrites with agonists ACEA or antagonists AM251, O 2050 to has clearly showed an effect on the dynamics of dendritic filopodia (Figure 10). . These experiments were performed on pyramidal neurons that were derived from E14 mouse cortex then dissociated and plated in borosilicate chambered cover glass, allowing the use of high magnification short working distance objectives and DIC optics (100X PlanApochromat, Olympus IX71). Measured stable cell cultures were maintained on the microscope in a chamber that controls CO2, humidity, and temperature.

E14 mouse cortical neurons were cultured for 8 DIV, allowing visualization of differentiated axons and dendrites. Pharmacological agents were then be added to culture media for 1 hr. Density of filopodia was found to increase compared to the control when neurons were treated with antagonist AM251 and inverse agonist O2050 and FSK. On the other hand, with ACEA agonist the density of filopodia was found to be below the control. Subsequently, we determined that cannabinoids modulates filopodia, precursors of spine formations.

Filopodia have been proposed to develop into dendritic spines. We assessed whether CB_1 receptor is required for spine formation. In mice, spine density in the hippocampus and neocortex is maximal at approximately 3 weeks after birth, a period of intense synapse formation. Using immunohistochemistry techniques synapse maturation and maintenance was monitored, dendrites were visualized with

glutamatergic spines with anti-GluR1 (polyclonal), and presynaptic terminals with anti-synaptophysin (monoclonal). The density and structure of dendritic spines of the cortical neurons were examined using microscopy.

Our recent observations suggest that endocannabinoids via the CB_1 receptors negatively regulate synaptogenesis (Figure 7) but the exact mechanism by which this occurs is still unrevealed. It has been demonstrated conclusively that stimulation of this Gi/Go-protein coupled receptor inhibits cAMP production and substantially decreases PKA activity. It was recently showed that activation of adenylate cyclase and PKA induced the translocation of DCC to the plasma membrane and produced a netrin-1-DCC dependent increase in synaptogenesis (Bouchard et al., 2008).

Long-term potentiation, a well studied form of synaptic plasticity, is associated with changes in synapse number and structure. Interestingly, the late stage of long-term potentiation (L-LTP) is critically dependent on the activation of PKA. L-LTP can be induced by PKA activation, and electrically induced L-LTP is blocked by PKA inhibition. L-LTP involves a PKA induced increase in the number of quanta released by a single action potential and an increase in the number of functional presynaptic terminals between hippocampal pyramidal neurons (Bear and Malenka, 1999).

We have shown that FSK stimulation caused DCC externalization into the synaptic plasma membrane and increased the presence of DCC at the synapse. To complete our results showing that netrin-1 and DCC contribute to the PKA dependent

synaptogenesis, membrane permeable direct activators of PKA, 1mM db-cAMP and 50μM Sp-cAMP should be applied to E14 cortical neuron cultures and single cell patch analysis additional to our immunocytochemical analysis (colocalization, and FM1-43) will further confirm that PKA activation affects synapse number and efficacy.

We have showed that FSK acts via the adenylate cyclase and PKA (200nM KT5720, 2μM H89) (Figure 8). Also, we have determined that netrin-1 and DCC are required effectors of PKA induced synapse formation by applying function-blocking antibodies. In order to validate these observations, a second set of experiments were realized using embryonic primary cortical neuron culture obtained from *dcc* and *netrin-1* KO mice (Figure 9 C-D).

We showed that PKA induced translocation of DCC is required for PKA mediated synapse modification. Cortical neurons were stimulated with ACEA, AM 251, or O 2050 for 15 min and cell surface protein expression levels were measured using by biotinylation and streptavidin pull-downs. AM 251, O 2050, and FSK significantly increased cell surface DCC 15 min after stimulation (biotinylated) and ACEA significantly decreased it. Application of KT 520 completely abolished the effect produced by AM 251. Membranes were stripped and reprobed for synaptophysin (SVP38) as a negative control to test the membrane permeability (Figure 8C).

Our results show that endogenous cannabinoids produced by neurons in culture, negatively modulate synapse formation. To determine if PKA, netrin-1 and DCC contribute to this effect, we added CB_1 receptor antagonists (AM251, O2050) to the neurons in culture for 1 day in the presence or not of antibodies that block DCC and netrin-1 function, and quantified synaptic density and functionality as pre and post-synaptic marker colocalization, FM1-43 staining. Another set of experiments were realized using embryonic primary cortical neuron culture obtained from dcc and netrin-1 knockout mice. Using pharmacological inhibitors, we confirmed that antagonizing CB_1 receptors increases adenylate cyclase and PKA activities (200nM KT5720, 2μM H89).

CB_1 is shown here to be a major player in synapse formation and to be an active modulator in development for brain wiring. It was previously known that many types of neurons can release endocannabinoids that act as retrograde signals to inhibit neurotransmitter release from presynaptic terminals (Freund et al., 2003). Here we report that synaptic development is evoked by endocannabinoid release and regulated by the CB_1 receptor. Further more we have linked this activity to the cAMP/PKA and netrin -1/DCC pathways uncovering a possible mechanism of synapse formation.
This leads us to come closer to the exciting possibility of arriving one day at the ability of interfering in the synapse formation stages and rewiring neurons of importance.

Understanding synaptogenesis and eventually regulating neuron wiring can be a huge step forward for the human race in controlling the brain complexities.

Suggested future investigations

Our results provide evidence that endocannabinoids regulate the density of synapses. However, our experiments have not undercovered various missing complexities in the underlying mechanisms of action. In order to further explore these results and to better understand the mechanisms, other experiments can be performed.

Genetic experiments: To determine if endogenous cannabinoids and their CB_1 receptor are absolutely required for synaptogenesis, culture cortical neurons derived from *cnr1* knockout mice can be examined. Cultures from dissociated cells will be treated with pharmacological agents, and synapse and filopodia densities will be quantified and compared to the wild type mice.

siRNA experiments: Another way of determing if CB_1 receptor is required for synaptogenesis is to block its expression using siRNA technology. Synapse and filopodia density can then be measured in these cultures and compared with cultures where the CB_1 expression is not silenced.

