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CHLORIDE INTRACELLULAR ION CHANNEL 1 AS A CUTTING EDGE
BETWEEN NEUROGENESIS AND NEURODEGENERATION.

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Chloride Intracellular Ion Channel 1 as a cutting
edge between neurogenesis and
neurodegeneration.

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Abstract

Chloride intracellular ion channel 1 (CLIC1) has peculiar properties compared to most known ion channels, in that it exists both as a soluble protein and as a membrane-integrated protein. Previous studies have shown that CLIC1 insertion into the plasma membrane is driven by different stimuli such as oxidation, cytoplasmic acidification, increased calcium concentration. Once inserted into the plasma membrane CLIC1 forms a chloride selective ion channel.

The best characterized mechanism leading CLIC1 into the plasma membrane is cell oxidation. Our laboratory has previously shown that in microglia cells stimulation by amyloid beta peptides, one of the most common hallmarks of Alzheimer disease (AD), determines the increase of CLIC1-mediated current.

Many studies have demonstrated that many AD patients show at first visual impairment rather than other cognitive deficits. AD patients show severe abnormalities in the structure and function of the retina, including retinal ganglion cells (RGCs) loss. Several cellular mechanisms precede RGCs degeneration, including oxidative stress. Therefore, in view of the oxidation-driven behavior of CLIC1, during my PhD, I analyzed CLIC1 expression in mouse retina. We found out that CLIC1 expression is increased in retinas of two different AD mouse models. Interestingly, CLIC1 increased expression is prior to the manifestation of the first cognitive symptoms, thus proposing CLIC1 as an early marker of neurodegeneration in AD.

Although CLIC1 expression is largely increased in AD mouse retinas, CLIC1 is also expressed in wild type retinas, mainly in the ganglionic layer, where the nuclei of RGCs, whose axons make up the optic nerve, are located. Thus, we wondered about the role of CLIC1 in the normal RGCs physiology. Our studies unraveled the crucial role of CLIC1-mediated current in axon outgrowth of RGCs, showing that the inhibition of the chloride current causes a reduced axon

outgrowth that is parallel to an altered morphology of the growth cones and a reduced co-localization of CLIC1 with the actin cytoskeleton. Our results also suggest that CLIC1 involvement in axon outgrowth might be due to CLIC1 modulation by cAMP. Taken together our data propose cAMP, or cAMP homologues, as a putative tool to modulate CLIC1 transient expression at the plasma membrane and consequently axon outgrowth.

Introduction

David Hubel, Nobel prize for Physiology and Medicine in 1991 for his studies on the information processing in the visual system, said:

“The brain is a tissue. It is a complicated, intricately woven tissue, like nothing else we know of in the universe, but it is composed of cells, as any tissue is. They are, to be sure, highly specialized cells, but they function according to the laws that govern any other cells. Their electrical and chemical signals can be detected, recorded and interpreted and their chemicals can be identified; the connections that constitute the brain’s woven network can be mapped. In short, the brain can be studied, just as the kidney can.”

Thus, although the great and beautiful complexity of the human brain, it is made up by cells, like every organ in the body, and it can be studied: its electrical and biochemical signals can be interpreted.

The last decades of the previous century was characterized by a mutual effort to explore the brain functioning and was defined as the “brain era”. Many advances were achieved in the study of the physiology and the pathophysiology of the brain. However, in spite of all the resources and manpower utilized in the neurophysiology research, our comprehension of the brain network mechanism remain marginal.

Since the pioneering studies of Alan Lloyd Hodgkin and Andrew Huxley on the electrical properties of excitable cells, our knowledge on the nature of the signals responsible for the communication between neuronal cells underwent fast steps ahead, allowing the study of these mechanisms at the molecular levels.

From the key role of ion channels in determining the bioelectrical properties of excitable membranes, to intracellular components and second messengers: it became clear the fascinating complexity and variability of the neuronal signals.

Every day a little piece to the “puzzle” of the brain functioning and neuronal interconnections is added by hundreds of experimental works performed all around the world. Each single protein has its role. Perfectly integrated in the neuronal networks, each molecule gives its small contribution to the complex organized neuronal physiology.

During my PhD I focused my attention on one of these small molecules, unraveling its own contribution to the brain physiology.

CLIC1 (Chloride Intracellular Ion Channel 1) was the first member identified of a family of intracellular chloride channels (Valenzuela et al., 1997). CLIC1 is a polymorphic protein, with a double nature as a cytoplasmic protein and as a membrane-inserted protein. It has been shown that upon different stimuli, such as increased calcium concentration, acidification, and altered redox conditions, CLIC1 undergoes major structural rearrangements that lead its translocation to the plasma membrane. Once inserted into the lipid bilayer, it has been shown to constitute a chloride selective ion channel (Averaimo et al., 2010).

Although since its cloning many biochemical and biophysical studies have been performed in order to demonstrate its ion channel identity, its physiological function has been only recently demonstrated.

In resting microglia, CLIC1 is present in its soluble form in the cytoplasm. However, upon stimulation with different pro-oxidant compounds, such as phorbol esters, H₂O₂, LPS, that alter the cellular redox state, CLIC1 inserts into the plasma membrane (Milton et al., 2008; Novarino et al., 2004).

Our laboratory recently demonstrated CLIC1 involvement in neurodegeneration in Alzheimer Disease (AD).

AD is a progressive neurodegenerative disease affecting more than 20% of people worldwide. From a medical point of view, it is characterized by memory loss and cognitive impairment, caused by a massive neurodegeneration mainly localized in the hippocampus and in the cortex. From a histological point of view, it is characterized by intraneuronal accumulation of the hyperphosphorylated form of the cytoskeletal protein tau, and deposits of amyloid beta protein in the extracellular milieu, surrounded by activated microglia (Zhang et al., 2011).

Microglia cells represent the immune system of the brain. In case of dangerous insults to the brain health, such as bacterial infections, ischemia, or, in AD, amyloid beta peptides, they become activated. Activated microglia secretes pro-inflammatory factors such as tumor necrosis factor alpha, nitrites, cytokines and reactive oxygen species (ROS) in an attempt to eliminate the “insult”. However, these factors are toxic to neurons. While in bacterial infection the damage is restricted to specific brain areas, in AD the insult represented by amyloid beta peptides, is diffused throughout the brain, and the incapability of microglia cells to completely phagocytose and eliminate it, causes a chronic inflammation that leads to a massive neurodegeneration and to the typical symptoms of AD (Block et al., 2007).

NADPH oxidase is the main enzyme responsible for ROS production by microglia cells. Once activated, NADPH oxidase catalyses the displacement of electrons from the intracellular donor NADPH to the extracellular oxygen, causing the formation of superoxide anion, which is highly reactive. The displacement of negative charges carried by electrons leads to a net outflow of negative charges that cause a cell depolarization, which in turn impairs the optimal functioning of the oxidase itself (Wilkinson and Landreth, 2006).

Our laboratory has recently demonstrated that CLIC1-mediated current provides the counter ion necessary to balance the charges lost during ROS production by the oxidase, allowing an optimal ROS production (Milton et al., 2008).

Several clinical studies have demonstrated a tight link between neurodegeneration typical of AD and visual deficits. Many patients, indeed, complain visual impairments before the manifestation of cognitive symptoms. In these patients, electroretinograms show strong abnormalities due to a down regulation of retinal ganglion cells functioning and a thickening of the RGC layer (Berisha et al., 2007;Milton et al., 2008). The axons of the RGCs are gathered to form the optic nerve, which project to the visual cortex (Wallace, 2011).

