UNIVERSITÀ DEGLI STUDI DI MILANO

Scuola di Dottorato in Scienze Farmacologiche

Dipartimento di Biotecnologie Mediche e Medicina Traslazionale

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ACTIVE ENDOCANNABINOIDS ARE RELEASED FROM MICROGLIA IN ASSOCIATION WITH EXTRACELLULAR VESICLES TO INHIBIT GABAERGIC TRANSMISSION

BIO/14

MARTINA GABRIELLI

MATRICOLA: R10167

TUTOR: Dott.ssa Mariaelvina SALA

CO-TUTOR: Dott.ssa Claudia VERDERIO

COORDINATORE: Prof. Alberto CORSINI

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“Happiness is a mood, not a destination,,

Julian Baker, One Three Hill

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ABSTRACT

Endocannabinoids (eCBs) are bioactive lipids which primarily influence synaptic communication within the nervous system. They are synthesized by neurons but also by microglia, especially under neuroinflammatory conditions. To exert their function, eCBs travel across the intercellular space. However, how eCBs move extracellularly remains obscure. Our recent evidence indicates that reactive microglia release extracellular vesicles (EVs), which may represent an ideal vehicle for the transport of hydrophobic eCBs. Hence, in this study we investigated whether microglial EVs carry eCBs and may influence neurotransmission.

First we analyzed the eCB content of EVs and found a clear enrichment of N-arachidonoylethanolamine (AEA) in EVs relative to parental microglia. This analysis revealed higher AEA levels in EVs shed from the plasma membrane (microvesicles), compared to those which originate from the endocytic compartment (exosomes). To bioassay the activity of vesicular AEA, we used patch clamp analysis of miniature inhibitory post-synaptic currents (mIPSC) on rat hippocampal primary culture. Exposure of neurons to microvesicles (MVs) induced a significant decrease in mIPSC frequency, mimicking the well-known inhibitory action of CB_1 receptor agonists. The involvement of vesicular AEA in this phenomenon was inferred from the ability of the CB_1 receptor antagonist SR141716A to block the reduction of mIPSC frequency evoked by MVs. Western blot analysis showed an increase in ERK phosphorylation in neurons exposed to MVs, which was completely inhibited by SR141716A. This indicate that CB_1 receptors activation by AEA-storing MVs translates into downstream signaling.

Finally, the use of biotin-AEA revealed an affinity of AEA for MV membrane, indicating that AEA travels in association with MVs surface. Consistent with a surface localization of AEA, MV membranes maintain their capability to decrease mIPSC frequency.

Overall, this study shows that microglial MVs carry AEA on their surface to stimulate CB_1 receptors on target GABAergic neurons and demonstrates that extracellular vesicular transport of eCBs play a crucial role in the modulation of inhibitory transmission.

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The electronic version of this abstract is the complete one and can be found online at:
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ABBREVIATION LIST

Aβ: β-amiloid

2-AG: 2-arachidonoylglycerol

ABDH-6: serine hydrolase α-β-hydrolase domain 6

AC: adenylyl cyclase

AD: Alzheimer's disease

AEA: anandamide, N-arachidonoylethanolamine

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AMT: AEA membrane transporter' protein

A-SMase: acid sphingomyelinase

ATP: adenosine-5'-triphosphate

Ca^{++}: calcium ions

cAMP: cyclic adenosine monophosphate

CB_1 receptors: cannabinoid type I receptors

CNS: central nervous system

CSF: cerebrospinal fluid

DAPI: 4',6-diamidino-2-phenylindole

DAG: diacylglycerol

DIV: days *in vitro*

DRG: dorsal root ganglion

EAE: experimental autoimmune encephalomyelitis

eCBs: endocannabinoids

ESCRT: endosomal sorting complex responsible for transport

ERK: extracellular signal-regulated kinase

EVs: extracellular vesicles

FAAH: fatty acid amine hydrolase

fGFP: farnesylated green fluorescent protein

FLAT: FAAH-like AEA transporter

GABA: γ-aminobutyric acid

GAD: glutamic acid decarboxylase

HD: Huntington's disease

ICAM: intercellular adhesion molecules

IL-1β: interleukin-1β

mEPSCs: miniature excitatory postsynaptic currents

mIPSCs: miniature inhibitory postsynaptic currents

LC-ESI-MS: liquid chromatography - electrospray ion source - mass spectrometry

MGL: monoacyl glycerol lipase

MHC: major histocompatibility complex

MS: multiple sclerosis

MVs: microvesicles

MVE: multivesicular endosomes

NAPE: N-arachidonoyl phosphatidylethanolamine

NMDA: N-methyl-D-aspartate

OEA: N-oleoylethanolamide

oATP: oxidized ATP

PD: Parkinson's disease

P-ERK: phosphorilated ERK

PKA: cAMP-dependent protein kinase

PLCβ: phospholipase C β

PLD: phospholipase D

PS: phosphatidylserine

KRH: Krebs-Ringer's HEPES solution

S1P: sphingosine-1-phosphate

SNAREs: Soluble NSF Attachment Protein Receptors

Sph: Sphingosine

TNFα: tumor necrosis factor alpha

TRPV1: transient receptor potential vanilloid type I receptors

TGFβ: transforming growth factor bet

VEPs: visual evoked potentials

INTRODUCTION

1. The synapse

The synapse is where communication between two neurons takes place. Synaptic communication occurs between a presynaptic terminal and a postsynaptic compartment, thanks to the excitable nature of neurons, which change their membrane potential in response to stimuli. In chemical synapses, when an action potential reaches the presynaptic terminal, it induces neurotransmitter release through activation of voltage-gated calcium (Ca^{++}) channels and the consequent fusion of neurotransmitter-storing synaptic vesicles with the presynaptic membrane. Neurotransmitters are hence released in the synaptic cleft (the intercellular space between pre and postsynaptic membrane) where they interact with their specific receptors placed on the postsynaptic membrane, thus inducing opening of ion channels and the consequent alteration of membrane potential (Sudhof, 2004). If the sum of generated postsynaptic currents reaches a specific threshold value, a new action potential is elicited in the postsynaptic neuron. (Figure I)

Excitatory synapses induce depolarization in the postsynaptic neurons, thus promoting the generation of an action potential. Instead inhibitory synapses induce hyperpolarization, inhibiting the generation of action potentials. In the central nervous system excitatory neurons typically release glutamate, while GABA is released from the great majority of inhibitory synapses in the brain.

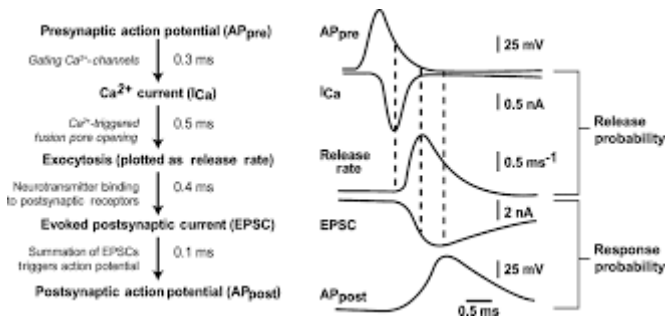


Figure I Synaptic transmission: diagram of reactions, on the left; electrophysiological traces relative to reactions, on the right (Sudhof, 2004)

Synaptic transmission is a highly regulated process. The correlation between an action potential, the release of neurotransmitter and the postsynaptic response is finely modulated by a plethora of intracellular and extracellular mediators, and is also altered by a repeated use of the synapse, a phenomenon named synaptic plasticity (Sudhof, 2004).

Glia (astrocytes, oligodendrocytes and microglial cells) play an important role in the regulation of neurotransmission. For many years considered as mere structural elements, glial cells are in fact capable of sensing signals and elaborating complex responses, exerting many biological functions, including the one of modulating synaptic transmission (Verderio and Matteoli, 2011). Astrocytes surround closely the synapses maintaining, supporting and modulating neuronal activity. Oligodendrocytes bind neuronal axons forming the myelin sheath (Verderio and Matteoli, 2011). Microglial cells are myeloid cells considered the macrophages of the brain. Beyond their immunological functions, there are growing evidence supporting new physiological roles for microglia and it is known that they release factors which alter synaptic activity (Kettenmann et al, 2013). [for a more detailed description of microglial functions see “Microglia” section]

2. The endocannabinoid system

The mediators involved in the regulation of synaptic transmission are countless. Among these are the endocannabinoids (eCBs) (Castillo et al, 2012): fatty acid metabolites of arachidonoyl lipids (Stanton et al, 2005).

eCBs are lipid mediators which play many different roles in neuromodulation. They primarily act as retrograde messengers: produced by neurons on demand, in response to high synaptic activity, eCBs move to presynaptic membrane where they activate their specific receptors, called CB_1 receptors, to inhibit neurotransmitter release (Ohno-Shosaku et al, 2001; Wilson and Nicoll, 2001; Katona et al, 2012) (Figure II). Retrograde eCB signaling happens at both excitatory and inhibitory synapses and it's responsible for different forms of short- and long-term plasticity (reviewed in Benarroch, 2014; Katona and Freund, 2012; Castillo et al, 2012). The typical purpose of retrograde signaling is to control over-excitability and preserving synaptic homeostasis. Furthermore eCBs are very important for correct neurogenesis, synaptogenesis and circuit formation (Harkany et al, 2008; Berghuis et al, 2007).

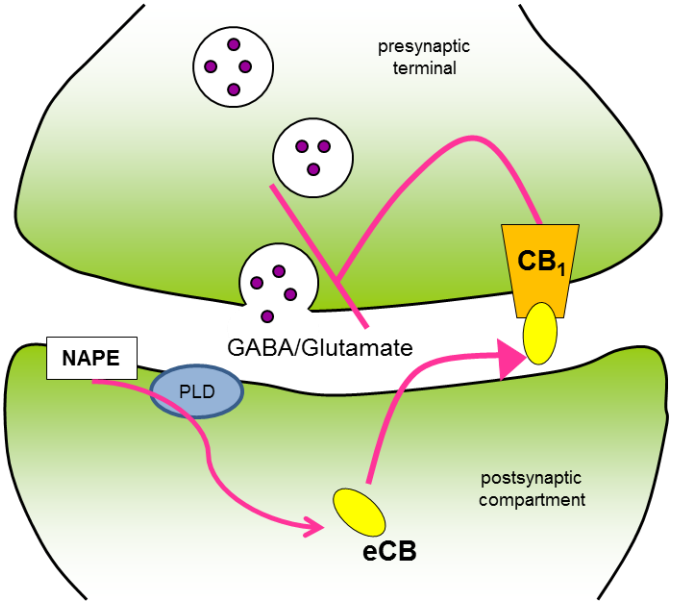


Figure II Retrograde signaling mediated by eCBs. eCBs are synthetized mainly in the postsynaptic compartment of neurons on demand and move to the presynaptic terminal to activate CB_1 receptors, responsible of inhibiting neurotransmitter release. NAPE: N-arachidonoylphosphatidilethanolamine; PLD: phospholipase D; CB_1: type I endocannabinoid receptor.

CBs are produced not only by neurons, but by astrocytes and microglial cells too (Carrier et al, 2004; Stella, 2010; Walter et al, 2002). During inflammation, primary microglia release even 20-fold the amount of eCBs compared to neuron and astrocytes, suggesting that microglial cells may represent the major producers of eCBs under inflammatory conditions (Stella, 2009). These molecules also mediate glia-neurons interaction, being involved in neuromodulation, regulation of inflammatory responses and neuroprotection (Benarroch, 2014; Navarrete and Araque, 2008).

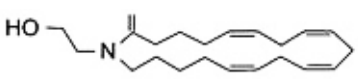
eCB signaling is involved in many different brain networks, affecting several brain functions such as learning, memory, cognition, emotion, addiction, pain perception, motor control, feeding behaviors (Benarroch, 2014).

eCB synthesis and metabolism

In neurons, eCBs are synthetized from membrane phospholipids of neurons, in response to synaptic firing: the trigger is the increase in intracellular Ca^{++} levels, due to depolarization-induced opening of voltage-gated Ca^{++}-channels, or the activation of $G_{q/11}$ coupled receptor, which in turn activate specific synthetic enzymes. (Benarroch, 2014). Glial cells too, express the enzymes necessary for eCB synthesis (Carrier et al, 2004; Stella, 2010; Walter et al, 2002). As eCBs are produced, they are rapidly released to exert their functions.

N-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG) (Figure III) are the two major eCBs. Both are present in the brain, with 2-AG at low micromolar and AEA at low nanomolar concentrations; AEA levels usually increase under inflammatory conditions (Nicolussi and Gertsch, 2015). They are produced and metabolized by different pathways.

anandamide (AEA)



2-arachidonoylglycerol (2-AG)

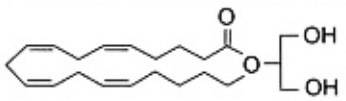


Figure III Chemical structure of N-arachidonoylethanolamine (anandamide, AEA) and 2-arachydonoylglycerol (2-AG)

AEA synthesis may involve different pathways but it's primarily generated by phospholipase D (PLD) starting from N-arachidonoylphosphatidilethanolamine (NAPE), which is synthetized by N-acyltransferase enzyme. After exerting its function, AEA is hydrolyzed in the postsynaptic compartment by the enzyme fatty acid amine hydrolase (FAAH), located inside organelles such as mithocondria. AEA enters organelles thanks to carrier systems.

2-AG mainly derives from phaphatidylinositol-4,5 biphosphate containing arachidonic acid: this lipid is hydrolyzed by phospholipase C β (PLCβ) enzyme in diacylglycerol (DAG), which is in turn metabolized in 2-AG by DAG lipase α. 2-AG is inactivated by monoacyl glycerol lipase (MGL), present in the presynaptic terminal, or by serine hydrolase α-β-hydrolase domain 6 (ABDH-6), located in the postsynaptic compartment. (Benarroch, 2014)

Once degraded, eCBs are incorporated into phospholipids (Nicolussi and Gertsch, 2015).

eCB receptors

Two kinds of eCB specific receptors have been described so far: CB_1 receptors (type I) and CB_2 receptors (type II).

CB_1 receptors typically mediate eCB signaling in the nervous system. They are mainly present presynaptically on GABAergic terminals, and only marginally on glutamatergic and other presynaptic terminals (Kano et al, 2009; Katona and Freund, 2012; Katona et al, 1999). They are expressed in specific nervous system areas, including hippocampus, depending on cell type and developmental stage. eCBs may also activate postsynaptic CB_1 receptors to reduce excitability. Relatively to glial cells, CB_1 receptors are expressed in astrocytes and oligodendrocytes. (Benarroch, 2014)

On the other hand, CB_2 receptors are expressed mainly in immune cells, while their presence in neurons is controversial (Malfitano et al, 2014). Among glial cells, they are expressed in microglia.

AEA functions as agonist also for the transient receptor potential vanilloid type I channel (TRPV1).

(Benarroch, 2014; Katona et al, 2012)

Both CB_1 and CB_2 receptors are G-protein-coupled receptors, coupled to $G_{i/o}$. Thus, when activated, they:

- inhibit adenylyl cyclase (AC), blocking cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) pathway (Gα subunit);
- inhibit presynaptic voltage-gated Ca^{++} channels, included Cav2.1 (P/Q-type) and Cav2.2 (N-type) channels (Gβ/γ subunit);
- activate inward rectifying and A-type potassium (K^+) channels (Gβ/γ subunit)

  (Benarroch, 2014)

PKA is involved in presynaptic LTP and cAMP-PKA pathway activation enhances neurotransmitter release in different brain areas, including hippocampus, acting at both excitatory and inhibitory synapses (Chavez-Noriega and Stevens, 1994; Trudeau et al, 1996; Wang, 2008). Putative targets of this cAMP-PKA-mediated effects are the proteins of neurotransmitter release machinery and their regulators (Wang, 2008).

P/Q- and N-type voltage-gated Ca^{++} channels activation triggers neurotransmitter release (Currie and Fox, 2002).

Inward rectifying K^+ currents are typically postsynaptic and stabilize resting potential, while A-type K^+ currents reestablish resting potential after action potential (Currie and Fox, 2002; Takigawa and Alzheimer, 1999; Ruschenschmidt et al, 2006).

Hence, it's clear how CB_1 receptors activation is related to an inhibition of neurotransmitter release.

Notably, it's known that CB_1 receptor activation induces extracellular signal-regulated kinase (ERK) phosphorylation in growth cones and adult neurons (Berghuis et al, 2007; Derkinderen et al, 2003; Dalton and Howlett, 2012). In the hippocampus, CB_1 receptor-induced ERK activation can be mediated by several different pathways, cAMP-pathway included (Derkinderen et al, 2003). Moreover, this phenomenon is followed by phosphorilated-ERK (P-ERK) accumulation in the nucleus of hippocampal CA1 and CA2 pyramidal cells and by the expression of immediate-early genes product: the protein c-Fos and Zif268 and BDNF mRNAs (Derkinderen et al, 2003).

CB_1 receptors can be homomers or form heterodimers with other receptors, possibly activating different downstream pathways as a consequence of this interaction.

eCB-mediated synaptic plasticity

eCB are responsible for two different types of inhibitory action: phasic and tonic.

The phasic action is an activity-dependent decrease of synaptic strength, while the tonic action consists in a basal and constant inhibitory tone.

The phasic action is the typical eCB retrograde signaling, in which eCBs are released on consequence of postsynaptic depolarization and act on presynaptic CB_1 receptors, mediating short- and long-term depression phenomena both homo- and heterosynaptically (Katona and Freund, 2012).

More specifically, they mediate:

- depolarization-induced suppression of inhibition (DSI): short-term depression of GABA release induced by postsynaptic depolarization (Ohno-Shosaku et al, 2001; Wilson and Nicoll, 2001);
- depolarization-induced suppression of excitation (DSE): short-term depression of glutamate release induced by postsynaptic depolarization (Kreitzer and Regehr, 2001);
- long-term depression (LTP) at both glutamatergic and GABAergic synapses.

Short-term plasticity forms are mediated by P/Q- and N-type Ca^{++} channels inhibition; long-term plasticity is mediated by cAMP-PKA pathway depression.

(Benarroch, 2014)

Tonic eCB signaling involve again CB_1 receptors and control basal neurotransmitter release probability, thanks to a constitutive release of eCBs from postsynaptic compartment (Benarroch, 2014; Katona and Freund, 2012). For example, in the hippocampus, a reduction in eCB inhibitory tone induces an increase in baseline synaptic transmission (Kim and Alger, 2010).