Electron Microscopy experiments: Although functional synaptic activity has been reported within minutes following contact between two excitable cells, a mature ultrastructural appearance takes days to develop. To determine the effect of cannabinoids on the ultrastructural characteristics of synapses, electron microscopy (EM) can be performed. Ultrastructural characteristics, number of synapses, total and docked vesicles per synapse can be assessed. Primary neurons obtained from *cnr1* KO animals can also be compared with those obtained from wild type animals.

Electrophysiological analysis: Conscious of the short term modulatory effect of CB_1 receptors on Ca^{2+} and K^+ channels (Deadwyler et al., 1993), long term physiological significance of the effect of cannabinoids on synapse function will be assessed 24 and 48 hrs after pharmacological manipulations. An increase in the number of glutamatergic synapses is predicted to increase the effectiveness of synaptic transmission in the neuronal network *in vitro.* Thus it would be interesting to analyze the recordings from cultured cells treated with the pharmacological drugs. In another set of experiments, *cnr1* KO animals can be compared to wild type animals. The endogenous cannabinoid system is thought to be an important neuromodulator in motivation, reward and motor control systems. Observations using animal models suggest that modulation of endogenous central cannabinoid signaling in mesolimbic pathways may be one component of the addiction process, particularly via interaction with opioid and dopaminergic systems.

The endocannabinoid system as a ubiquitous mediator of biological pathways in health and diseases. An understanding of the crucial role of cannabinoids in health and diseases has been rapidly gained from novel tools permitting the identification and functional characterization of cannabinoid receptors. Consequently, several studies have shown that, in addition to their anticipated CB_1-dependent central effects, cannabinoids also display a wide variety of CB_1- or CB_2-mediated peripheral functions, including regulation of energy balance, immune and inflammatory responses and bone mass, as well as antitumor properties and vasoregulatory and lipogenic effects Our preliminary findings suggest a novel mechanism by which endocannabinoids and their CB_1 receptors play a fundamental role in regulating synaptogenesis during neural development. Further investigation on the matter will contribute to our understanding of phenomena that regulate learning, memory, congenital, and neurodegenerative diseases. This will possibly permit the development of new therapeutic agents.

CB_1 is shown here to be a major player in synapse formation and to be an active modulator in development for brain wiring. It was previously known that many types of neurons can release endocannabinoids that act as retrograde signals to inhibit neurotransmitter release from presynaptic terminals (Freund et al., 2003). Here we report that synaptic development is evoked by endocannabinoid release and regulated by the CB_1 receptor. Further more we have linked this activity to the cAMP/PKA and netrin -1/DCC pathways uncovering a possible mechanism of synapse formation.

This leads us to come closer to the exciting possibility of arriving one day at the ability of interfering in the synapse formation stages and rewiring neurons of importance. Understanding synaptogenesis and eventually regulating neuron wiring can be a huge step forward for the human race in controlling the brain complexities.

Figures and Figure Legends

Figure 1. Filopodia and Spines: Actin network in neurons

(A) In migrating cells, filopodia, that are localized at the leading edge, probe the microenvironment and serve as pioneers in cell protrusion.

(B) Filopodia of epithelial sheets, which protrude from opposing cells, help the sheets of cells to align and adhere together.

(C) Filopodia participate in the guidance and migration of neuronal growth cones (top inset) and are precursors of the dendritic spine (bottom inset), which are the postsynaptic regions of most excitatory neuronal synapses. As a dendritic spine matures, its morphology changes from a filopodia-like protrusion to a mushroom-shaped structure.

From the following article:

Pieta K. Mattila & Pekka Lappalainen, *Filopodia: molecular architecture and cellular functions*, Nature Reviews Molecular Cell Biology June 2008, 9:446-454

Figure 2. Structure of netrin-1 and netrin-1 dependence receptors

Structure of netrin-1 and netrin-1 receptors. DCC is a type I transmembrane protein with an extracellular domain composed of four immunoglobulin domains, six fibronectin type III domains, a single transmembrane spanning region and a cytoplasmic domain including ADD. The four UNC5H receptors all have two immunoglobulin domains and two thrombospondin domains in the extracellular region and a ZU-5 domain (a domain of homology found in Zona Occludens-1 and UNC-5 protein). Their ligand, netrin-1, is a laminin-related secreted protein with V and VI domains (three epidermal growth factor domains) related to laminin and a positively charged carboxy (C)-terminal domain.

From the following article:

Mehlen P and Llambi F,*Role of netrin-1 and netrin-1 dependence receptors in colorectal cancers*, British Journal of Cancer (2005) 93, 1–6.

Figure 3. Natural cannabinoids

Cannabinoids are a group of substances that are structurally related to Δ^9-THC or that bind to cannabinoid receptors. Currently, there are natural and synthetic cannabinoids. Different types of natural cannabinoids are their structures are shown in this figure.

From the following website:
http://en.wikipedia.org/wiki/Cannabinoids

Figure 4. CB_1 and CB_2 receptors Structures

There are currently two known subtypes, CB_1 which is expressed mainly in the brain, but also in the lungs, liver and kidneys and CB_2 which is mainly expressed in the immune system and in hematopoietic cells. The protein sequences of CB_1 and CB_2 receptors are about 45% similar.

From the following article:

Straiker A, J Neurphysiol, 2003; Howlett AC, Pharmacol Rev, 2002; Howlett AC, Handb Exp Pharmacol, 2005

Figure 5. Inverse agonist, agonist, and antagonist of CB_1 receptors

The cannabinoid receptor type 1, often abbreviated to CB_1, is a G protein-coupled cannabinoid receptor that is found in the brain and is activated by the psychoactive drug cannabis and its active compound Δ^9-THC and by a group of endocannabinoid neurotransmitters including anandamide.

