

International PhD program in Neuroscience

XXX cycle

Direct neuronal and microglia-mediated effects of group I metabotropic glutamate receptors in neurodegenerative and neuroinflammatory processes

Doctorate thesis

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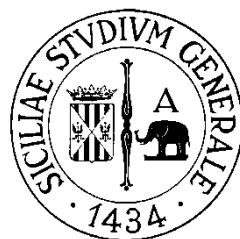


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Abstract

The group I of metabotropic glutamate (mGlu) receptor, mGlu1 and mGlu5 receptors are involved in neurodegenerative and in neuro-inflammatory processes. We have examined the role of mGlu5 receptor in a neuroinflammatory context. This receptor is expressed in glial cells and has been reported to play an anti-inflammatory role but also a pro-inflammatory effect. We were particularly involved in establishing whether mGlu5 receptor could modulate the inflammatory response in microglia, more specifically through the release of microvesicles (MVs). These have been shown to represent a more recent mode of intercellular communication and are extracellular vesicles, with a diameter of 100-1000 nm, that bud from the plasma membrane of cell of origin. MVs have been described in microglia where their formation occurs following activation of P2X7 purinergic receptors. We observed MVs development in pure microglial cultures, but, in the present study, they were more easily examined in the murine BV2 cell line. Activation of mGlu5 receptor attenuated the levels of pro-inflammatory cytokines, such as tumor necrosis factor (TNF) α , an effect that occurred only under conditions of activated microglia. Stimulation of mGlu5 increased also P2X7 receptor activation as shown by increased purinergic currents and by enhanced formation of MVs in response to the stable ATP analog, benzoyl-ATP. When MVs from mGlu5 receptor-stimulated microglia were transferred onto neurons, they increased neuronal toxicity induced by rotenone. Among possible factors released by MVs, in preliminary studies, we have identified miRNA146a as a potential mediator of this effect.

At the same time, the other member of group I mGlu receptor, mGlu1 receptor, was mainly analyzed considering its involvement in neurodegeneration. Specifically, we examined its role in an experimental setting of retinal ganglion cells (RGC) degeneration, a well-established model of several eye disorders including prematurity-induced retinal degeneration, glaucoma, and age-related macular degeneration. All these conditions are associated with excitotoxicity and it has been observed that activation of mGlu1 receptor significantly contributes to excitotoxic degeneration of RGCs. Thus, using postnatal day 9 wild type mice and knockout mice with a spontaneous recessive mutation

causing the lack of mGlu1 receptor protein (*crv4* mice), we observed that mGlu1 receptor is primarily involved in RGC excitotoxic death induced by monosodium glutamate (MSG). Treatment with the mGlu1 receptor negative allosteric modulator, JNJ16258695, was in fact protective against retinal degeneration induced by MSG. In addition, MSG treatment did not produce any toxic effect in *crv4* mice, lacking mGlu1 receptor.

Altogether these data confirm a role for mGlu1 and mGlu5 receptor in neurodegeneration and neuroinflammation, respectively and may serve as a starting point for the development of future possible pharmacological intervention targeting these receptors.

Introduction

Glutamate, the most abundant excitatory neurotransmitter in the central nervous system (CNS) acts through two distinct classes of receptors, ionotropic glutamate (iGlu) receptors and metabotropic glutamate (mGlu) receptors. iGlu receptors, namely N-methyl-D-aspartate (NMDA), α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors, are tetrameric ligand-gated ion channels and mediate fast responses to glutamate whereas mGlu receptors are G-protein coupled receptors (GPCRs) (Seeburg 1993; Pin and Duvoisin 1995; De Blasi 2001). Acting through these receptors glutamate is responsible for governing many processes in the brain such as fast and slow excitatory neurotransmission, control of basal neuronal activity and synaptic plasticity.

(Sladeczek et al., 1985; Nicoletti et al., 1986; De Blasi et al., 2001).

mGlu receptors have been traditionally associated with the regulation of synaptic transmission and recognized to play a modulatory role. This has been also due to their localization at pre- as well as post-synaptic levels and to their not central position at the synapse. In addition, several mGlu receptors are also expressed in glial cells, suggesting their potential, indirect participation to neuronal function. In the last several years, however, mGlu receptors have long been implicated in mechanisms of neurodegeneration and neuroprotection so that their involvement in several diseases, disorders and processes has been well recognized. These include pain, learning and memory, anxiety, epilepsy, drug addiction, Parkinson's disease, schizophrenia, hypoxic brain damage, excitotoxic neuronal death and efforts have been made to develop drugs targeting these receptors as efficacious treatment for several CNS disorders (Nicoletti *et al.*, 2011; Caraci *et al.*, 2012; Bruno *et al.* 2017;).

mGlu receptors structure and classification

As mentioned, mGlu receptors are GPCRs so they consist of an extracellular N-terminal domain, seven transmembrane-spanning domains, and an intracellular C-terminus. From a structural point of view, mGlu receptors are composed by a large extracellular N-terminal domain, also named Venus Flytrap domain (VFD) which contains two lobes able to create a clam shell-like structure. N-terminal

domain is characterized by the orthosteric binding site for glutamate. The two lobes responsible for the shell-like structure in this domain tend to close when the linkage between glutamate and its binding site occurs in VFD (Kunishima *et al.*, 2000; Kniazeff *et al.*, 2004; Muto *et al.*, 2007). At this level, all mGlu receptors constitutively can form stable dimers through a disulfide bond (Muto *et al.*, 2007). Further, by the presence of a cysteine-rich domain, it is possible to form an association between the VFD and the seven transmembrane-spanning domain modulating the signal transduction from the GPCRs (Kniazeff *et al.*, 2004; Doumazane *et al.*, 2013; Vafabakhsh *et al.*, 2015). Interestingly, the orthosteric binding site is characterized by a high level of conservation and this is the reason why mGlu receptors can be bound by more than one ligand (reviewed in: (O'Brien and Conn, 2015).

Several mGlu receptor selective ligands have been developed acting either at orthosteric or at allosteric sites, thus providing drugs with a modulatory function. Hence, positive allosteric modulators (PAM), able to potentiate the response of an orthosteric ligand, and negative allosteric modulators (NAM), or non-competitive antagonists, which induce the inhibition or a reduction of the response of an orthosteric ligand are now available (Gregory and Conn, 2015)

Through high affinity binding of NAMs and X-ray crystallography, 3D structures of the seven transmembrane-domain of the mGlu1 (Wu *et al.*, 2014) and mGlu5 (Doré *et al.*, 2014) receptors, it was possible to observe in more detail the allosteric binding region in the seven transmembrane domain. This finding provides an interesting platform for the development of new mGlu receptor ligands with more specific pharmacological profile and efficiency (Harpsoe *et al.*, 2015).

mGlu receptors form a family of eight subtypes (mGlu1 to -8), subdivided into three groups on the basis of amino acid sequence, pharmacological profile and transduction pathways (Table 1). They can be activated as individual, monomeric receptors but they also exist as dimers. In this case the formation of di-sulphide linkages between the N-terminal domains of the two individual receptors takes place (Romano *et al.*, 1996).

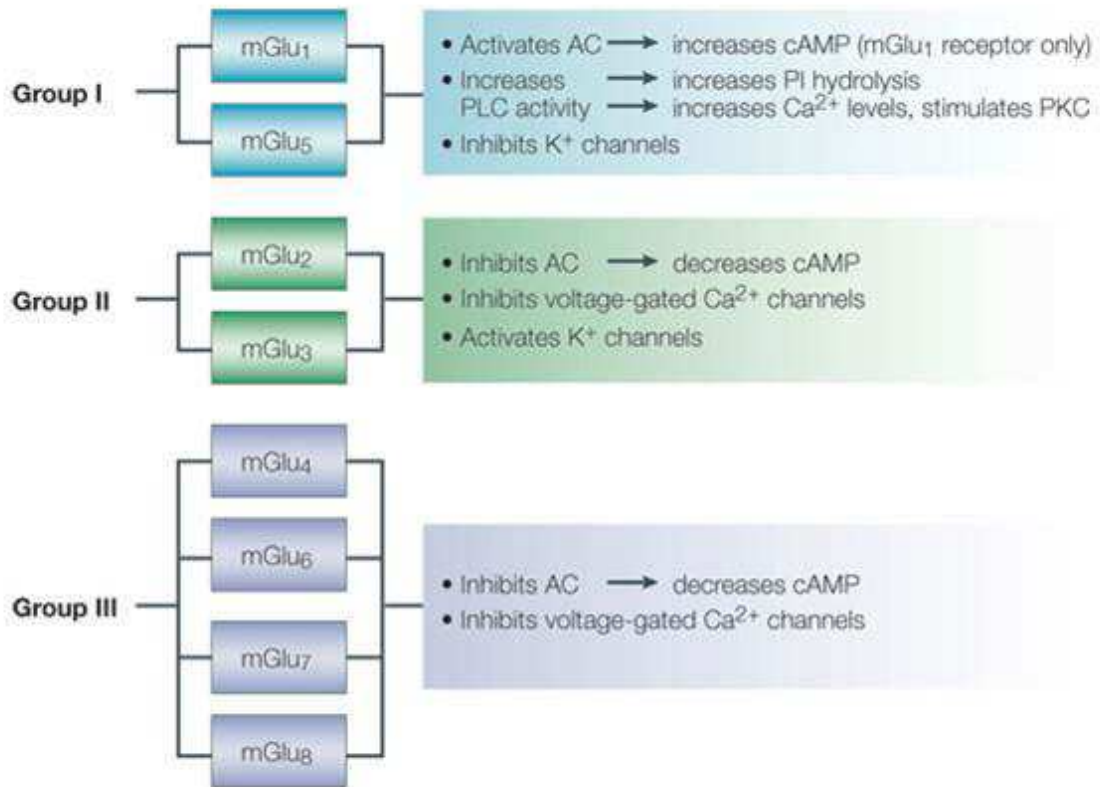


Table 1. mGlu receptor family includes eight subtypes (mGlu1 to -8), subdivided into three groups on the basis of amino acid sequence, pharmacological profile and transduction pathways (From Zhong et al., 2006; Nature Review).

Group I mGlu receptors

Group I includes mGlu1 and mGlu5 receptors, which are coupled to Gq/G11. These receptor subtypes are preferentially localized in the peripheral portion of dendritic spines (whereas all other mGlu receptor subtypes, with the exception of mGlu6 receptor, are preferentially localized in axon terminals where they negatively regulate glutamate release). In particular, high levels of mGlu5 receptor are found in forebrain regions such as the cerebral cortex, dorsal and ventral striatum (including the nucleus accumbens, olfactory bulb and tubercle, lateral septum, and hippocampus). (Romano *et al.*, 1993). In contrast, expression levels of mGlu1 receptor in these areas are relatively low, with the exception of the hippocampus where it is widely expressed primarily in the CA3 region and dentate gyrus. The cerebellum, thalamus, hypothalamus, and pallidum represent regions in which it is

possible to observe high expression levels of mGlu1 and low levels of mGlu5 receptor expression. (Shigemoto *et al.*, 1992).

Activation of the group I of mGlu receptors leads to the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PtdIns-4,5-P₂) with ensuing formation of inositol-1,4,5-trisphosphate (InsP₃) and diacylglycerol (DAG). InsP₃ determines the release of Ca²⁺ from intracellular stores whereas DAG is able to activate all isotypes of protein kinase C (PKC). The C-terminus domain of mGlu1 and mGlu5 receptors are associated, through homer proteins, to several membrane and intracellular proteins, such as signaling proteins (e.g., phospholipase-C β 3, and the PtdIns-3-kinase enhancer, PIKE-L) and ion channels (e.g., NMDA receptors, InsP₃ receptors, and TrpC6 channels) (Brakeman *et al.*, 1997). PIKE-L links mGlu1 and mGlu5 receptors to the activation of the PtdIns-3-kinase pathway, which is able to promote neuronal survival by inhibiting glycogen synthase kinase-3 β (GSK3 β) (Brazil and Hemmings, 2001). Although mGlu1 and mGlu5 receptors are in some cases involved in the same signal transduction mechanisms, they can exert very different behaviors in the context of brain injury. In fact mGlu1 receptor agonists are able to induce an increase of necrotic cell death (Allen *et al.*, 2000), whereas selective antagonists of the same receptors tend to exert a neuroprotective effect in vitro and in vivo (Faden *et al.*, 2001; Fei *et al.*, 2006; Szydłowska *et al.*, 2007). In contrast, stimulation of mGlu5 receptor has been shown to cause inhibition of caspase dependent neuronal apoptosis in cell culture models (Movsesyan *et al.*, 2004). (S)-3,5-Dihydroxyphenylglycine (DHPG) is the first agonist which showed a specific selectivity for group I of mGlu receptors. It can be considered a potent agonist for both mGlu1 and mGlu5 receptors (Wiśniewski *et al.*, 2002). In particular, DHPG exerts its agonist activity exclusively in the S-isomer. Some studies report that, under certain conditions, DHPG may also create an interaction with NMDA receptors. DHPG promotes PtdIns-4,5-P₂ hydrolysis in a dose-dependent manner either in adult and neonate hippocampus, decreases levels of cAMP in the adult and enhances it in the neonate. DHPG can induce an increase of [Ca²⁺]_i regulating multiple subtypes of Ca²⁺ channels and, being involved in a series of mechanisms, it can regulate the release of neurotransmitters. In

particular, based on the concentrations, DHPG can amplify or attenuate excitatory postsynaptic potentials (EPSPs) and it may induce long-term depression (LTD) and long-term potentiation (LTP) (Bear *et al.*, 1996).

(RS)-2-chloro-5-hydroxyphenylglycine (CHPG), an analog of DHPG is considered a selective agonist of mGlu5 receptor. The use of PAM allows receptor activation in a more physiological way determining less receptor desensitization and/or tolerance than classical agonists. PAMs for mGlu 1 receptor were developed (Knoflach *et al.*, 2001; Wichmann *et al.*, 2002): S)-2-(4-fluoro-phenyl)-1-(toluene-4-sulfonyl)-pyrrolidine (Ro 67-7476), diphenylacetyl-carbamic acid ethyl ester (Ro 01-6128) and (9H-xanthene-9-carbonyl)-carbamic acid butyl ester (Ro 67-4853). Specifically these compounds do not cause an intrinsic agonist activity but significantly promote an agonist-induced response (Knoflach *et al.*, 2001). The first positive allosteric modulators studied for mGlu5 receptor are 3,3'-difluorobenzaldazine (DFB) (O'Brien *et al.*, 2003) and *N*-{4-chloro-2-[(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)methyl]phenyl}-2-hydroxybenzamide (CPPHA) (O'Brien *et al.*, 2004). DFB, as well as CPPHA, do not exhibit an intrinsic agonist activity but are able to amplify agonist responses. 7-Hydroxyiminocyclopropan[*b*]chromen-1*a*-carboxylic acid ethyl ester (CPCCOEt) can be defined as the prototypic non-competitive mGlu1 receptor antagonist (Ott *et al.*, 2000). In particular, it acts selectively on mGlu1 receptor by inhibiting receptor activation non-competitively from a binding pocket into the transmembrane heptahelical domain. Since the discovery of CPCCOEt, (Hermans *et al.*, 1998; Litschig *et al.*, 1999), various selective NAMs for mGlu1 receptor were synthesized including (3*a*S,6*a*S)-6*a*-Naphthalen-2-ylmethyl-5-methyliden-hexahydro-cyclopental[*c*]furan-1-on (BAY36-7620), 1-(3,4-dihydro-2*H*-pyrano[2,3-*b*]quinolin-7-yl)-2-phenyl-1-ethanone (R214127) (Lavreysen *et al.*, 2003), 1-ethyl-2-methyl-6-oxo-4-(1,2,4,5-tetrahydro-benzo[*d*]azepin-3-yl)-1,6-dihydro-pyrimidine-5-carbonitrile (EM-TBPC) (Malherbe *et al.*, 2003a). Regarding negative modulators for mGlu5 receptor, 6-Methyl-2-(phenylazo)-3-pyridinol (SIB-1757) and (*E*)-2-methyl-6-(2-phenylethenyl)pyridine (SIB-1893) can be considered (Varney *et al.*, 1999). These led to the identification of 2-methyl-6-(phenylethynyl)-pyridine (MPEP), which can be

considered a more potent ligand (Gasparini *et al.*, 1999) and 3-[2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine (MTEP) which shows a better selectivity and CNS bioavailability (Cosford *et al.*, 2003b).

Group II mGlu receptors

Group II includes mGlu2 and mGlu3 receptors which are coupled to Gi/Go proteins. Their activation reduces intracellular cAMP formation and inhibits voltage-sensitive calcium channels. mGlu2 and mGlu3 receptors exhibit high homology and are predominantly expressed as heterodimers on presynaptic membranes of glutamatergic terminals where they negatively regulate the release of glutamate. However, localization of mGlu2 and -3 receptors in peripheral regions of the synapse has also been reported. Since these receptors are predominantly found as heterodimers, they are often indicated as mGlu2/3 receptors, although homodimers of each receptor subtype have been identified (Schoepp *et al.*, 2001; Tamaru *et al.*, 2001). mGlu2 and mGlu3 receptors are found in moderate to high abundance in the olfactory bulb, cerebral cortex, septal region, hippocampus, dorsal and ventral striatum, amygdala, and cerebellum (Ohishi *et al.*, 1993). Several agonists for mGlu2/3 receptors have been developed including (2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine (DCGIV); 1-carboxycyclopropyl-glycine (L-CCG-I); (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate (2R,4R-APDC); LY379268 and LY566332. Through some preliminary studies it has been discovered that Ro 67-6221 and Ro 71-8218 may exert an antagonist activity also for the group II of mGlu receptors. Moreover LY341495 and MGS 0039 are drugs that act as a selective antagonist blocking either mGlu2 and mGlu3 receptors. A series of mGlu2 receptor PAMs have been described (Johnson *et al.*, 2003) including LY487379 which causes an amplification of glutamate potency (Schaffhauser *et al.*, 2003; Koike *et al.*, 2011) whereas RO4491533 acts as a negative allosteric modulator of either mGlu2 and mGlu3 receptors (Campo *et al.*, 2011).