2-AG usually works in phasic signaling, while both AEA and 2-AG work in tonic signaling at GABAergic synapses in the hippocampus (Katona and Freund, 2012). Interestingly, an increase in 2-AG levels determines downregulation of CB_1

receptors, while this doesn't happen upon AEA level increase, which in contrast grant a sustained agonistic activity (Benarroch, 2014).

eCBs also act on postsynaptic receptors: CB_1 and TRPV1. Postsynaptic CB_1 receptors mediate postsynaptic K^+ channels activation, inducing hyperpolarization and inhibiting neuronal excitability. TRPV1 is activated by AEA and mediates postsynaptic LTD at excitatory synapses, while the effects at inhibitory synapses remain unknown (Benarroch, 2014).

CB_1 receptor-mediated effects in the hippocampus

As in most brain areas, in rodent hippocampus CB_1 receptors are mainly present presynaptically on a subpopulation of GABAergic terminals (i.e. cholecystokinin-positive cells), rather than on glutamatergic synapses (Tsou et al, 1999; Katona et al, 1999; Hajos et al, 2000; Hajos and Freund, 2002; Irving et al, 2000). Interestingly, the same results were found in human hippocampus (Katona et al, 2000).

Many major researches concerning CB_1 receptor-mediated eCB signaling have been performed in the hippocampus (Hajos and Freund, 2002). DSI, DSE and LTD mediated by CB_1 receptor has been reported in hippocampus using a specific electrophysiological protocol (Ohno-Shosaku et al, 2001; Wilson and Nicoll, 2001).

Other works along the years have revealed that the administration of the CB_1 receptor agonist WIN55-212,2 was able to suppress i) [H^3]GABA release evoked by electrical field stimulation (Katona t al, 1999); ii) evoked inhibitory postsynaptic current (eIPSC) amplitude; iii) spontaneous inhibitory postsynaptic current (sIPSC) frequency and amplitude in rodent hippocampal slices (Hoffman and Lupica, 2000). In hippocampal cultures, the same agonist was able to inhibit miniature inhibitory postsynaptic currents (mIPSCs) frequency with no alteration in their amplitude (Irving et al, 2000). The involvement of CB_1 receptor in these effects was proved by the ability of the CB_1 receptor antagonist SR141716A to totally abolish them (Katona et al, 1999; Hoffman and Lupica, 2000; Irving et al, 2000).

Some groups reported some of these WIN55-212,2-mediated effects at excitatory synapses too: for example, WIN55-212,2 is shown to suppress evoked excitatory postsynaptic currents (eEPSCs) in both cultures (Ohno-Shosaku et al,

2002; Sullivan, 1999) and slices (Takahashi and Castillo, 2006) and Dr J. M. Sullivan found, in autaptic hippocampal neurons in culture, that WIN55-212,2 decreases glutamate release probability and depresses mEPSC frequency (Sullivan, 1999), though the effect was little and very less prominent than the ones found by Dr I. J. Irving and colleagues at inhibitory terminals. However, the studies on CB_1 receptor-mediated effects on excitatory synapses are very controversial: '*in hippocampus, varying results have been obtained on the extent and site of cannabinoid actions on excitatory transmission, ranging from no effect to complete obliteration of synaptic responses'* (Bajo et al, 2009).

Notably, CB_1 receptor-mediated effects seem to differ depending on neuronal subtype and pathway: e.g. presynaptic inhibition of both GABA and glutamate release mediated by CB_1 receptors happens in fibers connected to CA1 pyramidal neurons, while GABA but not glutamate release is inhibited when the postsynaptic neuron is an interneuron (Hoffman et al, 2003).

eCB signaling and glial cells

Glial cells are central players in neuromodulation. They are involved in eCB signaling and they contain the synthetic machinery for eCB production (Castillo et al, 2012). Astrocytes and microglia are demonstrated to produce both 2-AG and AEA with microglia releasing high amounts during inflammation (Stella, 2009).

ATP administration stimulates 2-AG production in both astrocytes (Walter et al, 2004) and, through $P2X_7$ activation, in microglial cells (Witting et al, 2004). On the other hand, glial cells present functional CB1 and CB2 receptors, which regulate inflammation and play neuroprotective roles (Benarroch, 2014).

A *proof-of-principle* of the involvement of glial cells in eCB signaling to synapses has been provided by the fact that astrocytes, in response to neuronal eCBs and through activation of astrocytic CB_1 receptors, release glutamate, which in turn acts on synapses (Castillo et al, 2012). Astroglial CB_1 receptors control synaptic transmission and plasticity through different mechanisms (Oliveira da Cruz et al, 2015; Navarrete et al, 2014).

Also, microglial cells express functional CB_1 and CB_2 receptors (Stella, 2009). Microglial CB_2 receptor seem to mediate the acquisition of an alternatively activated, anti-inflammatory phenotype by microglial cells (Mecha et al, 2015). It's known that microglial cells can modulate synaptic activity through the release of neuromodulating factors (e.g. BDNF, TNFα) (Kettenmann et al, 2013). However it

has not been clarified whether eCBs produced by microglia can affect synaptic activity (Castillo et al, 2012). (for a more detailed description of microglial functions see “Microglia” section)

eCBs in pathology

In many pathological conditions, high glutamatergic firing induces eCB release, which may exert neuroprotective functions. This happens for example in neurodegenerative diseases and in traumatic or ischemic brain injuries, thanks to the inhibitory effects on glutamate release and the anti-inflammatory functions of eCBs. On the other hand, eCB system may play roles in the pathophysiology of several diseases, such as seizures, spasticity and movement disorders, pain and psychiatric disorders. (Benarroch, 2014)

For example, in some acute models of epilepsy, eCBs play neuroprotective and antiepileptic roles, while in other cases they increase seizure susceptibility. (Benarroch, 2014)

Alterations in eCB system have been found also in experimental models characterized by movement disorders. In Parkinson’s disease (PD), for example, eCB signaling seems to be detrimental for motor symptoms, while its stimulation reduces levodopa-induced dyskinesia. Altered eCB system may participate also in Hungtington’s disease (HD) pathogenesis, but in this case eCB signaling defect aggravates molecular, neuropathological and motor abnormalities. The capability of eCBs to reduce motor activity suggests that cannabinoids may be employed in the cure of hyperkinetic disorders, such as levodopa-induced dyskinesia or HD. Further studies will be necessary for better evaluating the possible use of cannabinoids in the therapy for these diseases. (Benarroch, 2014)

Recent findings point at the endocannabinoid system as a novel potential therapeutic target for Alzheimer’s disease (AD). Indeed, eCBs seem to be involved in AD pathogenesis. 2-AG signaling is upregulated, while AEA signaling is downregulated in the vicinity of Aβ plaques, and overall eCB signaling is likely decreased in AD. Although the mechanisms undergoing eCB roles in AD pathogenesis are poorly understood, cannabinoid treatment in AD seems to be beneficial. eCBs could in fact play anti-inflammatory and neuroprotective roles (e.g. reducing excitotoxicity), reduce Aβ plaques and intracellular neurofibrillary tangles formation and ameliorate learning and memory deficits (Bedse et al, 2015).

Again, there is growing evidence showing the involvement of eCB signaling in multiple sclerosis (MS), in both the neurodegenerative and the inflammatory part of the disease. eCB release is stimulated in active MS lesions. CB_1 receptors seem to be manly involved in neuroprotection, by inhibiting '*the synaptic mechanisms at the basis of the neurodegenerative damage*' (i.e. glutamate excitotoxicity; Rossi et al, 2011), while CB_2 has essentially anti-inflammatory functions (Rossi et al, 2010). Studies performed on the experimental model of MS (experimental autoimmune encephalomyelitis (EAE)) indicated that eCBs are able to restrain TNFα-induced excitotoxicity, and proposed it as a plausible mechanism for eCB-mediate modulation of neurodegenerative damage in MS (Rossi et al, 2011). Furthermore, eCBs have been reported to reduce spasticity in MS, by inhibiting excitatory wiring and inflammation in the spinal cord, and to effectively reduce central pain and painful spasms typical of this disease (Benarroch, 2014).

Accordingly, upon spinal cord and nerve injury as well as in neuropathic pain models, eCB signaling is upregulated and this seems to have an antinociceptive effects. (Benarroch, 2014)

Relative to psychiatric disorders, dysfunctions of the eCB system may participate to the pathogenesis of anxiety-related disorders and depression, while pharmacological enhancement of eCB signaling represent a promising therapy for the treatment of such disorders. Moreover, eCB neuromodulatory effects may play crucial roles in the psychotic-related behaviors observed in animal models of schizophrenia (e.g. social and cognitive deficits, altered emotionality). Cannabidiol, a non-psychotropic phytocannabinoid, is currently one of the best candidates for the therapy of many diseases, included neuropsychiatric disorders. (Ghosh and Basu, 2015)

These were just some examples of eCB roles in pathology, given their involvement in many brain functions, from memory and cognition to emotion, from pain perception to motor control, from feeding behaviors to addiction.

The effects of exogenous cannabinoid assumption (i.e. cannabis) have not been discussed here, as they represent a specific and separate subject.

3. Endocannabinoid transport

eCBs and membranes

Thanks to their lipidic nature, eCBs present high affinity for membranes. They are produced at the inner leaflet of cell membrane (Nicolussi and Gertsch, 2015). 2-AG is mainly associated with lipid rafts, highly specified domains rich in cholesterols where several proteins and receptors are located, while AEA can be associated with lipid rafts or not. This has been demonstrated, for example, in BV2 microglial cell line (Rimmerman et al, 2012; Fowler, 2013). Among membrane constituents, cholesterol seems to be very important for AEA uptake and translocation through bilayer membrane (Fowler, 2013).

eCB transport across the membranes and inside the cells

eCBs are released in the extracellular space and move in this hydrophilic environment to reach their target. A challenging and unsolved question is how highly hydrophobic fatty acid derivatives, such as eCBs, can cross cell membrane and move in the extracellular space (Stanton et al, 2005). Indeed, given AEA or 2-AG chemical properties, they *'would be unlikely to enter a hydrophilic solution in appreciable quantities unless the concentration gradient was huge'* (Stanton et al, 2005).

eCB uptake studies suggest that eCBs trespass cell membrane through facilitated (independent on either ATP or ion gradients) diffusion, in both directions. And studies on pharmacological block of AEA uptake, together with experiments on FAAH knock-out mice, suggest the existence of an 'AEA membrane transporter' (AMT) protein, able to specifically remove AEA from the extracellular space and relocate it within the cell (Nicolussi and Gertsch, 2015). However, the discovery that the inhibitors of AEA uptake was actually not selective, acting also on other intracellular AEA carrier and degrading enzymes, together with additional issues (e.g. researcher biases in these studies), motivated scientists to question the existence of an AMT (Nicolussi and Gertsch, 2015). An AMT protein has effectively not been cloned to date.

Dr C. J. Fowler claimed that data collected so far are consistent also with a simpler model, which doesn't reckon on the binding to a membrane carrier, and must be taken into account (Fowler, 2013). On the other hand, Nicolussi and

colleagues reported new findings consistent with the existence of an AMT, declaring that the molecular identification of the putative AMT would be probably just a matter of time (Nicolussi and Gertsch, 2015).

To date, different models concerning the mechanism of transport of eCBs across the cell membrane coexist. The subject is clearly matter of big debate (Nicolussi and Gertsch, 2015; Fowler, 2013).

Once inside the cell, AEA is sequestered by intracellular binding proteins and receptors or by catabolic enzymes (Fowler, 2013; Maccarrone et al, 2010). Many intracellular 'carrier' protein that mediate the transfer of AEA from cell membrane to intracellular target, have been identified: fatty-acid-binding proteins (FABP)-5 and -7, heat-shock protein 70 and albumin (Fowler, 2013; Maccarrone et al, 2010). FAAH-like AEA transporter (FLAT) has been proposed as AEA carrier as well (Fu et al, 2012), but its role as AEA transporter has been recently questioned. Indeed, new studies clarified that this protein actually never contacts plasma membrane, while it localizes primarily on intracellular membranes. Moreover, it seems that FLAT's role likely involves its enzymatic function rather than its carrier role (Leung et al, 2013).

A crucial player in AEA concentration gradient maintenance is the AEA degrading enzyme FAAH. AEA uptake and FAAH activity are indeed *'intrinsically coupled processes'*: FAAH degradation rate greatly affects AEA uptake constants in most cell types (Nicolussi and Gertsch, 2015).

Additionally, it has been recently demonstrated that AEA concentration gradient can be maintained also by lipid droplets (adiposomes), which sequester and metabolize AEA (Oddi et al, 2008; Kaczocha et al, 2010; Fowler, 2013; Maccarrone et al, 2010).

Starting from these knowledge, Nicolussi and Gertsch, 2015 listed the following models:

1. *Passive diffusion driven by FAAH activity*: FAAH degrades AEA thus creating a concentration gradient which favors AEA passive diffusion throughout plasma membrane. In this model, FAAH activity '*is the major driving force for AEA diffusion'*.
2. *Passive diffusion driven by carrier-mediated intracellular transport and sequestration*: an updated 'model 1' in which the driving force generated by

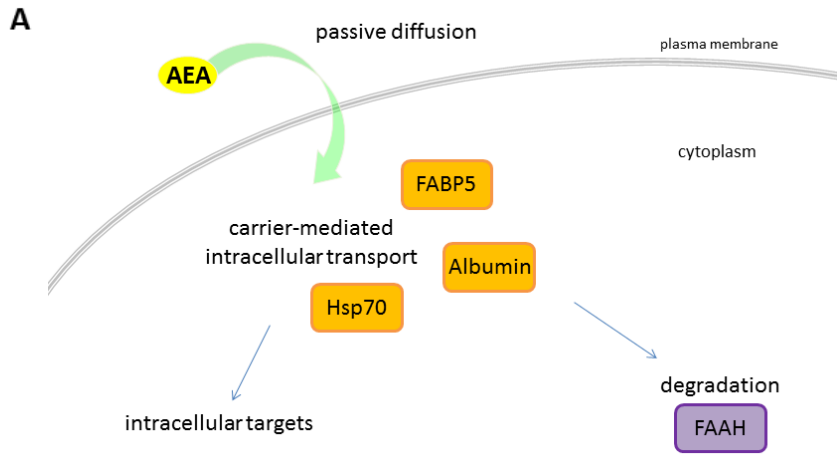
intracellular carrier activity is taken into account (Fowler, 2013) (Figure IVA).

3. *'Facilitated diffusion and carrier-mediated intracellular transport of AEA': 'AEA associates with the plasma membrane and accumulates in certain domains where it binds to a specific high-affinity membrane transporter protein* (AMT) *that facilitates translocation of its substrate rapidly toward the inner leaflet of the lipid bilayer'* (Nicolussi and Gertsch, 2015); once in the cytosol, AEA binds to intracellular carriers which drive them to targets or degrading enzymes (Figure IVB)
4. '*Endocytosis-mediated AEA uptake and intracellular sequestration'*: AEA binds to a carrier protein located within plasma membrane *lipids rafts* and it is internalized through caveolae-dependent endocytosis (Figure IVC) (Nicolussi and Gertsch, 2015)

Different routes can likely be used by cells for eCB uptake, for example '*depending upon whether they employ eCBs as signaling molecules or as a source of arachidonic acid'* (Fowler, 2013).

As you can see, most of the studies to date concern AEA rather than 2-AG cellular transport. One reason is the fact that 2-AG is more rapidly degraded than AEA and thus more difficult to be quantified (Nicolussi and Gertsch, 2015). However, according to literature, 2-AG cellular uptake seems to be very similar to the one of AEA (Nicolussi and Gertsch, 2015).

Figure IV Models of AEA transport through the plasma membrane: A. Passive diffusion driven by carrier-mediated intracellular transport and sequestration model; B. Facilitated diffusion and carrier-mediated intracellular transport of AEA; C. Endocytosis-mediated AEA uptake and intracellular sequestration. →


A
passive diffusion
plasma membrane
AEA
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FABP5
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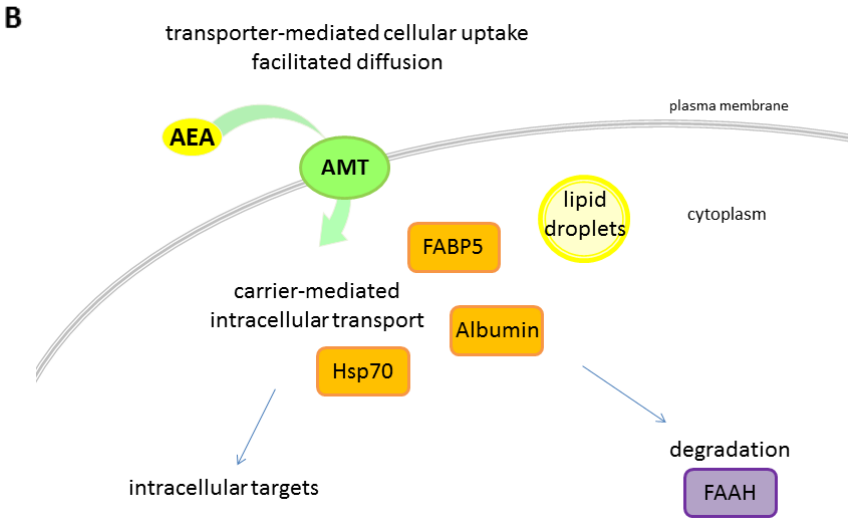
Albumin
Hsp70
degradation
intracellular targets
FAAH


B
transporter-mediated cellular uptake
facilitated diffusion
plasma membrane
AEA
AMT
lipid
droplets
cytoplasm
FABP5
carrier-mediated
intracellular transport
Albumin
Hsp70
degradation
intracellular targets
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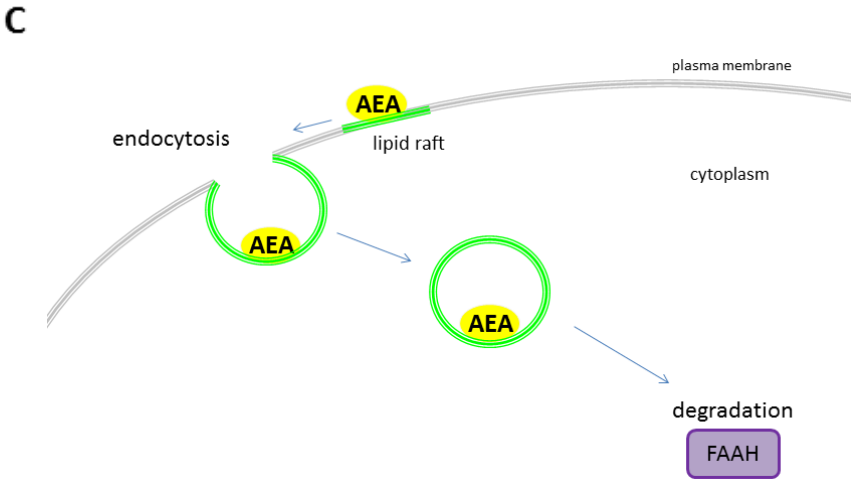
C
plasma membrane
AEA
endocytosis
lipid raft
cytoplasm
AEA
AEA
degradation
FAAH

eCB release and extracellular trafficking

Little is known about eCB release. We don't know whether it has to be considered as an uptake process in reverse or whether involves a separate route (Fowler, 2013), even if there are evidence strongly supporting the first hypothesis (Nicolussi and Gertsch, 2015).