Being unprotected by the skull, the eye represents the most accessible part of the central nervous system (CNS). Thus, these observations lead us to investigate the retina as a “mirror” of the physiopathological state of the CNS.

One of the main problems in the treatment of AD is that all the actual known treatments intervene when the disease symptoms are already manifested, which means that the patients already show clear cognitive impairments. At that point the neurodegenerative process is started and irreversible: dead neurons are not replaceable.

So in the last decade the biomedical research in this field is pointing out to the search for an early diagnosis. The possibility of intervening when the neurodegenerative process just started gets the possibility of rendering more effective the actual treatments.

In an attempt to identify a marker of neurodegeneration in AD, during my PhD I focused my attention on the retina.

Several studies have shown that CLIC1 is an ubiquitous protein (Ulmasov et al., 2007). It was first identified in the immune system, in particular in macrophages (Valenzuela et al., 1997).

Further studies have shown its localization in different areas of the brain, in monocytes, and our laboratory has recently shown its presence in tumor cells (Unpublished results).

During my PhD I demonstrated that CLIC1 is also expressed in the mammalian retina. Differently from what we were expecting, CLIC1 is not expressed in the retinal microglia. However, it was largely expressed in Muller cells (Mazzanti personal communication), which represent the main immune system cells in retina (Bringmann et al., 2006), and our studies demonstrated the unexpected strong expression of CLIC1 in retinal ganglion cells (RGCs).

In order to analyze the possibility of using CLIC1 as a marker of AD, I analyzed CLIC1 expression and localization in retinal sections from AD mouse models. Our results propose CLIC1 as an early marker of the physiopathology of AD.

Furthermore, the strong expression of CLIC1 both in adult and “young”, developing RGCs prompted us to analyze also its role in RGCs physiology. In order to understand the physiological function of CLIC1 in RGCs, we interfered with CLIC1-mediated current. We found out an involvement of CLIC1 in axon outgrowth, suggesting an involvement of CLIC1 both in neuronal maturation and degeneration, making us to speculate about a possible link between these two functions.

Chapter 1

Chloride intracellular Ion Channel 1.

Chloride intracellular Ion Channel 1 (CLIC1) is the first member identified of a 7 members family of intracellular chloride channels named CLIC 1-4, CLIC5A, CLIC5B, p64 and parchorin, which have a wide tissue and subcellular distribution in mammalian cells (Berryman and Bretscher, 2000;Duncan et al., 1997;Heiss and Poustka, 1997;Landry et al., 1993;Nishizawa et al., 2000;Qian et al., 1999;Ulmasov et al., 2007;Valenzuela et al., 1997). CLIC1 was first cloned in a macrophage cell line activated by phorbol ester (PMA) (Valenzuela et al., 1997). CLIC1 is a polymorphic protein, which can be present either as a soluble protein in the cytoplasm or as a membrane-inserted protein. Upon different stimuli, such as increased intracellular calcium concentration, pH acidification, and changes in the cellular redox state, it undergoes major structural changes and inserts into lipid membranes. Once inserted into the plasma membrane CLIC1 forms a chloride selective ion channel (Averaimo et al., 2010).

1.1 Biochemical properties of CLIC1.

CLIC1 is a small protein composed by 241 aminoacids, with a molecular weight of 27 KDa (Valenzuela et al., 1997). The crystal structure of the soluble form of the protein has been resolved few years ago by Harrop and colleagues (Harrop et al., 2001). CLIC1 has a reduced glutathione (GSH)-binding site in its N-terminal domain, which could render this protein sensitive to the oxidative state of the cell. The current hypothesis proposes that, upon oxidation, the conformational changes that bring the protein to the plasma membrane cause a detachment of the GSH from its binding site and an almost complete rearrangement of the N-terminal domain:

the GSH binding site is broken and the exposition of a hydrophobic region causes the interaction of CLIC1 with the plasma membrane (Goodchild et al., 2009).

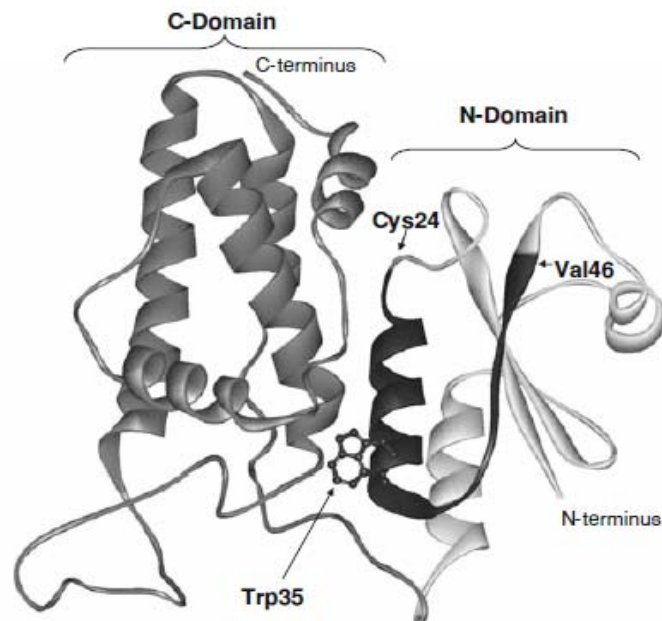


Figure 1.

Crystal structure of monomeric CLIC1. the C-terminal domain is shaded in dark grey; the putative TM domain is shaded in black and the two flanking residues (Cys 24 and Val 46) are indicated. The N-terminal domain is in light grey. Trp 35, used to demonstrate the existence of the TM is also indicated. Adapted from Goodchild et al., Eur Biophys J, 2009.

The N-terminus and the C-terminus domains are connected by a proline-rich region, in which proline 91 has been suggested to have a main role in the changes from the soluble to the membrane-inserted form of the protein, due to its change from *cis* to *trans* configuration (Harrop et al., 2001). Although the crystal structure of the membrane-inserted form has not been solved yet, bioinformatic studies have shown that CLIC1 has a single putative transmembrane (TM) domain between cysteine 24 and valine 46 (Harrop et al., 2001). Not only this region has the length and the hydrophobic characteristics necessary to cross the lipid bilayer, but also digestion experiments with proteinase K on a strictly related member of CLIC family, CLIC4, suggest that this region is protected by the digestion because is masked by the lipid membrane.