Group III mGlu receptors

Group III includes mGlu4, mGlu6, mGlu7 and mGlu8 receptors, which are also coupled to Gi/Go proteins. The expression levels of Group III receptors in the brain are more limited than those of group I and II mGlu receptors. mGlu6 receptor expression is confined to the retina whereas mGlu4 receptors have a limited distribution in the CNS, being mostly expressed on presynaptic terminals at high levels in the cerebellum, basal ganglia, thalamus, and hippocampus (Nakajima *et al.*, 1993); Corti *et al.*, 2002). In contrast, mGlu7 receptors are expressed much more widely throughout the CNS including the cerebral cortex, striatum, hippocampus, hypothalamus, and cerebellum, where they are predominantly located to presynaptic terminals. Finally, mGlu8 receptors have a restricted distribution in the brain, primarily being confined to olfactory bulb, piriform cortex, hippocampus and thalamus (Kinzie *et al.*, 1995; Saugstad *et al.*, 1997). L-AP4 is an orthosteric, highly selective agonist of group III mGlu receptors. Orthosteric antagonists have been developed that show high selectivity but low potency (Niswender, 2016). More interesting has been the development of PAMs and NAMs for these receptors. (-)-N-Phenyl-7-(hydroximino)cyclopropa[b]chromen-1a-carboxamide ((-)-PHCCC), an analogue of the mGluR1 antagonist CPCCOEt, has been described as a PAM of mGluR4 (Maj *et al.*, 2003; Marino *et al.*, 2003). The antagonists of mGlu5 receptor, SIB-1893 and MPEP, behave as positive allosteric modulator for mGlu4 receptor (Mathiesen *et al.*, 2003). In contrast, some mGlu5 receptor PAMs including DFB and CPPHA exert an antagonistic activity for mGlu4 and mGlu8 receptors (O'Brien *et al.*, 2003, 2004) but it is not yet clear the mechanism through which a competitive or non-competitive interaction is possible. Finally, VU0155041 and VU0080421 have been identified as structurally distinct mGluR4 PAMs (reviewed in Niswender and Conn, 2010).

Role of mGlu1 and mGlu5 receptors in neuronal damage

As mentioned above, both mGlu1 and mGlu5 receptors are found in the extrasynaptic portion of dendritic spines. In this region they can link NMDA receptors via a chain of scaffolding proteins which include the long isoforms of Homer, Shank, and PSD95 (Tu *et al.*, 1999). Specifically, mGlu5

receptors support the activation of NMDA receptors by relieving the Mg^{2+} blockade of the NMDA-gated ion channel or through other mechanisms (Doherty *et al.*, 1997; Attucci *et al.*, 2001; Mannaioni *et al.*, 2001; Pisani *et al.*, 2001). In this case it is legitimate to believe that pharmacological stimulation of mGlu1 and mGlu5 receptors determines neuronal cell damage by promoting the activation of NMDA receptors. However, the biological effects of these receptors are more complex. For this reason, the specific role of mGlu1 and mGlu5 receptors in mechanisms of neurodegeneration and neuroprotection has long been debated (Nicoletti *et al.*, 1999; Bruno *et al.*, 2001). Activation of mGlu1/5 receptors with DHPG or other orthosteric agonists may either increase or diminish excitotoxic neuronal death depending on several factors such as in vitro or in vivo models of neurodegeneration, nature of the toxic insult and functional state of the two receptors (reviewed by Nicoletti *et al.*, 1999). For example, in mixed cortical cultures (one of the most widely used in vitro models for the study of excitotoxicity) DHPG induces an amplification of NMDA toxicity when applied only once (either before or during the NMDA pulse), but it is also able to exert a neuroprotective effect when applied for the second time after a brief pre-exposure; this situation may be due to an activity-dependent switch in receptor signaling from the stimulation of PtdIns-4,5-P2 hydrolysis to the inhibition of voltage-sensitive Ca^{2+} channels (Herrero *et al.*, 1998; Bruno *et al.*, 2001). This dual role of group-I mGlu receptors has been confirmed in several studies. Thus, it has been shown that mGlu1 receptors can both stimulate intracellular Ca^{2+} release and activate the PI3K pathway only if the C-terminus domain is intact. A strong increase in intracellular Ca^{2+} levels, for example in the presence of a toxic stimulation of NMDA receptors, can in fact lead to a calpain-mediated cleavage of the C-terminal portion of the mGlu1 receptor, hindering the receptor from activating the PI3K pathway but, at the same time, allowing the stimulation of PtdIns-4,5-P2 hydrolysis. All this can provoke an imbalance in favor of the neurotoxic signal which results into neuronal death (Xu *et al.*, 2007).

A series of studies have shown that mGlu1 receptors may also interact with membrane estrogen receptors (ERs) in hypothalamic neurons, and this interaction is critical for the regulation of the

estrous cycle (Micevych and Mermelstein, 2008; Mermelstein, 2009). It has been observed that the interaction between mGlu1 receptors and membrane ERs occurs also in cortical neurons, where the protective effect of 17 β -estradiol against β -amyloid toxicity is prevented by pharmacological blockade of mGlu1 receptors, and the protective activity of DHPG is prevented by pharmacological blockade of ER α (Spampinato *et al.*, 2012). In contrast, interaction between the two receptors mediates an exacerbation of neurotoxicity, when stimulation of one or the other occurs after the toxic insult, such as that induced by a high concentration of NMDA (Spampinato 2012b). Taken together, these findings suggest that the precise role of mGlu1 and mGlu5 receptors in mechanisms of neurodegeneration and neuroprotection is very complex and depends heavily on the specific context. However, unlike the dual effect of receptor agonists, mGlu1 and mGlu5 receptor antagonists or NAMs are more consistently neuroprotective independently of the context and the nature of the toxic insult. For example, in *in vitro* and *in vivo* studies it has been shown that selective mGlu1 receptor antagonists exert a neuroprotective role by stimulating GABA release (Battaglia *et al.*, 2001; Cozzi *et al.*, 2002). It was also possible to observe a substantial protective action of mGlu1 receptor antagonists against hypoxic and ischemic neuronal damage (De Vry *et al.*, 2001; Cozzi *et al.*, 2002; Meli *et al.*, 2002; Moroni *et al.*, 2002; Pellegrini-Giampietro, 2003; Makarewicz *et al.*, 2006; Kohara *et al.*, 2008; Murotomi *et al.*, 2008, 2010). More recent studies show more specifically the mechanism through which mGlu1 receptor antagonists exert a protective effect by means of the release of GABA. Activation of mGlu1 receptors stimulates PtsIns-4,5-P2 hydrolysis with ensuing formation of DAG. This one is in turn converted by DAG lipase into the endocannabinoid, 2-arachidonylglycerol (2-AG), which diffuses back to presynaptic terminals and activates type-1 cannabinoid (CB1) receptors, thereby inhibiting GABA release. So, mGlu1 receptor blockade could attenuate the amount of 2-AG produced during the development of ischemic damage (reviewed by Landucci *et al.*, 2009). mGlu1 and mGlu5 receptors have also been shown to be involved in mechanisms of ischemic tolerance. Indeed, in organotypic hippocampal slices subjected to a protocol for "ischemic preconditioning", neuroprotection induced by a non-lethal 10-min pre-exposure to oxygen/glucose deprivation (OGD)

was canceled by treatment with mGlu1, but not mGlu5, receptor antagonists. DHPG pre-treatment exerted also a protective effect against neuronal damage induced by a subsequent lethal exposure to OGD (pharmacological pre-conditioning) (Werner *et al.*, 2007). In contrast, when hippocampal slices were subjected to a protocol of 'ischemic post-conditioning', in which a brief (3 min) period of OGD was delivered 5 min after a lethal episode of OGD, neuroprotection was abrogated by both mGlu1 and mGlu5 receptor antagonists. Stimulation of mGlu1 and mGlu5 receptors in the protocol of ischemic post-conditioning leads to neuroprotection through the activation of PI3K/Akt/GSK3 β pathway (Scartabelli *et al.*, 2008). Pharmacological blockade of mGlu5 receptors could induce a reduction of neuronal death in models of chronic neurodegenerative disorders, such as Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS). Indeed, higher levels of mGlu5 receptor immunoreactivity have been observed in the putamen of PD patients, and in the frontal cortex, hippocampus and putamen of patients with dementia with Lewy bodies, a disorder that has in common with PD the presence of intracellular aggregates of α -synuclein (Price *et al.*, 2010). Increased levels of mGlu5 receptors have been also found in α -synuclein transgenic mice. Moreover, in mice and monkeys subjected to parkinsonian neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), systemic exposure to mGlu5-receptor NAMs (MPEP and MTEP, respectively) exert a protective activity in nigro-striatal neuron against degeneration (Battaglia *et al.*, 2004; Masilamoni *et al.*, 2011). Treatment with MTEP also promotes an attenuation of MPTP-induced damage of noradrenergic neurons in the locus coeruleus of monkeys (Masilamoni *et al.*, 2011). Pharmacological blockade or genetic deletion of mGlu5 receptors has also a protective action against 6-hydroxydopamine lesions of the nigro-striatal dopaminergic pathway (Armentero *et al.*, 2006; Black *et al.*, 2010). On these bases, it has been suggested that treatment with mGlu5 receptor NAMs in PD patients is important to both attenuate motor symptoms and to reduce nigro-striatal degeneration.

mGlu receptors are not only expressed in neurons, but also in glial cells and to get a clearer view on the role of mGlu5 receptors in mechanisms of neurodegeneration and neuroprotection attention has

been paid to the cross-talk between neurons and astrocytes and the role of these receptors. Specifically, mGlu5 receptors are present in astrocytes and their expression in astrocyte cultures tend to increase when cells acquire a reactive-like phenotype. Interestingly, pharmacological blockade of mGlu5 receptors reduces excitotoxic death of spinal motor neuron grown in culture medium from enriched reactive astrocytes (D'Antoni *et al.*, 2011). Moreover, pharmacological blockade of these receptors delays the onset of motor symptoms in mice which express a mutated form of human superoxide dismutase, an animal model of familial ALS (Rossi *et al.*, 2008).

Retina and neurodegenerative processes

The retina can be defined as the sensory membrane of the eye which allows the vision through the reception of images by photoreceptors and that are then conveyed to the brain. The retina has a very complex organization, in fact it contains several neuronal cell types and interneurons (Hageman *et al.*, 1991). It is characterized by three layers of nerve cell bodies (the outer nuclear layer composed by cell bodies of the rods and cones, the inner nuclear layer with cell bodies of the bipolar, horizontal and amacrine cells and the ganglion cell (RGC) layer that contains cell bodies of RGCs and displaced amacrine cells (Fig. 1). Two layers of synapses are also present (Kolb *et al.*, 1991). Neurodegenerative processes of retinal cells are at the basis of several eye diseases including glaucoma (Sathyamangalam *et al.*, 2009), age-related macular degeneration (Shams Najafabadi *et al.*, 2017), retinitis pigmentosa (Bennis *et al.*, 2017), diabetic retinopathy (Jackson and Barber, 2010).

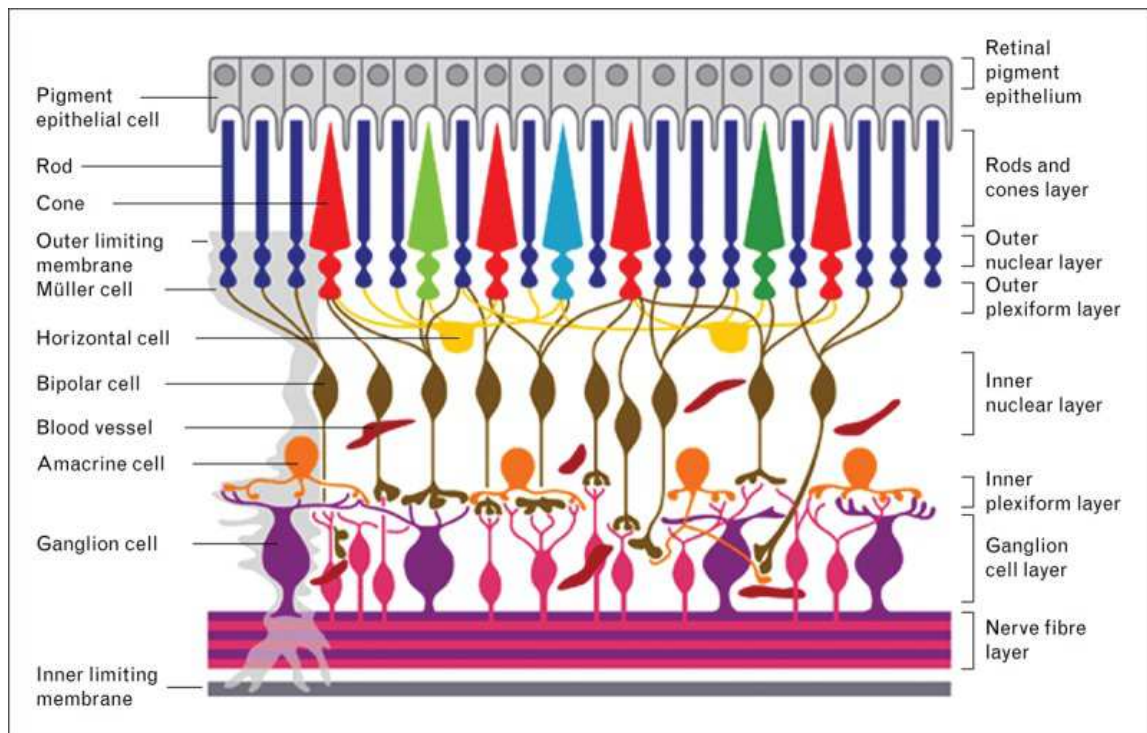


Fig. 1 Schematic representation of retinal organization: three layers of neural cells characterized by photoreceptor cells, bipolar cells, and ganglion cells, and a layer of pigmented epithelial cells (from Lorber et al., Curr Opin Ophthalmol 2016).

Within the retina mGlu1 and -5 receptors are present in the dendrites of ON-bipolar cells and in amacrine cells, but also in RGCs (Koulen *et al.*, 1997; Brandstätter *et al.*, 1998; Yang *et al.*, 2004). However, stimulation of PtdIns-4,5-P2 hydrolysis in RGCs occurs only when mGlu1, but not mGlu5, receptor is activated (Romano *et al.*, 2016). The increase of intracellular Ca^{2+} induced by mGlu1 receptor activation and its ability to modulate NMDA receptor function suggest a potential involvement of this receptor in excitotoxicity of RGCs.

Neurodegeneration and neuroinflammation

Growing evidence has clearly established that neurodegeneration is not merely a disease process that affects neurons, but that the surrounding environment plays a main role, taking an active part to neuronal fate with a complex event generally known as neuroinflammation. This may precede or accompany the neurodegenerative event and involves mainly glial cells but also peripheral cells of the immune system. Among glial cells, the most important role in neuroinflammation is played by microglia.

Microglia

Microglia are the immune cells of the CNS so their primary function is to maintain brain tissue homeostasis to provide the first line of defense during infection or brain injury and to promote tissue repair. Under normal conditions in the brain, microglia is generally named the 'resting' or 'surveillant' microglia (Nimmerjahn *et al.*, 2005; Kettenmann *et al.*, 2011). In this case microglial cells show a ramified morphology containing long and thin processes which allow the cells to communicate with surrounding neurons and other glial cells and continuously control the microenvironment to exert a guard function for incoming pathogens and brain alterations. When microglial cells detect a brain insult they act as inflammatory effector cell types and respond to pathogens and injury by becoming "activated". Specifically, in response to many types of alarm signals (cytokines, material from apoptotic cells and exogenous viral factors) surveillant microglia rapidly undergo a morphological change assuming an ameboid shape, proliferate and migrate to the site of infection or injury in which they phagocyte and destroy pathogens as well as remove damaged cells and protein aggregates, and provide the secretion of cytokines, chemokines, prostaglandins, nitric oxide and reactive oxygen species, which help to increase and govern the immune response. However, microglia are also involved in the resolution of the inflammatory response through the production of anti-inflammatory cytokines such as interleukin (IL)-10. Thus, depending upon the nature of stimuli, the entity of activation and the duration of environmental signals,

microglial cells acquire several phenotypes and can undergo a classical pro-inflammatory activation transforming into fully activated inflammatory effector cells, or an alternative activation, generally associated with tissue repair. These two states of microglia are commonly indicated as M1 (pro-inflammatory) and M2 (pro-regenerative) phenotypes (Mantovani and Locati, 2009; David and Kroner, 2011; Saijo and Glass, 2011; Brites and Vaz, 2014) The M2 phenotype inhibits inflammation and acts to restore homeostasis. This phenotype may be associated with the production of anti-inflammatory cytokines and trophic factors (Colton, 2009) and with phagocytosis and suppression of the innate immune response (Brites and Vaz, 2014). In contrast, the M1 phenotype is associated with the production of IL-1 β , tumor necrosis factor (TNF) α , interferon (IFN) γ , and inducible nitric oxide synthase (NOS) (Fig.2). Actually the concept of good and bad microglia is a bit inaccurate as microglia switch between these phenotypes and there are many intermediate states in which microglial cells may exist. Microglia have also been extensively studied for their harmful roles in neurodegenerative diseases and brain injuries, such as Alzheimer's disease (AD), Parkinson's disease, ischemic injury, and traumatic brain injuries (TBI) (Tuttolomondo *et al.*, 2008).

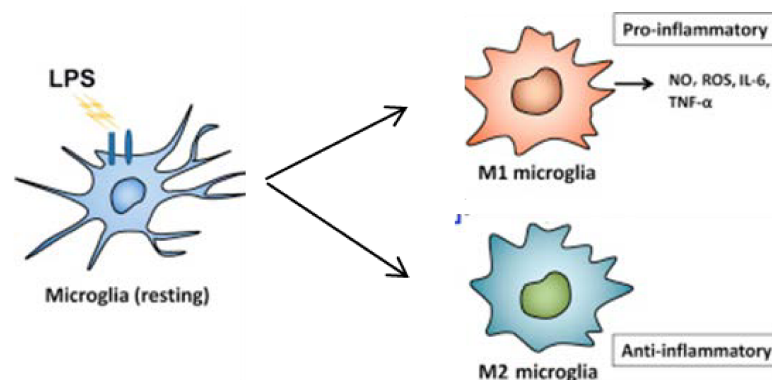


Fig. 2. Activation of resting microglia by inflammatory stimuli and its subsequent morphological and functional change leading to two possible states of microglia indicated as M1 (pro-inflammatory) and M2 (pro-regenerative) phenotypes.