In this figure, the structures of selective inverse agonist, agonist and antagonist for CB_1 are shown. From the following website:

http://www.tocris.com/

Figure 6. CB_1 receptors are expressed in cortical neuron cultures. They are distributed along dendrites and axons in polarized pyramidal cortical neurons in vitro.

(A) Western blot analysis of CB_1 receptor, synaptophysin (SVP38), AMPA receptor subunit GluR1, and NCAM protein expression in primary cortical neurons cultured for 1 to 21 days in vitro (DIV). There is an increase of CB_1 receptor protein intensity levels around DIV 13.

(B-C) Cellular distribution of CB_1 receptor *in vitro*. Primary cultures of cortical neurons were grown under serum free conditions for 3 DIV **(B)** or 15 DIV **(C)**, fixed and stained for CB_1 receptor (**B and C** (red)) and GluR1 (Chemicon, AB1504), NFM (Chemicon, MAB1621, AB1987) or MAP2 (Sigma M1406) (green).

(B) Distribution of CB_1 receptors in immature non-polarized neurons (DIV3). All neurites were positives for either GluR1 or NFM.

(C) CB_1 protein distributes to dendrites in mature cortical neurons as indicated by the colocalization of CB_1 with MAP2. CB_1 receptor also localizes to MAP2-negative axons (arrowhead). It is confirmed by its colocalization with NFM positive axons and NFM negative dendrite (arrowhead) Scale bar = 15 μm.

Figure 7. CB_1 receptors modulate functional synapses in vitro.

(A-B) Immunocytochemical analysis of 10 DIV cortical neurons shows that 24 hrs application of 300 nM AM 251, a cannabinoid CB_1 receptor inverse agonist, significantly increases the number of synaptophysin immunoreactive punctae closely apposed to GluR1 immunoreactive dendrites **(B)** when compared to control **(A)**. GluR1 staining is shown in green and was used as a dendritic marker. Synaptophysin stain (red) labels presynaptic boutons. Isolated GluR1 positive neurons were selected randomly and quatification was performed blindly. Scale bar = 15 μm.

(C) Average number of immunoreactive synaptic puncta per μm^2 dendritic area plotted as Δ % control.* $p < 0.05$ vs ctrl. # $p < 0.05$ vs AM 251, O 2050.

Stimulating with 300 nM AM 251 and 1uM O 2050, a pure antagonist of CB_1 receptor, produced a significant increase of synaptic punctae. **(D)** Mean dendritic thickness, measured as the ratio of dendrite surface area to dendrite length and plotted as % control, was not appreciably affected by ACEA, AM 251, O 2050, or FSK stimulation.

(E) Western blot analysis of lysates obtained from cultured cortical neurons treated with agonist or antagonist/inverse agonist of CB_1 receptors or FSK for 24 hrs did not reveal any significant changes in the protein expression levels of GluR1 or SVP 38.

(F-G) Average denisty of active vesicules in AM 251 treated cells **(G)** increased compared to control **(F)**. Active presynaptic sites are identifed by monitoring the endocytosis and release of the fluorescent styryl dye FM1-43. **(H-I)** These panels represent differential interference contrast (DIC) pictures ofcells **(F and G)** respectively.

(J) Significant differences in the density of active vesicles were found between control cultures and cultures treated with ACEA, AM 251, O 2050, or FSK.

Figure 8. Cannabinoids modulate the number of synaptic punctae in vitro: Link between Cannabinoids, PKA, and synaptogenesis

Immunocytochemical analysis of 10 DIV cortical neurons shows that 24 hrs application of 300 nM AM 251, a cannabinoid CB_1 receptor inverse agonist, significantly increases the number of synaptophysin immunoreactive punctae closely apposed to GluR1 immunoreactive dendrites. This effect was reversed when neurons were pre-treated with KT5720 of H89 both inhibitors of PKA

(A) Quantification of 10 DIV cortical neurons shows that 24 hrs application of 300 nM AM 251, and 1uM O 2050 significantly increases the number of synaptophysin immunoreactive punctae closely apposed to GluR1 immunoreactive dendrites when compared to control. This effect was reversed when neurons were pretreated with KT5720 and H89, two inhibitors of PKA.

Activation of adenylate cyclase and PKA using 10μM forskolin (FSK) induced an increase in the number of presynaptic punctae closely apposed to dendrites. This response was blocked by 50 nM ACEA, an agonist of CB_1 receptors and by KT5720 and H89.

Average number of immunoreactive synaptic puncta per μm^2 dendritic area plotted as Δ % control. * $p < 0.05$ vs ctrl. # $p < 0.05$ vs AM 251, O 2050, or FSK.

(B) Mean dendritic thickness, measured as the ratio of dendrite surface area to dendrite length and plotted as % control, was not appreciably affected by AM 251, O 2050, H89, KT5720 or FSK stimulation.

(C) Cortical neurons were stimulated with ACEA, AM 251, or O 2050 for 15 min and cell surface protein expression levels were measured using by biotinylation and streptavidin pull-downs. AM 251, O 2050, and FSK

significantly increased cell surface DCC 15 min after stimulation (biotinylated) ACEA significantly decreased it.

Application of KT 520 completely abolished the effect produced by AM 251 .

Membranes were stripped and reprobed for synaptophysin (SVP38) as a negative control to test the membrane permeability.

Figure 9. CB_1 receptors modulate synapse formation *in vitro* and this effect is dependent of DCC receptor and PKA

Immunocytochemical analysis of 10 DIV cortical neurons shows that a pre-treatment of neurons with DCCfb antibody blocks the effect of 300nM AM 251 or 1uM O 2050 on the number of synaptophysin immunoreactive punctae closely apposed to GluR1 immunoreactive dendrites.

(A) Average number of immunoreactive synaptic puncta per μm^2 dendritic area plotted as Δ % control.* $p < 0.05$ vs ctrl. # $p < 0.05$ vs AM 251, O 2050.