The mechanism of CLIC1 insertion into the plasma membrane is still not completely understood. According to the model proposed by Littler in 2004, *in vitro*, upon oxidation, CLIC1 undergoes structural changes that expose hydrophobic residues which promote the protein dimerization, in order to minimize the contact with the aqueous solution. *In vivo*, the hydrophobic region could act as a membrane docking region, promoting CLIC1 insertion into the plasma membrane, and protein dimerization could be only successive

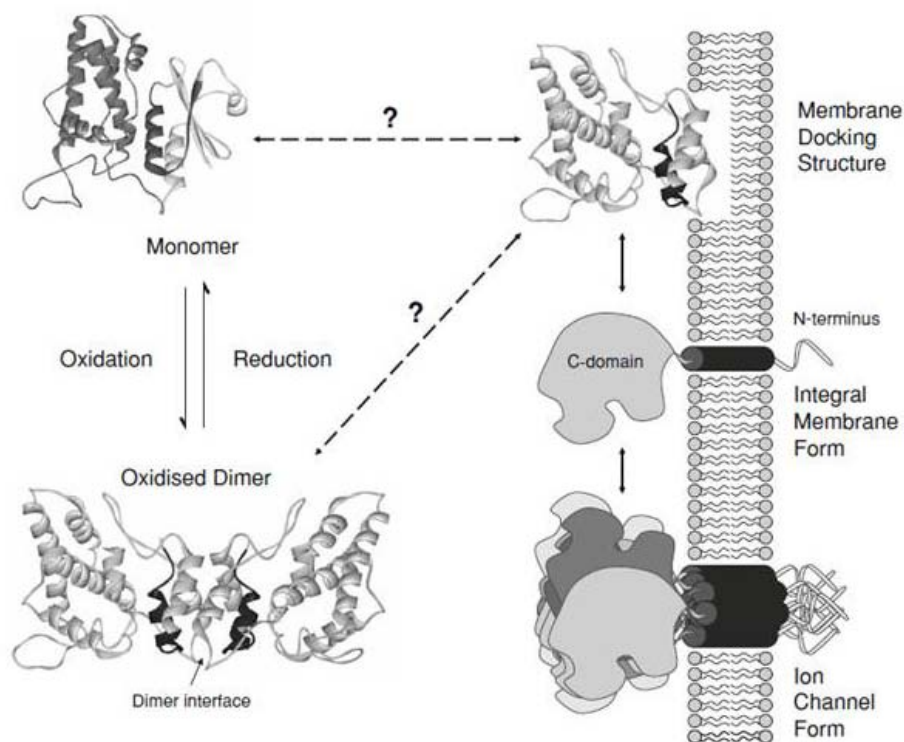


Figure 2.

Current model of CLIC1 insertion into the plasma membrane upon oxidation. The oxidation-driven conformational change of CLIC1 monomer leads it either to the dimerization or to the interaction with the plasma membrane with the N-terminal domain facing the extracellular solution and the cytoplasmic C-terminal domain. Association of 2 or 4 CLIC1 protein may lead to the formation of the fully functional ion channel. Adapted from Goodchild et al., Eur biophys J, 2009.

Cysteine 24 and 59 seem to be crucial for the transition of the monomeric form to the dimeric form, probably due to the formation of a disulfide bridge between these two residues to stabilize the dimeric form (Littler et al., 2004).

Goodchild and colleagues demonstrated that the dimerization is not necessary for the protein interaction with the plasma membrane, but the monomer itself can first interact with the lipid bilayer, and only successively the oxidation could promote the dimerization and the structural changes leading to the insertion of CLIC1 into the plasma membrane and the formation of a functional ion channel. However, it is still not clear how CLIC1 can interact with the plasma membrane before oxidation if the docking-membrane region is masked as part of the GSH binding site (Goodchild et al., 2009).

Only recently, based on FRET experiments Goodchild and colleagues proposed a new model stating that to make up a fully functional ion channel, six or even eight CLIC1 subunits need to associate together to constitute the hydrophilic ionic pathway (Goodchild et al., 2011).

Once inserted into the plasma membrane CLIC1 exposes its N-terminal domain to the extracellular side, while the C-terminus remains on the intracellular side (Milton et al., 2008; Tonini et al., 2000).

1.2 Biophysical properties of CLIC1 ion channel.

At the beginning, CLIC1 was identified in transfected cells, mainly in the nucleoplasm and in the nuclear membrane (Valenzuela et al., 1997). Later, it has been demonstrated that its subcellular localization varies strongly amongst the different cell types. For example, in non-polarized cells it appears diffused throughout the cytoplasm, while in polarized cells it localizes primarily at the apical domain (Ulmasov et al., 2007).

Due to the main properties of the protein, such as the unusual 3D structure, the actual identity of CLIC1 as an ion channel has been largely debated. It seems indeed unlikely that a protein undergoes so large conformational changes to insert into the plasma membrane. Thus, many scientist proposed that CLIC1 could work as a regulator of other ion channels resident in the plasma membrane. However, several studies with different experimental approaches have been performed in order to demonstrate its ion channel identity, either in artificial bilayer, in cell expression systems or in endogenously CLIC1 expressing cells (Novarino et al., 2004;Singh and Ashley, 2006;Tonini et al., 2000;Tulk et al., 2002;Valenzuela et al., 1997;Valenzuela et al., 2000;Warton et al., 2002). Purified CLIC1 protein reconstituted in artificial bilayer allowed the recording of ion channel openings, with the same biophysical features of those recorded in patch clamp on single cells, either in transfected CHO (Chinese Hamster Overy) cells or in microglia cells expressing endogenous CLIC1 (Milton et al., 2008;Novarino et al., 2004;Tonini et al., 2000). Expression of CLIC1 carrying a tag at the N-terminus of the protein demonstrated that CLIC1 not only docks the plasma membrane, but it also spans the lipid bilayer forming the ion channel, and the ionic flow can be inhibited by antibodies specifically directed against the N-terminus of the protein (Tonini et al., 2000). CLIC1 ion selectivity has been measured by several groups, being $I^- > SCN^- > Cl^- > NO_3^{2-} > Br^- > F^-$ (Singh and Ashley, 2006). The reversal potential of the current varies accordingly to the changes in chloride concentration (Novarino et al., 2004). The single channel conductance of CLIC1 is strictly dependent on chloride concentration. In equimolar 140 mM chloride CLIC1 conductance is 30 pS, while in patch clamp experiments, where intracellular chloride concentration is 20 mM and the extracellular concentration is 140 mM, the single channel conductance is 7 pS. CLIC1 is slightly voltage dependent and slightly inwardly rectifying (Novarino et al., 2004).

The only effective ion channel inhibitor so far identified is IAA94, which seems to be quite specific at a concentration of 50 μ M, with an EC_{50} of 8.6 μ M (Tulk et al., 2002). A

crystallographic study demonstrated that the structure of IAA94 is very similar to the ethachrinic acid, the inhibitor of the GST protein family (Cromer et al., 2002). The current hypothesis is that upon oxidation, CLIC1 loses the GSH and rearranges its structure. After the spanning of CLIC1 through the plasma membrane the binding site is faced on the extracellular side, accessible to the channel blocker (Harrop et al., 2001; Littler et al., 2004).

Exogenous expression of wild type (WT) and mutated protein in cell systems allowed to record CLIC1 single channel activity on the plasma membrane of transfected cells (Milton et al., 2008; Novarino et al., 2004; Tonini et al., 2000). We have previously shown that point mutations of two residues in the putative TM domain significantly alter the biophysical features of the ion channel, like the open probability and the single channel conductance, further demonstrating that CLIC1 is actually an ion channel (Unpublished results).

1.3 Physiological role of CLIC1 in microglia cells.

Although many laboratories are actively working to analyze the biochemical and biophysical properties of CLIC1, its physiological role has been only recently demonstrated.

The tight regulation of CLIC1 by redox conditions (Singh and Ashley, 2006; Tulk et al., 2002; Warton et al., 2002) led to investigate its role in oxidative cellular processes.