In the latter case, the distinction between the M1 and M2 phenotype is not so clear. TBI determines in fact a wide range of responses both at pro-inflammatory and anti-inflammatory level so that after TBI a concomitant expression of pro- and anti-inflammatory genes can be observed. Interestingly, this microglial oscillatory profile between M1 and M2, has also been observed in LPS-stimulated conditions and ALS models of neuro-inflammation and spinal cord injury (Chiu *et al.*, 2013). Therefore, microglia as well as macrophages do not exclusively assume an M1 or M2 phenotype but display a mixed phenotype likely due to the complex signaling events surrounding them (Morganti *et al.*, 2016).

mGlu receptors in neuroinflammation

mGlu receptors are expressed in glial cells and a role for these receptors in neuroinflammation has also been considered. Accordingly, mGlu receptors have been identified as potential targets for the treatment of MS, the prototype of CNS disease with a prominent neuroinflammatory component. An overexpression of different subtypes of mGluRs in the brain of MS patients has been observed. mGlu1 receptors show in fact an enhanced axonal immunoreactivity and are expressed also in reactive microglia and astrocytes. Similarly, mGlu2/3 receptors are markedly overexpressed in astrocytes in acute and chronic active MS lesions and mGlu5 receptor expression is enhanced in reactive astrocytes. mGlu4 receptor also is overexpressed at the rim of chronic active lesions and a role for this receptor subtype has been characterized in detail using an animal model of multiple sclerosis, the experimental autoimmune encephalomyelitis (EAE) model, obtained in mice after immunization with the oligodendrocyte protein, Myelin Oligodendrocyte Glycoprotein (MOG)-35-55. The absence of the mGlu4 receptor enhances the severity of EAE, whereas treatment with a selective mGlu4 receptor enhancer is protective against EAE. This activity has been ascribed to a peripheral role of mGlu4 receptors on cells of the immune system, dendritic cells (DCs) and T lymphocytes. The activation of mGlu4 receptors is in fact able to modify the differentiation of naive

T cells towards the immune tolerant Treg cells, with a concomitant reduction of autoreactive TH17 cells. Interestingly, other mGlu receptors are expressed in cells of the immune system and may play a regulatory role as well. The protective effect mediated by mGlu4 receptor, however, are not exclusively due to immune cells as mGlu4 receptors are also expressed in glial cells and their activation protects oligodendrocytes against excitotoxicity (Spampinato *et al.*, 2015).

Looking more specifically to group I mGlu receptors, it has been reported that mGlu5 receptor stimulation with the selective agonist, CHPG, causes a reduction of microglial activation and a decrease of associated pro-inflammatory factors release in vitro, which is mediated in part through inhibition of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. These effects of delayed CHPG administration were associated with reduction of chronic neuroinflammation and ensuing neurodegeneration after experimental traumatic brain injury in mice. (Byrnes *et al.*, 2009, 2009b).

In microglial cells, the selective mGlu5 selective agonist, CHPG, has been reported to reduce the production TNF α induced by LPS. On the other hand, a correlation between LPS and mGlu5 receptor has been suggested. LPS in microglial cells can in fact induce [Ca²⁺] oscillations in a Toll-like receptor 4 (TLR4)-independent manner, an effect sensitive to blockade by the mGlu5 receptor antagonist MTEP. These observations, together with immunocytochemistry showing that LPS co-localizes with mGlu5 receptor, suggest that mGlu5 receptor may mediate a proinflammatory action in microglia and that it may represent a novel target for modulating microglia-dependent neuroinflammation. (Byrnes *et al.*, 2012; Wang *et al.*, 2012, 2013; Liu *et al.*, 2014).

Cell communication through extracellular vesicles

Brain functions depend on coordinated interactions between neurons and glial cells that include microglia, astrocytes, and oligodendrocytes. These cells, either in normal or pathological condition, are able to release extracellular vesicles (EVs) (Chivet *et al.*, 2014) which represent an important mode of intercellular communication. In fact they may represent a vehicle for transfer and delivery

of membrane and cytosolic proteins, lipids, mRNAs, and microRNAs (miRNAs) between cells (Lin *et al.*, 2015). Formation of EVs is not restricted to CNS as they can be released by several cell types and are present in many biological fluids including blood, urine, saliva, amniotic fluid, breast milk and medium of cell cultures (Théry *et al.*, 2002, 2006). EVs include exosomes and microvesicles (Fig 3).

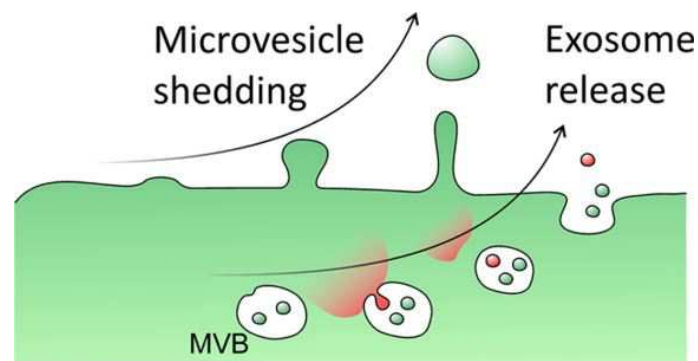


Fig. 3. Differences in the formation of microvesicles and exosomes.

Exosomes

Exosomes are endocytic membrane-derived vesicles with a diameter between 30–100 nm. These small vesicles are contained in multivesicular bodies (MVBs) within the endosomal system and then they are secreted through MVB fusion with the plasma membrane (Prada *et al.*, 2013). Although MVB fusion with the plasma membrane has not been described in detail yet, this process seems to involve several Rab GTPases, such as iRab5, Rab27, and Rab35 (Shifrin *et al.*, 2013; Yuyama *et al.*, 2012; Mittelbrunn *et al.*, 2011). Exosomes are associated with a lot of cellular components among which messenger RNAs (mRNAs), microRNAs (miRNAs) and proteins. Exosomes derived from microglial cells express specific markers including major histocompatibility complexes (MHCs) class

II molecules, enzymes (cathepsin S) and integrins, which are involved in antigen presentation and pattern recognition receptors during innate immune response. Moreover exosomes are packed with tetraspanins, a family of transmembrane proteins including CD9, CD14, CD63 and CD81, which are particularly enriched in exosomes derived from microglia. Further, in microglia-derived exosomes, there is a high expression of lactate monocarboxylate transporter 1MCT-1 and CD13 (the surface-bound aminopeptidase which degrades enkephalins). Extrusion of Na, K-ATPase is also frequent in exosomes derived from several populations of cells. Stressful events in astrocytes, like heat or oxidative stress, were shown to promote the release of exosomes carrying the heat shock protein 70 (Hsp70) and synapsin 1 with ensuing pro-survival effect on neurons (Taylor *et al.*, 2007). In addition exosomes may contain a distinct set of proteins, such as cytoskeletal proteins and the glycolytic protein glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Microvesicles

Microvesicles (MVs) are vesicles larger than exosomes (100–1000 nm) which bud directly from the plasma membrane of donor cells (Turola *et al.*, 2012). Shedding of MVs usually involves a budding process in which surface blebs selectively accumulate cellular constituents that are packaged into MVs (They *et al.*, 2009). These vesicles present a lot of cell surface receptors, intracellular signaling proteins and genetic materials derived from the cell of origin. Because MVs originate from different cell types, they are characterized by a different molecular profile which in turn represents the mirror of the differential proteins expression of various cells of origin. The heterogeneity of composition and biological activity of MVs is dependent on the state (resting or stimulated) of donor cells and on the agent employed for stimulation (Bernimoulin *et al.*, 2009).

Blebbing of MVs can be induced by purinergic P2X7 receptor activation. Indeed a specialized type of MVs shedding has been found in cells which typically express the ATP receptor P2X7. P2X7 receptor is an ATP-gated ion channel highly expressed in immune cells. It is characterized by a long cytoplasmic C-terminus in which there are a lot of protein-protein interaction motifs. Dramatic

morphological changes occur in cells expressing P2X7 receptor during and subsequent to receptor stimulation. In particular these changes consist in rapid formation of membrane blebs associated to several intracellular signaling events such as the activation of protein kinases and other effector enzymes (Duan and Neary, 2006). In particular P2X7-induced blebbing is dependent upon p38 and ROCK activation. This process leads to local disassembly of cytoskeletal elements associated with the P2X7 C-terminus (Budagian *et al.*, 2003; Morelli *et al.*, 2003; Verhoef *et al.*, 2003). Surface blebbing is preceded by loss of plasma membrane symmetry and exposure of phosphatidylserine (PS) at the outer leaflet of the plasma membrane, a process that occurs within the first few minutes of P2X7 receptor activation and is reversible (Hugel *et al.*, 2005). Acidic sphingomyelinase (A-SMase) is also involved in MVs biogenesis. This enzyme hydrolyzes sphingomyelin (SM) to the sphingolipid ceramide. After P2X7 stimulation, p38MAP kinase activation determines translocation of A-SMASE to the plasma membrane outer leaflet in which ceramide is formed. MVs budding can be caused by reorganization of ceramide in the bilayer and by local accumulation of the cone-shaped sphingolipid into the inner leaflet of the membrane. Thanks to its natural negative curvature, ceramide can promote the formation of subdomains with curvature different from the adjacent planar membrane. Further, hydrolysis of SM, which is known to present a considerable affinity for cholesterol could determine an increase of the efflux of cholesterol. This is considered the main factor implicated in membrane fluidity and structural integrity of the plasma membrane. This condition can contribute to membrane destabilization and promote the process of blebbing and MVs secretion (Bianco *et al.*, 2009).

The expression of P2X7 receptor is evident in macrophages (Steinberg *et al.*, 1987), mast cells (Cockcroft and Gomperts, 1979) and microglia (Visentin and Levi, 1997), where it controls the release of inflammatory cytokines, including IL-1 β and IL-18 (Ferrari *et al.*, 2006). This is accompanied by assembly of inflammasome which represents the protein complex able to activate IL-1 β processing enzyme, caspase 1. In this way a rapid cytokine secretion occurs (Bianco *et al.*, 2009). MVs blebbing occurs in reactive microglia (Fig. 4), for example following exposure to

LPS, and cytokines can be released into the environment in a ATP-dependent manner (Bianco *et al.*, 2005).

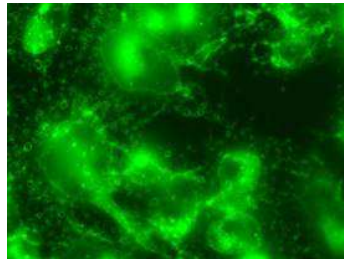


Fig. 4. MVs shed from cultured primary microglia from rat cerebral cortex as shown by labelling with the fluorescent dye FM1-43 (Beneventano *et al.*, 2017).

However, sustained P2X7 receptor activation can also be responsible for cell death. In contrast, several studies have shown that shedding of MVs from microglia, is not only induced by stimulation of P2X7 receptor through exogenous ATP but also by ATP endogenously released from healthy astrocytes in astrocyte-microglia co-cultures. Hence, although ATP is generally considered an alarm signal, these studies suggest that MVs can be released from microglia even in normal conditions, in the absence of cellular damage.

MVs as containers of bioactive molecules modulating cell functionality

One of the reasons for increasing interest on MVs is because the cells have several advantages when release cytokines through MVs rather than exporting these molecules directly. For example, secreted MVs containing IL-1 β , can deliver the cytokine at considerable distance from the cell of origin, thus avoiding dispersal, degradation and dilution of the cytokine in the extracellular space. In addition, several different factors can be contained in MVs, so that biogenesis of MVs represent an important mechanism to mediate the assembly of multiple factors regulating biological responses. Microglia-

released MVs influence brain cell functions, by propagating inflammation and causing neurodegeneration (Fig. 5).

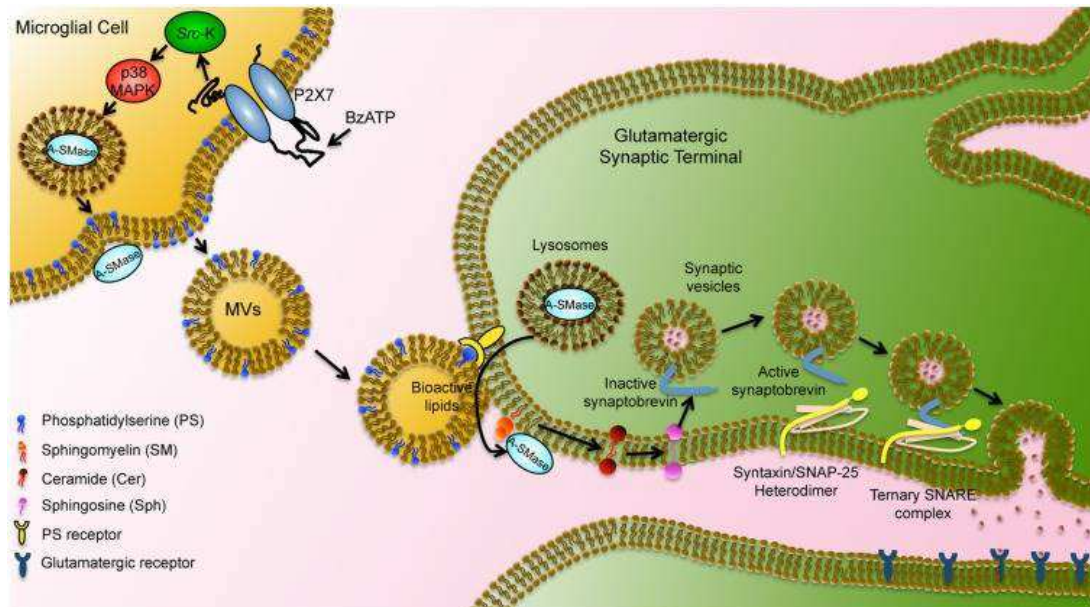


Fig. 5. Schematic representation of microglial MVs interaction with neurons: blebbing of MVs induced by P2X7 stimulation by BzATP, preceded by loss of plasma membrane symmetry and exposure of PS at the outer leaflet of the plasma membrane; hydrolysis of SM to ceramide by ASMase which is translocated to plasma membrane outer leaflet after p38 MAPK activation; released MVs can then interact with target neuronal cell (from Turola *et al.*, Front Physiol. 2012).

It was observed that MVs from reactive microglia induce an inflammatory reaction in target cells (Verderio *et al.*, 2012a) and cause an immune-stimulatory activity in recipient microglial cells that determines an up-regulation of CD86 and expression of inflammatory genes (Verderio *et al.*, 2012). Indeed, in several pathological conditions, a role for microglia-derived MVs has been recognized. For example, microglial MVs participate in the progression of AD by inducing the production of soluble amyloid β species and the propagation of such toxic forms (Joshi *et al.*, 2014). In addition it has been observed that MVs derived from microglial cells are present in cerebrospinal fluids of rodents and interestingly, their concentration tend to increase in the course of EAE (Verderio *et al.*, 2012). Accordingly, injection of MVs into the brain of mice with subclinical EAE promotes recruitment of inflammatory cells at the site of delivery whereas a reduction of MVs formation, as occurs in A-SMase knock out mice, protects against EAE (Verderio *et al.*, 2012). In addition, microglia-released MVs enhance excitatory neurotransmission. In fact, MVs shed from microglia,

are able to interact with the plasma membrane of neurons evoking and increasing excitatory transmission. Interestingly, it was found that MVs modulate neurotransmission by inducing sphingolipid metabolism in neurons (Antonucci *et al.*, 2012).

MicroRNA

MicroRNAs (miRNAs) are a class of small noncoding RNAs (containing about 22 nucleotides) which represent key post-transcriptional regulators of gene expression, hindering the translation of their target mRNA or promoting their degradation. Besides, they may also play a role as translation activators or impair transcription by binding to gene promoters (Faravelli and Corti, 2017). The majority of miRNAs are transcribed as pri-miRNAs by RNA polymerases II and III in the nucleus and cut into pre-miRNAs by the Drosha complex. The last stage of the process involves exportin 5 and Ran GTPase which transport pre-miRNAs to the cytoplasm where they undergo final processing by the RNase III enzyme Dicer. In this way the formation of 22 nt double-stranded mature miRNAs with a 5' phosphate end takes place. The mature miRNA is then incorporated into the RNA-induced silencing complex (RISC), which become able to repress translation. Although most of miRNAs are localized within the cell, it has been observed that some miRNAs, commonly termed extracellular miRNAs or circulating miRNAs, may occupy extracellular environment, comprising biological fluids and cell culture media.

miRNAs can modulate the development and function of the nervous system (Karthikeyan *et al.*, 2016). Neural miRNAs are implicated in several steps of synaptic development, such as dendritogenesis (involving miR-132, miR-134 and miR-124), synapse generation and synapse maturation (miR-134 and miR-138 seem to be related to these events). Moreover it has been found that miRNA expression is modified in schizophrenia, as well as in bipolar disorder and major depression and anxiety disorders. Recently, some studies have led to the consideration of a link between miRNAs and inflammation. Recently it has been found that miR-92a is down-regulated after

activation of TLRs, and exerts a significant role in inflammatory contexts being essential for the production of cytokines in pro-inflammatory macrophages (M1 phenotype). miR-124 is implicated in the secretion of cytokines from reactive pro-inflammatory effector microglial cells (Ponomarev *et al.*, 2011). In addition, genes associated to reactive microglial cells, among which genes encoding proteins related to TGF- β pathway (SMAD2), may represent targets of miR-155 (Louafi *et al.*, 2010). In contrast, miR-Let7, under inflammatory conditions, can play an important role in microglial cell's functions being responsible of anti-inflammatory effects through repression of genes targeting downstream signaling pathways.

MiR-146a is widely expressed in different species and tissues, and is involved in immunity, inflammation and viral infections by regulating different target genes (Li *et al.*, 2010).