Extracellular eCB carriers have been proposed to be responsible of removing eCB molecules from donor cell membrane and facilitating their interaction with specific receptors on target cell membrane. These are probably circulating binding proteins, with albumin and lipocalins as putative molecules. Indeed, albumin is known to work as a carrier for fatty acids moving from one cell to the other in the blood and it has been demonstrated that this protein is very effective in delivering eCB-based drugs *in vitro* (Stanton et al, 2005). Lipocalins are a family of proteins capable of binding small hydrophobic molecules, interacting with specific plasma membrane receptors and creating complexes with soluble macromolecules (Flower, 1996). However, to my knowledge, a fatty acid binding protein specific for eCB has not been identified yet.

Another hypothesis that has been proposed through the years is that eCBs might bind transsynaptic proteins, such as Eph-Ephrin or neurexin-neuroligin pairs in their hydrophobic domains to reach the other side of the synapse, or that this may happen by a process similar to trans-endocytosis (Stanton et al, 2005).

4. Extracellular vesicles (EVs)

In my laboratory, we are studying extracellular vesicles (EVs): a newly emerged and very important mechanism of cell-to-cell communication (Cocucci and Meldolesi, 2015; Raposo and Stoorvogel, 2013). Often visualized by electron microscopy, EVs had been long considered as mere cellular *debris*. However, we now know they play crucial roles in intercellular cross-talk in prokaryotes till plants and higher eukaryotes (Yanez-Mo et al, 2015; Gyorgy et al, 2011; Cocucci and Meldolesi, 2015).

EVs are membrane structures capable of collecting all sorts of bioactive molecules (protein, nucleic acids, lipids) from a donor cell and delivering them to specific target cells (Figure V). Recipient cells may be in the vicinity or even far away from their origin (Yanez-Mo et al, 2015); indeed, membrane shell of EVs preserve their contents from degradation (Cocucci and Meldolesi, 2015). EVs are released by virtually every cell type and their components, and the signals transmitted, reflect the nature and activation state of the donor cell (Cocucci et al, 2009; Fruhbeis et al, 2013).

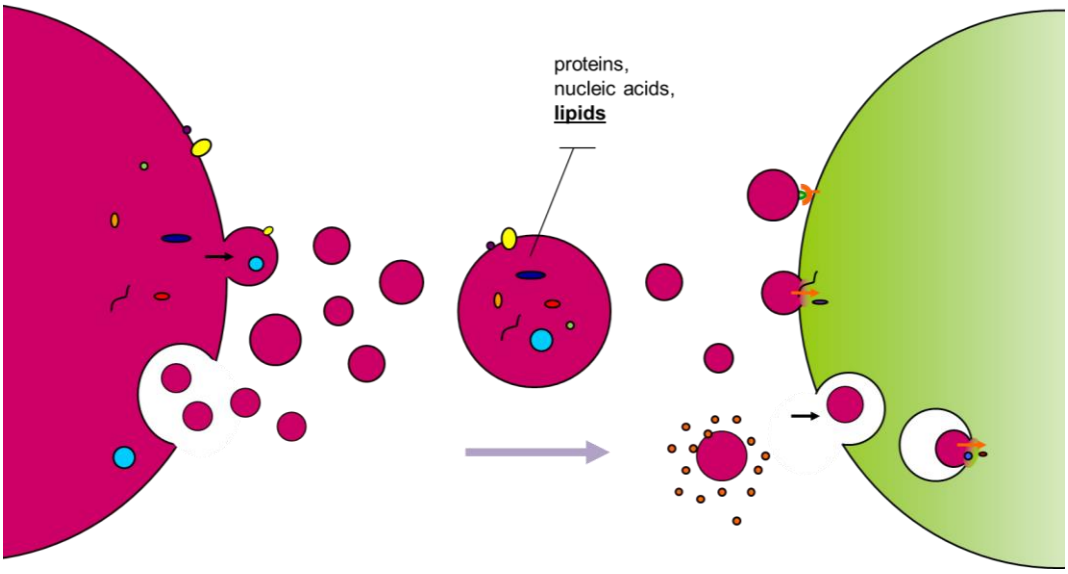

Figure V **EVs** are membrane structures capable of collecting all sorts of bioactive molecules (protein, nucleic acids, lipids) from a donor cell and delivering them to specific target cells

EVs have been found to be secreted also *in vivo* (Raposo and Stoorvogel, 2013; Verderio et al, 2012); and they have been detected in several body fluids, including cerebrospinal fluid (Verderio et al, 2012), blood, urine, faeces, saliva, seminal fluid, breast milk, amniotic fluid, synovial fluid, broncho alveolar fluid, nasal secretion, ascites fluid and bile (Raposo and Stoorvogel, 2013; Yanez-Mo et al, 2015).

Hence, no wonder that they are reported to influence several physiological and pathological processes (Yanez-Mo et al, 2015), such as neuromodulation, inflammation, coagulation, angiogenesis, tumor progression and others, yet depending on the nature and state of parental cells (Antonucci et al, 2012; Nakano et al, 2015; Rajendran et al, 2014; Smith et al, 2015). For that reason, they have been proposed as prognostic (Ratajczak et al, 2006) and diagnostic tools (e.g. markers of inflammation, Colombo et al, 2012; Verderio et al, 2012), therapeutic agents or target agents (Thery et al, 2009; Verderio et al, 2012), and as drug delivery systems (Ratajczak et al, 2006), thanks to their dimensions and ability to access several biological fluids (Gyorgy et al, 2011).

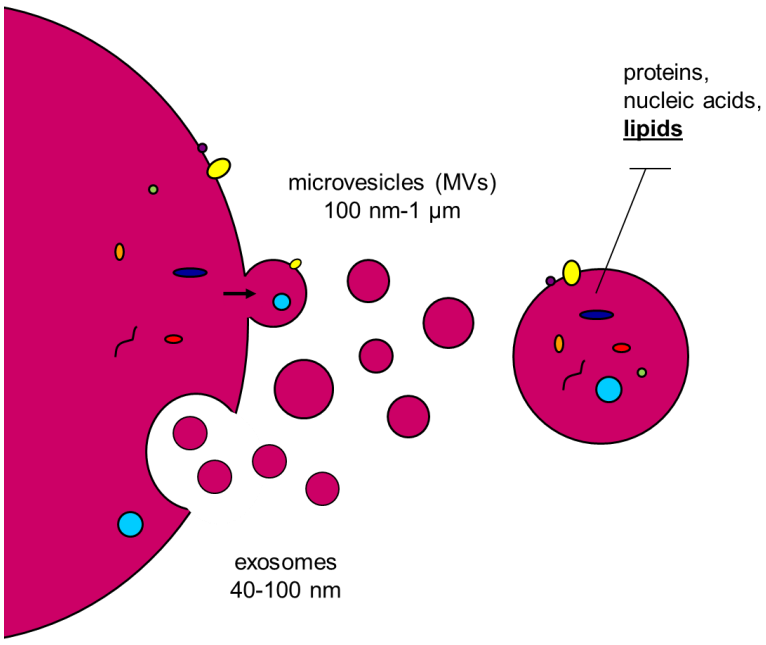

Figure VI MV and exosome origin. MVs bud directly from the plasma membrane through a process called shedding. Conversely, exosomes originate inside multivesicular bodies.

Two kinds of EVs have been described so far: microvesicles (MVs) and exosomes. MVs shed directly from the plasma membrane. They are more heterogeneous and larger then exosomes, displaying a diameter of 100 nm to 1 μm (Cocucci and Meldolesi, 2011; Mause and Weber, 2010; Raposo and Stoorvogel, 2013). Exosomes, on the other hand, originate inside multivesicular bodies and are released after fusion of multivesicular bodies themselves with the plasma membrane. They are more homogeneous and smaller compared to MVs, ranging from 40 to 100 nm in diameter (Mause and Weber, 2010; Raposo and Stoorvogel, 2013; Simons and Raposo, 2009). (Figure VI) Exosomes are constitutively released, while MV secretion undergoes a drastic increase after stimulation. The two populations have distinct characteristics and may exert distinct functions, in spite of several overlapping roles (Gabrielli and Verderio, *in print*; Cocucci and Meldolesi, 2015).

Biogenesis and sorting

Recent studies enlightened the role of proteins and membrane lipids in the budding and pinching off of EVs. For example, tetraspanins, endosomal sorting complex responsible for transport (ESCRT) complexes (with a crucial role for the ESCRT-I subunit Tsg101 and for ESCRT-III) and their accessory proteins (e.g. Alix), the membrane lipid ceramide and its synthetic enzyme sphingomyelinase, other integral proteins and glycoproteins have been reported to be involved in EV biogenesis (Raposo and Stoorvogel, 2013). EV pinching off is likely triggered by raising of intracellular Ca^{++} concentrations and requires cytoskeleton remodeling, involving microtubules, actin and the molecular motors myosins and kinesins, together with small GTPase protein (i.e. Rab11, Rab27 and Rab35) and a SNARE (Soluble NSF Attachment protein REceptors) fusion machinery (Raposo and Stoorvogel, 2013). On the other hand, we know from literature that stimulated release of MVs happens, at least in some cell types (i.e. microglia, macrophages and dendritic cells), in response to adenosine-5'-triphosphate (ATP)-dependent activation of the $P2X_7$ purine receptor (Bianco et al, 2005; Cocucci and Meldolesi, 2015). However, the mechanisms underlying these processes seem to be complex and remain largely unclear (Raposo and Stoorvogel, 2013).

The same applies to the sorting process, by which specific molecules segregate inside EVs. During vesicle assembly, proteins, lipids, various RNAs (mRNAs, siRNAs, long non coding RNAs, miRNAs), and all the molecules which

will be included in EVs, gather in the vicinity of the budding area, into small microdomains, through a not yet completely defined process. (Raposo and Stoorvogel, 2013; Cocucci and Meldolesi, 2015)

What we know is that cholesterol, sphingomyelin and its metabolite ceramide are usually sorted in both types of EVs. This is not the case for other lipids and membrane proteins, which are specific of MVs or exosomes or present only on EVs derived from certain cell types. Cargo protein segregation happens more for MVs rather than for exosomes. And, in the case of MVs, this process requires ESCRT complexes and the binding of cytoplasmic proteins to the cell membrane thanks to plasma membrane anchors (e.g. palmitoyl, myritroyl residues) or after their polymerization. Other proteins bind these anchored complexes to be sorted as well. (Cocucci and Meldolesi, 2015)

Membrane proteins enriched in microdomains, such as tetraspanins (CD63, CD81, CD82, CD53 and CD37), are usually highly present in EVs, as well as protein associated with lipid rafts, such as flotillin and glycosylphosphatidylinositol-anchored proteins. On the other hand, a pure EV sample will be free of serum proteins and intracellular compartment contaminants, such as mithocondria, endoplasmic reticulum and nucleus components. (Raposo and Stoorvogel, 2013)

Interaction with donor cell

EVs can deliver their signals in different ways (Figure VII). Some of them break down and release soluble molecules in the extracellular space: in this way are release, for example, interleukin-1β (IL-1β) and other tissue and growth factors, including transforming growth factor beta (TGFβ). Other EVs remain intact and can move far away from the releasing point: these are the so called navigating EVs, detected in body fluids.

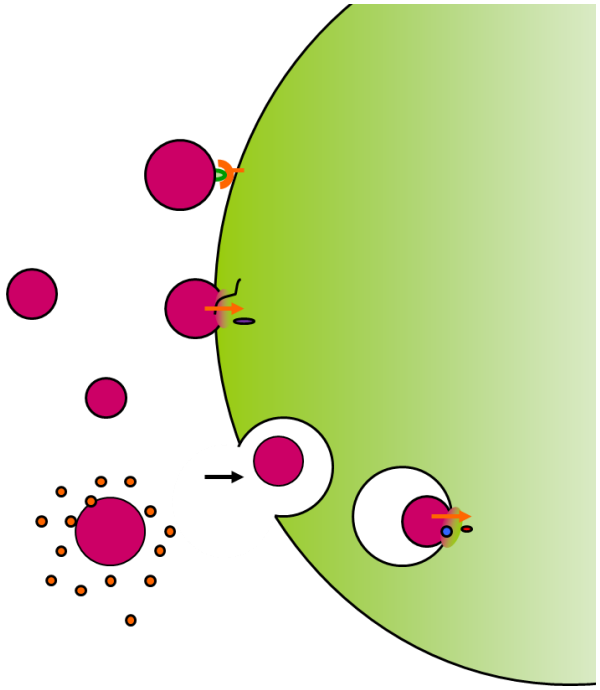

Figure VII EV interact with donor cells in different ways: they can undergo a ligand-receptor interaction with specific membrane molecules present on neuronal surface; they can fuse with the plasma membrane and transfer their cargoes directly inside the cytoplasm of donor cell; or they can be internalized through endocytosis and subsequently fuse with endosomal membrane to release their content in the cytosol. Alternatively, they can break down and release soluble factor in the extracellular space.

EVs can also make contact with donor cells. (Figure VII) The binding to the recipient cell is target-specific and is likely mediated by adhesion molecules (e.g. integrins, ICAM-1, MHC class II) present on EV surface. Tetraspanin complexes and galactins are also involved in target cell specificity (Raposo and Stoorvogel, 2013). The binding of EVs with surface proteins or receptors is usually preceded by the rolling of the vesicle over cell surface (Cocucci and Meldolesi, 2015). After the binding to the target cell, EVs can stay or dissociate. They can fuse with the plasma membrane and transfer their cargoes inside the cytoplasm of donor cells. This is called a direct transfer and happens usually in the case of genetic material and cytoplasmic proteins. In case of fusion, receptors and other molecules present on EV surface are transferred to donor cell membrane too. Finally, EVs can undergo endocytosis, through different mechanisms (e.g. Clatrin-dependent, phagocytosis, macropinocytosis). Internalized EVs can then be released in the cytosol or face lysosomal degradation.

5. Microglial MVs

Within the CNS, neurons (Doeuvre et al, 2009), oligodendrocyte, astrocytes and microglial cells all have been reported to release EVs. According with literature, EVs participate in neurite outgrowth and neuronal survival and in myelin formation (Raposo and Stoorvogel, 2013). Several molecules involved in the pathogenesis of brain diseases, such as prion protein, β-amiloid (Aβ) protein, α-sinuclein, are secreted in association with EVs (Raposo and Stoorvogel, 2013).

In my laboratory we are focusing our studies on EVs released by glial cells.

A few years ago the lab reported how glial cells release MVs upon ATP activation (Bianco et al, 2009; Bianco et al, 2005) (Figure VIII, left). To induce MVs release ATP must activate $P2X_7$ receptors, which respond to high ATP concentration (Farber and Kettenmann, 2006; Lalo et al, 2011) reached usually under inflammatory conditions (Davalos et al, 2005; Fiebich et al, 2014).

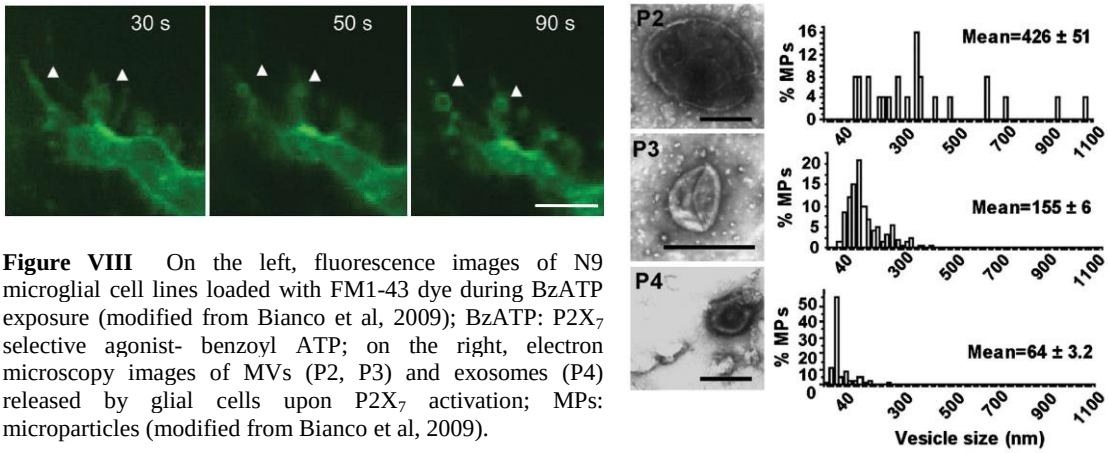

Figure VIII On the left, fluorescence images of N9 microglial cell lines loaded with FM1-43 dye during BzATP exposure (modified from Bianco et al, 2009); BzATP: $P2X_7$ selective agonist- benzoyl ATP; on the right, electron microscopy images of MVs (P2, P3) and exosomes (P4) released by glial cells upon $P2X_7$ activation; MPs: microparticles (modified from Bianco et al, 2009).

Subsequently, microglial MVs have been deeply characterized through several different methods: electron microscopy, fluorescence microscopy, fluorescence activated cell sorting (FACS), western blotting, spectrometric analysis (Bianco et al, 2009; Bianco et al, 2005) (e.g. see Figure VIII, right).

MV release was preceded by repeated protrusion and retraction of membrane blebs and by phospholipid phosphatidylserine (PS) exposure on the outer

leaflet of plasma membrane. Although these are both apoptotic signals, MV release was demonstrated to be independent from programmed death phenomena (Bianco et al, 2009; Bianco et al, 2005). PS residues function is likely to facilitate MVs contact with neuronal surface, through a ligand-receptor interaction (Antonucci et al, 2012) (Figure X).