Many cellular processes involve changes in the redox state of the cell, such as duplication, migration, differentiation and maturation. Reactive oxygen species (ROS), indeed, may act as second messengers. Many proteins have redox-activated sites, containing cysteine residues that are modified by changes in the redox state (Menon and Goswami, 2007). Cell homeostatic systems stabilize a precise balance between ROS produced and eliminated.

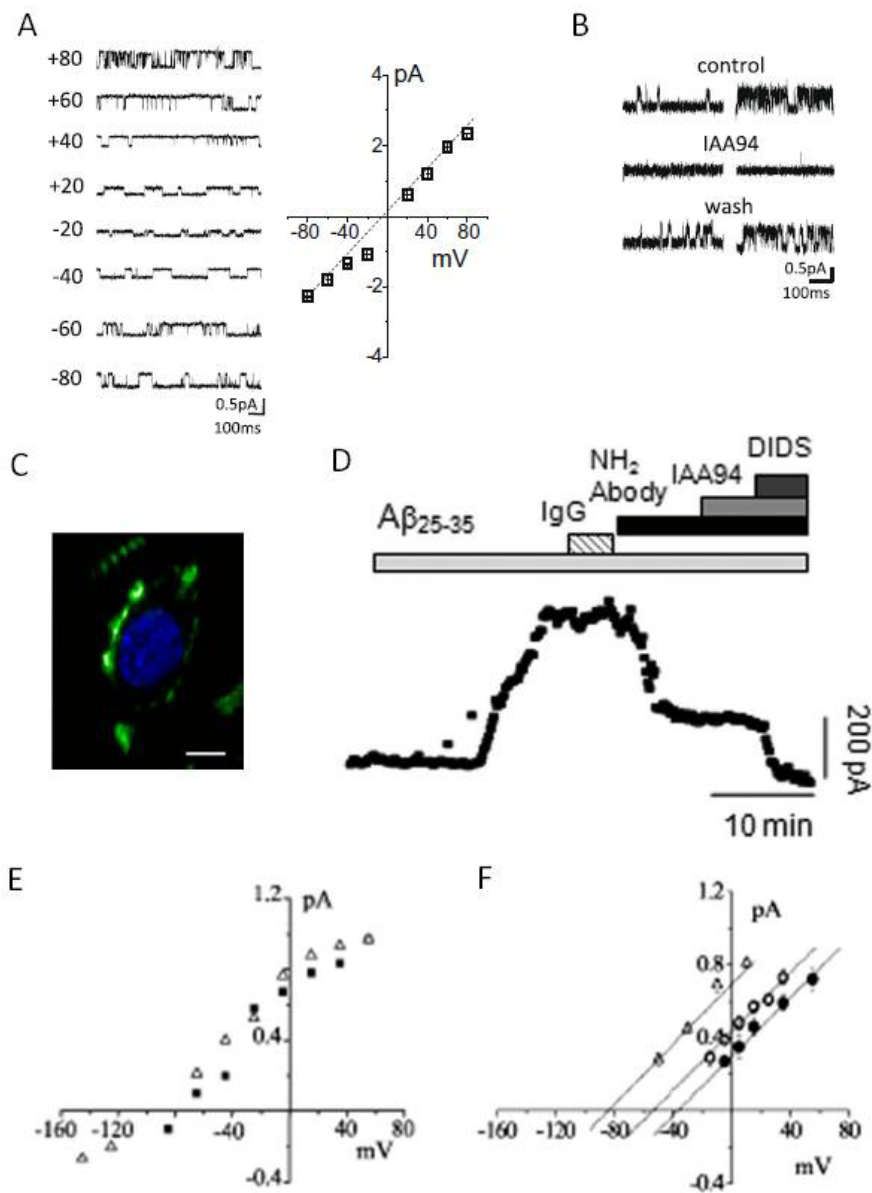


Figure 3.

A) Single channel recordings of CLIC1 in tip dip at the different voltages applied (left) and relative *iV* relationship (right). B) outside out single channel block by IAA94 in BV2 cells. C) Unpermeabilized stimulated BV2 cell probed with anti NH₂ antibody confirmed CLIC1 localization in the plasma membrane, exposing the NH₂-terminus on the extracellular side. D) CLIC1 mediated current recorded in patch clamp on BV2 cell stimulated with Ab is specifically reduced by the perfusion of NH₂-antibody. E) *iV* curves of CLIC1 mediated current in a BV2 cell (Δ) and in primary microglia (■). F) linear fit of single channel recordings obtained at the different voltages at 149.5 (Δ), 60 (○) and 30 (●) mM chloride in the cell-attached pipette. Adapted from Milton et al., *the Journal of Neuroscience*, 2008; Novarino et al., *the Journal of neuroscience* 2004; Averaimo et al., *FEBS letters*, 2010.

However, when, for any reason, ROS production overwhelms its removal, a condition of oxidative stress is established. Several pathological conditions are characterized by deep alterations of the cellular redox state (Behrend et al., 2003;Boillee and Cleveland, 2008;Gao et al., 2003;Thomas et al., 2007;Wu, 2006).

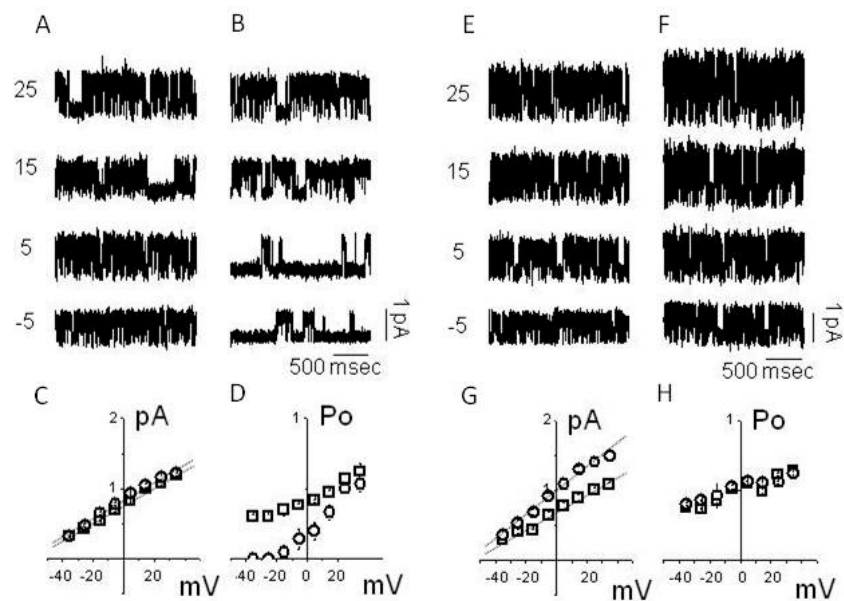


Figure 4.

Single-channel current traces are shown for wild type (A and E), R29A mutant (B), and K37A mutant (F) transfected cells. On the bottom of the figure: single-channel iV relationship for R29A (C) and K37A (G) and open probability of R29A (D) and K37A (H) mutant, compared to the control.

A pathological condition associated with oxidative stress is Alzheimer Disease (AD). Activated microglia surrounding deposits of amyloid beta ($A\beta$) protein in the central nervous system, one of the main hallmarks of AD, secretes several neurotoxic factors, including ROS, in order to eliminate the dangerous cue. CLIC1 plays a crucial role in ROS production by activated microglia (Meyer-Luehmann et al., 2008).