When implicated in the regulation of inflammation and other processes that involve the innate immune system, miR-146, acts as a mediator of inflammation along with miR-155. Indeed the expression of miR-146 is upregulated by inflammatory factors such as IL-1) and TNF- α (Sheedy, and O'Neill, 2008). miR-146 dysregulates a number of targets which are mostly involved in toll-like receptor pathways that bring a cytokine response (Sonkoly *et al.*, 2008). Accordingly, miR-146a appears to be preferentially expressed in cells of microglial lineage. A great up-regulation of miR-146a was observed in immortalized murine microglial cell line, BV2 cells, following TLR2 or TLR4 activation. It has also been suggested that miRNA146a can be involved in neurodegenerative disorders such as AD. In this pathological condition, phagocytosis of β -amyloid (A β) peptide by activated microglia is significantly increased in the presence of ligands that activate TLRs. In particular, TLR4 stimulation of BV2 cells resulted in activation and in the transcription of miR-146a. Then miR-146a may represent a potent modulator of microglial function by regulating the activation state of microglial cell during neurodegeneration (Saba *et al.*, 2012). Accordingly, miRNA146a is upregulated in AD patients (Zhang *et al.*, 2016) and in this condition, its targets include the low-density lipoprotein receptor-related protein-2 (Lrp2), a member of the LDLR family which exerts a

protective role in AD (Marzolo and Farfan, 2011). Overexpression of miRNA-146a in neuroblastoma SH-SY5Y cells induced in fact a significant reduction of Lrp2 expression, with ensuing decrease of Akt activation and induction of pro-apoptotic caspase-3, thereby causing cell apoptosis.

Another target of miR-146a is superoxide dismutase 2 (SOD2) which represents an endogenous antioxidant enzyme able to exert a protective role against reactive oxygen species (ROS) (Valko *et al.*, 2006). Through interaction with SOD2 mRNA, miR-146a is involved in the post-transcriptional regulation of SOD2 expression (Pogue *et al.*, 2011). Hence, miR-146a is up-regulated by H₂O₂-induced stress and down-regulates SOD2 protein expression whereas an antisense-miR-146a reverses the decrease of both the SOD2 level and cell viability in H₂O₂ treated PC12 cells (Ji *et al.*, 2013).

Main objectives of the research project

My research has focused on the role of group I mGlu receptors, namely mGlu1 and mGlu5 receptors, in neurodegeneration and in neuroinflammation. The research has been carried out following two independent lines.

The major part of the work has been centered on the role of mGlu5 receptor in neuroinflammation, based mainly on the discrepancy existing in literature regarding a potential anti-inflammatory activity, but also a possible pro-inflammatory effect, mediated by this receptor subtype. Using microglia cells in culture, my aim was then to establish whether mGlu5 receptor activation in microglia could modify the neuroinflammatory process and mainly to establish whether this effect could involve MVs that are recognized as important novel mediators of cell-to-cell and also glia-to-neuron communication.

The other part of the study has been centered on the role of mGlu1 receptor in neurodegeneration. In particular, we focused on neurodegenerative processes occurring during eye disorders and affecting RGC such as prematurity-induced retinal degeneration, glaucoma, and age-related macular degeneration. The main goal of the study was to establish the real role of mGlu1 receptor in excitotoxicity that is responsible for RGC death. For this purpose we used wild type and *crv4* mice, that, because of spontaneous recessive mutation of the *Grm1* gene, do not express mGlu1 receptors.

Shedding of microvesicles from microglia contributes to the effects induced
by metabotropic glutamate receptor 5 activation on neuronal death

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Running title: mGlu5 receptor activation increases microglial microvesicles

Keywords: microglia, miRNA, extracellular vesicle; mGlu5; neuroinflammation; CHPG

Funding:

This work was supported by personal grants to MAS (University of Catania cod. 20130143075).

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Abstract

Metabotropic glutamate (mGlu) receptor 5 is involved in neuroinflammation and has been shown to mediate reduced inflammation and neurotoxicity and to modify microglia polarization. On the other hand, blockade of mGlu5 receptor results in inhibition of microglia activation. To dissect this controversy, we investigated whether microvesicles (MVs) released from microglia BV2 cells could contribute to the communication between microglia and neurons and whether this interaction was modulated by mGlu5 receptor. Activation of purinergic ionotropic P2X7 receptor with the stable ATP analog benzoyl-ATP (100 μ M) caused rapid MVs shedding from BV2 cells. Ionic currents through P2X7 receptor increased in BV2 cells pretreated for 24 h with the mGlu5 receptor agonist CHPG (200 μ M) as by patch-clamp recording. This increase was blunted when microglia cells were activated by exposure to LPS (0.1 μ g/ml for 6 h). Accordingly, a greater amount of MVs formed after CHPG treatment, an effect prevented by the mGlu5 receptor antagonist MTEP (100 μ M), as measured by expression of flotillin, a membrane protein enriched in MVs. Transferred MVs were internalized by SH-SY5Y neurons where they did not modify neuronal death induced by a low concentration of rotenone (0.1 μ M for 24 h), but significantly increased rotenone neurotoxicity when shed from CHPG-treated BV2 cells. miR146a was increased in CHPG-treated MVs, an effect concealed in MVs from LPS-activated BV2 cells that showed *per se* an increase in miRNA146a levels. The present data support a role for microglia-shed MVs in mGlu5-mediated modulation of neuronal death and identify miRNAs as potential critical mediators of this interaction.

Introduction

Metabotropic glutamate receptors (mGlu) receptors are G protein coupled receptors involved in a variety of functions and processes at the central nervous system (CNS) including synaptic plasticity, neurodevelopment, neuronal excitability, pain, addiction, neurodegeneration, neuroinflammation (Conn & Pin, 1997; Nicoletti *et al.*, 2011). Among the three classes of mGlu receptor subtypes, group 1 mGlu, which includes mGlu1 and mGlu 5 receptors, is highly expressed throughout the brain (Martin *et al.*, 1992; Romano *et al.*, 1995) and mGlu5 receptor shows high density also in the spinal cord (Berthele *et al.*, 1999). They are both Gq-coupled receptors, thus their activation causes increase of intracellular Ca^{2+} and predominate at post-synaptic level where they participate to the modulation of synaptic excitability (Shigemoto *et al.*, 1993; Lujan *et al.*, 1996; Biber *et al.*, 1999). Although initial studies made questionable the function of mGlu5 receptor with a dual role, either neuroprotective or neurotoxic, depending on neuronal type and experimental conditions, it is now clearer that mGlu5 receptor interacts with NMDA receptors and likely exerts a permissive role in NMDA excitotoxicity. Hence, several antagonistic modulators of mGlu5 receptor activity exhibit neuroprotective properties whereas mGlu5 receptor agonists are neurotoxic (Parmentier-Batteur *et al.*, 2014). Among others, mGlu5 receptors are not only expressed in neurons, but also in glial cells (Biber *et al.*, 1999). Interestingly, in the cortex, expression of this receptor subtype is even higher in astrocytes than in neurons (Zhang *et al.*, 2014) and appears upregulated in reactive astrocytes (Aronica *et al.*, 2000; Ferraguti *et al.*, 2001; Dolan *et al.*, 2003., Ribeiro *et al.*, 2017). Cultured microglia also express mGlu5 receptor, and its activation has been reported to be associated with reduced inflammation and neurotoxicity (Byrnes *et al.*, 2009) as well as changes in microglia polarization (Loane *et al.*, 2014). However, again, anti-inflammatory effects secondary to activation of mGlu5 receptor are rather controversial as opposing results with reduction of microglia activation by blockade of group 1 mGlu receptors have also been reported (Hsieh *et al.*, 2012). Surprisingly, mGlu5 receptor has also been suggested to act as an alternative binding site for lipopolysaccharide (LPS), thus mediating activation of microglia toward an inflammatory phenotype (Liu *et al.*, 2014).

Microglia affects neuronal function by the release of several factors, but in more recent years an additional way of intercellular communication has been described in several cell types, including glial cells. Extracellular vesicles, either as microvesicles (MVs) or exosomes that differ in size, content and origin, form under basal conditions, or as in the case of microglia, upon stimulation of the purinergic P2X7 receptor. In particular, microglial-derived MVs, ranging from 100 to 1000 nm in diameter, are shed from the extracellular membrane and play as a cargo for cytokines, RNA, miRNA, etc. to neighboring cells. Although the precise kinetics of this interaction are not completely known, shedding of MVs from microglia is a well-established event (Turola *et al.*, 2012; Prada *et al.*, 2013) reported to mediate neurotoxicity by transferring and propagation of pro-inflammatory cytokines (Bianco *et al.*, 2005), or through modulation of β -amyloid aggregation status (Joshi *et al.*, 2014). Even more, MVs can be detected and are increased in the cerebrospinal fluid of humans and rodents under conditions of brain inflammation, suggesting their role in the development of the disease, by spreading the inflammatory process (Verderio *et al.*, 2012).

On these bases we decided to investigate whether MVs could take part to the effects elicited by mGlu5 receptor activation in microglia and whether this alternative means of interaction between cells could account for the controversial response observed following activation of this receptor.

Materials and methods

Drugs and reagents

Cell culture plastics were provided by BD Falcon (Milan, Italy). Media, media supplements, serum, trypsin and antibiotics, were from Invitrogen Srl (Milan, Italy). (RS)-2-chloro-5-hydroxyphenylglycine (CHPG) Sodium Salt, 3,5-dihydroxyphenylglycine (DHPG), and 3-((2-Methyl-1,3-thiazol-4-yl)ethynyl)pyridine (MTEP), were from Tocris (North Point, UK). 2'(3')-O-(4-Benzoylbenzoyl)adenosine-5'-triphosphate tri(triethylammonium) salt (Bz-ATP), lipopolysaccharide

(LPS), rotenone were from Sigma-Aldrich (St. Louis, MO, USA). The following primary antibodies were used: rabbit anti-metabotropic glutamate receptor 5 (1:500; *Millipore*, Billerica, MA, USA), rabbit anti-flotillin (1:500; Santa Cruz Biotechnology, CA, USA); goat anti-tumor necrosis factor (TNF) α , (1:100; Santa Cruz Biotechnology), FITC-conjugated mouse anti-TNF α , (1:10; Miltenyi Biotec, Macs, Berisc-Gladbach, Germany).

Cell cultures

Immortalized murine microglial BV2 cells were cultured at 37 °C, with 5% CO₂, in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), penicillin/streptomycin (100 U/ml-100 μ g/ml). For the experiments cells were plated in 35 mm tissue culture dishes (200 k/ each). The human neuroblastoma SH-SY5Y cell line was cultured at 37 °C with 5% CO₂, in DMEM F-12 supplemented with 10% FBS and penicillin/streptomycin (100 U/ml-100 μ g/ml). For experiments, cells were plated in 35 mm culture dishes (300 k/each) or in 96 well plates (60 k/each). To achieve BV2 cells conditioned medium (CM), BV2 cells were exposed to either vehicle, CHPG (200 μ M), LPS (0,1 μ g/ml) or their combination, for 24 h, then washed and incubated with fresh medium for further 6 h. This particular paradigm was chosen in order to allow exposure to treatments for a reasonable time to induce effects before removal so that transferred CM did not contain any drug.

Shedding of microvesicles

BV2 cell cultures were treated with CHPG (200 μ M, for 24 h), the selective mGlu5 receptor antagonist MTEP (100 μ M added 30 min before CHPG), and with LPS (0,1 μ g/ml, for 6 h). They were then stimulated with Bz-ATP 100 μ M, 20 min at 37° C in Krebs-Ring-buffer (Sigma-Aldrich) and the supernatant was subjected to differential centrifugations at 4° C: 5 min at 300 x g to discard cells and debris (P1 pellet); 20 min at 1200 x g to obtain P2 vesicle fraction (P2); 60 min at 10 000 \times g to obtain vesicle population (P3). The resulting pellets corresponding to microvesicles (P3; MVs)

was resuspended in 30 μ l of protein solubilisation buffer MPER (Invitrogen srl) supplemented with protein inhibitors and loaded for gel separation by western blotting analysis. For this specific experiment, the total amount of protein from 8 x 35-mm dishes, resuspended in a small volume was then loaded into the gel in order to assess the whole flotillin, expression of MVs formation. Alternatively, MVs were processed for microRNA extraction or resuspended in DMEM/F12 medium and transferred on SH-SY5Y cells. In the latter case four 35-mm dishes per treatment group were used and, after centrifugation, resuspended in a final volume of 1200 μ l that were then divided into 8 wells of a 96-well multiwell plate (150 μ l/well). To achieve experimental consistent conditions, MVs were collected from BV2 cells plated and grown always at the same density and transferred on SH-SY5Y cells also maintained under constant conditions.

Western blot analysis

Protein extraction was performed using the M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Grand Island, New York) and protein concentration was determined with the Bradford reagent (Sigma-Aldrich). Forty to 50 μ g of protein extract for each sample were diluted in loading buffer, denatured by boiling at 95 °C for 5 min. In the case of proteins extracted from MVs, an equal volume of the extracts was subjected to electrophoresis. Polyacrylamide-SDS gel at 10% or 4-15% were used. The proteins were then transferred onto nitrocellulose membranes (Hybond ECL, Amersham) through a semi-dry transfer apparatus for 60 min at 0.8 mA/cm². After transfer, membranes were blocked with Odyssey blocking buffer (LI-COR Biotechnology GmbH, Bad Homburg, Germany) diluted 1:1 with phosphate-buffer, and then incubated with primary antibodies overnight, at 4 °C. Immunodetection was performed using specific fluorescent secondary antibodies IRDye[®]680 or IRDye[®]800-conjugated secondary anti-bodies (LI-COR). Detection of specific bands was carried out using the Licor Odyssey[®] infrared Imaging System (LI-COR). Band intensity was analyzed using the image processing software “ImageJ” developed by NIH and in public domain.

Densitometric analysis of band intensity was carried out with the Image J Software freely available on the web.

Cell viability assay

SH-SY5Y cell cultures were exposed to rotenone in the presence of either BV2-derived CM or MVs derived from BV2-treated cells. To test SH-SY5Y cell culture viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed after 18 h treatments. Cells were exposed to MTT solution (5 mg/ml) for 90 min at 37 °C. DMSO was added to dissolve formazan crystals. The absorbance was measured at 540 nm using Varioskan™ LUX multimode microplate reader (ThermoFisher, Milan, Italy).

Flow cytometry

After treatments BV2 cells were harvested, fixed in PFA 4% and then washed and centrifuged at 300 x g for 10 min. Cells were incubated for 20 min at RT with 300 µl of Inside Stain Kit (Miltenyi Biotec) and then stained with FITC-conjugated anti-TNF α (1:10, Miltenyi Biotec) according to the manufacturer's instruction for 15 min, at RT. Cells were then washed and resuspended in 20 µl of PBS. Analyses were carried out using Amnis® imaging flow cytometer (Millipore).

RNA extraction

RNA extraction from BV2-derived MVs was performed using The RNeasy Micro Kit (Qiagen, Milan, Italy) according to the manufacturer's instruction. About 20 ng of RNAs were used for miRNA-specific reverse transcription (RT) to obtain miRNA-specific cDNAs by using TaqMan™ MicroRNA Reverse Transcription Kit (Thermo Scientific), according to the manufacturer's instructions. Four-fifths of the cDNA total volumes were analyzed with quantitative real-time polymerase chain reaction (RT-PCR) using TaqMan MicroRNA Assays (Thermo Scientific). RT-PCRs were performed on a 7900 HT Fast Real Time PCR System (Applied Biosystem – Thermo Scientific), by using TaqMan™

Universal Master Mix II, no UNG (Thermo Scientific), according to the manufacturer's instructions. SnRNA U6 was used as reference gene. Expression fold changes were calculated by the $2^{-\Delta\Delta Ct}$ method.

Reactive oxygen species determination

Determination of reactive oxygen species (ROS) was performed using the 2',7'-dichlorodihydrofluorescein diacetate (H₂-DCFDA) assay (Sigma-Aldrich). SH-SY5Y cell cultures, plated in 96 well plates and treated for 24 h as described in the result section, were loaded with carboxy-H₂DCFDA at a final concentration of 1 μ M and incubated for 30 min in the dark at 37 °C, with 5% CO₂. After dye's removal, intracellular fluorescence (excitation 485 nm, emission 530 nm) was measured using Varioskan TM LUX multimode microplate reader. Results are given as fluorescence intensity normalized for the number of viable cells as measured by MTT assay.

Whole-cell patch-clamp recordings and analysis

Cells plated in 35 mm dishes were transferred from cell culture medium to an extracellular recording solution containing (in mM) the following components: 138 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, 10 HEPES, at pH 7.25 with NaOH. Borosilicate patch pipette were pulled with P-1000 puller (Sutter Instruments, USA) and resistance was 3-5 M Ω when filled with an intracellular solution containing (in mM): 115 K⁺ gluconate, 20 KCl, 2 EGTA, 10 HEPES, 2 Mg-ATP, and 0.3 GTP-Na₂, pH 7.25 adjusted with KOH. P2X7 receptor current was recorded in a low divalent cation extracellular solution to avoid blocking action of Ca²⁺ and Mg²⁺. This solution contained (in mM): 138 NaCl, 4 KCl, 0.1 CaCl₂, 10 glucose, 10 HEPES at pH 7.25 with NaOH. Bz-ATP (100 μ M) was added to this solution and application and removal of P2X7 receptor selective agonist and other drugs (as indicated in figure legends) were made locally by a gravity-driven multibarrel local perfusion system operated by solenoid valves (ValveBank II, Automated Scientific, USA) and controlled by computer. Exchange times were measured by the change in junction currents at the tip of an open

patch pipette and were estimated in 126.2 ± 9 ms. Experiments were performed at RT using an EPC7 Plus amplifier (HEKA Elektronik, Germany) in voltage-clamp configuration (holding potential, -70 mV). Data were acquired at 5 kHz and filtered at 1 kHz using a 7-pole Bessel filter and digitized with a low noise data acquisition system, Digidata 1440A (Molecular Devices, USA). Data were recorded and analyzed in pClamp 10 (Molecular Devices) and peak current was normalized to membrane capacitance (Cm) using Microsoft Excel.

Statistical analysis

All data are presented as mean \pm SEM and are always derived from at least three independent experiments. Data were analyzed by Student's t-test or, where appropriate, one-way ANOVA followed by Neuman-Keuls test for significance. Plots, bar diagrams and statistical analysis were finalized with GraphPad Prism 5 (GraphPad Software, USA).