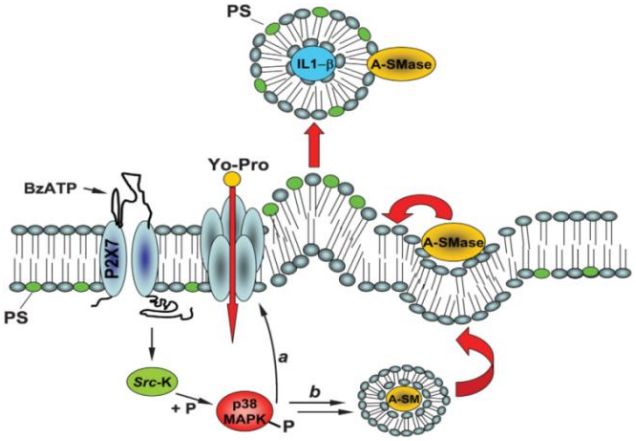

Figure IX Model for MV release mediated by the pathway downstream $P2X_7$ activation in glial cells. See the text for description. BzATP: $P2X_7$ selective agonist- benzoyl ATP; A-SMase, A-SM: acid sphingomyelinase (Bianco et al, 2009)

Subsequent experiments performed in the lab enlightened the molecular mechanisms underlying MV release from glial cells (Figure IX). ATP-$P2X_7$ pathway activation involves Src kinase and induces P38-MAPK phosphorylation. This determines the translocation of the enzyme acid sphingomyelinase (A-SMase) from the lysosomal compartment to the outer membrane leaflet, wherein it facilitates MV budding, thanks to the conical geometry of its product ceramide. The budding of MVs likely happens in the lipid rafts. (Bianco et al, 2009)

Microglial MVs contain the key cytokine of acute inflammation IL1β, which is produced from its precursor, pro-IL1β, inside the vesicles and subsequently released in the extracellular space in a regulated manner (Bianco et al, 2005). Furthermore, ATP-induced MV release was increased in reactive microglia,

exposed to endotoxin LPS or TH1 cytokines, responsible of driving microglia towards an inflammatory phenotype (Verderio et al, 2012).

In vivo existence of microglial MVs was also reported by my laboratory. Indeed, "MVs positive for typical microglial markers were detected by electron microscopy and confocal microscopy in the cerebrospinal fluid (CSF) collected from rodents and humans" (Verderio et al, 2012; Gabrielli and Verderio, in press). Interestingly, higher levels of MVs are concomitant with activated microglia status: MV concentration was found increased in the CSF of mice with experimental autoimmune encephalomyelitis (EAE; mouse model of multiple sclerosis), during symptomatic phases, and in the CSF of multiple sclerosis patients in the acute phase of the disease (Verderio et al, 2012). More importantly, data indicated that MVs released from reactive microglia spread inflammatory signals (such as pro-inflammatory cytokine IL-1β) to other glial cells, thus contributing to neuroinflammation (Verderio et al, 2012; Gabrielli and Verderio, in press).

Again, electrophysiological recordings on cultured rat hippocampal neurons showed that MVs, released from microglia upon ATP activation, are able to induce an increase in glutamate release probability. And we demonstrated that MV-induced potentiation of excitatory transmission was mediated by the sphingolipid Sphingosine (Sph) whose production in neurons is increased by microglial MVs themselves (Antonucci et al, 2012) (Figure X).

Furthermore, a *proof-of-principle* that microglia MVs can acutely alter neurotransmission also *in vivo* came from electrophysiological recordings of the rat visual cortex after injection of microglial MVs (Antonucci et al, 2012). Microglial MVs induced a significant increase in the amplitude of visual evoked potentials (VEPs) evoked by high contrast stimuli, which reflects the sum of excitatory currents induced by sensory stimulation (Porciatti et al, 1999; Restani et al, 2009). In addition, single unit recordings revealed an increase in neuron receptive fields after MV injection, which is typically determined by an alteration of excitatory/inhibitory balance (Benali et al, 2008; Liu et al, 2010). (Gabrielli and Verderio, in press).

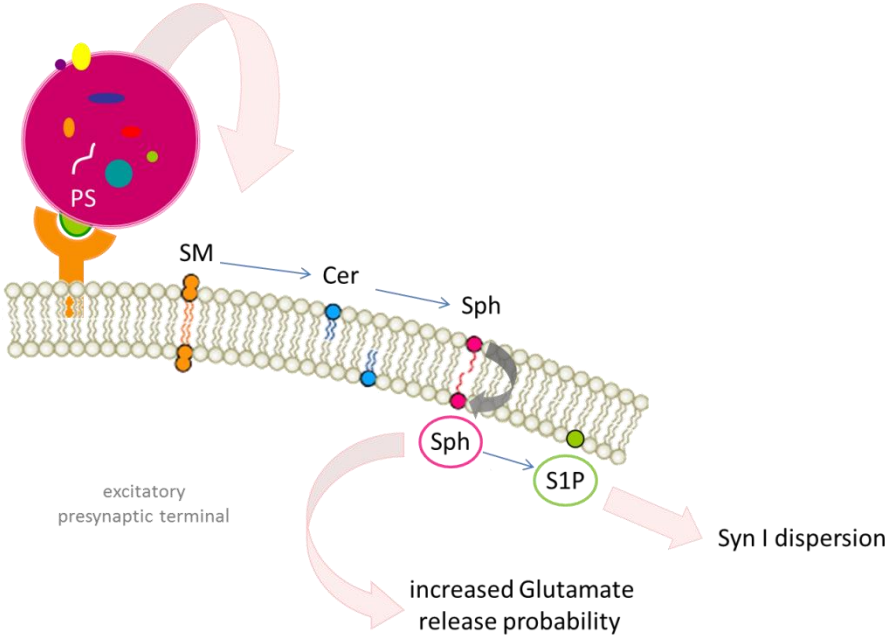

Figure X MV effects on excitatory synapses. Phosphatidilserine (PS) residues exposed on MV surface bind to their specific neuronal receptor, thus facilitating MV interaction with neuronal surface. Subsequently, an unknown lipid present on MV membrane stimulates sphingolipid metabolism in neurons. The sphingolipid sphingosine (Sph) and sphingosine-1-phosphate (S1P) are the effectors, responsible for increasing glutamate release probability and inducing synapsin I (Syn I) dispersion respectively (Antonucci et al, 2012; Riganti et al, *submitted*). SM: sphingomyelin; Cer: ceramide.

Additionally, we showed that the sphingolipid cascade induced by MVs goes up to sphingosine-1-phosphate (S1P), which participates to presynaptic stimulation by inducing dispersion into the cytoplasm of synapsin I (SynI), a presynaptic phosphoprotein that regulates the availability of synaptic vesicles for exocytosis (Riganti et al, *submitted*) (Figure X). Both Sph and S1P increase the number of synaptic vesicles of the readily releasable pool, ready to be exocytosed upon action potential.

Microglia-derived MVs communicate with neurons also through another mechanism: the direct transfer of their cargo. Recent findings from my laboratory indicate that miR-146a is transferred from microglial MVs to neurons, decreasing the expression of synaptotagmin I, a validated miR-146a target (Prada et al, *unpublished*).

Further evidence indicates that microglial MVs participate to degeneration in Alzheimer's disease thanks to i) formation of soluble (more toxic) Aβ species by MV lipids from extracellular insoluble aggregates; ii) the shuttling of neurotoxic Aβ species by MVs produced by Aβ-containing microglia (Joshi et al, 2014).

Again, microglial MVs have been found to promote oligodendrocytes precursors (OPC) differentiation in culture and myelin deposition in OPC-DRG neurons co-coltures (M. Lombardi and C. Verderio, *unpublished data*).

Finally, we took advantage of optical tweezers technique combined to live imaging to drive single microglia-derived EVs on the surface of astrocytes, microglia or neurons in culture and study their behavior (Prada et al, *in print*; Prada et al, *unpublished data*). Through these experiments we confirmed that PS residues, externalized on the outer membrane of MVs, play an important role for MV recognition by target cells. In fact, the cloaking of these residues effectively reduced MV interaction with both glial and neuronal surface (Prada et al, *in print*; Prada et al, *unpublished*). Ongoing optical manipulation experiments on MV interaction with neuronal surface are revealing an unexpected and complex trafficking of MVs outside the surface of neurons, which may underlie the activation of contact-mediated signaling pathways and regulate the transfer of MV cargo in specific neuronal compartments (Prada et al, *unpublished*).

6. Microglia

Microglial cells are the innate immunity cells resident in the brain. They are myeloid cells, similar to macrophages in features and functions. (Kettenmann et al, 2013)

In the normal brain, microglia are in a resting/surveillant status, characterized by a ramified morphology. In response to specific stimuli, they get activated: they assume an amoeboid morphology and specialize for operate in a pathological environment, becoming active phagocytes. (Kettenmann et al, 2013) (Figure XI)

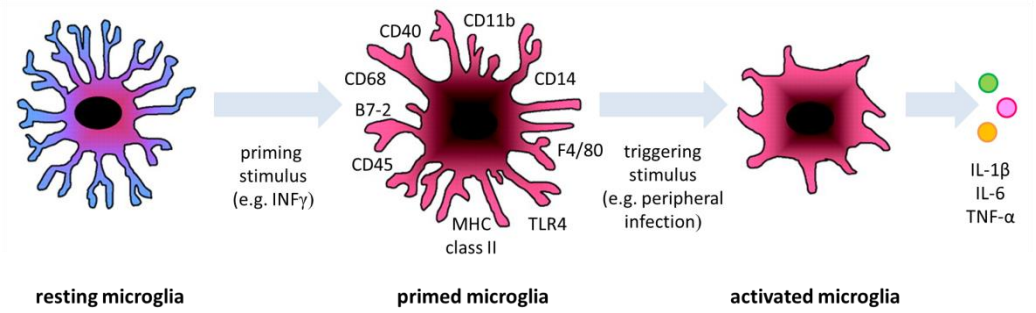

Figure XI Microglial cell activation. Resting microglial cells are stimulated by different stimuli to reach first a primed state, characterized by an increase in the expression of cell-surface markers, and after a triggering stimulus an activates state, characterized by the release of high doses of pro-inflammatory cytokines (modified from Dilger and Johnson, 2008).

Microglia respond to two classes of signals: 'on signals', which push them toward activation, and 'off signals', which turn them away from activation (Biber et al, 2007; Kettenmann et al, 2013). ATP, together with other purines, chemokines and glutamate, are typical 'on signals'. They are usually released from damaged tissues, but also from neurons which are hyperactive in a regulated manner (Biber et al, 2007). On the other hand, a typical 'off signal' is the binding of neuronal surface proteins with their specific microglial receptors, a signal of neuronal integrity (Biber et al, 2007). Notably, microglia sense neuronal activity, thanks to the plethora of receptors for neurotransmitter, and other neuromodulator, which express (Pocock and Kettenmann, 2007).

Microglial cells integrate all the signals they receive, showing an heterogeneous range of activation states: microglia may show several different phenotypes, both pro- and anti-inflammatory, depending on the stimuli they receive, on the kind of disease and on its phase (Kettenmann et al, 2013; Kettenmann et al, 2011; Hanisch and Kettenmann, 2007).

In pathology, microglial cells exert different functions. Acquiring an amoeboid phenotype, they move, following chemotactic gradients, to the site of damage. They release chemotoxic agents to fight pathogens, recall other immune cells and work as antigen presenting cells to T cells. Finally, they clean up from dead cells and *debris*.

All brain pathologies are characterized by microglia activation. However, in some case, microglia activity is neurotoxic and promotes the progression of the pathology, while in others it seems to be beneficial, important for resolution and tissue regeneration. This dualism appears to be due to different activation signals. (Hanisch and Kettenmann, 2007; Kettenmann et al, 2011; Graeber and Streit, 2010; Perry et al, 2010)

Although microglia play central roles in all the inflammatory processes which take place in the CNS, there is growing evidence that these cells play also several important physiological roles (Kettenmann et al, 2013).

During development, 'resting' microglial cells release growing factors which promote synaptogenesis and mediate the controlled elimination of synapses (e.g. synaptic pruning), which is essential for the constitution of a functional neuronal network (Kettenmann et al, 2013; Paolicelli et al, 2011; Paolicelli and Gross, 2011; Zhan et al, 2014).

In the adult brain, microglia release several molecules which affect neurons, such as cytokines, trophic factors, neurotransmitters, even in physiological situations. (Kettenmann et al, 2013) Microglia can affect synaptic plasticity. For example, on consequence to a prolonged change in synaptic activity, they release low doses of tumor necrosis factor alpha (TNFα) which adapt the strength of the synapses in the network to the change (i.e. synaptic scaling) (Stellwagen and Malenka, 2006). Furthermore, microglia regulate the integration of newly generated neurons in the net, contributing to adult neurogenesis, and remove unnecessary and apoptotic neurons. (Kettenmann et al, 2013)

Finally, it has been recently reported that resting microglia, far from being inactive, have very mobile processes able to sense signals from their environment (Davalos et al, 2005; Nimmerjahn et al, 2005). In the normal brain, microglial processes typically target synapses, making several brief but repetitive contact which are likely activity-dependent (Wake et al, 2009; Tremblay et al, 2010; Fontainhas et al, 2011), while Davalos et al, 2005 provided evidence of their ATP-mediated nature (Kettenmann et al, 2013).

AIM OF THE THESIS

Active endocannabinoids (eCBs) are important lipid mediators in the brain. They are secreted by neurons and their main function is to inhibit GABA and glutamate release from presynaptic terminals acting on their specific receptors, called CB_1 receptors. Glial cells produce eCBs too, with microglia likely representing the main source under inflammation. How these highly hydrophobic molecules can cross cell membrane and move in the extracellular space to exert their function was still largely unknown.

On the other hand, in my laboratory we work on extracellular vesicles (EVs) of microglial origin. EVs may represent an ideal vehicle for the transport of hydrophobic mediators in the brain. They are membrane structures which work as shuttles for active molecules, from a donor to a target cell. Two kinds of EVs have been described so far: exosomes, which originate in the endocytic compartment; and microvesicles (MVs), which bud directly from the plasma membrane. Hence, in this thesis we aimed at exploring the possibility that eCBs may be released from microglial cells in association with EVs, thus influencing neurotransmitter release.

RESULTS

EV quality control

Extracellular vesicles (EVs), released in the supernatant of microglial cells after 1h stimulation with 1 mM ATP, were collected through differential centrifugation, after clearing by cell debris, as described in "Materials and Methods". For some of the experiments, a mixed EV population, containing both microvesicles (MVs) and exosomes, and obtained through a 100,000g centrifugation, was used; for other sets of experiments, MV- and exosome-enriched pellets, obtained by centrifuging at 10,000g and 100,000g respectively, were employed.

EV isolation is a crucial step in order to avoid the presence of contaminants (i.e. subcellular organelle components, vesicles produced by damaged cells, etc) in the samples, which may create artifacts. Moreover, EV yield can vary among different cell preparations. Hence, although microglial EVs had been already extensively characterized in the laboratory (see "Introduction"), EV quality control has been carried out routinely. EV production and sample purity have been checked through i) Nanoparticle Tracking Analysis (Figure 1A); ii) quantification of protein content through Bicinchonic acid (BCA) assay (Figure 1B); iii) western blot analysis for the EV markers Tsg101 (Joshi et al, 2014; Nabhan et al, 2012), flotillin and Alix, and for markers of subcellular organelles and lipid droplets (Figure 1C).

Nanoparticle Tracking Analysis of MV- and exosome-enriched pellets reflected data reported in literature (Raposo and Stoorvogel, 2013) (Figure 1A). Tsg101 stained both MV and exosome fractions, flotillin was found enriched in MVs, while Alix stained preferentially exosomes. The absence of immunoreactivity for typical markers for nucleus (SP1), mitochondria (TOM20), Golgi reticulum (GS28) and lipid droplets (Adipophilin; Straub et al, 2013) excluded the presence of contaminants in the samples.

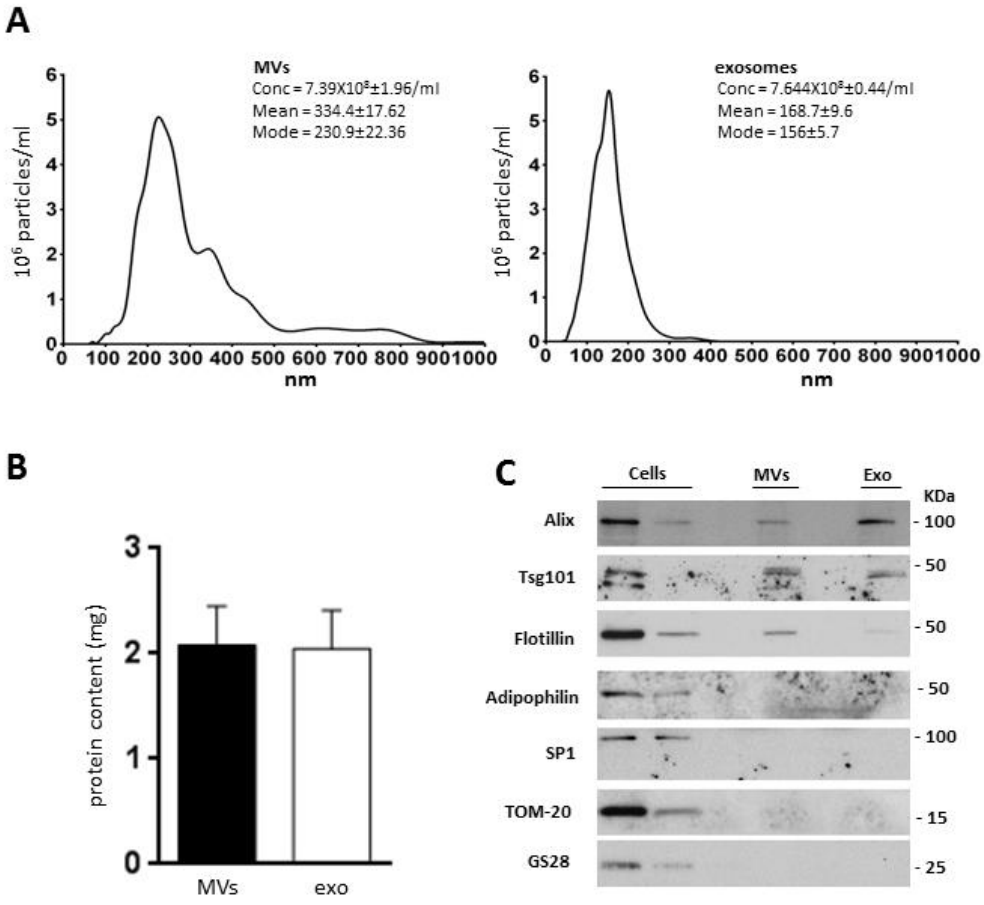

Figure 1 EV quality control

A. Representative plots for particle size distribution of MV- (left) and exosomes- (right) enriched fractions pelleted from 1 × 10^6 primary microglia and resuspended in 400 µl of 0.1-µm-filtered sterile KRH. Normalized EV concentration: MVs = 1 ± 0.05, exosomes = 1.2 ±0.2 (N = 3).