ROS production in microglia cells is mainly due to the activation of the NADPH oxidase (Bedard and Krause, 2007). The enzyme catalyzes the displacement of electrons from the intracellular donor NADPH to the extracellular oxygen (Wilkinson and Landreth, 2006). The outflow of negative charges carried by electrons causes a cell depolarization (Decoursey et al., 2003; Henderson et al., 1987). The excessive depolarization could in turn impair the functioning of the oxidase itself, weakening an efficient ROS production (Decoursey et al., 2003). Indeed, it has been demonstrated that in other phagocytic cells NADPH oxidase activity starts to decline already around 0 mV and it completely shuts down when the membrane potential reaches 200 mV (Decoursey, 2003). So the cell needs a mechanism to balance this leak of negative charges and set the membrane potential to voltages that allow an optimal functioning of the NADPH oxidase and an optimal ROS production. Many ionic currents have been proposed to be involved and set the membrane potential, such as potassium channels (Reeves et al., 2002), swelling-activated chloride channels (Moreland et al., 2006), and proton channels (Decoursey, 2003; Decoursey and Ligeti, 2005; Eder and Decoursey, 2001; Ramsey et al., 2009). Many studies performed in neutrophils and eosinophils stimulated by PMA proposed proton current as the main responsible for this balancing (Decoursey et al., 2003; Decoursey and Ligeti, 2005; Eder and Decoursey, 2001; Ramsey et al., 2009). In symmetrical pH proton current has an activation threshold around +40 mV (Eder and Decoursey, 2001). When the cells are activated the depolarization reaches levels sufficient to activate proton current and thus counteract the inward current mediated by the oxidase. Moreover, knock out cells for the proton channel Hv1 show a reduced ROS production (Ramsey et al., 2009).

The situation is a little different in microglia cells. Once activated by A β , microglia cells undergo a depolarization, due to the activation of the NADPH oxidase. However, in physiological conditions, the membrane potential never reaches positive potentials (Milton et al., 2008). This can be possibly due to the much more hyperpolarized resting potential of microglia cells

compared to neutrophils and eosinophils, being on average around -70 mV (Milton et al., 2008;Novarino et al., 2004;Schilling and Eder, 2007). Upon A β stimulation membrane potential rarely goes over -40 mV. Therefore, it seems unlikely that in these cells proton channels can be activated and able to counteract the NADPH oxidase functioning.

Chloride ions are the main ions available to counteract the charge movement across the cell membrane at the membrane voltage values reached after oxidase activation.

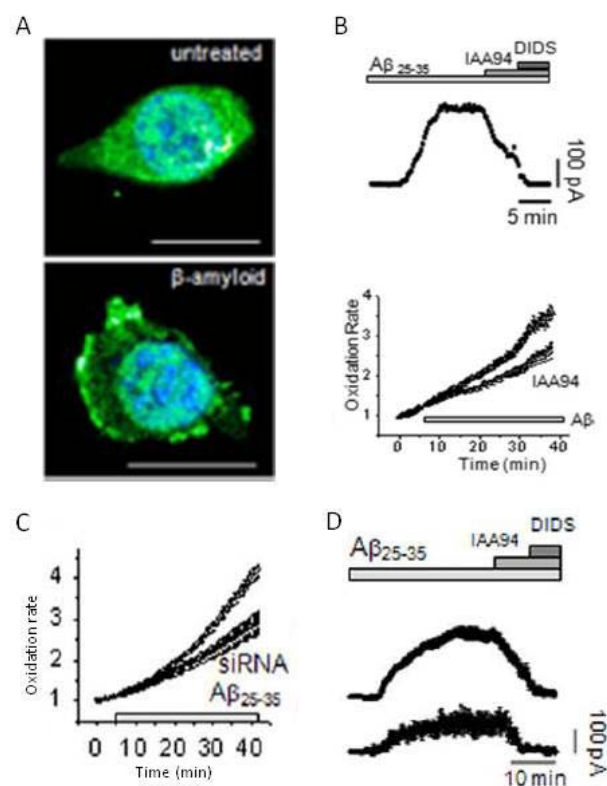


Figure 5

A) *CLIC1* translocation from the cytoplasm to the plasma membrane in Ab-stimulated BV2 cell. B) Patch clamp recording on the top, and ROS production on the bottom, showing the inhibitory effect of IAA94 on Ab elicited current and the concomitant reduction of ROS production in primary microglia. C) Reduced ROS production in Ab-stimulated primary microglia cells transfected with siRNA against *CLIC1*, compared to control cells. D) Reduced *CLIC1* mediated current in siRNA transfected cells fails to induce a DIDS-sensitive current upon Ab stimulation. Adapted from Averaimo et al., *FEBS letters*, 2010; Milton et al., *the journal of Neuroscience* 2008.

Indeed, our laboratory has previously shown that the membrane depolarization prompted by A β stimulation is exacerbated by the inhibition of CLIC1-mediated current with IAA94, by eliminating the main counter ion. ROS production is also reduced by knocking down CLIC1 expression with specific siRNA (Milton et al., 2008). Thus, CLIC1 role seems to be crucial in microglia activation during A β stimulation. A β stimulation of microglia cells at first causes an oxidative peak by activating the NADPH oxidase. This unbalanced oxidation causes the migration of CLIC1 from the cytoplasm to the plasma membrane. Then, CLIC1 mediates a chloride current that, through the inflow of negative charges brought by chloride ions, counteracts the ones lost by the displacement of electrons from the NADPH to extracellular oxygen. In this way, excessive membrane depolarization is avoided and the oxidase is able to work efficiently and produce ROS as a brain defense mechanism (Milton et al., 2008). Changes in pH can be easily counteracted by cellular buffering systems (De et al., 2008). However, the initial acidification could contribute, together with the oxidation, leading CLIC1 to the plasma membrane (Tulk et al., 2002;Warton et al., 2002). Finally, the importance of chloride contribute over proton's in microglia activation in AD is strengthened by the observation that amyloid plaques in AD brain are rich in zinc (Oddo et al., 2006), which is one of the well known inhibitors of proton current that act by shifting the activation threshold of proton current towards more positive potentials (Eder and Decoursey, 2001). Thus, if microglia cells surrounding amyloid plaques are in contact with inhibitors of proton channels, is unlikely that they could contribute in setting the membrane potential like they do in neutrophils, leaving this issue to the main ion available at those membrane potentials: chloride. CLIC1-mediated current is promptly followed by a DIDS-sensitive component, which is activated only after IAA94 sensitive current, suggesting a trigger role of CLIC1-mediated current versus the DIDS-sensitive current (Milton et al., 2008;Novarino et al., 2004). Previous studies have demonstrated, indeed, a role of increased intracellular chloride concentrations in modulating other chloride channels (Jentsch et al., 2002).

DIDS-sensitive current could then contribute to set the membrane potential together with CLIC1-mediated current.

Mounting evidence suggest an interaction of the members of the CLIC family with the actin cytoskeleton. CLIC5 is enriched in actin-containing fractions of the placental microvilli, and many studies performed in artificial bilayer showed the inhibitory effect of F-actin on the ion channel activity of CLIC1. Indeed, the addition of F-actin from the “intracellular” side causes an almost complete inhibition of ion channel activity, while the addition of depolymerizing agents like Latrunculin or Cytochalasin leads to a complete recovery (Berryman et al., 2004;Singh et al., 2007;Suginta et al., 2001).