Results

Expression of mGlu5 receptor in microglial BV2 cells was examined by Western blot analysis. As shown in Fig 1A, microglial BV2 cells expressed mGlu5 receptor and the protein levels were not modified by activation of BV2 cells after exposure to LPS (0.1 μ g/ml) for 24 h. The antiinflammatory effect mediated by mGlu5 receptor was confirmed by assessment of TNF- α expression by Western blot and flow cytometry. Treatment for 24 h with the selective mGlu5 receptor agonist CHPG (200 μ M) and exposure to LPS (0.1 μ g/ml) during the last 6 h, significantly counteracted the LPS-induced increase of TNF- α expression as by Western blot (Fig. 1B) and by flow cytometry (Fig 1C,D) analyses. This specific time point (6 h LPS treatment) was chosen based on published data showing that a significant increase of proinflammatory cytokines (Minogue *et al.*, 2012) and of FoxP3 (Chung *et al.*, 2010) is observed within 6 h following LPS exposure in primary microglia and in BV2 cells, respectively. Specificity of this response was shown by prevention of the effect by the mGlu5 receptor antagonist, MTEP (100 μ M), added 30 min before the agonist (Fig 1C,D). Changes induced by mGlu5

receptor stimulation in microglial cells modified the response of SH-SY5Y cells to a non specific toxic insult such as the mitochondrial electron transport inhibitor, rotenone. Transferring of CM from CHPG-pretreated BV2 cells, significantly increased SH-SY5Y neuronal death induced by rotenone (100 nM for 24 h; Fig. 1E). This effect was observed only with CM from quiescent microglia, but not after activation by exposure to LPS (0.1 $\mu\text{g/ml}$).

In order to dissect the large discrepancies existing in literature regarding the real role of mGlu5 receptor in neuroinflammation, we decided to investigate whether modulation of mGlu5 receptor could also affect shedding and content of MVs involved in the crosstalk between microglia and neurons. To this purpose, BV2 cells were exposed to the ATP stable analog Bz-ATP (100 μM), a P2X7 receptor agonist, for 20 min, a condition that causes blebbing of cell membrane and ensuing formation of MVs (Antonucci *et al.*, 2012).

Before assessment of MVs formation, patch-clamp recording was performed in order to test purinergic current through ionotropic P2X7 receptors. BV2 cells exposed to vehicle or CHPG (200 μM) for 24 h were stimulated with Bz-ATP (100 μM) for 3 s in a solution with low divalent ions (0.1 mM Ca^{2+} , 0 mM Mg^{2+}) to avoid blocking action on channels (Norenberg *et al.*, 2010). Currents elicited by P2X7 receptor activation were significantly increased in BV2 cells pre-treated with CHPG (Fig. 2A, upper panel and B). In contrast, when BV2 cells were activated by exposure to LPS for 6 h, pre-treatment with CHPG (200 μM , for 24 h) did not elicit any significant increase in current (Fig. 2A, lower panel and B). LPS *per se* was not able to modify P2X7 currents as well (Fig. 2A, lower panel and B). Membrane capacitance (C_m) showing no change in cell size following treatments is reported (Fig. 2C).

MVs shedding from BV2 cells was monitored by time-lapse microscopy following staining of cell membranes with the fluorescent dye FM1-43 (10 μM). MVs formation started soon after addition of Bz-ATP (1 min) and was monitored by sequential captures for up to 20 min (Fig 3A). Shed MVs were visible, close to the cell surface for the time points examined. Sequential images did not reveal any eye-detectable change in MVs shedding following mGlu5 receptor activation with the mixed

agonist DHPG (100 μ M) for 24 h (Fig 3A). However, expression of flotillin, a protein that is enriched in MVs, was significantly increased in MVs shed from CHPG-treated BV2 cells, when an equal volume, and not an equal concentration, of protein were loaded for gel electrophoresis (Fig. 3B). This effect recapitulates the increased response to Bz-ATP observed under conditions of mGlu5 receptor activation when P2X7 receptor currents were analyzed, as described above. The increase in flotillin expression was counteracted by the mGlu5 receptor antagonist MTEP (100 μ M), added 30 min before CHPG (Fig. 3B). In contrast, flotillin levels were not changed in LPS-activated BV2 cells (Fig. 3C). When isolated fluorescent MVs from control and CHPG-treated BV2 cells were transferred on top of SH-SY5Y neuronal cultures, they were rapidly internalized and still visible inside recipient neurons for hours (Fig. 3D). Pretreatment of BV2 cells with DHPG (100 μ M, 24 h) did not modify the interaction of MVs with SH-SY5Y cells as shown by epifluorescent microscopic analysis (not shown).

To assess the effect of microglia-derived MVs on the viability of neuronal cultures, rotenone was applied to SH-SY5Y cells for 24 h and viability was analyzed by MTT assay. Rotenone, at the low concentration used, (0.1 μ M for 24 h) induced about 25-30% neuronal death, either alone or in the presence of MVs from untreated BV2 cells (Fig. 4A). However, when MVs were from CHPG- or DHPG-treated BV2 cells, a significant increase of rotenone-induced death was observed (Fig. 4A). This effect was prevented by pre-incubation (30 min) with the selective mGlu5 receptor antagonist MTEP (100 μ M) (Fig 4A). To analyze whether MVs could interfere with the ability of rotenone to induce oxidative stress, ROS formation was assessed in SH-SY5Y cells exposed to rotenone in the presence of MVs shed from BV2 cells either untreated or exposed to CHPG. In the latter condition, ROS generation appeared significantly increased when compared to rotenone challenge in the presence of MVs from control BV2 cells (Fig. 4B). Interestingly, potentiation of rotenone toxicity observed in the presence of MVs from CHPG-treated cells was not observed in the presence of MVs shed from LPS-treated microglia (Fig. 4C). Searching for cellular factors mediating the observed effects and transferred through shed MVs to the neuronal culture, attention was focused on cytokines.

Specifically, we tried to detect TNF- α and interleukin-6 in protein extracts from MVs. However, under the experimental conditions used in the present study, no cytokine was measurable, consistently, in MVs protein extracts by Western blot or by flow cytometry (not shown). In contrast, miRNA could be isolated and, following an initial screening, our preliminary results identified miR146a as a potential mediator of the observed effects. Its expression appeared in fact significantly modified in MVs from CHPG-treated BV2 cells. More specifically, exposure to CHPG increased the expression of miR146a in BV2 cell-shed MVs (Fig. 5A), an effect partially counteracted by pretreatment with MTEP (Fig 5B). This effect was significantly modified in LPS-stimulated conditions (0.1 μ g/ml for 4 h), where CHPG did not affect the increase of miR146a expression induced by LPS itself (Fig 5A).

Discussion

The present study was undertaken with the primary aim to elucidate the role of mGlu5 receptor in neuroinflammation and its modulatory role in microglia. To this purpose we used BV2 cells, that, for many aspects including gene transcription, protein production and functional attitude, are superimposable to cultured primary microglia, even under activated conditions that mimic an inflammatory state (Henn *et al.*, 2009; Orihuela *et al.*, 2016). The use of a cell line allows also availability of a great amount of cells, hardly warranted by microglia pure cultures. In addition, looking for MVs, it was crucial to deal with a homogeneous cell population and pure cultures of microglia, although highly enriched (90-95% purity), never reach 100% purity and contaminating astrocytes are also a source of MVs.

The BV2 cell line expresses functional mGlu5 receptors (Loane *et al.*, 2009) and has already been used for the study of the signaling pathway activated by this receptor (Chantong *et al.*, 2014). mGlu5 receptor appears the only group 1 mGlu receptor expressed in microglia in vivo and also present in microglia cultures. In contrast, mGlu1 receptor is lacking or barely detectable in cultured microglia (Byrnes *et al.*, 2009), although it has been detected in selected microglia cell populations in multiple

sclerosis brain (Klaver *et al.*, 2013). Activation of mGlu5 receptors in primary microglia or in BV2 cell cultures has already been shown to exert anti-inflammatory effect, as revealed by reduction of LPS-induced NO and ROS production, TNF α release, and microglial proliferation (Byrnes *et al.*, 2009; Loane *et al.*, 2009).

In our conditions, we confirmed an anti-inflammatory role for mGlu5 receptor following LPS activation, as suggested by the reduction of the pro-inflammatory cytokine TNF α , by western blot and flow cytometry. However, this anti-inflammatory effect did not produce any neuroprotective outcome, as transferred CM to neuronal cultures was not able to modify neuronal death induced by a low concentration of rotenone. A slight increase of rotenone toxicity was rather observed in neurons exposed to CM from CHPG-treated BV2 cells. This appears in contrast with previous reports showing that CHPG could prevent the neurotoxic effect obtained by co-culturing LPS-activated microglia with neurons (Byrnes *et al.*, 2009). Much higher concentrations of CHPG (20 fold higher) were however needed to induce neuroprotection against LPS-stimulated BV2 cell toxicity (Byrnes *et al.*, 2009). Hence, our results indicate the ability of mGlu5 receptor to modify the inflammatory phenotype induced by LPS exposure without however any positive outcome on neuronal viability.

Despite the literature and our own observation that indicate anti-inflammatory effect mediated by mGlu5 receptor, when attention is turned to neuropathological conditions such as Alzheimer's (AD) and Parkinson (PD) diseases, in which activated microglia accumulate around brain lesions and play a role in the neurodegenerative process, the scenario dramatically changes. In vivo studies do not support in fact a protective effect mediated by mGlu5 receptor (Liu & Hong, 2003; Town *et al.*, 2005). More specifically, activation of mGlu5 receptor has been linked to increased synaptotoxicity induced by β -amyloid peptide in AD and is reported to exert detrimental effect in animal models of PD (reviewed in Bruno *et al.*, 2017). All this large body of evidence suggests that the anti-inflammatory effect mediated by mGlu5 receptor on microglia may be vanished by effect of drugs acting on this receptor directly on neurons or that it may be not strong enough or contrasted by other factors to elicit any protective effect on neurons. In an attempt to elucidate this aspect, we tried to investigate the

mGlu5-mediated modulation of MVs that have recently gathered increasing attention for their function as an alternative/additional way of cell-to-cell and, as in this case, glia-to-neuron communication (Turola *et al.*, 2012; Verderio *et al.*, 2012; Fruhbeis *et al.*, 2013; Prada *et al.*, 2013; Tetta *et al.*, 2013; Carandini *et al.*, 2015; Cocucci & Meldolesi, 2015). MVs are shed from microglia upon activation of P2X7 receptor with the stable receptor agonist Bz-ATP (Turola *et al.*, 2012; Prada *et al.*, 2013). Our results show that the current elicited by P2X7 receptor activation in microglia is significantly increased following stimulation of mGlu5 receptor with CHPG. This effect is in line with the reported potentiation of NMDA-evoked currents in selected neuronal populations (Awad *et al.*, 2000; Yang *et al.*, 2011) and suggests a greater response of microglia cells in CHPG-stimulated conditions. Accordingly, CHPG treatment is able to induce shedding of a greater amount of MVs, indirectly measured by the expression of flotillin in CHPG-derived MVs, when equal volumes of proteins were loaded. Our microscopic observation instead was not accurate enough to let us appreciate such changes in MVs production by eye. Of note, LPS-activated microglia behaved differently, as no potentiation of NMDA-evoked currents by CHPG was observed. From our data we cannot derive any explanation for this different behavior, but it is known that microglial LPS- and P2X7-mediated responses are related (reviewed in (Kettenmann *et al.*, 2011) and this interdependence can probably account for the modified sensitivity to CHPG-induced effect. According to the increased response induced by CHPG, a greater amount of MVs is formed and, when transferred to neuronal cultures, the toxic effect induced by rotenone, either as cell viability or as ROS formation, is increased.

Microglial-derived MVs have been reported to carry the pro-inflammatory cytokine interleukin-1 β (Bianco *et al.*, 2005) that may mediate the interplay with other microglia and neurons to spread and propagate the inflammatory reaction (Verderio *et al.*, 2012; Prada *et al.*, 2013), thus enforcing the neuroinflammatory process (Colombo *et al.*, 2012). We could not observe any change in rotenone-induced neuronal damage in the presence of MVs and this may be related to the amount of transferred MVs or to the ratio MVs: neurons achieved in our conditions. However, when MVs were derived

from CHPG-treated microglia, toxicity induced by rotenone was significantly increased, suggesting again that MVs-mediated glia-to-neuron signal can be amplified by stimulation of mGlu5 receptor in microglia.

miRNA are among factors that have gained, and are expected to yield, increasing attention for their role in glia to neuron communication (Su *et al.*, 2016). miRNA transferred through extracellular vesicles, either from microglia or astrocytes, have been shown to affect neuronal viability (Saba *et al.*, 2012; Mao *et al.*, 2015; Tang *et al.*, 2016., Hu *et al.*, 2012; Coleman *et al.*, 2017). We focused our attention on miR146a that is highly expressed in glial cells (Li *et al.*, 2011), is up-regulated by pro-inflammatory signals (Lukiw *et al.*, 2008) and negatively controls several target genes involved in the inflammatory response (Lukiw *et al.*, 2008; Li *et al.*, 2011). In addition, transfection with miR146a mimics has been shown to promote apoptosis in SK-N-SH neurons (Li *et al.*, 2017). We here report that stimulation of mGlu5 receptor in BV2 microglia cells up-regulates expression of miR146a that, upon P2X7 receptor stimulation, can be packaged into shed MVs and diffuse to neighboring cells. Our data are rather preliminary and further studies are needed to identify the real significance of miR146a up-regulation in MVs from CHPG-treated microglia. Interestingly, also LPS-stimulated microglia up-regulates miRNA-146a in MVs and, once again, under these activated conditions, the effect of CHPG is blunted. Since this up-regulation is accompanied by different effects on neuronal death induced by CHPG- and LPS-shed MVs, we have to take into account that a greater number of MVs is formed upon CHPG stimulation and that, together with miR146a, other miRNAs or other MVs components can be responsible for changes in the fate of neuronal cells in response to rotenone. In addition, the similar response induced by mGlu5 receptor agonist and LPS points out the common pathway that is potentially activated, suggesting that mGlu5 receptor may function as a mediator of inflammatory processes in microglia, as already reported (Liu *et al.*, 2014).

In our opinion, at least four different conclusions can be drawn from our results. First of all we confirmed the modulatory role of mGlu5 receptor on neuroinflammation that however does not have an obvious, ensuing influence on neuronal survival. Even more important, these effects may involve

not only conventional interplay between glial cells and neurons, but also novel mediators represented by MVs shed from microglia that function as a cargo for neuromodulators. Third, in our experimental conditions, MVs are formed after stimulation of ATP and shedding and content of MVs seem to vary depending on microglia level of activation. Finally, miRNA transferred through MVs may contribute to the interaction between microglia and neurons thus representing a novel, targetable mediator of neuroinflammation.

Figure legends

Figure 1. mGlu5 receptor mediates anti-inflammatory effect on BV2 microglial cells. Western blot analysis of expression of mGlu5 receptor in BV2 cells treated with LPS (0.1 $\mu\text{g/ml}$) for 24 h (A). BV2 cells were activated with LPS (0.1 $\mu\text{g/ml}$ for 6 h) after pretreatment with CHPG (200 μM for 24 h) and the selective mGlu5 receptor antagonist MTEP (100 μM added 30 min before CHPG). TNF α expression was evaluated by western blot (B) and flow cytometry (C,D). More specifically, assessment of the percentage of TNF α -positive cells (C) and the level of fluorescence expressed as mean fluorescent intensity (MFI; D) are shown. A representative image acquired by imaging flow cytometry showing cells in their shape and size (brightfield; BF), complexity (side scatter; SS) and TNF α immunostaining (green) is also reported (E). MTT assay on SH-SY5Y cells exposed to rotenone (100 nM for 24h) in the presence of conditioned medium (CM) from BV2 cells activated with LPS (0.1 $\mu\text{g/ml}$ for 6 h) after pretreatment with CHPG (200 μM for 24 h) (F). Student's t-test (A) and one-way ANOVA followed by Newman-Keuls test for significance (B-E) were applied. * $p < 0.05$ vs C; ** $p < 0.05$ vs LPS (Vhl); § $p < 0.05$ vs CHPG+LPS; ° $p < 0.05$ vs CM rot.

Figure 2. CHPG treatment increases purinergic current through P2X7 receptors in basal but not LPS-activated conditions. In A, representative traces of purinergic current recorded in BV2 cells in the following conditions: basal (C), after 24 h treatment with CHPG 200 μM , after 6h treatment with LPS 0.1 $\mu\text{g/ml}$ and after co-treatment of CHPG for 24 h and LPS added during the last 6 h. Currents were elicited after stimulation of P2X7 receptors with the selective agonist Bz-ATP, 100 μM . Summary of purinergic current elicited from cells stimulated as in A are reported in B. Data are presented as current (pA) obtained by each cell and normalized to membrane capacitance (Cm), an estimation of cellular surface area. Bars are mean \pm SEM (n = 11 for C; n = 8 for CHPG; n = 9 for LPS and LPS + CHPG), * $p < 0.05$ by one-way ANOVA followed by Newman-Keuls post hoc test. Summary of Cm measured for each cell in different conditions (C). Bars are mean \pm SEM of corresponding cells used for bar diagram in B.

Figure 3. mGlu5 receptor activation increases MVs release from BV2 microglial cells. BV2 cells were treated with DHPG (100 μ M for 24 h) and labeled with the fluorescent dye FM1-43 (10 μ M for 5 min). MVs formation following addition of Bz-ATP (100 μ M) was monitored with time-lapse microscopy up to 20 min. Representative images are shown in A. Western blot of flotillin of equal volume of MVs' protein extracts from BV2 cells treated with CHPG (200 μ M for 24 h) and MTEP (100 μ M for 24 h; B), or with LPS (0.1 μ g/ml for 6 h; C). Representative images of MVs shed from FM1-43-stained BV2 cells, transferred on top of SH-SY5Y cultures (D). Nuclei were stained with DAPI. Magnification = 40 X (A), 63 X (D). Scale bars = 20 μ m (A) and 10 μ m (D). * p <0.05 vs control. One-way ANOVA followed by Newman-Keuls test for significance (B) and Student's t-test (C) were applied to detect statistically significant differences.

Figure 4. MVs shed from CHPG-treated microglia potentiate rotenone-induced neurotoxicity. In A, MTT assay on SH-SY5Y cells exposed to rotenone (100 nM for 24 h) in the presence of MVs derived from BV2 cells treated with CHPG (200 μ M for 24 h), DHPG (100 μ M for 24 h), and MTEP (100 μ M for 24 h) and transferred to SH-SY5Y cells after rotenone (100 nM for 24 h) exposure. Determination of ROS levels in SH-SY5Y cells exposed to rotenone (100 nM for 24 h) in the presence of MVs derived from CHPG-treated BV2 cells (B). MTT assay on SH-SY5Y cells (100 nM for 24 h) in the presence of MVs from LPS-activated BV2 cells (0.1 μ g/ml for 6 h) pretreated with CHPG (200 μ M for 24 h; C). * p <0.05 vs control; ** p <0.05 vs rot, § p <0.05 vs DHPG vhl. One-way ANOVA followed by Newman-Keuls test for significance was applied.