B. Protein quantification of MVs and exosomes produced by 1×10^6 primary microglia (N = 14).

C. Western blot analysis of MV- and exosome-enriched fractions produced from 1×10^7 primary microglia using the specified antibodies. Notably, there was no immunoreactivity for nuclear, mitochondrial and Golgi markers (SP1, TOM-20, GS28 respectively) and for the lipid droplet marker Adipophilin. In the first two lanes lysates from 2×10^5 and 0.4×10^5 cells was loaded.

AEA is enriched in MVs and exosomes released from microglial cells

First aim of the project was to evaluate whether endocannabinoids (eCBs) may be released by microglia in association with EVs. To address this question, eCB content in mixed EV pellets and in MV- and exosome-enriched fractions were measured through mass spectrometry. Detectable amounts of N-arachidonoylethanolamine (anandamide, AEA) were found in mixed EV pellets produced by N9 microglia cell line and primary microglia, as well as in corresponding parental cells; while 2-arachidonoylglycerol (2-AG) levels were not detectable in all the samples tested (the limit of detection of the instrument is 0.2 pmol/mg of protein). Notably, a clear enrichment in AEA was found in EVs compared to donor N9 (~2900-fold enrichment) or primary microglial cells (~120-fold enrichment) (Figure 2A). These data indicate that the eCB AEA is released from microglial cells carried by EVs.

Interestingly, AEA content was higher in MVs compared to exosomes (Figure 2B). On consequence, we focused our attention on MVs, which were used in all subsequent experiments.

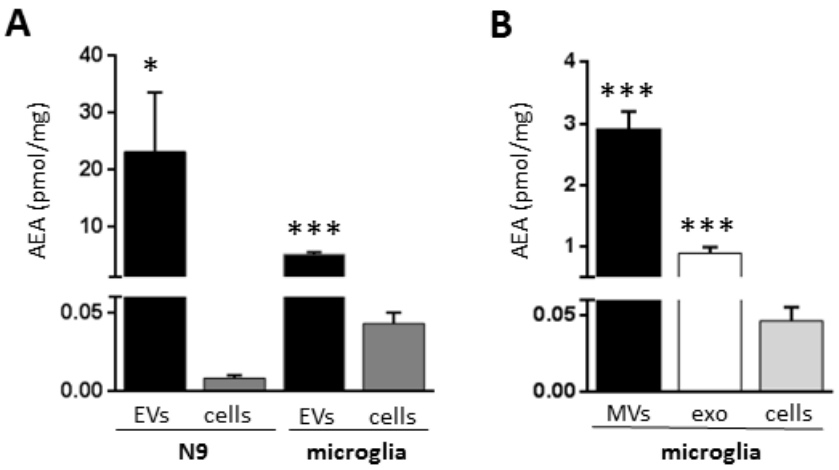

Figure 2 AEA is enriched in MVs and exosomes released from microglial cells

A. AEA content in EVs versus corresponding donor N9 cells (N = 2), or primary rat microglia (N = 4). Student's t-test, $P < 0.05$, $P < 0.001$

B. AEA content in MVs and exosomes versus parental primary microglia (N = 3). One-way ANOVA, $P < 0.0001$, followed by Bonferroni test for comparison among groups, $P < 0.0001$

Hippocampal primary cultures are a suitable system to bioassay eCB activity

A well-known function of AEA is to inhibit GABA release acting on presynaptic CB_1 receptors on inhibitory terminals and electrophysiological recordings are usually used as a readout for AEA activity (Benarroch, 2014; Katona and Freund, 2012). Hence, in order to explore the possibility that eCBs present in MVs may induce a functional response in neurons, patch clamp analysis of spontaneous inhibitory postsynaptic currents (mIPSC) on cultured hippocampal neurons were employed to bioassay vesicular AEA activity.

However, since the majority of the studies in this field were performed on slices, hippocampal primary cultures were first validated as a suitable system to detect AEA activity. According to literature (Tsou et al, 1999; Katona et al, 1999; Hajos et al, 2000; Hajos and Freund, 2002; Irving et al, 2000), CB_1 receptors are expressed in a subpopulation of GABA-ergic interneurons rather than in glutamatergic neurons, as indicated by the triple-stained immunofluorescence analysis showed in Figure 3A-B. Consistent with CB_1 receptor expression pathway and with literature, the acute administration of the CB_1 receptor agonist WIN55,212-2 (1 μM) induced a strong reduction of mIPSC frequency, while wasn't able to induce any major alteration on mEPSCs (miniature excitatory postsynaptic currents) (Figure 4A).

As a further proof of CB_1 receptor functionality in culture, calcium imaging analysis of calcium responses after acute administration of the CB_1 receptor agonist WIN55,212-2 was performed. Indeed, we knew from literature that WIN55,212-2-induced CB_1 receptor activation drives to an increase of intracellular calcium levels (Lauckner et al, 2005). The acute application of WIN55,212-2 (1 μM) to hippocampal cultures induced calcium transients in a subpopulation of neurons (Figure 4B); according to the other evidence.

Thus, we concluded that hippocampal primary cultures maintain several aspects of *in vivo* physiology, included the expression of functional CB_1 receptors on interneuron presynaptic terminals.

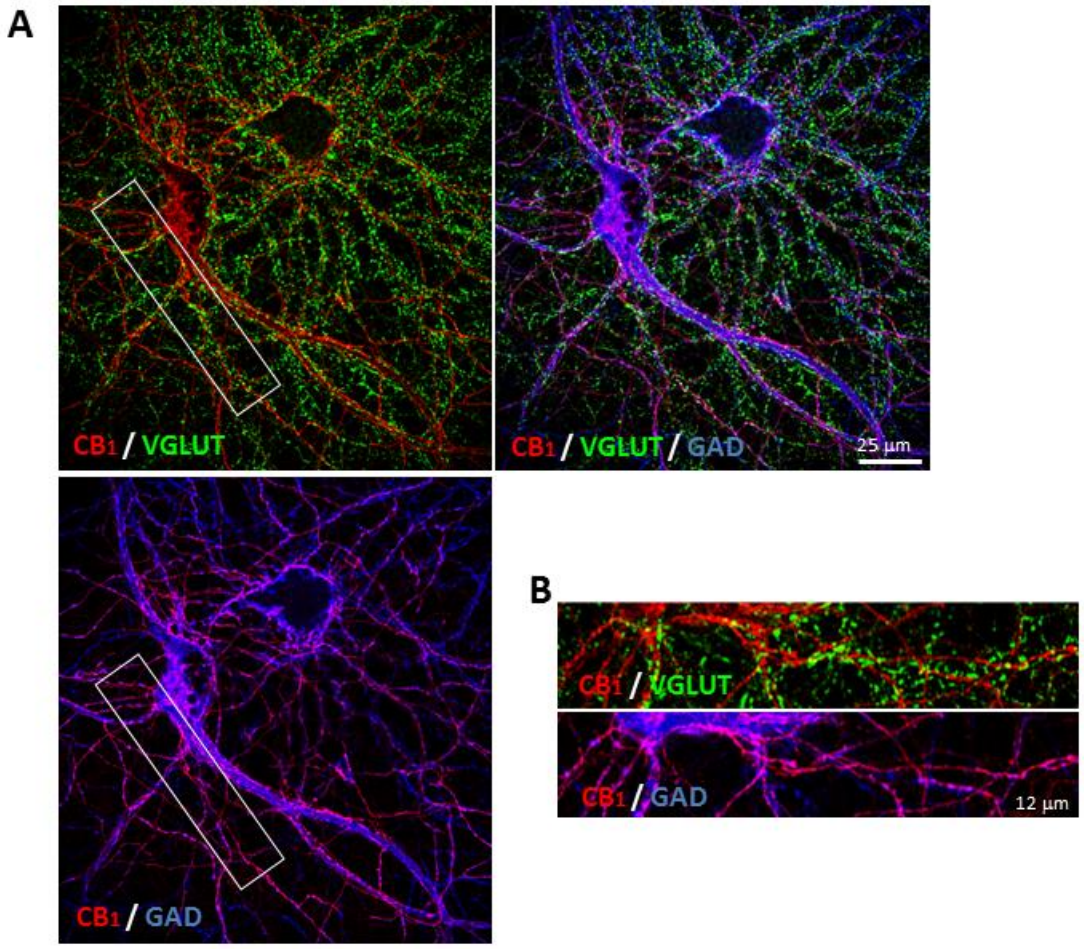

Figure 3 Hippocampal primary cultures express CB_1 receptors on a subpopulation of GABAergic neurons rather than on glutamatergic neurons

A. Immunofluorescence images of 17 DIV hippocampal neurons with CB_1 receptor in red, the glutamatergic marker VGLUT in green and the GABA synthetic enzyme GAD (glutamic acid decarboxylase) in blue. CB_1 receptors colocalize with a subpopulation of GAD-positive neurons rather than with VGLUT-positive neurons; scale bar: 25 µm;

B. Areas selected in A at higher magnification; scale bar: 12 µm

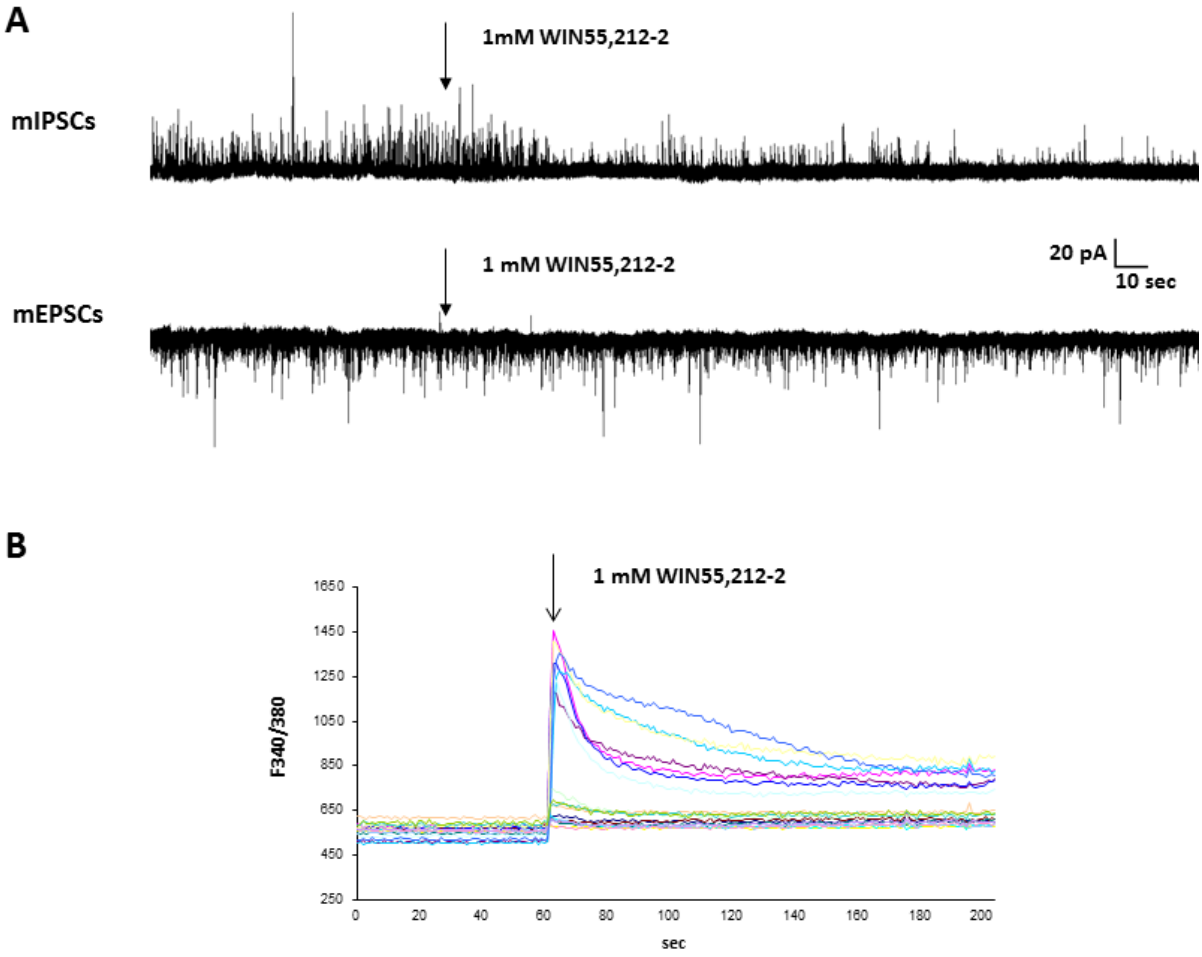

Figure 4 Functional CB_1 receptors are expressed in a subpopulation of GABAergic neurons

A. Effects of CB_1 receptor agonist WIN55,212-2 (arrow) acute administration on mIPSC (top) and mEPSC (bottom): after WIN55,212-2 (1 μM) application a strong reduction of mIPSC frequency was found; while no visible alteration of mEPSC was detected after treatment

B. Representative calcium imaging plot of calcium responses in hippocampal neurons after WIN55,212-2 acute application (arrow)

Microglia MVs inhibit GABA spontaneous release through activation of CB_1 receptors

After validating hippocampal cultures as biosensor for eCB activity, we evaluated whether eCBs present in MVs were able to induce a functional response in neurons in culture taking advantage of patch-clamp technique. Rat hippocampal neurons were exposed for 40-45 min to MVs produced by primary microglial cells (1.2 μg/ml, 10,000g pellet) and mIPSC were recorded. The results showed a significant reduction of mIPSC frequency in MV-treated neurons compared to control cells (Figure 5A-B), while no alteration in mIPSC amplitude was found after MV administration (Figure 5A,C), according with a presynaptic nature of MV action. Notably, the effects of MV treatment mimicked the ones of CB_1 receptor agonist WIN55,212-2 (see Figure 4A).

The role of eCBs present in MV-enriched fraction in this inhibition of GABA spontaneous release was indicated by the capability of the selective CB_1 receptor antagonist SR141716A (Rimonabant, 1 μM) to completely abolish MV-mediated depression of mIPSC frequency (Figure 6A). The administration of SR141716A (1 μM) alone was ineffective on mIPSC frequency (Figure 6A), according with literature (Irving et al, 2000).

eCBs present in 10,000g MV-enriched fraction and responsible for this action may still have been released in association with other structures which may co-purify with MVs, such as lipid droplets or micelles. To rule out the possibility that eCBs could be release through a non-vesicular pathway, we pharmacologically blocked MV release by administrating to microglia the $P2X_7$ receptor antagonist oxidized ATP (oATP, 100 μM; Bianco et al, 2005) and then proceeded with the standard protocol for MV isolation. oATP blocks ATP-mediated release of MVs (Bianco et al, 2005) allowing constitutive release only. The obtained 10,000g pellet was completely ineffective on mIPSC frequency (Figure 6B), thus demonstrating that the eCBs accounting for mIPSC frequency decrease are actually carried by MVs.

These findings demonstrate that microglial cells release active eCBs through a vesicular pathway and that eCBs stored in MVs play a crucial role in the modulation of inhibitory transmission in the brain.

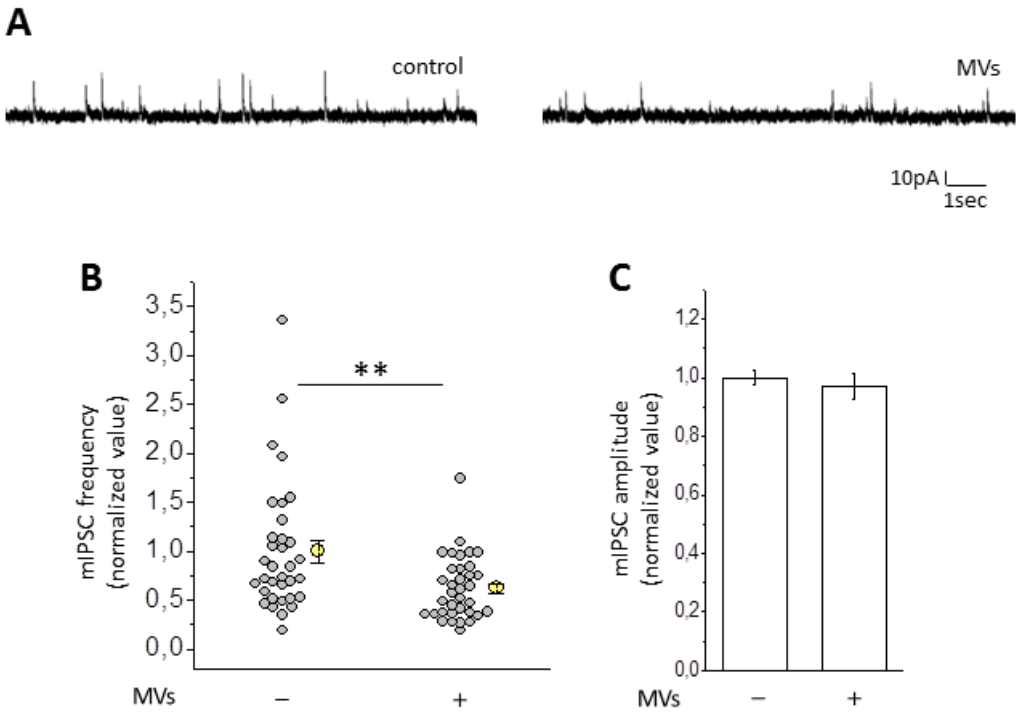

Figure 5 Microglia MV treatment depresses mIPSC frequency

A. Representative mIPSC traces of control and MV-treated 16-17 DIV hippocampal neurons

B. Effect of microglial MV treatment (10,000 g pellet from primary microglia, incubation lasted 40-45 min) on mIPSC frequency in 16-17 DIV hippocampal cultures (N=5, normalized data); Mann–Whitney Rank-sum test, P = 0.004; Ctrl n = 35 cells, MVs n = 35 cells

C. Effect of microglial MV treatment on mIPSC amplitude (N=5, normalized data); Mann–Whitney Rank-sum test, P = 0.428, Ctrl n = 35 cells, MVs n = 35 cells