Thus, CLIC1 is a protein with the unique feature of being transiently expressed on the plasma membrane, being accessible from the outside and thus an easier target for eventual drug treatments. It works as a sensor of cell oxidation, and as an executor itself. However, differently from other ion channels resident in membrane and part of the normal cell physiology, CLIC1 is functionally expressed only in “stress” conditions. Therefore, CLIC1 might be a perfect tool to control specific cell mechanisms.

Chapter 2

Alzheimer's disease.

One of the largest apprehensions of contemporary life is population aging. During the last century the great advances in medical sciences and the healthier living conditions have led to an increased life expectancy. As a consequence, medical problems linked to aging have become very common. In this scenario, neurodegenerative disorders play a crucial role for their social impact.

Alzheimer's disease (AD) is the most common cause of dementia in the elderly. It is a progressive neurodegenerative disease that causes a cognitive decline, with deep alterations of behavior and personality that end up in dementia (Gotz and Ittner, 2008;Zhang et al., 2011).

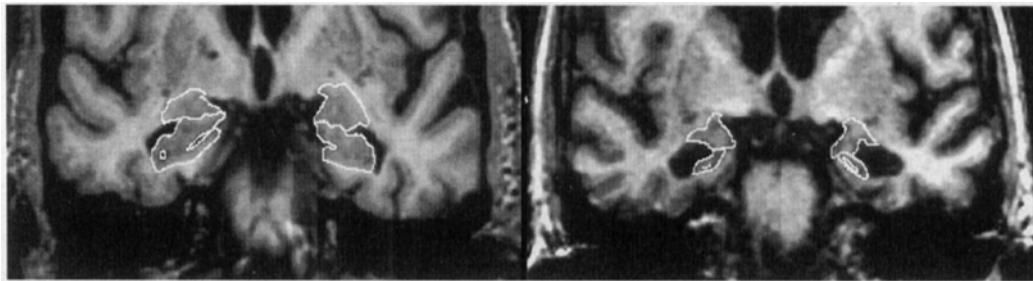


Figure 1.

On the left panel oblique coronal MR image throughout the temporal lobe of a 75-year old female control subject, while on the right from a 73-year old female AD patient. Adapted from Jack et al., Neurology 1997.

AD was first diagnosed by the German neuropathologist Alois Alzheimer in the beginning of the 20th century. In 1901 Alzheimer observed the first symptoms of what was later called “dementia”: strange behavioral symptoms, including short-term memory loss. When in 1906 the patient died, Dr. Alzheimer used staining techniques to identify amyloid plaques and

neurofibrillary tangles in his brain. The amyloid plaques were positive to the *congo red* staining. In 1906 he presented for the first time the neuropathological analysis of this neurodegenerative disorder (Puchtler and Sweat, 1966).

From the clinical point of view AD is characterized by cognitive deficit and memory loss, due to neurodegeneration mainly localized in the hippocampus and in the cortex. From the histological point of view, the main hallmark of AD are intraneuronal accumulation of the cytoskeletal protein tau, and deposits of amyloid beta ($A\beta$) protein in the extracellular milieu, called senile plaques. The senile plaques are surrounded by activated microglia (Gotz and Ittner, 2008).

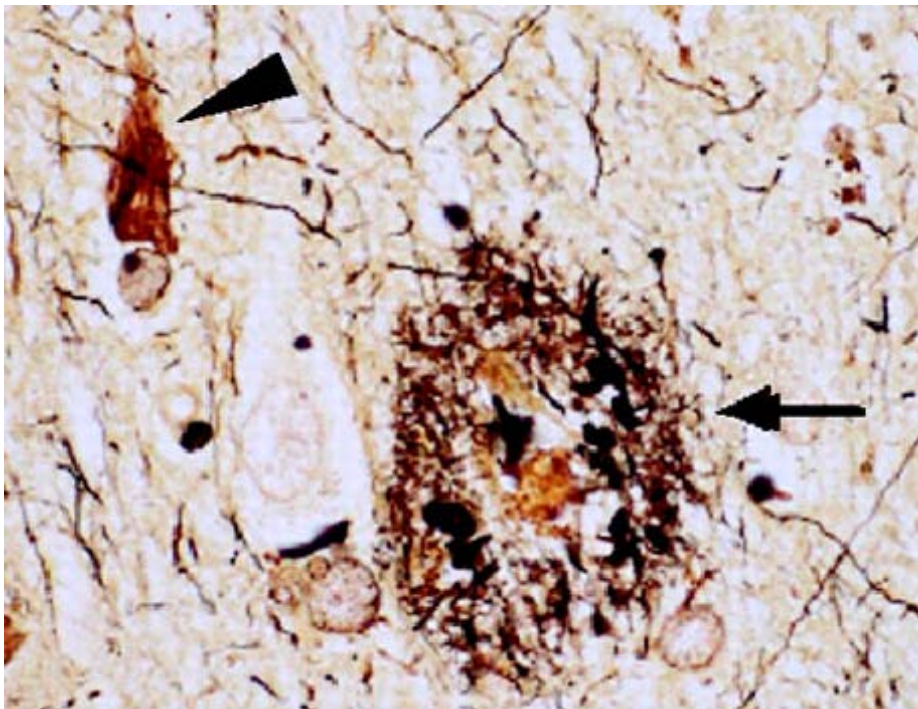


Figure 2.

Extracellular aggregation of amyloid beta protein into senile plaques (arrows) and neurofibrillary tangles (arrowhead), typical hallmarks of Alzheimer disease. Adapted from Taylor et al., Science 2002.

2.1 Amyloid beta.

A β protein is a 42 aminoacid peptide, belonging to the Amyloid Precursor Protein (APP) which is a transmembrane (TM) glycoprotein. APP is synthesized in the endoplasmic reticulum and then transported through the Golgi apparatus and the trans-Golgi-network to the cell surface. APP physiological function is still obscure, although it has been proposed a role as a receptor, and many evidences link APP to cell adhesion. APP can be alternatively cleaved in an amyloidogenic pathway and in a non-amyloidogenic pathway, by α or β secretases respectively, and successively by γ secretases.

α secretases, acting from the outside, cut the extracellular part of the protein giving rise to a TM fragment, and the sAPP α . The latter is soluble and has been demonstrated to have several neuroprotective properties. The cleavage by α secretase precludes the generation of A β , as the cleavage site is within the A β domain.

In the amyloidogenic pathway, APP is first cleaved by β secretases and successively by γ secretases. BACE1 (beta-site APP-cleaving enzyme) and BACE2 are two beta secretases so far identified, although BACE1 is the major β secretase and its expression in neurons is higher compared to BACE2. BACE1 is a membrane-bound aspartyl protease. Its expression is found increased in the temporal cortex of AD brains. Mounting evidence show that BACE is a stress-induced protease. Its expression, indeed, is increased in cells exposed to oxidative stress (Tamagno et al., 2002) and in *in vivo* models of cerebral ischemia (Wen et al., 2004) and traumatic brain injury (Blasko et al., 2004). BACE1 has been proposed as a putative therapeutic target in AD by several studies. Indeed, BACE1 knock out mice do not generate detectable levels of A β , showing no severe phenotypic abnormalities. Moreover, BACE1 deficiency in AD mice is able to rescue neuronal loss and reduce A β levels (Luo et al., 2001; Ohno et al., 2007; Tesco et al., 2007). Thus, for instance, regulation of BACE1 levels and its β secretase activity through a

mechanism specifically involving the small BACE1-interacting protein GGA3 may be the cause of increased BACE and subsequently A β levels in AD brain (Tesco et al., 2007), proposing GGA3 as a possible target in AD treatment.