Figure 5. CHPG increases miRNA146a levels in BV2 cells-shed MVs. BV2 cells were activated with LPS (0.1 μ g/ml for 6 h) after pretreatment with CHPG (200 μ M, total exposure 24 h). MVs were isolated, miRNA extracted and levels of miRNA146a were analyzed by real time PCR (A). In B, levels of miRNA146a were analyzed in MVs derived from MPTEP pretreated-cells (100 μ M added 30 min before CHPG). Results are expressed as percentage of CHPG. One-way ANOVA followed by Newman-Keuls test (A) and Student's t-test (B) were applied for significance. * p <0.05 vs control (A); * p <0.05 vs CHPG (B).

Author contributions

MB, SS, SM, PP performed all the experiments, collected and analyzed all data. MC performed the electrophysiological studies. MR, Mp carried out the miRNA studies. FN contributed to extension and revision of the ms. MS designed the experiments and wrote the manuscript with the contribution of all other authors.

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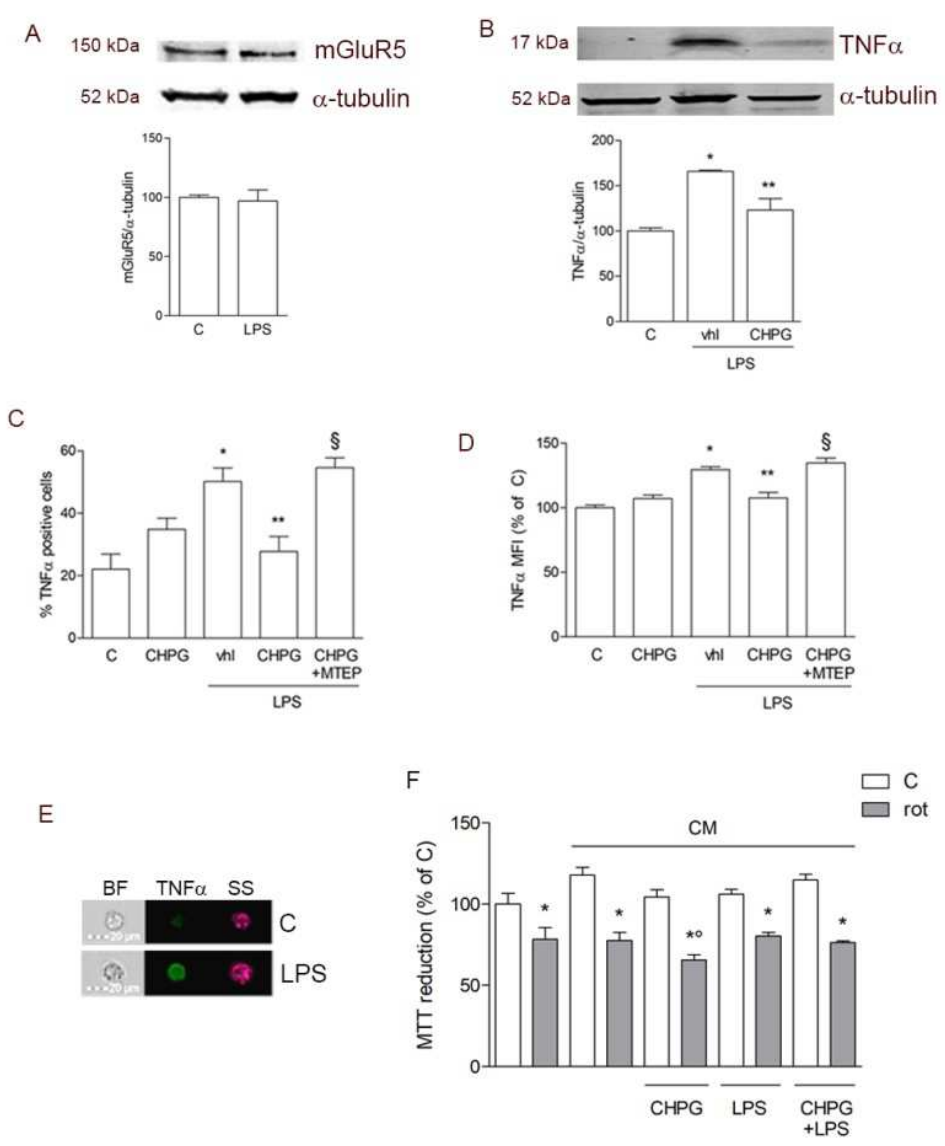
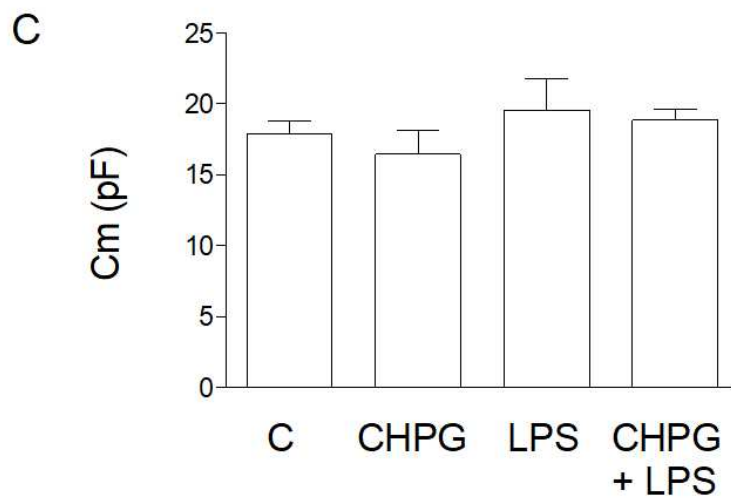
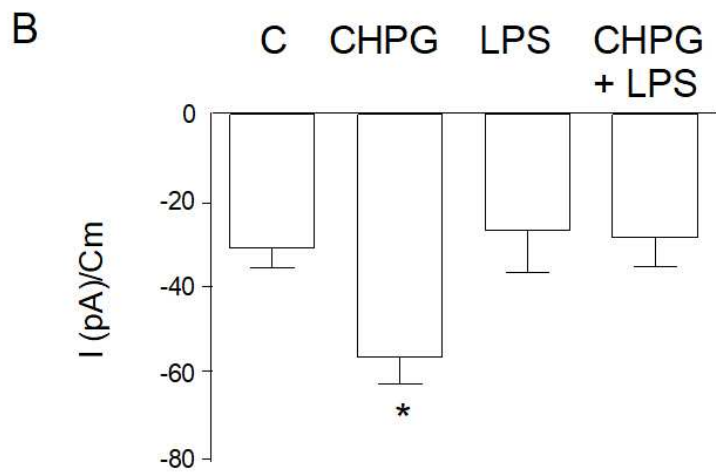
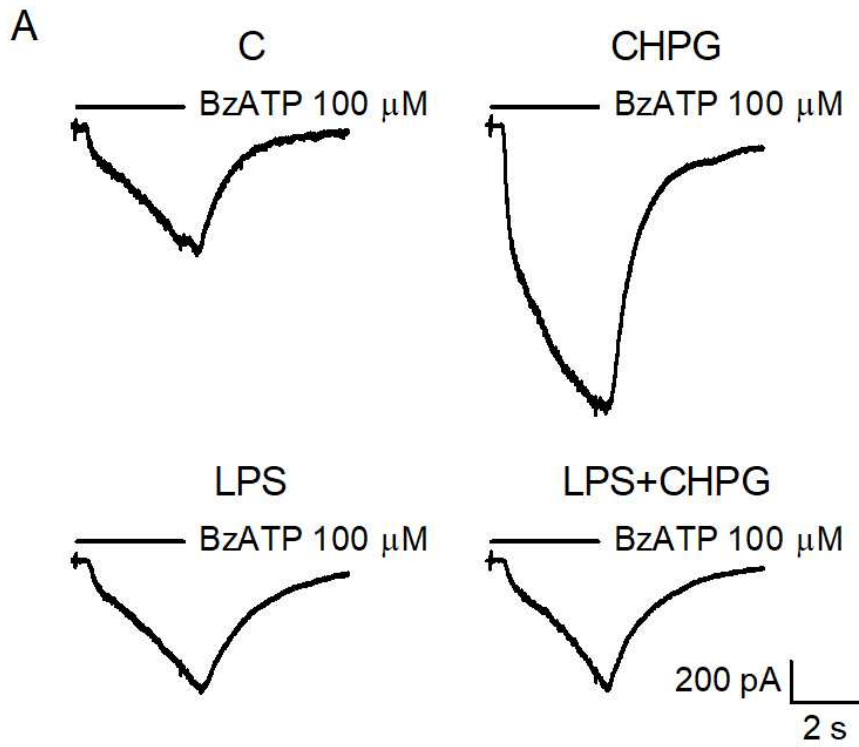


Fig. 1



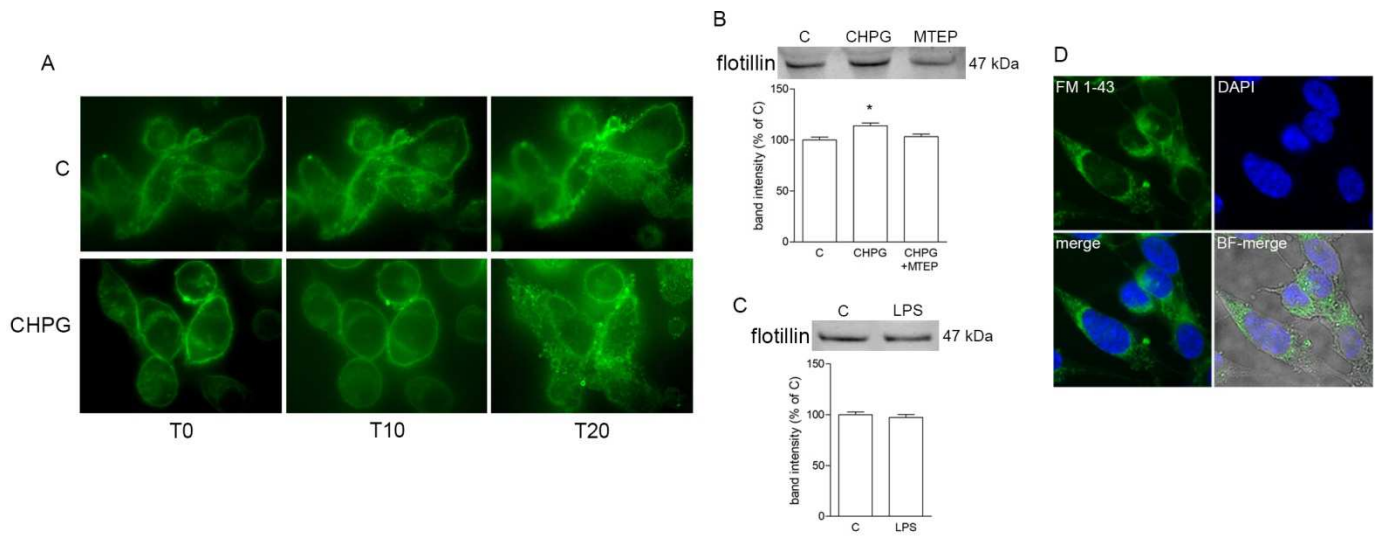
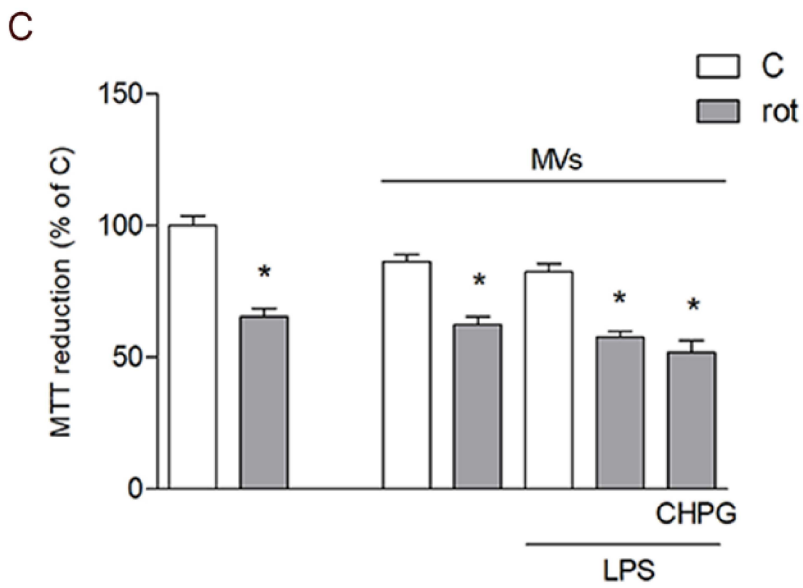
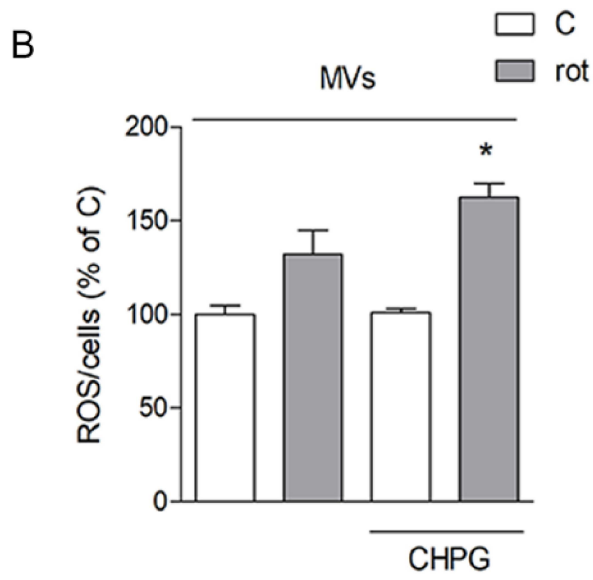
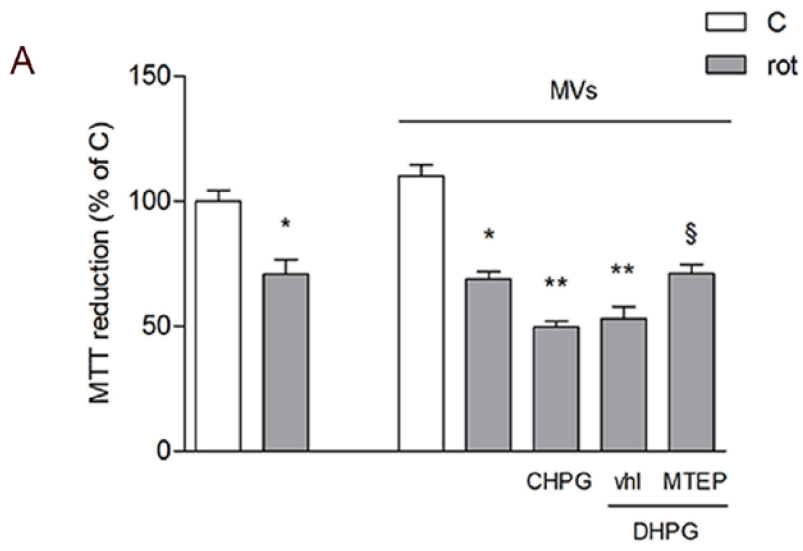


Fig. 3



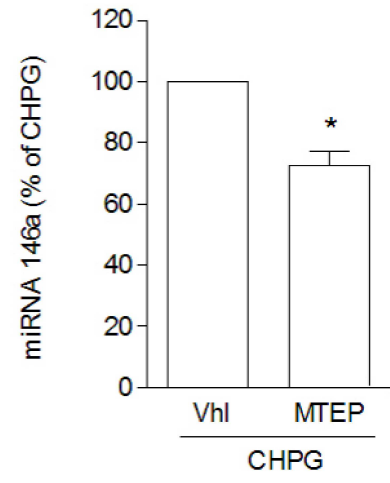
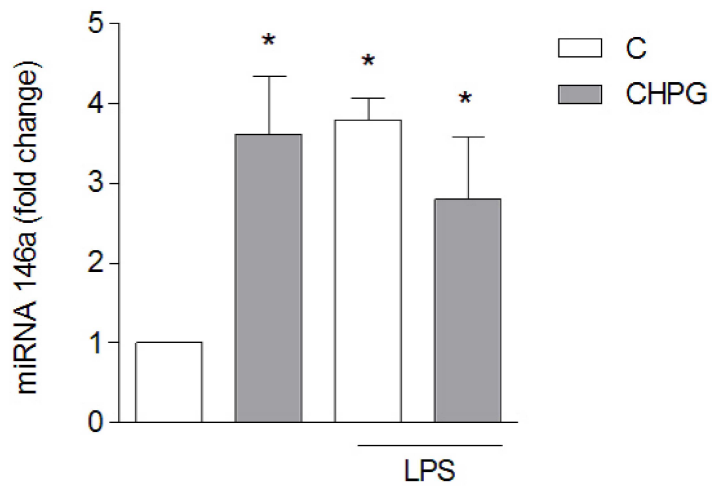


Fig. 5

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Manuscript Draft

Manuscript Number:

Title: PHARMACOLOGICAL BLOCKADE OF MGLU1 METABOTROPIC GLUTAMATE RECEPTORS IS PROTECTIVE AGAINST EXCITOTOXIC RETINAL DEGENERATION

Article Type: Research Paper

Section/Category: Disease-Oriented Neuroscience

Keywords: Retinal ganglion cells; mGlu1 receptors; monosodium glutamate; JNJ16258695; crv4 mice

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Abstract: Neuroprotection is an unmet need in eye disorders characterized by retinal ganglion cell (RGC) death, such as prematurity-induced retinal degeneration, glaucoma, and age-related macular degeneration. In all these disorders excitotoxicity is a prominent component of neuronal damage, but clinical data discourage the development of NMDA receptor antagonists as neuroprotectants. Here, we show that activation of mGlu1 metabotropic glutamate receptors largely contributes to excitotoxic degeneration of RGCs. Mice at postnatal day 9 were challenged with a toxic dose of monosodium glutamate (MSG, 3 g/kg), which caused the death of >70% of Brn-3a+ RGCs. Systemic administration of the mGlu1 receptor negative allosteric modulator (NAM), JNJ16258695 (2.5 mg/kg, s.c.), was largely protective against MSG-induced RGC death. We also injected MSG to crv4 mice, which lack mGlu1 receptors because of spontaneous recessive mutation of the Grm1 gene. MSG did not cause retinal degeneration in crv4 mice, whereas it retained its toxic activity in their wild-type littermates. These findings demonstrate that mGlu1 receptors play a key role in excitotoxic degeneration of RGCs, and encourage the study of mGlu1 receptor NAMs in models of retinal neurodegeneration.