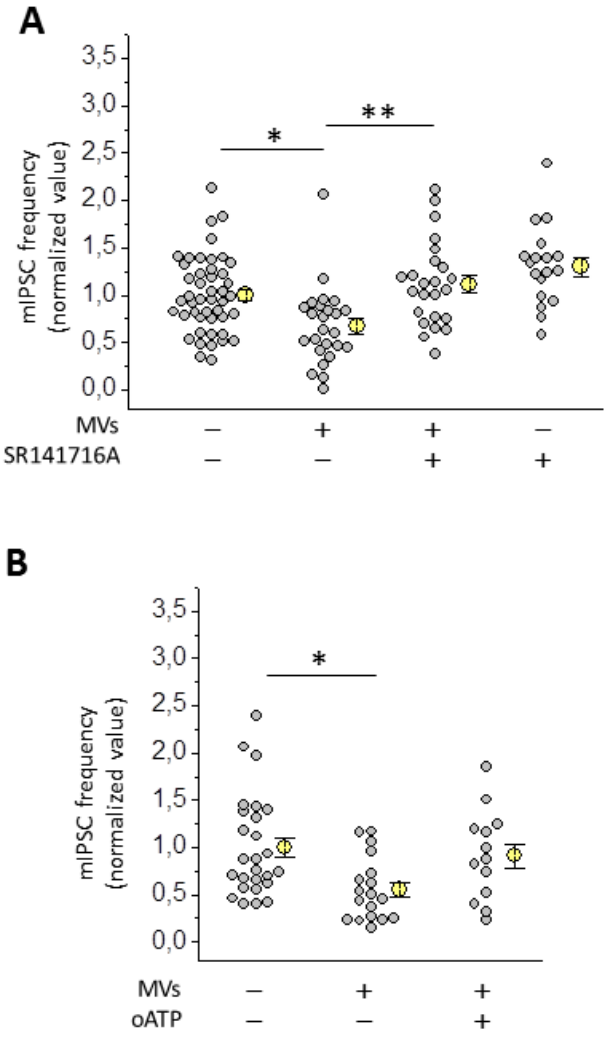

Figure 6 Microglia MV treatment depresses mIPSC frequency through activation of CB_1 receptors

A. MV action on mIPSC frequency is blocked in the presence of the CB_1 receptor antagonist SR141716A , administrated at the effective dose of 1 μM for 15 min before and together with MV treatment (N =5); One-way ANOVA, $P < 0.001$, followed by Bonferroni test for comparison among groups, $P < 0.05$, $P < 0.01$

B. oATP inhibit MV release through blockade of $P2X_7$ receptor, responsible for MV shedding; 10,000 g pellet produced from primary microglia pre-treated with oATP (100 μM), lacking of MVs, wasn't able to induce any alteration in mIPSC frequency (normalized data; N = 3); Kruskal–Wallis ANOVA, $P = 0.006$, followed by Dunn's test for comparison among groups, $P < 0.05$

MV-mediated CB_1 receptor activation drives to a downstream signaling in neurons

It's known from literature that CB_1 receptor activation leads to ERK phosphorylation (Dalton and Howlett, 2012). Hence, in order to investigate whether MV-induced activation of CB_1 receptor could drive to an intracellular signaling pathway activation, we performed western blot analysis for total and phosphorylated-ERK (P-ERK). The results revealed an increase in P-ERK levels 5 min after MV exposure (Figure 7). This effect was completely blocked by co-administration of the CB_1 receptor antagonist SR141716A (1 μM) (Figure 7).

These data corroborate the fact that eCBs present in MVs are active on neurons and indicate that MV-mediated activation of CB_1 receptor actually translates into downstream signaling in neurons.

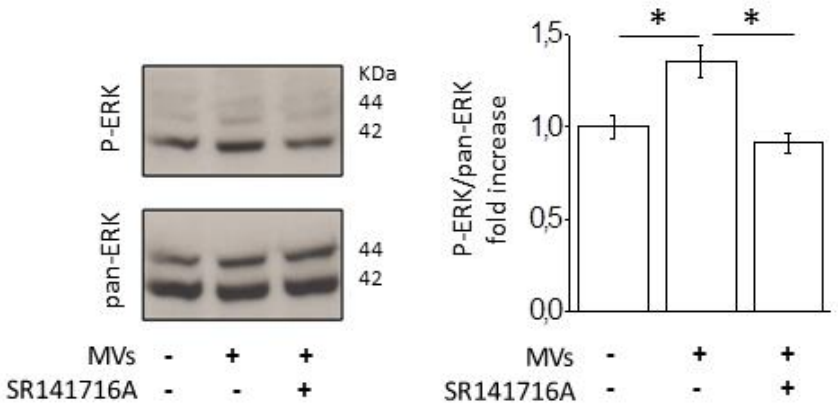

Figure 7 CB_1 receptor activation induced by MVs drives to intracellular signaling pathway activation

MV administration induced an increase in P-ERK levels. This effect is prevented by co-administration of the CB_1 receptor antagonist SR141716A (1 μM) (normalized data; N =3); Kruskal–Wallis ANOVA, P = 0.002, Dunn's test for comparison among groups, P < 0.05

eCBs are carried on the surface of microglial MVs

We can speculate that CB_1 receptor activation can happen only if (or when) eCBs are exposed on the surface of MV. We've already provided evidence of the fact that MVs and neurons can interact by surface-to-surface contact in Antonucci et al, 2012. Here we show a representative confocal image, complete with x-y projections, of neurons exposed to farnesyl-GFP–positive MVs (f-GFP-MVs) for 3 h and repeatedly washed, where MVs appear to bind neuronal surface (SNAP25-positive) without undergoing internalization (Figure 8A). Farnesyl-GFP labels MV membranes.

To verify eCB localization on MVs, AEA detection was necessary. Since no good antibody against AEA is available at the moment, we took advantage of a tool specifically designed for AEA visualization: a biotinylated analog of AEA, labelled with Cy3-streptavidin. As shown in Figure 8B, biotin-AEA localized on MVs surface (calcein-positive), indicating that AEA has affinity for MV membrane. Control MVs, incubated with Cy3-streptavidin but not with biotin-AEA, didn't show any reactivity for Cy3-streptavidin (Figure 8B), ruling out the possibility that the reactivity we saw in biotin-AEA–treated samples were due to artifacts, generated by streptavidin molecule aggregation and interaction with MV plasma membrane.

Finally, we showed that MV membranes, obtained from MVs broken by hipo-osmotic stress and depleted of their luminal cargo, maintained their capability to depress mIPSC frequency (Figure 8C), supporting the idea of a surface localization for AEA on MVs.

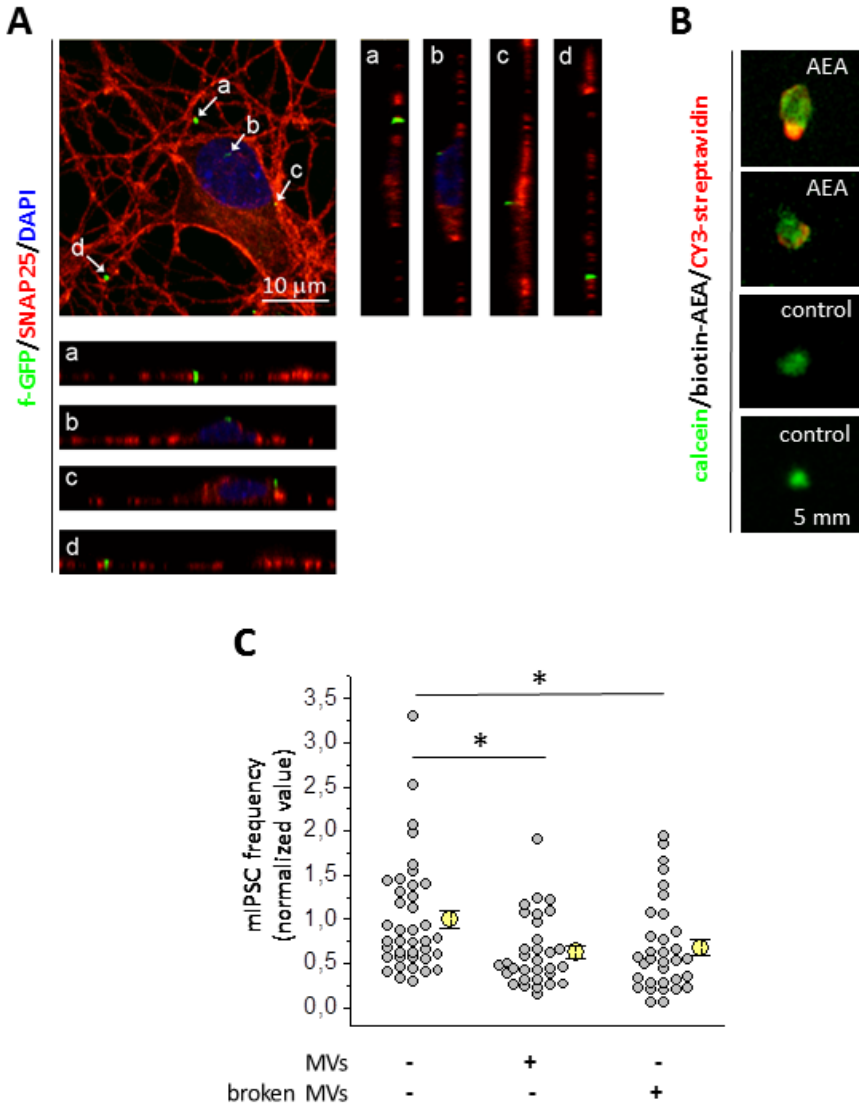

Figure 8 AEA localizes on the membrane of microglial MVs

A. Confocal images of cultured neurons after a 3h incubation with fGFP labelled MVs (in green) and several washes. MVs derived from primary glia transfected with fGFP and corresponded to the 10,000g pellet; after fixing, neuronal surface was imaged using an antibody against SNAP25 (in red), while DAPI dye (in blue) labels cell nuclei. The binding of MVs with the membrane is more clearly shown in the x-y projections

B. Confocal images of MVs produced by calcein loaded microglia (in green), incubated with biotin-AEA (5 µM) and Cy3-streptavidin (in red). Biotin-AEA colocalizes with MVs surface showing an heterogeneous distribution pattern. No immunoreactivity for Cy3-streptavidin was detectable on MV membrane when Cy3-streptavidin alone was administrated (control).

C. Effect of MVs broken by hipo-osmotic stress and emptied of their cargo on mIPSC frequency (normalized data; N = 3). Kruskal–Wallis ANOVA, P = 0.003, followed by Dunn's test for comparison among groups, $P < 0.05$

Microglial MVs generate excitation-inhibition unbalance in the brain

In our previous publication, we reported that microglial MVs enhance glutamate release probability from hippocampal excitatory terminals through stimulation of sphingolipid metabolism in neurons (Antonucci et al, 2012). Hence, microglial MVs are responsible for opposite but synergic effects on excitatory and inhibitory synapses, enhancing excitatory transmission and depressing inhibitory transmission, thus generating an excitation-inhibition unbalance in the brain.

In order to investigate whether there is a cross-relation between the two pathways activated by MVs (i.e. sphingolipid cascade and CB_1 receptor), we performed electrophysiological experiments to evaluate: i) MV effects on excitatory synapses when CB_1 receptors are antagonized; ii) MV effects on inhibitory synapses under blockade of the sphingolipid cascade. In both cases MV effects were not perturbed. Indeed, MVs maintained their capability to enhance mEPSC frequency when co-administrated with the CB_1 receptor antagonist SR141716A (1 μM) (Figure 9 A-B), demonstrating that MVs directly potentiate excitatory transmission and that this action is not dependent on MV-induced depression of inhibitory tone. SR141716A (1 μM) alone has no effect on mEPSC frequency (N = 3; normalized values: Ctrl = 1.000±0.107, n = 18; SR141716A-treated = 1.151±0.151, n = 13 cells; Student's t-test, P = 0.407).

On the other hand, MV-mediated enhancement of mIPSC frequency was not perturbed by the presence of the sphingolipid cascade inhibitor N-oleoylethanolamine (OEA, 37.5 μM) (Figure 9 C). OEA alone was ineffective on mIPSC frequency (N = 3; normalized values: Ctrl = 1.000 ±0.117, n = 22 cells; OEA-treated = 1.186±0.173, n = 19 cells; Mann–Whitney Rank-sum test, P = 0.539). Incidentally, we must say that OEA is an endogenous peroxisome proliferator-activated receptor and GPR119 agonist, which acts independently of CB_1 receptors (Hansen, 2010).

Taken together these data indicate that MVs are able to deliver different signals depending on the glutamatergic or GABAergic nature of target neurons.

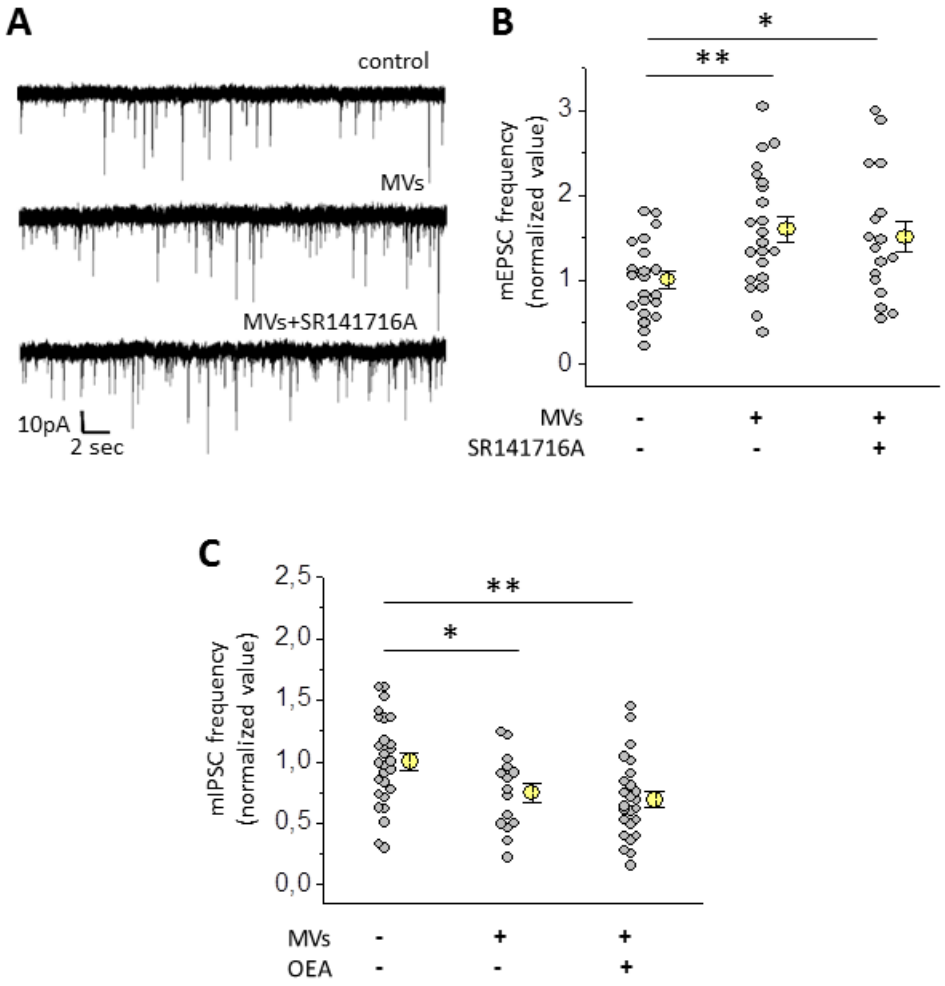

Figure 9 Microglial MVs act on excitatory and inhibitory synapses through distinct molecular pathways

A. Representative mEPSC traces of untreated (control) 13-15 DIV hippocampal neurons and neurons treated with MVs alone or in co-administration with the CB_1 receptor antagonist SR141716A (1 μM)

B. MV effect on mEPSC frequency was preserved in the presence of SR141716A (1 μM) (normalized data; N = 4). One-way ANOVA, P = 0.007, followed by Holm-Sidak's test for comparison among groups, $P < 0.05$, $P < 0.01$

C. MV effect on mIPSC in the presence of the sphingolipid cascade inhibitor OEA (37.5 μM) (normalized data; N = 5). One-way ANOVA, P = 0.003, followed by Holm–Sidak's test for comparison among groups, $P < 0.05$, $P < 0.01$

DISCUSSION

eCBs are very important bioactive molecules in the brain, being involved in synaptic plasticity, synaptogenesis and synaptic circuit formation and in several brain functions (Benarroch, 2014). However, how they cross cell membranes and move in the extracellular space is a challenging and still unsolved issue, become a hot topic in eCB research (Maccarrone et al, 2010; Nicolussi and Gertsch, 2015; Fowler, 2013).

Whether eCBs trespass cell membrane through passive diffusion, carrier-mediated transport or other mechanisms is still matter of debate (Maccarrone et al, 2010; Nicolussi and Gertsch, 2015; Fowler, 2013). Proteins and other structures that bind AEA intracellularly have been identified (i.e. FABP-5 and 7, heat-shock protein 70, albumin, lipid droplets), while the role of FLAT as intracellular AEA transporter cannot be endorsed given current results (Fowler, 2013; Kreitzer and Regehr, 2001; Leung et al, 2013). Relative to extracellular transport, AEA could likely bind circulating proteins. Lipocalins and albumin are putative extracellular carriers (Piomelli, 2003; Stanton et al, 2005).

On the other hand, my laboratory recently reported how reactive microglia release EVs upon ATP stimulation (Bianco et al, 2009; Bianco et al, 2005). EVs are an important mechanism of intercellular communication: they shuttle active molecules from a donor to a recipient cell or release them in the extracellular environment (Cocucci and Meldolesi, 2015; Raposo and Stoorvogel, 2013). EVs can contain all kinds of signaling molecules and we demonstrated that a not yet identified membrane constituent of microglial MVs increase glutamate release probability in cultured hippocampal neurons, acting on sphingolipid cascade (Antonucci et al, 2012). Additionally, microglia do produce eCBs and the production increases greatly under inflammation (Stella, 2009).

Hence, we explored the possibility that active eCBs may be released from microglial cells in association with EVs and thus signal to neurons.

Here we show that the eCB AEA is enriched in EVs released from microglia (both MVs and exosomes) as compared to donor cells, thus providing evidence that AEA may be released in association with EVs. We noted that in this study lower AEA absolute concentrations in microglial cultures relative to previous studies have been detected (Carrier et al, 2004; Walter et al, 2002). We attributed this to

the fact that eCBs are labile substances, difficult to be measured, and it's known that the detected levels vary among different experimental conditions.