Stimulating with 300 nM AM 251 and 1uM O 2050, a pure antagonist of CB_1 receptor, produced a significant increase of synaptic punctae. This effect was antagonized by an antibody blocking DCC function (DCCfb).

(B) Mean dendritic thickness, measured as the ratio of dendrite surface area to dendrite length and plotted as % control, was not appreciably affected by ACEA, AM 251, AM 251+DCCfb, O 2050, O 2050 + DCCfb or FSK stimulation.

(C-D) 10 DIV cortical neurons derived from DCC receptor knockout (-/-) compared to their wild type (+/+) littermates display no increase in synaptic density, revealed by the close apposition between GluR1-positive dendrites and synaptophysin-positive presynaptic boutons when treated with CB_1 inverse agonist, antagonist or FSK for 24 hrs.

Figure 10. Cannabinoids regulate axonal and dendritic filopodia number via a mechanism dependent of the cAMP/PKA pathway and of DCC

(A) Control (untreated) and AM 251 treated **(B)** 8 DIV cortical neurons stained for GAP-43. Axonal or dendritic filopodia were identified as GAP-43 protrusions stemming from the axon or the dendrite. **(C-D)** Close-up of the dendrites of control verses AM251 treated neuron. Scale bar = 15 μm for upper panels and 5 μm for lower panels.

(E) Average number of filopodia per μm^2 dendritic area plotted as Δ % of control. Significant differences were found between Ctrl cultures and cultures treated with ACEA, AM 251, O2050, or FSK. Addition of PKA inhibitors (K5720, H89) or of DCC function blocking antibody reverse the increase in filopodia number induced by AM 251, O 2050 or FSK. * $p <$ 0.05 vs ctrl, # $p < 0.05$ vs AM 251, O 2050, or FSK.

Mean dendritic thickness, measured as the ratio of dendrite surface area to dendrite length and plotted as % control was compared between the different treatments and the control. No statistical differences were observed. (Results not shown).

(F-G) 8 DIV cortical neurons derived from DCC receptor knockout (-/-) compared to their wild type (+/+) littermates display no increase in filopodia density, revealed by the GAP43-positive dendritic and axonal neurites when treated with the inverse agonist, antagonists or FSK for 1hr.

Figure 11. Putative mechanism by which endogenous cannabinoids decrease synaptic density

(A) The overall hypothesis is that acting on the CB_1 receptor; cannabinoids decrease cytosolic cAMP concentration, inhibiting PKA, and block the recruitment of DCC to the neuron membrane, thereby decreasing the formation of axonal and dendritic filopodia formation and synaptogenesis.

(B) By activating their CB_1 receptors, endogenous cannabinoids decrease the number of synapses.

(C) Our results suggest that endogenous cannabinoids via the activation of their CB_1 receptors reduce axonal and dendritic filopodia. This mechanism is dependent of PKA and DCC. Dendritic filopodia differentiate into spines following their contact with an axon. Following the initial contact, a stable synaptic adhesion site is established and the axonal and dendritic compartments differentiate into pre- and postsynaptic specializations. Therefore axonal and dendritic filopodia have an essential role in the synaptogenesis.

(D) The decrease in axonal and dendritic filopodia number reduces the contact probability between pre and postsynaptic counterparts and therefore decrease synapse density.

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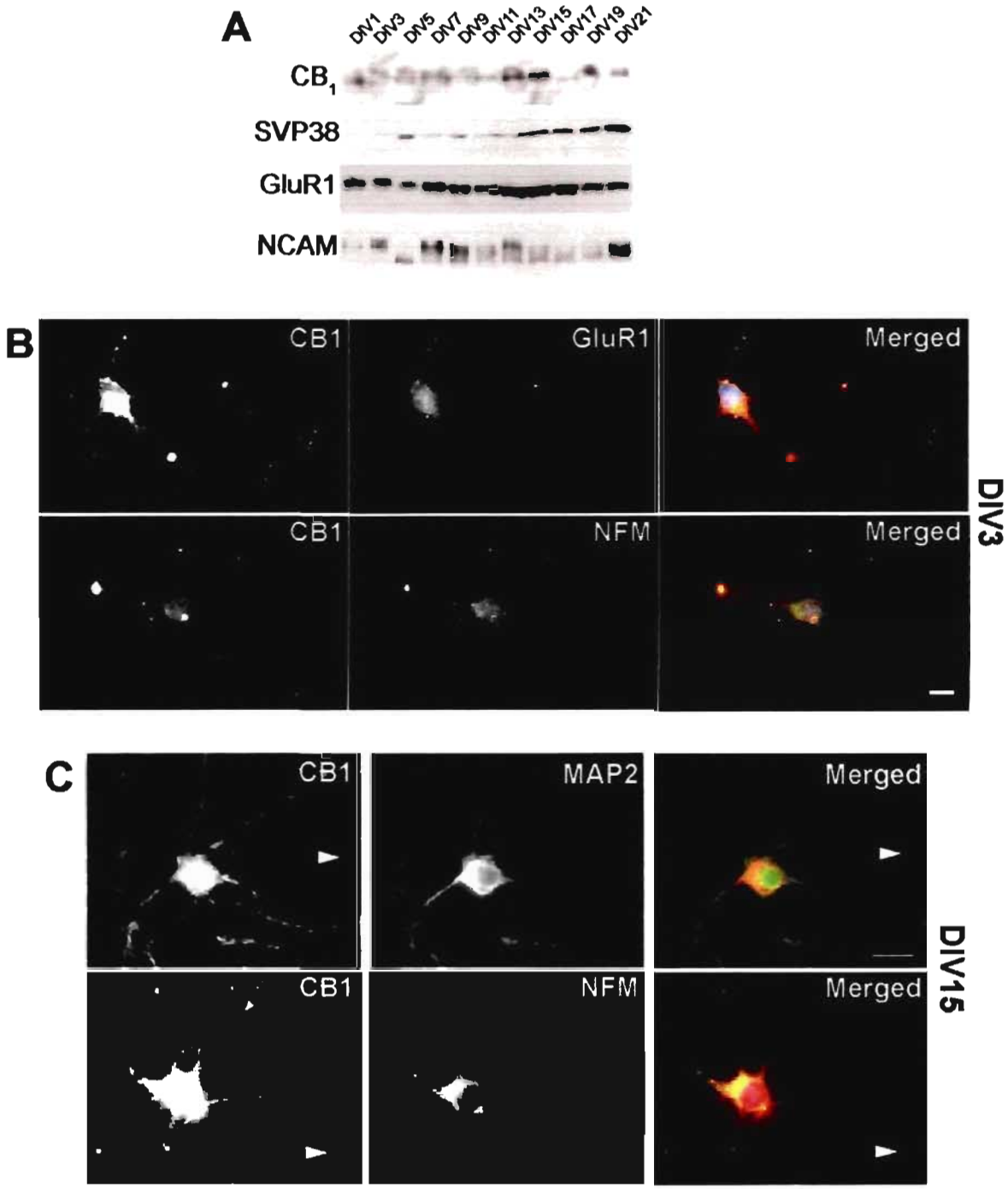
Appendix