Highlights

- We used the monosodium glutamate model in mice to examine the role of mGlu1 receptors in retinal neurodegeneration.
- Systemic pharmacological blockade of mGlu1 receptors was protective against MSG-induced retinal ganglion cell loss.
- *crv4* mice lacking mGlu1 receptors were resistant to MSG-induced retinal damage.
- mGlu1 receptor antagonists are promising candidate drugs for the treatment of retinal neurodegenerative disorders.

PHARMACOLOGICAL BLOCKADE OF MGLU1 METABOTROPIC GLUTAMATE RECEPTORS IS PROTECTIVE AGAINST EXCITOTOXIC RETINAL DEGENERATION

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Abstract

Neuroprotection is an unmet need in eye disorders characterized by retinal ganglion cell (RGC) death, such as prematurity-induced retinal degeneration, glaucoma, and age-related macular degeneration. In all these disorders excitotoxicity is a prominent component of neuronal damage, but clinical data discourage the development of NMDA receptor antagonists as neuroprotectants. Here, we show that activation of mGlu1 metabotropic glutamate receptors largely contributes to excitotoxic degeneration of RGCs. Mice at postnatal day 9 were challenged with a toxic dose of monosodium glutamate (MSG, 3 g/kg), which caused the death of >70% of Brn-3a⁺ RGCs. Systemic administration of the mGlu1 receptor negative allosteric modulator (NAM), JNJ16258695 (2.5 mg/kg, s.c.), was largely protective against MSG-induced RGC death. We also injected MSG to *crv4* mice, which lack mGlu1 receptors because of spontaneous recessive mutation of the *Grm1* gene. MSG did not cause retinal degeneration in *crv4* mice, whereas it retained its toxic activity in their wild-type littermates. These findings demonstrate that mGlu1 receptors play a key role in excitotoxic degeneration of RGCs, and encourage the study of mGlu1 receptor NAMs in models of retinal neurodegeneration.

Key words: Retinal ganglion cells, mGlu1 receptors, monosodium glutamate, JNJ16258695, *crv4* mice.

Abbreviations: mGlu, metabotropic glutamate receptor; MSG, monosodium glutamate; NAM, negative allosteric modulator; NMDA, N-methyl-D-aspartate; PI, polyphosphoinositide; PND, post-natal day; RGC, retinal ganglion cell; s.c., subcutaneous.

INTRODUCTION

Retinal ganglion cells (RGCs) collect the visual information from photoreceptors *via* bipolar and amacrine cells, and transfer the information to the brain through the optic nerve. Excitotoxic degeneration of RGCs is involved in the pathophysiology of several retinal disorders, such as glaucoma (Lipton, 2003; Seki and Lipton, 2008), age-related macular degeneration, and prematurity-induced retinal degeneration (Hinton et al., 1986; Tenhula et al., 1992; Lipton, 2003; Casson, 2006; Kaur et al., 2008; Seki and Lipton, 2008; Hernández and Simò, 2012). It is generally believed that glutamate-induced RGC damage is mediated by the activation of N-methyl-D-aspartate (NMDA) receptors and the resulting increase in intracellular Ca^{2+} (Ferreira et al., 1996; Niwa et al., 2016). However, the value of NMDA receptors as candidate drug targets for retinal neuroprotection was questioned by the disappointing clinical data with the NMDA channel blocker, memantine, in the treatment of glaucoma (Childlow et al., 2007; Osborne, 2009). The lack of efficacy of memantine might reflect the opposite role of synaptic and extrasynaptic NMDA receptors in mechanisms of neurodegeneration/neuroprotection (reviewed by Hardingham and Bading, 2010). Alternatively, glutamate receptor types other than NMDA receptors may contribute to excitotoxic retinal degeneration.

Group-I metabotropic glutamate receptors (mGlu1 and mGlu5 receptors) are linked to NMDA receptors through a chain of anchoring proteins, and their activation enhances NMDA receptor function (Tu et al., 1999; Awad et al., 2000). mGlu1 and mGlu5 receptors are coupled to $G_{q/11}$. Their activation stimulates polyphosphoinositide (PI) hydrolysis, a transduction mechanism that leads to increased intracellular free Ca^{2+} and activation of protein kinase C (Nicoletti et al., 2011). Both effects might contribute to neuronal degeneration under pathological conditions. The role of mGlu1 and mGlu5 receptors in mechanisms of neurodegeneration/neuroprotection is discussed in numerous review articles (Nicoletti et al., 1999; Bruno et al., 2001, 2017).

In the retina, mGlu1 and mGlu5 receptors are present in the dendrites of ON-bipolar cells and in amacrine cells. In addition, mGlu1 receptors are also found in RGCs (Koulen et al., 1997; Brandstätter et al., 1998; Yang et al., 2004). We found recently that mGlu1, but not mGlu5, receptors are coupled to PI hydrolysis in the mouse and bovine retina (Romano et al., 2016). This suggests that, in pathological conditions, mGlu1 receptors might be involved in mechanisms of retinal degeneration. We tested this hypothesis using the monosodium glutamate (MSG) model of

retinal degeneration (Blood et al., 1969; Lowe et al., 1970). This model is a milestone in the field of excitotoxicity, offering one of the first demonstration that excessive glutamate can be toxic to neurons. Using both genetic and pharmacological approaches we now demonstrate that activation of mGlu1 receptors largely contributes to MSG-induced RGC death.

EXPERIMENTAL PROCEDURES

Animals

C57BL/6J mice (Charles River, Calco, Italy), *crv4* mice and their BALB/c wild-type littermates (provided by A.P.) were used in this study. *crv4* mice have a spontaneous recessive mutation consisting of a retrotransposon long terminal repeat (LTR) insertion that disrupts the splicing of the *Grm1* gene determining the absence of the mGlu1 receptor protein (Conti et al., 2006). *crv4* mice were generated by heterozygous breeding. Mouse genotyping was performed in DNA extracts from the mouse tail as reported previously (Conti et al., 2006). The lack of mGlu1 receptor protein in *crv4* mice was confirmed by Western blot analysis in the cerebellum and immunohistochemical analysis in the retina. Pregnant dams had free access to food and water and were maintained in cages in an environmentally controlled room with a temperature of 22°C, a humidity of 55%, and a 12-hour light/dark cycle. The offspring was used at postnatal day (PND) 9, with no sex distinction.

All efforts were made to reduce the number of animals used. All experiments were carried out in agreement with the Statement for the Use of Animals in Ophthalmic and Vision Research and in compliance with the Italian law on Animal Care No. 26/2014 and the Directive 2010/63/EU. The protocols were approved by the local Animal Care and Use Committee of Neuromed Institute.

Experimental design

C57BL/6J mice at PND9 (n = 3-9 mice per group) were treated as follows. Two groups received a subcutaneous (s.c.) injection of sesame oil, followed, 30 min later, by s.c. injection of either saline or MSG (3 g/kg) (Sigma-Aldrich, Milan, Italy); two additional groups were treated with 3,4-dihydro-2H-pyrano[2,3-b]quinolin-7-yl-(cis-4-methoxycyclohexyl)-methanone

(JNJ16258695), dissolved in sesam oil (2.5 mg/kg) (Tocris Bioscience, Bristol, UK) followed, 30 min later, by a s.c. injection of either saline or MSG. In another set of experiments, four groups of *crv4* mice or their wild-type littermates (n = 5-7 mice per group) were injected s.c. with either saline or MSG (3 g/kg). Homozygous *crv4* and wild-type mice were genotyped prior to the experiment.

All mice were killed 18 hours after drug injection. The eyes and cerebella were quickly removed. Cerebella were stored at -80°C. Eyes were placed in 4% (w/v) paraformaldehyde for 5 hours at 4°C. After extensive washing in water for 5 min, eyes were placed in 70% ethanol at 4°C until paraffin inclusion.

Immunohistochemical analysis and RGC counting

Retina sections (20 µm) were incubated overnight with polyclonal goat anti-Brn-3a antibody (1:100; sc-6026, Santa Cruz Biotechnology, Dallas, TX, USA) and then for 1 hour with secondary biotin-coupled anti-mouse antibody (1:200; Vector Laboratories, Burlingame, CA, USA). 3,3-diaminobenzidine tetrachloride (Sigma, Milan, Italy) was used for detection. Before incubation with primary antibody, sections were treated with citrate buffer, and heated in a microwave for 15 min for antigen retrieval. Negative control was performed without primary antibody.

Neuronal density values (number of RGCs/mm²) were calculated in retinal tissue slides stained with Brn-3a antibody. Cells were counted at 100x magnification within a rectangular area (disector) on a computer monitor of 2500 mm². This rectangular area was positioned randomly by the software within an area of interest, drawn by the operator at 2.5x magnification, over the ganglion cell layer of the retina slides.

Confocal microscopy analysis of mGlu1α receptors in the retina

For immunofluorescence analysis, 30 µm retinal sections were incubated with a mouse anti-mGlu1α primary antibody (1:200, BD Bioscience, Milan, Italy). Serial sections were then incubated with a secondary antibody conjugated to Alexa Fluor 488 (1:200, Invitrogen, Carlsbad, CA, USA). Slides were then coverslipped with mounting medium (Vector). All coverslipped mounted tissue sections were scanned using a LSM 5 Pascal confocal laser scanning microscope with a Zeiss ECPLAN-NEOFLUAR 40 × /1.30 M27 oil immersion objective (Carl Zeiss

Microimaging Inc.). We used a 488 nm argon laser to excite Alexa Fluor 488.

Western blot analysis of mGlu1 α receptors in *crv4* and wild-type mice

Western blot analysis of mGlu1 α receptors was performed in the cerebellum of *crv4* mice and their wild-type littermates. Tissue was homogenized and lysed on ice with RIPA buffer containing a cocktail of protease inhibitors (Santa Cruz Biotechnology) for 30 min and an aliquot was used for protein determination. Lysates were separated on 8% sodium dodecyl sulphate-PAGE at 100 V for 1 hour and transferred onto Immuno PVDF membranes (Bio-Rad, Milan, Italy) for 7 min using the Trans Blot Turbo System (Bio-Rad). The membrane was incubated for 2 hours in blocking buffer (TBS, 0.05% Tween-20 and 5% nonfat milk) and, then, overnight with a mouse monoclonal anti-mGlu1 α antibody (1:700, BD Biosciences). Filters were washed three times in Tris-Tween buffered saline and then incubated for 1 hour with secondary peroxidase-coupled antibody (anti-mouse 1:7000, Calbiochem, Milan, Italy). Immunostaining was revealed by enhanced chemiluminescence luminosity (GE Healthcare Life Science, Milan, Italy). The blots were reprobated with monoclonal anti- β -actin antibody 1:50000 (Sigma-Aldrich).

Statistical analysis

Data are presented as means \pm S.E.M. Statistical analysis was performed by one-way ANOVA + Newman-Keuls test to isolate the differences. A *p* value < 0.05 was considered as statistically significant.

RESULTS

Pharmacological blockade of mGlu1 receptors is protective against MSG-induced neuronal death in the mouse retina

We performed immunohistochemical analysis using antibodies directed towards Brn-3a, a reliable marker for the identification and quantification of RGCs (Nadal-Nicolàs et al., 2009). Accordingly, Brn-3a immunostaining was confined to RGCs and was not observed in other retinal layers (Fig. 1, Fig. 2).

A single systemic injection of MSG (3 g/kg) to PND9 C57BL/6J mice caused extensive retinal degeneration reducing the number of Brn-3a⁺ RGCs by >70% (Fig. 1A,B). Pre-treatment with the selective mGlu1 receptor NAM, JNJ16259685 (2.5 mg/kg), markedly attenuated MSG-induced retinal damage. In mice treated with JNJ16259685, MSG lowered the number of RGC by less than 20% (Fig. 1A,B), suggesting that activation of mGlu1 receptors largely contributes to excitotoxic RGC damage. JNJ16259685 treatment did not affect RGC number in the absence of MSG (Fig. 1A,B).

MSG-induced retinal damage is attenuated in mice lacking mGlu1 receptors

To confirm that mGlu1 receptors are involved in mechanisms of retinal degeneration, we used *crv4* mice and their wild-type littermates. The lack of mGlu1 receptor protein in *crv4* mice was confirmed by Western blot analysis in the cerebellum, a region in which mGlu1 receptors are expressed at high levels (Nicoletti et al., 2011). Immunoblots of mGlu1 α receptor protein showed the expected band at 140 kDa corresponding to receptor monomers, and a higher molecular size band, corresponding to receptor dimers (Fig. 2A). The mGlu1 α receptor was absent in the cerebellum of *crv4* mice, as expected (Fig. 2A). We also examined mGlu1 α receptor immunoreactivity in the retina of wild-type and *crv4* mice by confocal analysis. In wild-type mice, mGlu1 α immunoreactivity was detected in the RGCs and inner plexiform layers (Fig. 2B) in agreement with previous reports (Koulen et al., 1997; Dhingra and Vardi, 2012). mGlu1 α immunoreactivity was not detected in the retina of *crv4* mice (Fig. 2B).

There was no difference in RGC number between *crv4* mice and their wild-type littermates (Fig. 3A,B). MSG administration to wild-type BALB/c littermates reduced RGC number to a lower extent than in C57BL/6J mice (compare Fig. 3 with Fig. 1), indicating that excitotoxic retinal damage is strain-dependent. MSG-induced RGC degeneration was abolished in *crv4* mice (Fig. 3A,B), confirming that, at least in the MSG model, activation of mGlu1 receptors is necessary for the induction/development of excitotoxic retinal damage.

DISCUSSION

Our data demonstrate that mGlu1 receptors are recruited in mechanisms of excitotoxic retinal degeneration and their activation is necessary for MSG-induced RGC death. This could not be

automatically inferred from our knowledge of mGlu1 receptor signaling because the existing data on mGlu1 receptors and neurodegeneration are controversial. Most of the studies carried out in *in vitro* or *in vivo* models have shown a neuroprotective activity of mGlu1 receptor antagonists against excitotoxic or hypoxic/ischemic neuronal death (Bruno et al., 1999; Pellegrini-Giampietro et al., 1999; Battaglia et al., 2001; De Vry et al., 2001; Cozzi et al., 2002; Meli et al., 2002; Moroni et al., 2002; Murotomi et al., 2008, 2010; Smialowska et al., 2012; Li et al., 2013). However, mGlu1 receptor knockout mice were as vulnerable as their wild-type counterparts to focal cerebral ischemia and kainate-induced neurotoxicity (Ferraguti et al., 1997), and pharmacological activation of mGlu1 receptors was found to protect motor neurons against kainate-induced toxicity (Valerio et al., 2002). Thus, mGlu1 receptors can be either neurotoxic or neuroprotective depending upon the cellular context and the experimental paradigm of neurotoxicity. For example, Michel Baudry and his colleagues have shown that mGlu1 receptors may activate either a neuroprotective signal (the phosphatidylinositol-3-kinase pathway) or a toxic signal (intracellular Ca^{2+} release), and the concomitant activation of NMDA receptors shifts the balance towards the neurotoxic signal (Xu et al., 2007; Zhou et al., 2009). In cultured neurons, mGlu1 receptors behave as a «dependence receptors», supporting cell survival in response to agonist activation, and causing apoptotic death in the absence of glutamate (Pshenichkin et al., 2008). Another variable is the heterodimerization of mGlu1 receptors with mGlu5 receptors (Doumazane et al., 2011), which are also coupled to PI hydrolysis and have a dual role in mechanisms of neurodegeneration/neuroprotection (Nicoletti et al., 1999).

At least the latter component is absent in the retina, where only mGlu1 receptors appear to be coupled to PI hydrolysis (with the detection limits of the PI assay). Accordingly, using both bovine retinal slices and intact mouse retinas, we were able to demonstrate that stimulation of PI hydrolysis by the mGlu1/5 receptor agonist, 3,5-dihydroxyphenylglycine (DHPG), was sensitive to mGlu1 receptor blockade, but was not influenced by mGlu5 receptor blockade. In addition, we found no PI response to DHPG in retinas of *crv4* mice lacking mGlu1 receptors, whereas the response was intact in retinas of mGlu5 receptor knockout mice (Romano et al., 2016). This provides an unique example of a tissue in which a detectable PI response to DHPG is exclusively mediated by mGlu1 receptors. Our present findings indicate that mGlu1 receptors are key players in mechanisms of RGC degeneration triggered by toxic concentrations of glutamate. This has potential translational value in the treatment of prematurity-induced retinal damage, in which

excitotoxicity largely contributes to the pathophysiology of RGC death (see Introduction and References therein). Pituitary adenylyl cyclase activating peptide (PACAP) and the potassium channel activator, diazoxide, have shown neuroprotective activity in the MSG model (Tamàs et al., 2004; Racz et al., 2007; Atlasz et al., 2007, 2008). However, both compounds were tested after intravitreal administration, and whether systemic administration of PACAP or diazoxide may confer neuroprotection against retinal damage is unknown. JNJ16259685 shows central bioavailability in rodents (Lavreysen et al., 2004), and, because of its hydrophobicity, is expected to cross the blood-retinal barrier and reach sufficient concentrations to achieve neuroprotection in the developing human retina. By definition, NAMs bind to an allosteric region of mGlu1 receptors, and, therefore, they inhibit mGlu1 receptor activation regardless of the ambient concentrations of glutamate. Thus, JNJ16259685 and other mGlu1 receptor NAMs might be potential candidates for the treatment of prematurity-induced retinal degeneration. Our data also encourage the study of mGlu1 receptor NAMs as potential disease modifiers in the treatment of glaucoma and other chronic neurodegenerative disorders of the retina. The study, however, requires a chronic systemic treatment with mGlu1 receptor NAMs, which might have negative consequences on motor learning and motor coordination by restraining the activity of mGlu1 receptors in cerebellar Purkinje cells (Aiba et al., 1994; Conquet et al., 1994; Ichisa et al., 2000; Ohtani et al., 2014; Kano and Watanabe, 2017). Thus, accurate pharmacological studies in experimental animal models of glaucoma and other degenerative eye disorders are needed to establish whether mGlu1 receptor blockade might be considered as a valuable and safe strategy to slow the progression of these disorders.