Furthermore, our findings indicate that eCBs present in MVs are biologically active on neurons. In fact, eCB-storing MVs are able to activate presynaptic CB_1 receptors and trigger their downstream signaling, thus inhibiting mIPSC frequency and activating ERK pathway in GABAergic neurons in culture (Figure XII).

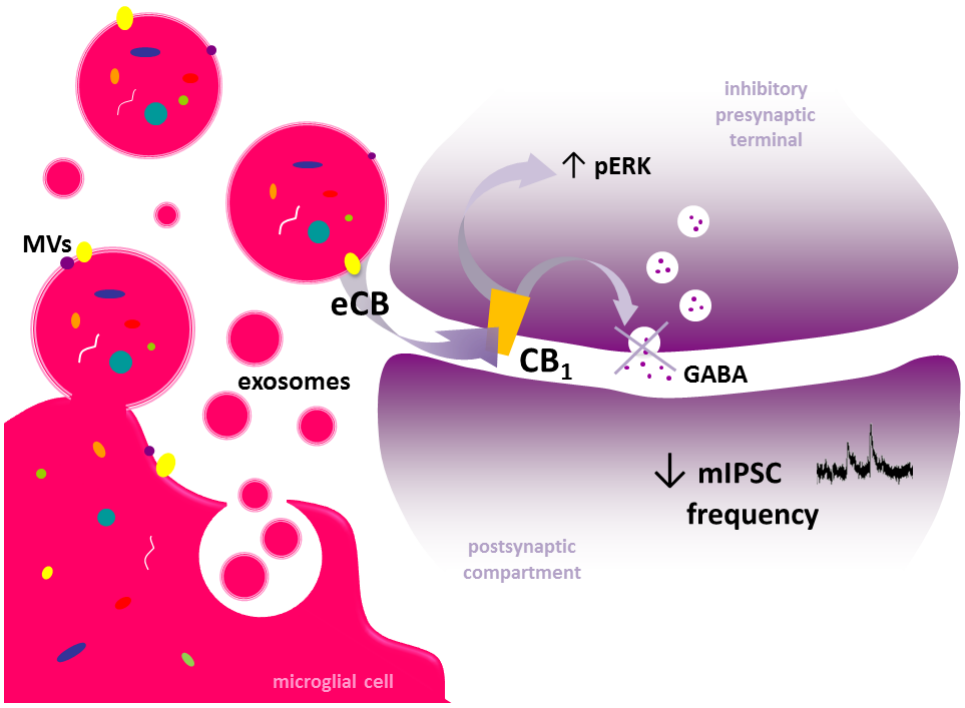

Figure XII Microglial MV effects on inhibitory synapses. Microglial cells release eCBs in association with EVs. Microglial MVs are able to activate CB_1 receptors on inhibitory presynaptic terminals thus inhibiting GABA release and activating ERK pathway.

It's known that microglial cells release active molecules which affect neuronal functions and regulate network formation (Zhan et al, 2014; Schlegelmilch et al, 2011; Kettenmann et al, 2013). We can speculate that microglia, through the release of eCB-carrying EVs, can regulate axon guidance and circuit formation of GABAergic neurons in the developing brain.

Notably, microglial EVs contain other molecules important for brain development, such as the morphogen Wnt3a (Hooper et al, 2012), a hydrophobic

lipid-modified protein (Zecca et al, 1996). Thus we can hypothesize that microglia can secrete different hydrophobic molecules, involved in brain circuit constitution, through EVs. Further experiments will be necessary to explore this possibility.

eCB receptors and hippocampal neurons in cultures

Our findings show that CB_1 receptors are expressed on GABAergic rather than glutamatergic hippocampal neurons in culture and CB_1 receptor agonist effects on spontaneous synaptic activity reflect their expression pattern. These results appear in line with immunocytochemical and electrophysiological evidence reported in literature.

Indeed, although Sullivan and colleagues showed a significant increase in mEPSC frequency in hippocampal cultures after WIN55-212,2 administration (Sullivan, 1999), this effect was very small, not comparable to that reported on mIPSC frequency (Irving et al, 2000). Actual CB_1 receptor-mediated effects on hippocampal excitatory synapses remain in fact controversial, since data reported in literature are very heterogeneous, "*ranging from no effect to complete obliteration of synaptic response"* (Bajo et al, 2009). Possible difference between the results can be due to different experimental conditions.

Some claim that usual techniques are not sensitive enough to detect the probably very low levels of CB_1 receptors present on adult glutamatergic terminals in the hippocampus (Irving et al, 2000). And, effectively, *in situ* hybridization experiments for mRNA detection, performed by Marsicano and Lutz, report the existence, at much lower levels than in inhibitory cells, of CB_1 receptor mRNAs in non-GABAergic neurons which, according to them, must be excitatory glutamatergic cells (Marsicano and Lutz, 1999).

On the other hand, Yasuda and colleagues have recently reported how in the hippocampus excitatory synaptic plasticity mediated by CB_1 receptors is prerogative of immature synapses rather than of adult ones. In fact, according to them, CB_1 receptors can actually be expressed at glutamatergic synapses but they are detectable only at early development stages (Yasuda et al, 2008; Benarroch, 2014).

We didn't explored the possibility that vesicular eCBs may activate also CB_2 receptors. This was beyond the aim of this study, which was primarily to

evaluate whether eCBs were present in MVs, using patch-clamping of neurons expressing CB_1 receptors as an eCB readout. CB_2 receptors are primarily express in immune cells, while their presence in neurons is still highly controversial (Malfitano et al, 2014).

eCBs are released on the surface of EVs

The lack of appropriate antibodies prevented us to directly detect eCBs on microglial MV surface. However our findings indicate that eCBs are carried on the membrane of MVs, since MV membrane fraction maintains the capability to activate pre-synaptic CB_1 receptors. Moreover, in order to activate eCB receptors, eCBs must be exposed on the outer leaflet of MV membranes. Supporting this theory, our confocal microscopy evidence shows that MVs make stable contact with neuronal surface and that the biotinylated analog of AEA has affinity for MV outer membrane. Further experiments will be necessary to describe more precisely eCB localization on MVs and to characterize the mechanisms of vesicular eCB interaction with CB_1 receptors. Yet, cholesterol may be a good candidate as mediator of a possible AEA translocation through EV membrane bilayer (Fowler, 2013).

Microglial MVs alter excitation-inhibition balance in the brain

A *proof-of-principle* of the fact that microglia MVs increase excitation-inhibition balance in the brain was provided by our previous *in vivo* electrophysiological evidence after injection of MVs in the rat visual cortex (Antonucci et al, 2012). Accordingly to *in vivo* results, *in vitro* we found that MVs are able to promote excitatory transmission on one hand (Antonucci et al, 2012) and to depress inhibitory transmission on the other (Gabrielli et al, 2015; the present study) (Figure XIII).

These effects could be detrimental: MV-mediated potentiation of excitatory transmission may be pathological, participating to excitotoxicity phenomena which occur in neuroinflammatory diseases characterized by microglia activation (e.g. multiple sclerosis) (Centonze et al, 2009; Centonze et al, 2010). Indeed, it's known from literature that microglial cells secrete mediators able to modulate excitatory and inhibitory transmission in the brain under inflammatory conditions (e.g. TNFα; Centonze et al, 2009; Centonze et al, 2010; Kettenmann et al, 2011; Kettenmann et

al, 2013) and that this may drive to excessive stimulation of neurotransmission and excitotoxic damage: the so called glutamate excitotoxicity.

This hypothesis would be in line with the capability of MVs to propagate inflammation in multiple sclerosis patients (Verderio et al, 2012). In addition, MV release is stimulated by $P2X_7$ receptor activation (Bianco et al, 2005), which responds to high extracellular ATP concentrations (Farber and Kettenmann, 2006; Lalo et al, 2011). High ATP concentrations represent a typical danger signal (Davalos et al, 2005; Fiebich et al, 2014), and $P2X_7$ activation is usually connected to damage, neurodegeneration and inflammation (Färber and Kettenmann, 2006; Lalo et al, 2011; Abbracchio et al, 2008).

On the other hand, we cannot exclude that MV effects on synaptic activity may have a protective function, representing a homeostatic mechanism devoted to the restoration of correct network excitability in case of a deficit in synaptic transmission. In this case, microglia would sense the deficit in neuronal activity, thanks to their mobile processes, and react to this releasing MVs to mediate an overall increase of excitatory firing. This would be in line with the fact MVs do not seem to cause any toxic effect on neurons (authors' observations). Counter, Jimok Kim and Bradley E. Alger recently reported that the homeostatic response to chronic inactivity is a decrease, instead of an increase, in eCB tone, responsible for specifically straightening the subpopulation of CB_1-expressing interneurons present in hippocampus (Kim and Alger, 2010).

Further experiments will be necessary to clarify whether the release of MVs from microglia has to be considered as part of a pathological or physiological process.

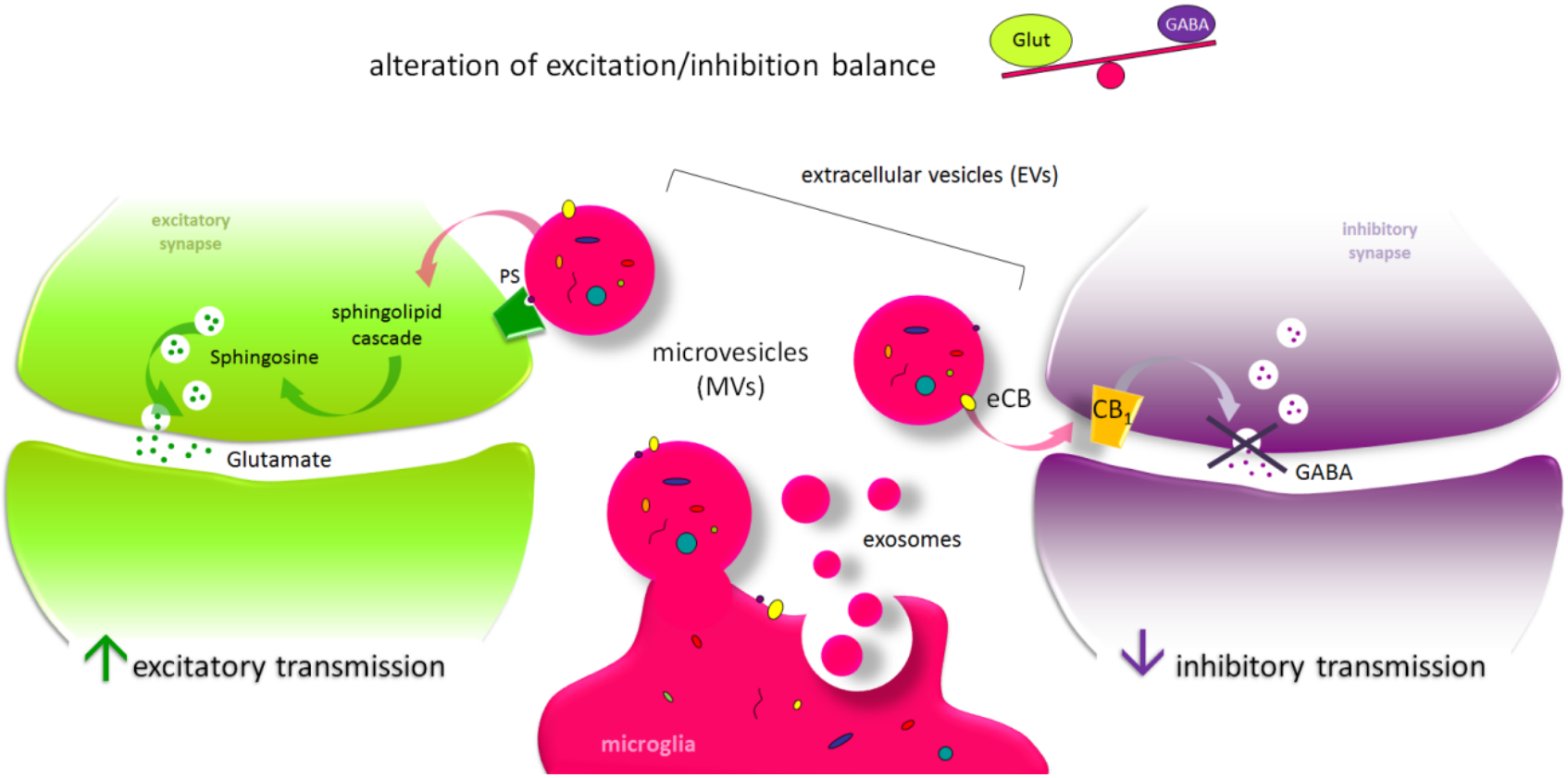

Figure XIII MVs alter excitation-inhibition balance. MVs are able to promote excitatory transmission by stimulating sphingolipid cascade on one hand, and to depress inhibitory transmission through activation of presynaptic CB_1 receptors on the other, thus altering excitation-inhibition balance (modified from Gabrielli and Verderio, in print).

Remarkably, the amount of MVs employed was that produced by a double number of microglial cells compared to neurons (microglia-to-neurons ratio 2:1): i.e. a concentration close to that present in the rodent brain, where microglia are about as common as neurons. However, more work will be necessary to define unequivocally whether this concentration is actually typical for physiological conditions or it is reached only in a pathological status, when activated microglia proliferate (Graeber and Streit, 2010) and release higher amounts of MVs (Bianco et al, 2009).

Microglial MVs act on excitatory and inhibitory synapses through distinct pathways

The evidence collected so far corroborates the notion that microglial MVs represent an important mechanism of cell-to-cell communication. Furthermore this study shows that MVs are able to deliver distinct messages and activate distinct signaling pathways depending on the GABAergic or glutamatergic nature of recipient neurons (Figure XIII). They inhibit GABA release through CB_1 receptor activation specifically on inhibitory neurons, and they directly potentiate glutamate release through stimulation of the sphingolipid cascade in excitatory neurons. This is indicated by the cross-check experiments with the sphingolipid cascade inhibitor and the CB_1 receptor antagonist on inhibitory and excitatory synapses respectively. Indeed, pharmacological blockade of sphingolipid metabolism doesn't prevent the down-regulation of inhibitory transmission evoked by MVs, while MV-mediated effects on excitatory transmission are maintained upon inhibition of CB_1 receptors.

The sphingolipid Sphingosine accounts for MV-mediated action on excitatory synapses, as previously demonstrated by us (Antonucci et al, 2012). Besides, a recent paper reported that the sphingolipid sphingosine-1-phosphate (S1P) is an antagonist of CB_1 receptors (Selley et al, 2013). Thus, it is possible that the increased sphingolipid production, that we registered after MV treatment (Antonucci et al, 2012), may not only directly induce the potentiation of excitatory wiring (through Sphingosine) but also mask an hypothetical vesicular eCB-mediated inhibition of glutamate release (through S1P).

EV isolation

EVs are mostly isolated through differential centrifugation, although other protocols are sometimes used. A standardize EV isolation and purification protocol is still lacking and the extraction of exosomes and MVs appears a complex issue by now (Cocucci and Meldolesi, 2015; Raposo and Stoorvogel, 2013). Consequently, it's very important to characterize the obtained exosome- or MV-enriched fractions using multiple approaches, by taking advantage of different techniques such as immunoblotting, imaging, electron microscopy and the more innovative Nanoparticle Tracking Analysis (Nanosight®) or Tunable Resistive Pulse Sensing (Izon qNANO®) (Raposo and Stoorvogel, 2013; Yanez-Mo et al, 2015).

Here we provided a deep characterization of EVs, MV- and exosome-enriched fractions used in our experiments, isolated through differential centrifugation 10,000g/100,000g after clearing by cell debris (see "Materials and Methods"). And we integrated the characterization carried out previously by the laboratory (Bianco et al, 2009), using different and updated methodologies.

CONCLUSIONS

In conclusion, our study demonstrates that:

- eCB AEA is enriched in EVs, both MVs and exosomes, produced by microglia;
- vesicular eCBs are biologically active, as MVs activate CB_1 and its downstream signaling and inhibit mIPSC frequency;
- MVs are able to deliver distinct signals depending on the glutamatergic or GABAergic nature of the recipient neuron.

In addition, these data suggest that microglia-derived MVs may affect excitatory-inhibitory balance in the adult nervous system. In fact, MVs increase excitatory transmission and directly downregulate inhibitory transmission.

More broadly, results from these experiments identify EVs as an ideal vehicle for the transport of hydrophobic signaling molecules which, like eCBs, move across the nervous system to exert their biological activity.

IMPORTANT NOTES

This thesis cites the following article, of which I'm the first author:

Active endocannabinoids are secreted on extracellular membrane vesicles

Gabrielli M, Battista N, Riganti L, Prada I, Antonucci F, Cantone L, Matteoli M, Maccarrone M, Verderio C.

EMBO Rep. 2015 Feb;16(2):213-20. doi: 10.15252/embr.201439668. Epub 2015 Jan 7.

PMID: 25568329

MATERIALS AND METHODS

Animals

All the experimental procedures involving animals followed the guidelines established by the European Legislation (Directive 2010/63/EU) and the Italian Legislation (L.D. no 26/2014). All efforts were made to minimize the number of animals used and their suffering.

Cell cultures

Glial cell cultures

Primary glial cell cultures were established from P2 Sprague Dawley rats (Charles River Italia) as previously described in (Bianco et al, 2005). Briefly, cortex and hippocampi were extracted from pups and subjected to enzymatic digestion with 0.25% Trypsin (Gibco) in the presence of 0.5 g/L DNAsi (Sigma-Aldrich) (15 min at 37°C, twice) in an HBSS-based solution composed of 10% HBSS-10X, 10 mM HEPES (pH 7.3), 1% antibiotics (Pen/Strep, Gibco) in dH_20. HBSS solution ensures pH stability and sample preservation. In order to remove not-dissociated tissue, the supernatant obtained from digestion was collected and filtered through a nytex membrane (Millipore) and cells were subsequently pelleted. The pellet was dissociated and cells were plated in 75 cm^2 flasks pre-treated with poly-L-lysine (10 µg/ml, Sigma-Aldrich). Glial cells were maintained in standard conditions (37°C, 5% CO_2), in medium containing MEM (Gibco), 20% FBS (Fetal Bovine Serum, Gibco), 5.5 g/L glucose (Sigma-Aldrich) and 1% antibiotics (Pen/Strep, Gibco).