Figure 6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SVP38
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B
CB1
GluR1
Merged
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Merged
DIV3
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MAP2
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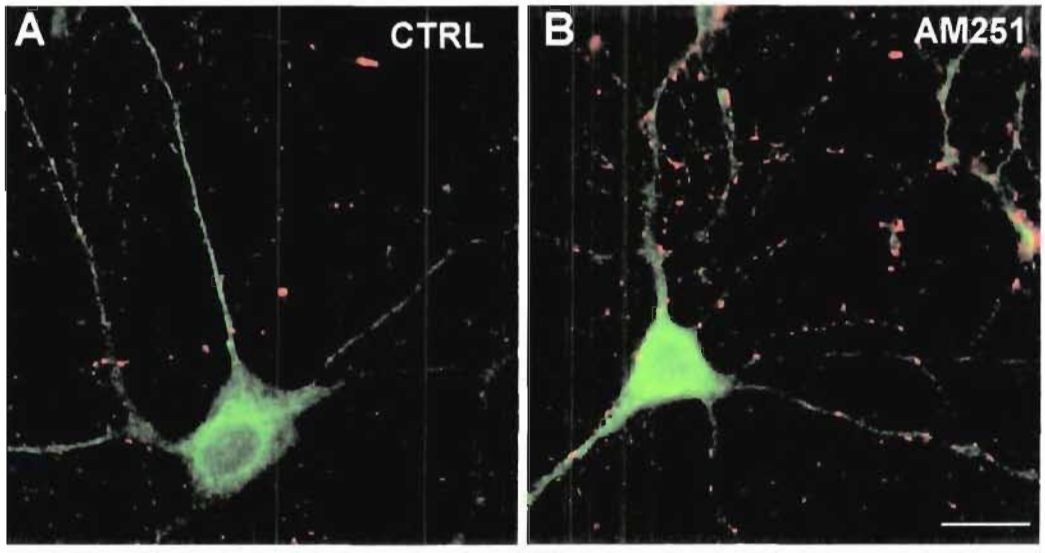
CB1
NFM
Merged
DIV15

Figure 7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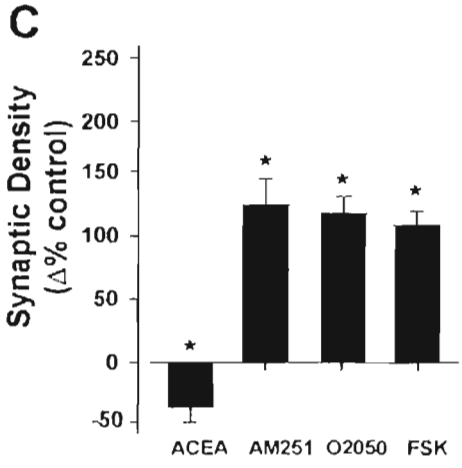
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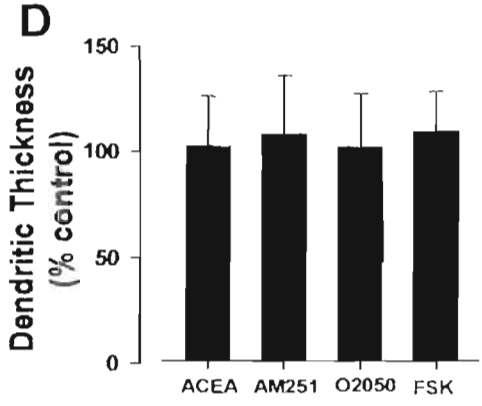
100
50
0
-50
ACEA
AM251
O2050
FSK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100
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0
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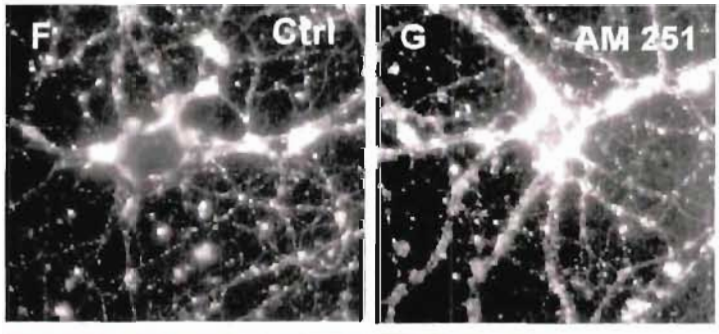
AM251
O2050
FSK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G
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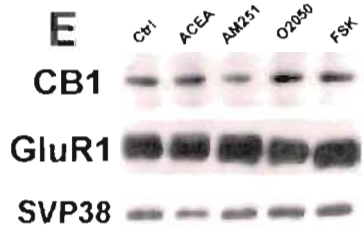
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O2050
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CB1
GluR1
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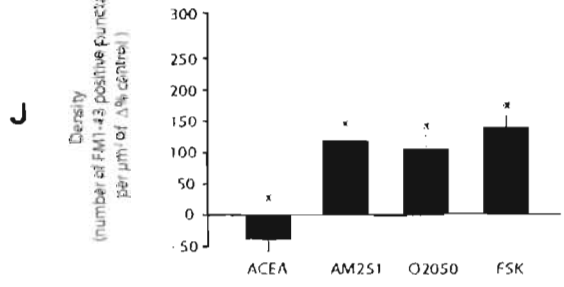
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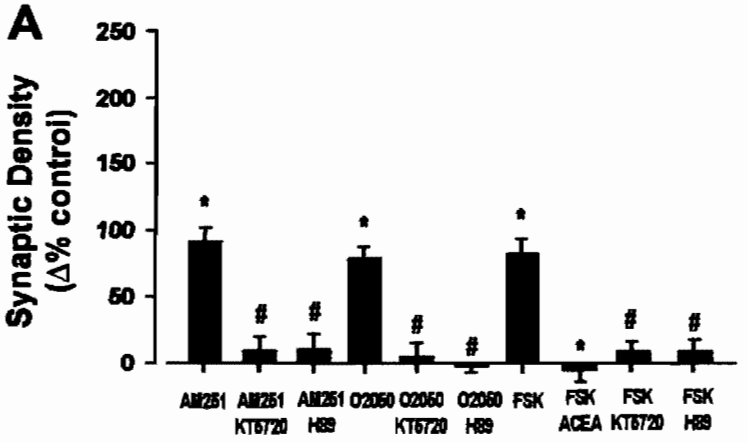
ACEA
AM251
O2050
FSK