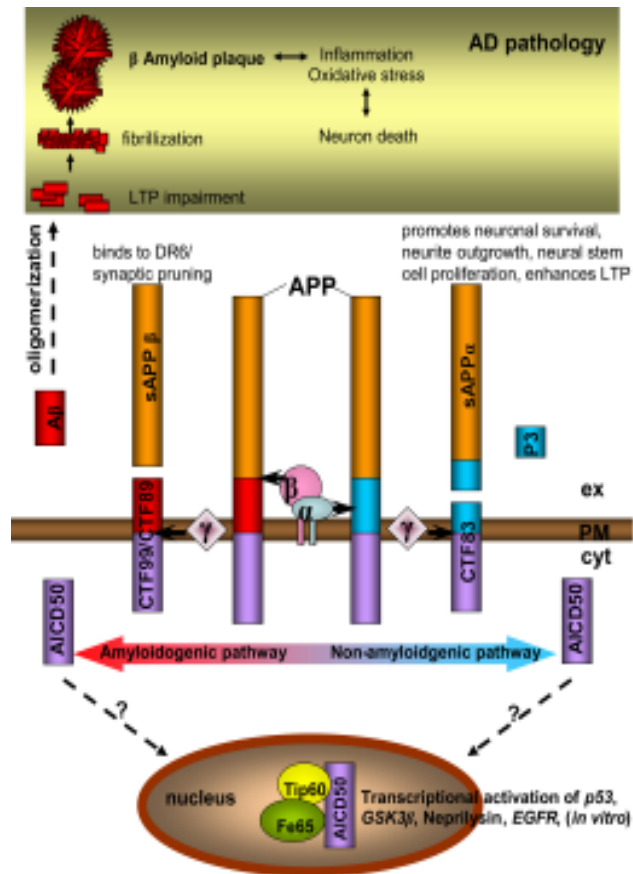


Figure 3.

Schematic representation of the amyloidogenic and non-amyloidogenic cleavage pathway of amyloid precursor protein (APP) by the different secretases. Adapted from Chow et al., Neuromolecular medicine 2010.

The fragments belonging either from the cleavage of APP by β or α secretases are the target of γ secretase action. The sequential cleavages by α and γ secretases result in a non toxic fragment. On the other hand, the cleavage of APP by β and γ secretases gives rise to two main isoforms: A β 1-40 and A β 1-42, the latter being more toxic than the first one. The hydrophobic nature of

these peptides leads to abnormal aggregation of these fragments in insoluble structures known as senile plaques (Ling et al., 2003;Zhang et al., 2011).

The familiar form of AD, which accounts for less than 1% of the total number of AD cases, has been associated with mutation in three genes: the gene coding for the APP, presenilin 1 (PSEN1) and presenilin 2 (PSEN2). Presenilins are membrane component of the γ secretase complex. In sporadic AD different “susceptibility genes” have been identified, including Apolipoprotein E (APOE). Mutations in the APP gene are associated with an increased production of A β , resulting in a major probability of developing the pathology. The APP gene localizes on the chromosome 21. In individuals affected by down syndrome (trisomy of chromosome 21) there is a major probability of developing AD, due to the higher amount of APP expressed (Liang et al., 2008).

Recent studies correlate the mechanisms of A β degradation with increased levels of A β in the brain. ApoE is involved in A β degradation, by positively modulating Nephrylisin and Insulin Degrading Enzyme (IDE), both responsible for the A β deposits removal respectively in the extracellular and intracellular environment. Mutations in the gene coding for ApoE, indeed, correlate with increased number and size of amyloid plaques in the brain (Ling et al., 2003).

The action mechanism underlying A β toxicity is not completely understood. Many studies suggest that A β could insert into neuronal lipid membranes forming non selective pores that mediate calcium inflow. The resultant increase of intracellular calcium could activate the apoptotic pathway, starting with an initial synapse loss, followed by the loss of dendritic spines, in the end leading to cell degeneration (Green and LaFerla, 2008). However, several studies suggest that the specific interaction of A β with AMPA, NMDA and metabotropic glutamate receptors expressed on the neuronal surface can be responsible of impaired synaptic transmission and in the end leading to neuronal death (Gasparini and Dityatev, 2008).

According to the “classical” model, amyloid plaques are the main responsible for neurodegeneration in AD. However, more recent studies suggest that soluble oligomers and protofibrils may be more dangerous. Indeed, not always the neurodegeneration correlates with the presence of senile plaques, but neuronal loss is often localized in brain regions far away from amyloid plaques (Dahlgren et al., 2002). On the other hand, oligomers interfere with signal transduction in long term potentiation (LTP), leading to memory impairment typical of the first stages of the disease. Protofibrils cause oxidative stress and rapid changes of the electrophysiological properties of the neuronal plasma membrane (Chen et al., 2000; Soderling and Derkach, 2000).

2.2 Microglia role in Alzheimer disease.

A β deposits are surrounded by activated microglia and recent studies strongly support the hypothesis of a neurodegenerative process mainly due to a microglial hyper-activation caused by the spread presence of A β in the central nervous system (CNS). Microglia cells represent the brain immune system. They represent 12% of the cells in the brain and their density varies by brain regions: they are mostly present in the gray matter, with the higher concentrations in the hippocampus, *substantia nigra*, olfactory telencephalon and the basal ganglia. Microglia is generally present in a resting state with a typical ramified morphology, with long extensions monitoring the brain. However, upon activation by different cues, such as bacteria or damaged neurons after brain ischemia, they readily become activated. They undergo main morphological changes becoming amoeboid. They express different surface molecules including the major histocompatibility complex (MHC), duplicate, migrate towards the site of injury and start secreting several substances, such as chemokines, cytokines, nitrites, reactive oxygen species (ROS), nitrites, Tumor Necrosis Factor (TNF) α , in order to recall other microglia cells and eliminate dangerous cues through phagocytosis (Block et al., 2007).

In AD, the incapability of microglia to efficiently remove the senile plaques, which represent the “insult”, leads to a chronic microglia activation. The chronic and diffuse inflammation causes a massive neurodegeneration, determining the typical symptoms of the disease (Meyer-Luehmann et al., 2008).