Primary microglial cultures were obtained and maintained as reported in Bianco et al, 2005. Mixed glial cultures have were shaken for 30 min on orbital shaker, in order to detach microglial cells often settled on the layer of astrocytes. The supernatant containing microglial cells was collected and pelleted. Microglial cells were plated at a density of ~90 cells/mm^2 in tissue culture dishes pre-treated with Poly-DL-ornithine hydrobromide (50 mg/L, Sigma-Aldrich) and maintained in glial cultured medium (described above) supplemented with GM-CSF (Granulocyte macrophage colony-stimulating factor) supplement. GM-CFS supplement was obtained from X-63 cell lines transfected with a GM-CSF plasmid. Cells were cultured in standard condition (37°C, 5% CO_2).

N9 is an immortalized murine microglial cell line. N9 cells were maintained in IMDM medium (Gibco) with 10% FBS (Gibco), 1% L-glutamine (Gibco) and 1% antibiotics (Pen/Strep, Gibco) in standard culture condition (37°C, 5% CO_2).

Primary cultures of hippocampal neurons

Primary cultures of hippocampal neurons were established from E18 Sprague Dawley rat embryos, as previously described in Bartlett and Banker, 1984. Briefly, hippocampi surgically removed from E18 embryos were subjected to enzymatic digestion with 0.25% Trypsin (15 min at 37°C) and then to mechanic dissociation. During the whole procedure tissues were kept in an HBSS-based solution (see above). Cells were plated at a density of ~400 cells/mm^2 on 24 mm glass coverslips pre-treated with poly-L-lysine (1 mg/ml, Sigma Aldrich). Neuronal cultures were maintained in standard culture condition (37°C, 5% CO_2) in a medium with Neurobasal Medium (Gibco), 2% B27 supplement (Invitrogen), 0.5 mM L-glutamine (Gibco), 12 μM glutamate (Sigma-Aldrich) and 1% antibiotics (Pen/Strep, Gibco). After 3 days *in vitro* (DIV) the medium was partially replaced with glutamate-free fresh medium.

Isolation of MVs, exosomes and mixed EV population

MVs, exosomes and mixed EV population were isolated from N9 cultures at 80% confluence or 1-2 DIV primary microglial cultures (plated at a density of $1x10^6$ cells per ø 60 mm culture dish; ~90 cells/mm^2). After washing twice with PBS (Phosphate Buffer Saline) and once with KRH (Krebs-Ringer's HEPES solution; 125 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO, 25 mM HEPES/NaOH, 6 mM D-glucose and 2 mM CaCl, pH 7.4), microglia or N9 cells were incubated with 1 mM ATP in KRH for 30 min/1 h, to stimulate MVs release. The EV-containing supernatant was collected and subjected to a double centrifugation at 300g for 10 min at 4°C (ALC 4227 R centrifuge, rotor ALC 5690) to clear it from cells and *debris*. MV- and exosome-enriched fractions were then separated by differential centrifugation: MV-enriched pellet was obtained through a 10,000g x 30 min centrifugation at 4°C (VWR CT15FE centrifuge, rotor Hitachi T15A61 or Beckman Optima L-90K Ultracentrifuge, rotor SW41Ti); the residual supernatant were subsequently centrifuged at 100,000g for 1h at 4°C (Beckman Optima L-90K Ultracentrifuge, rotor SW41Ti) to obtain an exosome-enriched

pellet. Otherwise, to obtain a mixed EVs population, the supernatant was centrifuged at 100,000g immediately after 300g pre-clearing.

To perform electrophysiological recordings, nanoparticle tracking analysis (Nanosight) and fluorescent microscopy experiments, the pellets of vesicles were resuspended and used immediately after isolation. For mass spectrometry analysis, the dry pellets were stored at -80°C. While for western blots the pellets were resuspended in SDS sample buffer, as described below.

Nanoparticle tracking analysis (Nanosight)

Vesicles present in MV- and exosome-enriched pellets were analyzed for particle size distribution and concentration by Nanoparticle Tracking Analysis. Nanoparticle tracking is an innovative system for the visualization and analysis of particles in liquids: particles are tracked by video one-by-one simultaneously; the size of each particle is then evaluated by a software from their Brownian motion; particle concentration can be directly estimated too.

For our experiments, a Nanosight LM10-HS system, complemented with an EMCDD camera (Hamamatsu Photonics) and a 405 nm laser was used. Video recording and analysis were performed using NTA-software (version 2.3): camera shutter speed was 20.01 ms and camera gain 350, while minimal expected particle size, minimum track length and blur setting were all set to automatic. MV and exosome pellets were resuspended in 0,1µm-filtered sterile KRH (400 µl) and 5 x 30 sec recordings were performed for each sample. 0,1µm-filtered sterile KRH was analyzed and no particle was detected. All measurements were performed at RT (25-28°C).

Bicinchonic acid (BCA) assay

Protein content of EVs and donor cells was assessed by bicinchoninic acid (BCA) assay, using Micro BCA protein assay kit and BCA respectively (Thermo Fischer Scientific). In BCA method, BCA molecules chelate cuprous ion Cu^{+1}, formed from Cu^{2+} reduction by proteins in alkaline environment. This chelation generates a purple-colored reaction product which has a strong absorbance at 562 nm, proportional to protein concentration in the sample.

Sample absorbance wes read through a spectrophotometer (Victor^2 - 1420 multilabel counter, Wallac) set to 550 nm.

Western blot analysis

MV and exosomes pellets were resuspended in 1X Sample Buffer (from 5X solution: 15% SDS, 0.575 M Sucrose, 0.325 M TrisHCl, 0.5% β-mercaptoethanol, pH 6.8). Cells were scraped and collected in Solubilization Buffer (290 mM Sucrose, 1% SDS, 62.5 mM Tris) complemented with protease inhibitors and phosphatase inhibitors (1:1000); then Sample Buffer was added. Samples were first boiled at 100°C for 5-10 min, in order to denature ate proteins. Samples components were separated according to their molecular weights by SDS-PAGE electrophoresis (in reducing condition) and then transferred on a nicrocellulose membrane. After 1h blocking with 5% non-fat dry milk in TBS (Tris-buffered saline) with 0.1% Tween20 at RT, we incubated the membrane with primary antibodies directed against the proteins of interest, diluted in 5% non-fat milk, TBS, 0.1% Tween20. The following primary antibodies were used: mouse anti-Tsg101 (1:500; Abcam), rabbit anti-alix (1:500; Covalab), mouse anti-flotillin (1:1000; BD Transduction), mouse anti-SP1 (1:5000; Upstate), rabbit anti-TOM-20 (1:500; Santa Cruz Biotechnology), rabbit anti-adypophilin (1:200; Santa Cruz Biotechnology), mouse anti-GS28 (1:1000; BD Transduction), rabbit anti-ERK 1/2 and anti-P-ERK 1/2 T202/Y204 (1:1000; Cell Signalling). After washing with TBS 0.1% Tween20 (3 x 10 min), the membranes were incubated with species-specific secondary HRP antibodies in 5% non-fat milk, TBS, 0.1% Tween20. After washing with TBS 0.1% Tween20 (3 x 10 min), with TBS 0.3% Tween20 (3 x 10 min) and with TBS (10 min), immunoreactive bands were detected using ECL kit (Thermo Scientific) and quantified using ImageJ software.

LC-ESI-MS

Endogenous levels of AEA and 2-AG were detected through LC-ESI-MS.

In LC-ESI-MS the sample is first separated into analyte(s) (molecule(s) of interest) and other sample components by liquid chromatography (LC), then the analyte is detected, identified and quantified by mass spectrometry (MS). Electrospray ion source (ESI) is an interface connecting the LC, which works with liquid samples, with the MS, which needs high vacuum. Thus ESI's role is to convert molecules in the liquid phase into ions in the gas phase, compatible with MS system.

AEA and 2-AG contents were measured in EVs from $50x10^6$ N9 cells and from $15x10^6$ primary microglial cells and in donor cells; and in MVs and exosomes

from 30 x10^6 primary microglial cells and in donor cells. Samples were subjected to lipid extraction with chloroform/methanol (2:1, v/v), in the presence of d_8-AEA and d_8-2-AG as internal standards, used to improve the accuracy and reliability of the measurement. The organic phase was dried and analyzed by LC-ESI-MS, using a Perkin Elmer LC system (Perkin Elmer) associated with a single quadrupole API-150EX mass spectrometer (Applied Biosystems). Quantitative analysis was performed by selected ion recording over the respective sodiated molecular ions, as previously reported (Francavilla et al, 2009).

These experiments were performed in collaboration with Natalia Battista (University of Teramo and European Center for Brain Research/IRCCS Santa Lucia Foundation) and Mauro Maccarrone (University of Rome and European Center for Brain Research/IRCCS Santa Lucia Foundation) at University of Teramo.

Immunocytochemistry

Cells plated at a density of ~400 cells/mm^2 on 24 mm glass coverslips were fixed with 4% paraformaldehyde 4% sucrose for 15-20 min and washed with PBS (Phosphate Buffer Saline, Sigma Aldrich). After fixing, samples were then washed with low salts (3 x 5 min; 150 mM sodium chloride, 10 mM phosphate buffer pH 7.4) and high salts solutions (3 x 5 min; 500 mM sodium chloride, 20 mM phosphate buffer pH 7.4) and incubated with GSDB/Triton blocking solution (Goat Serum Dilution Buffer; containing goat serum, Triton X-100, phosphate buffer and sodium cloride) for 30-45 min at RT. For the detection of specific antigens, coverslips were incubated with primary antibodies diluted in GSDB/Triton. The following primary antibodies were used: rabbit anti-CB_1 (1:500; Synaptic System), guinea pig anti-vGLUT1 (1:1000; Synaptic System), human anti-GAD (kindly gift from Prof M. Solimena, Medical School, TU Dresden). For CB1 receptor detection, an anti-CB_1 antibody directed against the C-terminus (intracellular) epitope of the protein was used, which, according to Hajos et al, 2000, shows a significantly higher reactivity on axons. After washing with GSDB/Triton, samples were incubated with species-specific fluorochrome-conjugated secondary antibodies: Alexa-488, Alexa-555 or Alexa-633 fluorophores (Invitrogen). Coverslips were then washed in high salts (3 x 5 min) and low salts solutions (3 x 5 min) and with 5 mM Phosphate Buffer (1 x 10 min) and mounted on microscope slides using Vectashied (Vector Laboratories Incorporated) as mounting medium. Images were

acquired with a Leica TCS SPE confocal microscope and processed with ImageJ software.

Visualization of MV-neuron interaction

Farnesyl-GFP-expressing MVs were isolated from glial cultures transfected with pEGFP-F Vector (BD Biosciences Clontech). Cells transfected with pEGFP-F Vector express GFP on the cell membrane, thanks to the genetic fusion between the fluorescent protein and a farnesylation sequence (which target the plasma membrane). (Harvey et al, 2001). Neurons were detected using a mouse anti-SNAP-25 (SMI 81 1:500; Sternberger) primary antibody and with Goat anti-GFP FITC-conjugated antibody (Novus Biologicals) Images were acquired with a Leica TCS SPE confocal microscope and processed with ImageJ software.

AEA visualization

AEA visualization was performed taking advantage of a specifically developed tool: a biotinylated AEA (biotin-AEA, Tocris; Fezza et al, 2008). First, microglia was loaded with calcein-AM (Sigma-Aldrich), a non-fluorescent cell-permeant dye, which become fluorescent and cell-impermeant when hydrolyzed by intracellular esterases. Then they were stimulated with ATP to produce MVs. MV-enriched fraction were incubated with 5 μM biotin-AEA or maintained in 1 ml KRH (control) for 10 min at 37°C. After dilution in KRH (12 ml), MVs were pelleted at 10.000g, and re-suspended in KRH (50 μl) containing Cy3-streptavidin (1:200) for 30 min. Control samples were incubated with Cy3-streptavidin but not with biotin-AEA. MV samples were then diluted in KRH (12 ml) and re-pelletted. After resuspension in 100 μl KRH, MVs were spotted on a glass coverslip and observed using a Leica TCS SP5 confocal microscope. Images were acquired using the same microscope and processed with ImageJ software.

Calcium imaging

Calcium imaging set-up included: an Axiovert 100 inverted microscope (Zeiss), Polychrome V light source (TILL Photonics GmbH) and a CCD Imago-QE camera (TILL Photonics GmbH). For the analysis, a TILLvisION 4.01 software was used.

Hippocampal 14-16 DIV cultured neurons were loaded with 2 mM Fura-2/AM (Invitrogen) for 30 min in the incubator at 37°C, washed with KRH and visualized.

Fura-2/AM is a ratiometric calcium indicator: it reaches absorption maxima at two different excitation wavelengths depending on its conjugated or not conjugated state. If Fura-2 is bounded to Ca^{++} ions, it is excited at 340 nm, while if it's unbounded, it is excited at 380 nm. Emission was acquired at the unitary Fura-2 emission length of 505 nm at 1 Hz. Acetoxymethyl (AM) ester form make Fura-2 molecule hydrophobic enough to trespass cell membrane. In this way, the ratio between emission after excitation at 340 nm and emission after excitation at 380 nm, will be a value proportional to Ca^{++} levels inside the cells and normalized on dye internalizaion. 340/380 fluorescence ratio (F340/F380) was measured in the soma of neurons (in regions of interest). Basal Ca^{++} levels were recorded for at least 100 secs before stimulus and traces with basal Ca^{++} levels exceeding normal physiologic values were excluded.

EV and drug treatments

Treatments for electrophysiological recordings

Neurons were exposed to MVs in an amount of 1.2 µg/ml for 40-45 min at 37°C. 1.2 µg/ml is the amount of MVs produced by a double number of microglia cells compared to the number of neurons treated. MV pellet were carefully resuspended in a specific amount of conditioned medium (400 µl) and the single coverslip was incubated.

The CB_1 receptor agonist WIN55,212-2 (Sigma-Aldrich), kindly provided by Dr Mariaelvina Sala (University of Milan), was acutely administrated to neuronal culture at the active concentration of 1 µM in the electrophysiology external solution (see "Electrophysiological recordings"), after previous dilution in a solution of saline (0.9% NaCl) and Tween-80 (2%) (Schulz et al, 2013). The CB_1 receptor antagonist SR141716A (Rimonabant, 1 µM, Tocris) was pre-incubated for 15 min and then co-incubated together with MVs for 40-45 min in culture medium. OEA (37 µM, N-oleoylethanolamide, Sigma-Aldrich) was pre-incubated for 15 min and then co-incubated together with MVs for 40-45 min as well, accordingly with (Antonucci et al, 2012).

Broken MVs were obtained through hypo-osmotic stress by resuspending MV-enriched pellet in 7.3 mM Phosphate Buffer at 4°C for 30 min on a rotating wheel. MVs broken and depleted of their luminal cargo (thus, MV membrane only) were collected through centrifugation at 100,000*g* for 1h.

oATP (100 µM) blocks ATP-mediated release of MVs (Bianco et al, 2005) allowing only constitutive release. Microglia was pre-treated with oATP for 10 min and then incubated with both oATP and ATP (1 mM) for 30 min, before standard 10,000g pellet isolation.

Treatments for western blot analysis

Neurons were exposed to an amount of MVs (7 µg/ml). Incubations, which lasted for different time periods (specified in the text), were performed in incubator at 37°C.

CB_1 receptor antagonist SR141716A (1 µM, Tocris), also called Rimonabant, were pre-administrated to neurons for 15 min and then co-administrated together with MVs.

Electrophysiological recordings

Miniature postsynaptic currents were recorded using whole-cell patch-clamp technique, in voltage-clamp mode and in the presence of 1 µM tetrodotoxin (TTX, Tocris). TTX inhibits sodium channel-mediated action potential generation and allows the user to record only spontaneous activity. Each miniature event represents the spontaneous release of a single synaptic vesicle.

Whole-cell recordings were performed at RT (20-25°C) using a MultiClamp 700A amplifier (Axon Instruments), a 1320A Digidata (Axon Instruments) and the software P-Clamp 10 (Molecular Devices), on an inverted Axiovert 200 microscope (Zeiss). Signal sampling frequency was always set at 10 kHz, while filtering frequency was 4 kHz. Recording pipettes were pulled from patch-clamp borosilicate capillary glass (World Precision Instruments) to a tip resistance of 3–5 MΩ using a two-stage vertical puller (Narishige). Series resistance was monitored at the beginning and during each recording.

All the experiments were performed on neurons plated at a density of ~400 cells/mm^2 and using KRH with the addition of 1 µM TTX (Tocris) as external solution.

mIPSC recordings were performed on 16-20 DIV hippocampal neurons, setting +10 mV as holding potential and using a Cesium Gluconate internal solution (130 mM CsGluc, 8 mM CsCl, 2 mM NaCl, 10 mM HEPES, 4 mM EGTA, 4 mM MgATP, 0,3 mM Tris-GTP; pH 7.3, adjusted with CsOH). Cesium ions block potassium currents which can occur when recording at positive holding potentials. The analysis of mIPSC traces was performed using the electrophysiological analysis software Clampfit (Molecular Devices). Each event was selected manually setting the threshold value at 5 pA.

mEPSC recordings were performed on 13-15 DIV hippocampal neurons, setting -70 mV as holding potential and using a Potassium Gluconate internal solution (130 mM KGluc, 10 mM KCl, 1 mM EGTA, 10 mM Hepes, 2 mM $MgCl_2$, 4 mM MgATP, 0.3 mM Tris-GTP; pH 7.4, adjusted with KOH), as previously described in (Antonucci et al, 2012). The analysis of mEPSC traces was performed using MiniAnalysis Program (Synaptosoft Inc.). Each event was selected manually setting the threshold value at 10 pA.

Event frequency and peak amplitude were taken into account. Event frequency ("frequency") is the number of events in a unit of time and is expressed in Hertz (Hz = event/sec); peak amplitude ("amplitude") is expressed in pA and is the maximum amplitude from the baseline reached by each event. Our control cultures showed an average mIPSC and mEPSC frequency of ~1,4 Hz and an average mIPSC amplitude of ~14 pA. Data shown are normalized values.

Statistical analysis

Data are presented as mean ± SEM, unless otherwise stated. 'n' indicates the number of cells, while 'N' indicates the number of independent experiments. Statistical analysis were performed using SigmaStat 3.5 (Jandel Scientific, San Jose, CA, USA) software. Data were first tested for normal distribution and variance, and the appropriate statistical test was performed accordingly to data characteristics (see Figure Legends), i.e. to test the difference between the means of two normally distributed independent variables, unpaired t-test was used; to test differences among more than two normally distributed independent variables, one-way ANOVA was used, followed by a specific post-hoc test in an all pairwise multiple comparison. Differences were considered significant when $P<0.05$, indicated by a single asterisk; $P<0.01$ is indicated by two asterisks and $P<0.001$ by three asterisks. The power of each test was calculated and taken into account.

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