Figure 8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(Δ% control)
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0
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FSK
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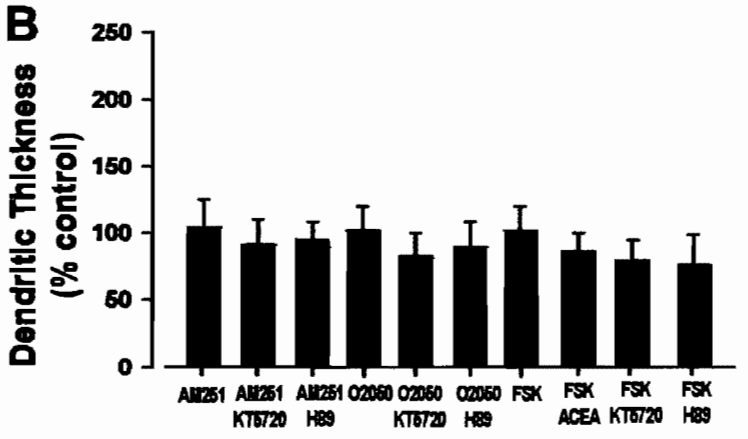
FSK KT5720
FSK H89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Dendritic Thickness
(% control)
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200
150
100
50
0
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AM251 KT5720
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O2050
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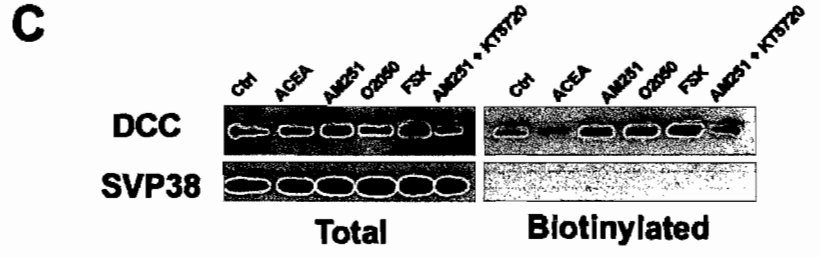
O2050 H89
FSK
FSK ACEA
FSK KT5720
FSK H89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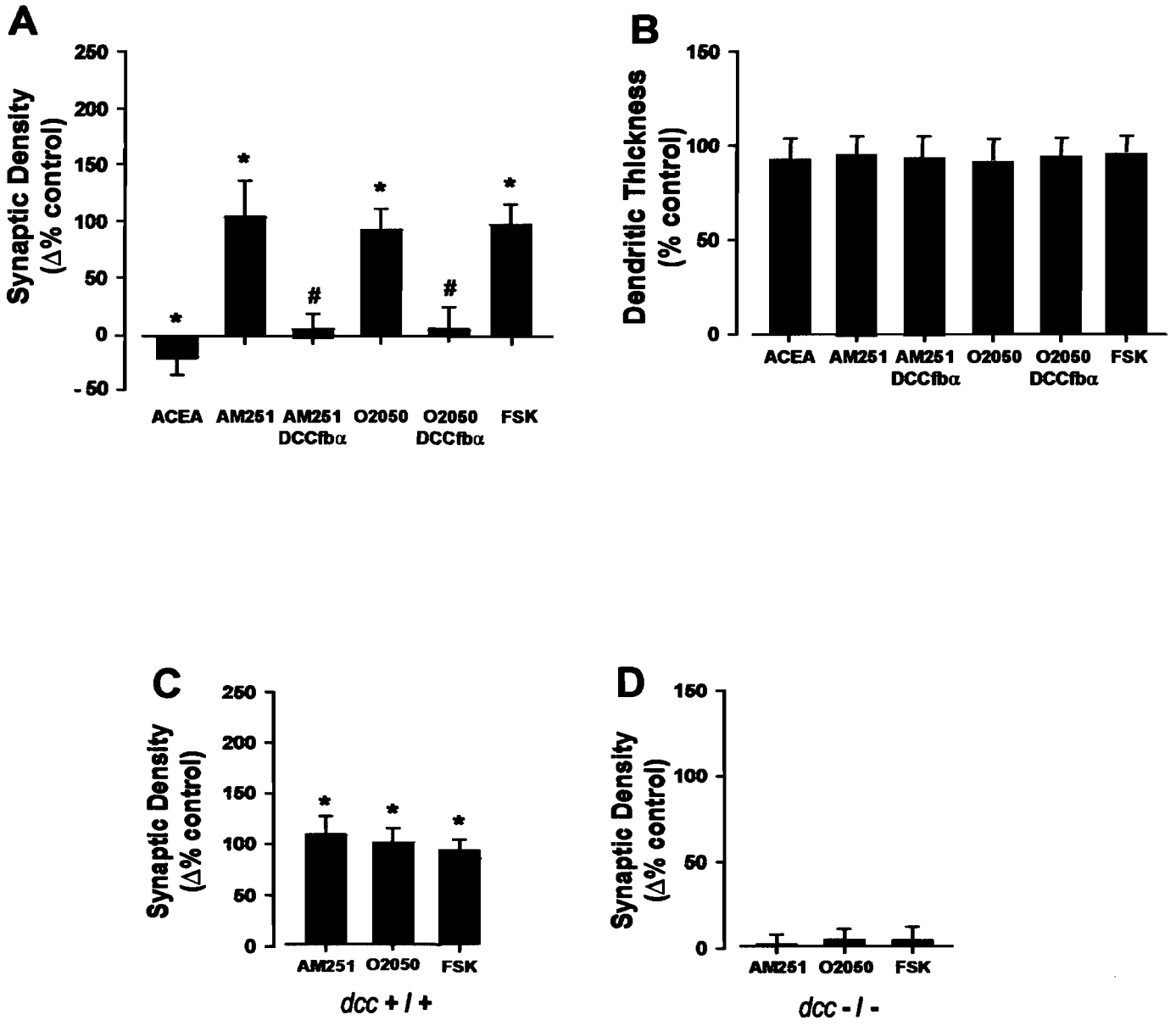
Ctrl
ACEA
AM251
O2050
FSK
AM251 + KT5720
DCC
SVP38
Total
Biotinylated