Conflict of interest

The authors do not have any conflict of interest

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FIGURE LEGENDS

Fig. 1 – Systemic pharmacological blockade of mGlu1 receptors is protective against MSG-induced RGC damage.

Representative images of Brn-3a⁺ RGCs of mice treated s.c. with vehicle + saline, JNJ16259685 (2.5 mg/kg) + saline, vehicle + MSG (3 g/kg), and JNJ16259685 + MSG are shown in (A). Cell counts are shown in (B), where values are means \pm S.E.M. of 3-9 retinas per group (one retina per individual mouse). $p < 0.05$ (One-way ANOVA + Neumann-Keuls post-hoc test) vs. (*) control mice (i.e., mice treated with vehicle + saline) or (#) mice treated with vehicle + MSG. $F_{(3,21)} = 35.6$.

Fig. 2 – Lack of mGlu1 receptors in the cerebellum and retina of *crv4* mice used for toxicity experiments.

Western blot analysis of mGlu1 α receptor protein in the cerebellum of wild-type littermates of Balb/c strain and *crv4* mice is shown in (A). The representative immunoblot (n = 4-5 per group) shows the absence of mGlu1 α receptor in *crv4* mice, as expected.

Representative confocal images of mGlu1 α receptor protein in the retina of *crv4* mice and their wild-type littermates of Balb/c strain are shown in (B). mGlu1 α receptor expression in C57/Black mice is also shown for comparison. Note that in wild-type mice mGlu1 α receptor immunoreactivity was mainly detected in the ganglion cell layer (GCL), and that no immunoreactivity was detected in *crv4* mice.

Fig. 3 – *crv4* mice lacking mGlu1 α receptors are protected against MSG-induced RGC damage.

Representative images of Brn-3a⁺ RGCs of wild-type and *crv4* mice treated s.c. with vehicle or JNJ16259685 (2.5 mg/kg) are shown in (A). Cell counts are shown in (B), where values are means \pm S.E.M. of 5-7 retinas per group (one retina per individual mouse). * $p < 0.05$ (One-way ANOVA + Neumann-Keuls post-hoc test) vs. all other groups. $F_{(3,22)} = 5.03$.

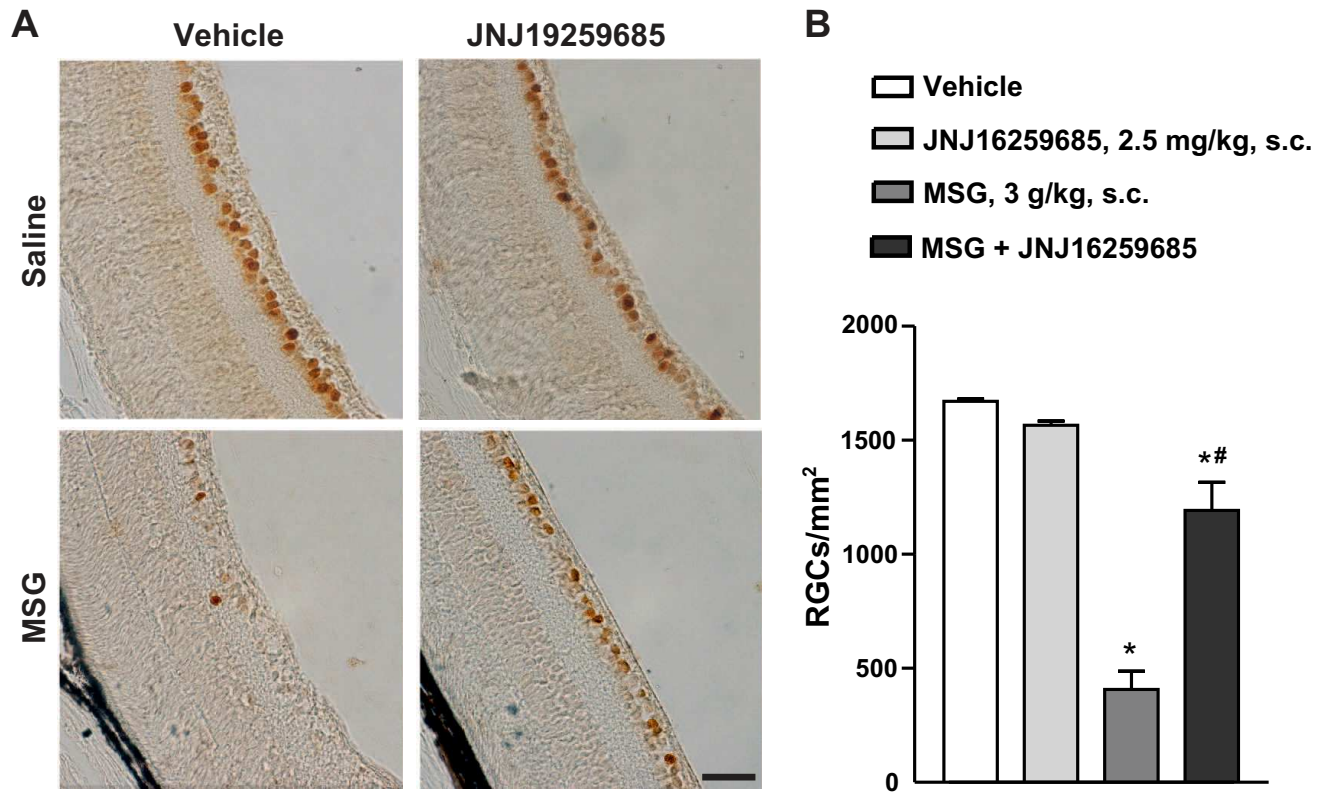
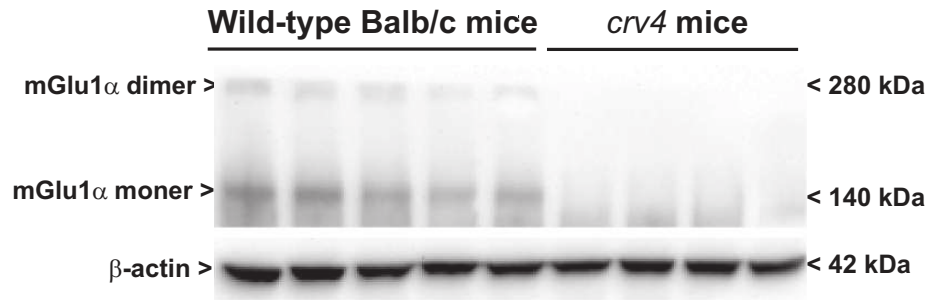


FIGURE 1

A



B

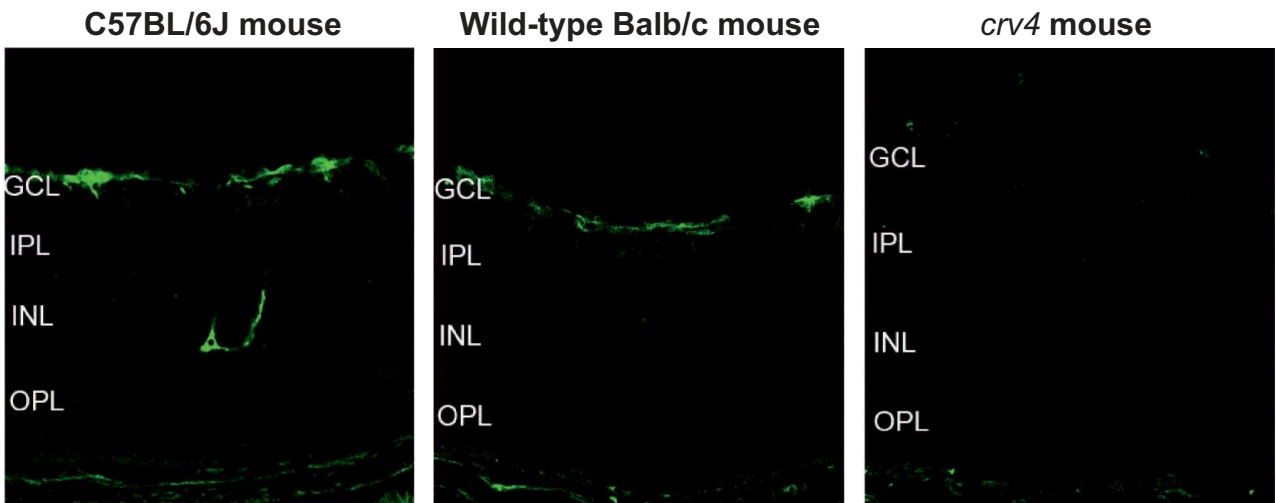


FIGURE 2

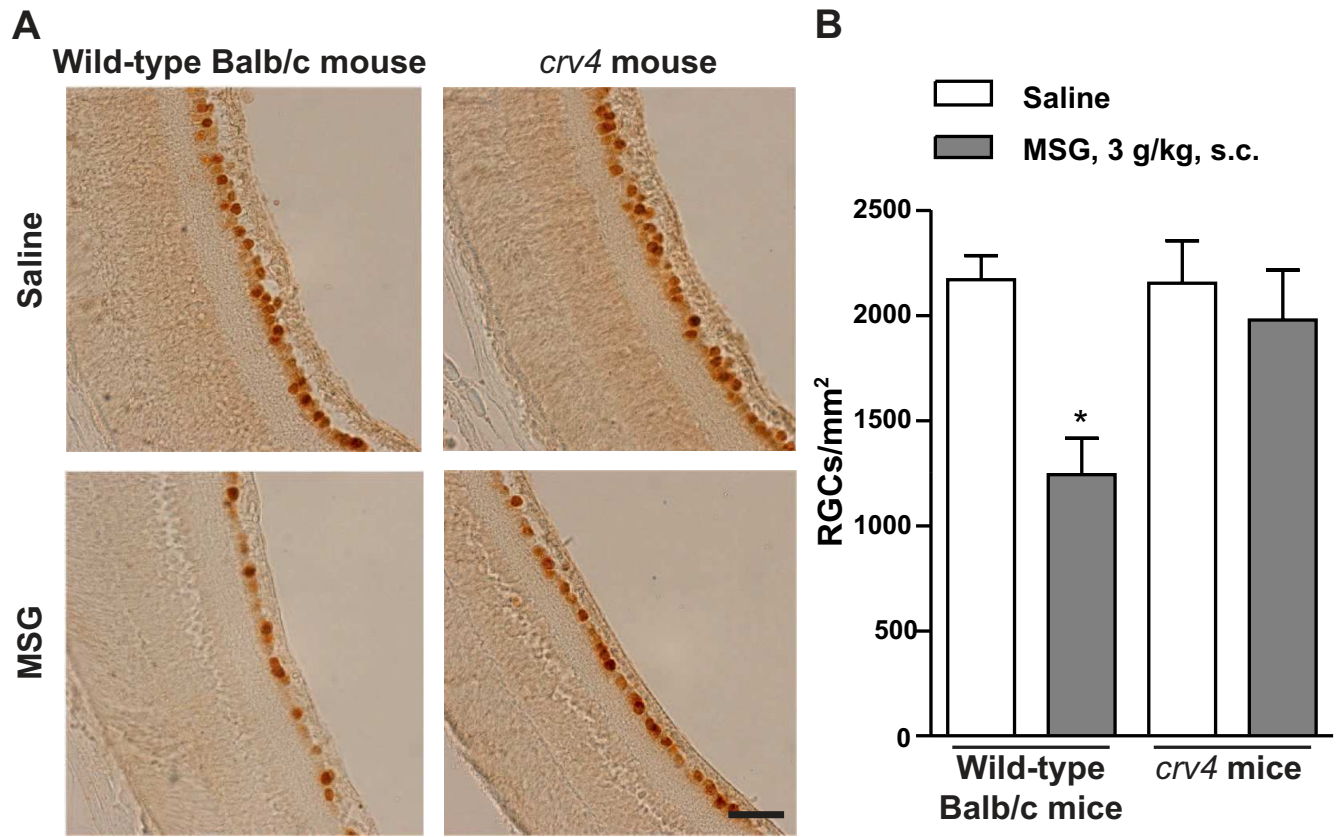


FIGURE 3

Concluding remarks

Group I mGlu receptors includes mGlu1 and mGlu5 receptors which have been largely shown to be involved in neurodegenerative and in neuroinflammatory processes. What captured our attention and was found particularly intriguing was that, despite the large literature in this regard, the role of both receptors is still debated and drugs acting either activating or blocking mGlu1 and mGlu5 receptors have been proposed as effective pharmacological tools in conditions of neurodegeneration/neuroinflammation. Our research has then been carried out in two different directions, considering more specifically a neuroinflammatory context to define the role of mGlu5 receptor and a more excitotoxic/neurodegenerative environment to assess the function of mGlu1 receptor. In particular, in several recent studies, mGlu5 receptor has been shown to exert either an antiinflammatory activity or a pro-inflammatory effect (Byrnes KR et al., 2009; Liu F. et al., 2014.). To understand more in depth the dual role of mGlu5 receptor, we focused on microglia that, as brain resident myeloid cells, represent the major cell type in the neuroinflammatory response and that had already been reported to express mGlu5 receptor, whereas mGlu1 receptor is only expressed at low or undetectable levels. We were particularly interested in establishing (1) whether mGlu5 receptor activation in microglia could modulate neuronal survival in response to toxic stimuli, and (2) whether this modulatory effect could take place through MVs shed from microglia that could operate as a cargo for glia-to-neuron communication. In the past few years, this novel mode of interaction, relying on the exchange of extracellular vesicles between cells, has become evident and seems to play a role in place of, or alternative to, classical ways of cell-to-cell interactions. Following activation of the ATP-gated ion channel, P2X7 receptor, by ATP or the selective agonist Benzoyl-ATP, cells undergo a dramatic morphological change in the plasma membrane. During their formation, MVs selectively accumulate various cellular components, including soluble and integral proteins, lipids and nucleic acids, reflecting the composition of donor microglia. MVs may then contain bioactive molecules which are able to influence neuron function

and also modulate the activity of surrounding non-neuronal cells. Initially we set up our experimental model to assess MVs formation using pure primary culture of rat microglia, obtained from a mixed glia cultures from the cortex of 1 to 3 days-old rat pups, as previously described by our own group (Spampinato et al., 2015). MVs could be obtained in response to stimulation of the purinergic P2X7 receptor and sequential captures, by time lapse microscopy, allowed us to follow their formation and shedding. Although MVs could be clearly detected in pure microglial cultures (see Fig. 4), we decided to switch to a microglial cell line, the BV2 murine microglial cells, in order to deal with technical issues, mainly the heterogeneity of the primary microglial culture and the possibility that astrocytes, that express mGlu5 receptor as well, could be present in the preparation and affect the observed response. Using this cellular model we could demonstrate that activation of mGlu5 receptor causes an apparent anti-inflammatory effect, suggested by reduction of the levels of TNF- α in activated microglia that however was not able to affect the viability of neurons exposed to a toxic challenge. MVs formation and function was clearly modified by mGlu5 receptor stimulation, as an increased amount of MVs occurred, likely due to potentiation of the purinergic currents by mGlu5 receptor activation. In addition, transferring of MVs from mGlu5 receptor activated-microglia resulted in an increased toxic effect. Interestingly, however, both these effects occurred under basal, but not activated conditions. Hence, our data point out an amplification of neuroinflammation by mGlu5 receptor activation, although this effect is not univocal and appears variable, depending on the cell status. This seems to be in line with the reported duality of mGlu5 receptor, evidencing, even within the same cellular system, how underlying conditions can influence its behavior and affect the final response. Regarding the content of MVs, we focused our attention on miRNA and identified miRNA146a as a potential mediator of mGlu5 receptor activation. However, results of this part of the study are rather preliminary, but appear promising and the study is still ongoing. Focusing on the function of mGlu1 receptor, we looked at RGCs, retinal neurons that represent the final ring of a chain to convey the external information, the image, to the CNS. In this cell type mGlu1 receptor has been shown to be the only group I mGlu receptor

to be functionally linked to PtdIns-4,5-P2 hydrolysis. To carry out this part of the project, I took advantage of the possibility to spend part of the time in an external lab, so that I joined for few months, at the University of Rome, the lab headed by Prof Ferdinando Nicoletti, who is a leading scientist in the field of mGlu receptors. This part of the study gave me the chance to perform an in vivo study and to work also with transgenic animals that do not express the *grm1* gene, so that they lack mGlu1 receptor. Our data demonstrate that mGlu1 receptor is critical for the excitotoxicity induced by exposure to MSG, as suggested by significantly decreased retinal damage in mice treated with a selective mGlu1 receptor NAM. Interestingly, the retinal damage in response to the toxic stimulus was reduced in *crv4* mice bearing the spontaneous recessive mutation that disrupts the splicing of the *Grm1* gene causing the absence of the mGlu1 receptor protein. These data suggest in a clearly defined way a negative function of mGlu1 receptor in the model of retinal degeneration we have used. In conclusion, the data obtained in our project identify and confirm group I mGlu receptors as a potential pharmacological target in different cell types, specifically microglia and retinal neurons. They also indicate an alternative way of modulating neuronal function, i.e. the control of shedding of MVs from microglial cells, thus widening the possibility of pharmacological intervention through mGlu receptors.

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Acknowledgements

I would like to thank Prof. Filippo Drago, Prof. Salvatore Salomone, Prof. Ferdinando Nicoletti and Prof. Agata Copani for giving me the chance to start an interesting and fascinating research work within this PhD program.

In particular I would like to thank Professor Maria Angela Sortino for giving me the opportunity to undertake an exciting research activity that has allowed me to grow in all aspects of my life. Thank you for your passion, and the dedication through which you followed me during my PhD period.

I would like to thank Dr. Sara Merlo and Dr. Simona Spampinato for teaching me step by step all I need to do to carry out a research activity in the right way. They have been for me teachers of life and it will be really hard to meet better colleagues of them in the future. I have a great esteem towards them and I consider them great friends. A very important figure from both human and scientific point of view was also that of Dr. Paola Platania who was immediately passionate about the project of my PhD and with great passion helped me to proceed in this research activity.