Figure 9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O2050 DCCfbα
FSK
B
Dendritic Thickness
(% control)
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ACEA
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AM251 DCCfbα
O2050
O2050 DCCfbα
FSK
C
Synaptic Density
(Δ% control)
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Synaptic Density
(Δ% control)
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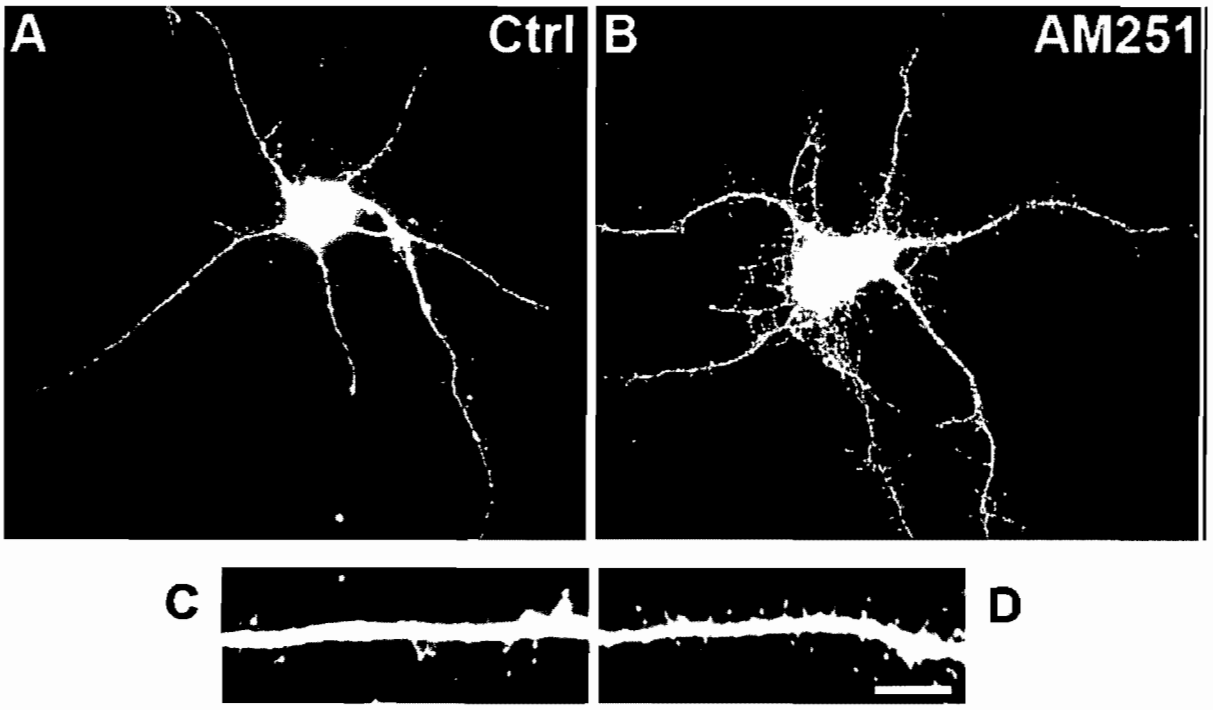
Figure 10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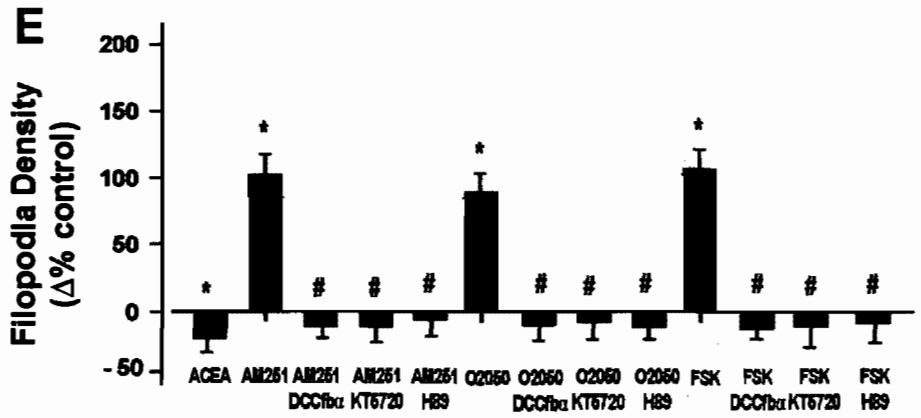
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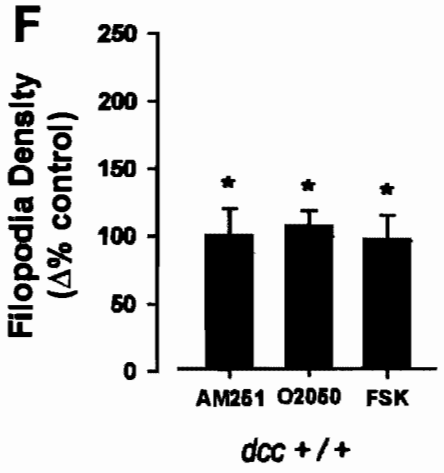
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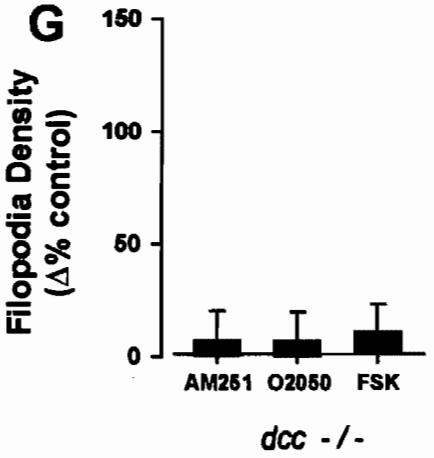
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Filopodia Density
(Δ% control)
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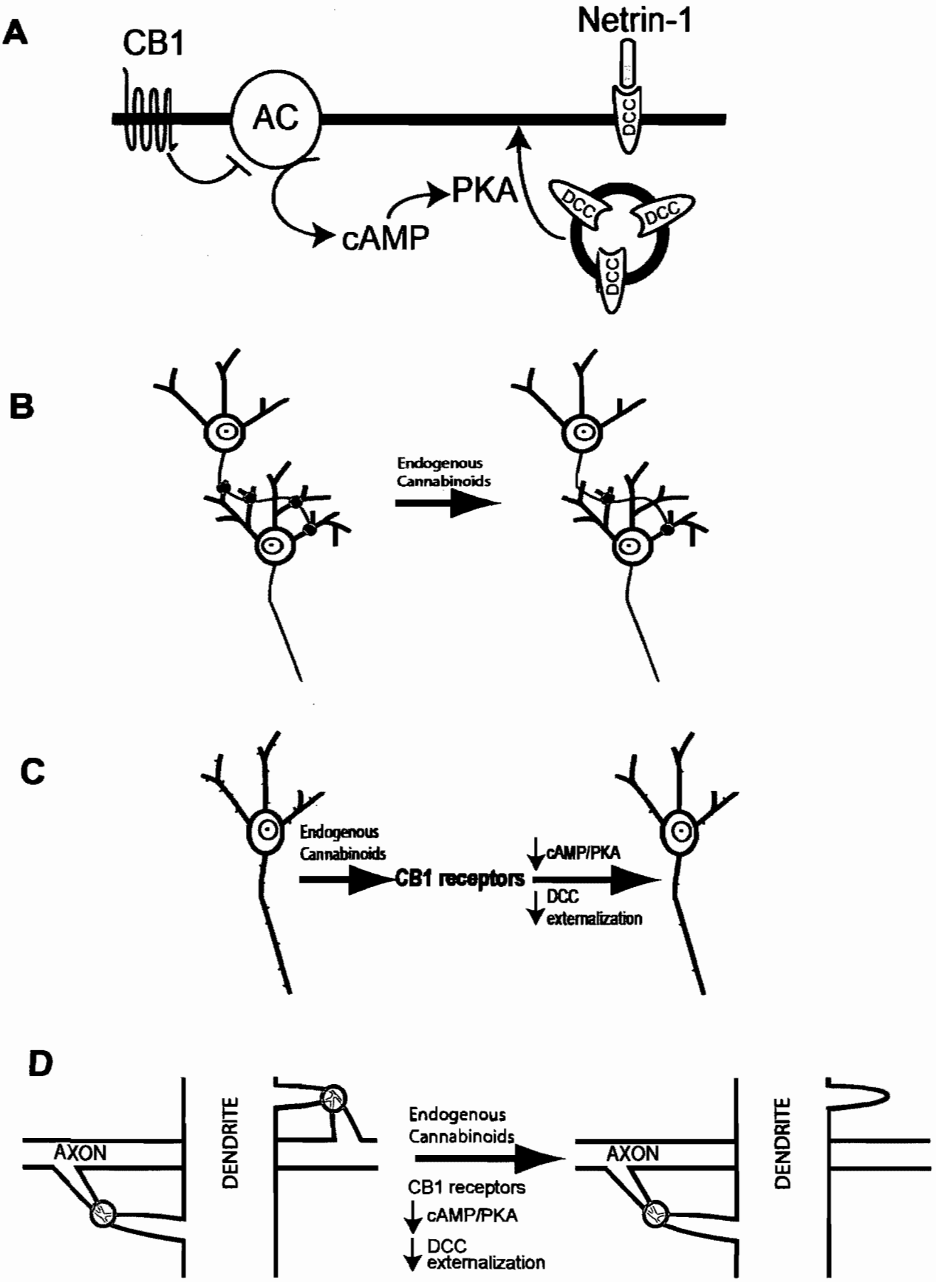
Figure 11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CB1 receptors
cAMP/PKA
DCC
externalization
D
AXON
DENDRITE
Endogenous
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CB1 receptors
cAMP/PKA
DCC
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AXON
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