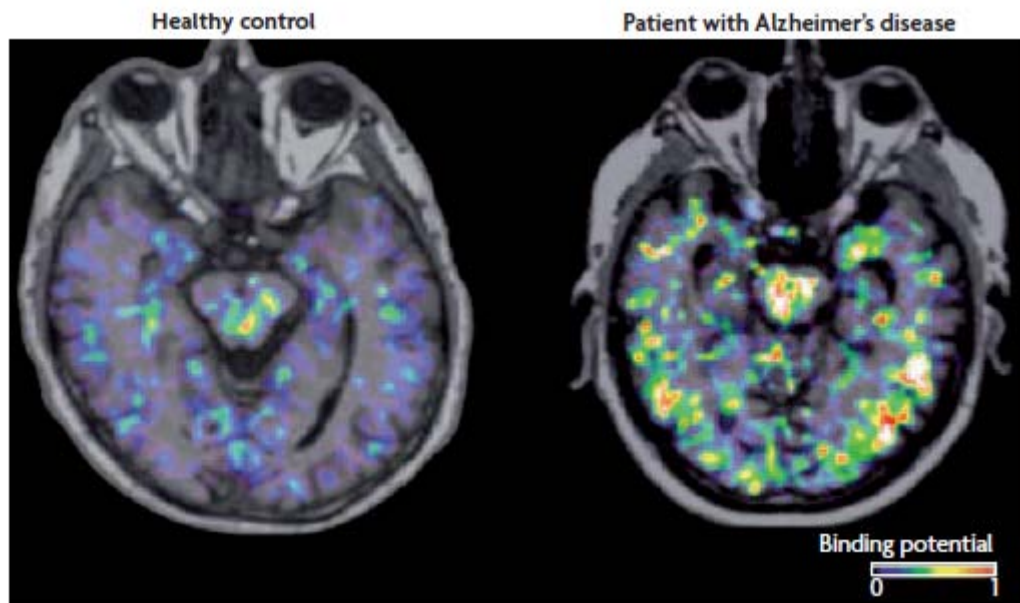


Figure 4.

PET (positron emission tomography) imaging showing microglial activation in 74 years old healthy subject (left) and 65 years old AD patient. Microglia activation was visualized taking advantage of the uptake of a radiolabeled ligand usually taken up by a benzodiazepine receptor overexpressed in activated microglia. The binding potential is an esteem of microglia activation, that is widespread in the patient whereas is negligible in the healthy subject.

ROS production in microglia cells is mainly due to the activation of the NADPH oxidase. The NADPH oxidase isoform expressed by the phagocytic cells, including microglia, is NOX2. The NADPH oxidase is a multimeric enzyme, made up by two transmembrane (TM) subunits: gp91phox, which is the catalytic subunit (named NOX2 in phagocytic cells), and p22phox. It is also composed by four cytoplasmic regulatory subunits: p47, p67, p40 and Rac-GTP. These last

four components associate with the cell membrane only upon different stimuli, such as the enhancement of internal calcium concentration, phosphorylation by protein kinase C (PKC) and temperature changes. Once assembled, they constitute the functional protein complex that produces superoxide anion, which is released extracellularly during the microglial immune reaction.

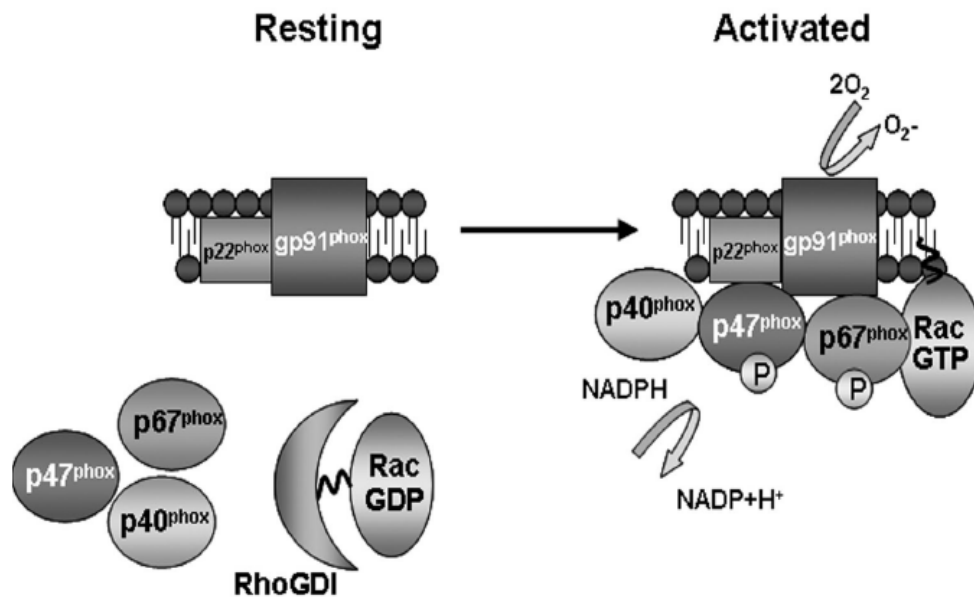


Figure 5.

Schematic representation of the constituent subunits of the NADPH oxidase. In resting conditions (on the left) and upon activating stimuli (on the right). Adapted from Wilkinson and Landreth, Journal of neuroinflammation. 2006.

gp91^{phox} is a highly glycosylated TM protein. The N-terminal domain is made up by six α helices; the third one and the fifth one coordinate the heme group. At the C-terminal domain there is the binding sites for FAD and NADPH. So, gp91^{phox} contains the whole redox machinery that displaces the electrons from the NADPH, through FAD and the two heme domains, to the extracellular side. The protein complex constituted by gp91 and p22 represents the flavocytochrome b558. In resting conditions, the proline-rich region at the C-terminus of gp91 interacts with the SH3 domain of p47, avoiding the binding between p22 and gp91, and

thus the subsequent enzyme activation. The interaction amongst all the subunits is necessary for the functioning of the oxidase, because the interaction of the activation domain of p67phox with a region of the flavocytochrome b558 allows the displacement of electrons from the NADPH to FAD. p22 is a TM protein with a C-terminal tail rich in prolines that acts as an anchoring site for p47. Upon phosphorylation of p47 at multiple serine residues by PKC, the protein undergoes conformational changes of the inhibitory domain that allow the interaction of p22 and gp91. Then, the C-terminal domain of p47 interacts with the C-terminus of p67, bringing it to the plasma membrane. p67 binds to Rac. In resting conditions Rac forms a heterodimeric complex with the inhibitor protein RhoGDI in the cytoplasm. Upon stimuli, Rac is released from the complex, brought to the plasma membrane and converted into its active form, binding GTP instead of GDP. Rac-GDP conversion to Rac-GTP is due to the action of GEFs (guanine-nucleotide exchange factors) proteins. The interaction of the active form of Rac with the amino terminal domain of p67 is crucial for the activation of gp91. Indeed, p67, activated by the binding of Rac, directly interacts with gp91, allowing ROS production. Finally, p40 also positively regulates gp91 activation, by associating with p67, without affecting p47 interaction (Lambeth, 2004;Sumimoto, 2005;Wilkinson and Landreth, 2006).

The role of microglia in the brain is thus crucial for the CNS health. However, when microglia results to be hyper-activated, for example around A β deposits, the continue secretion of these neurotoxic factors in the end results in vascular damage, neuronal damage and neurodegeneration (Block et al., 2007).

Thus, defining microglia cells as beneficial or detrimental for neuronal survival is not a trivial issue. Microglia is important in bacterial infections, or in ischemia, but it becomes a great enemy in AD. The key point is likely to be the over-activation of microglia cells.

Although AD was the first pathology to be linked to microglia over-activation, a chronic inflammation state has now been shown to be involved in many other neurodegenerative

disorders, such as Parkinson disease, amyotrophic lateral sclerosis, multiple sclerosis, Huntington disease (Behrend et al., 2003;Boilley and Cleveland, 2008;Gao et al., 2003;Thomas et al., 2007;Wu, 2006). Microglia activation not only exacerbates ongoing neurodegeneration, but it also can cause neurodegeneration by itself (Block et al., 2007).

2.3 Tau protein in Alzheimer disease.

The key role of Tau protein in AD has been fully demonstrated. AD is indeed also referred to as a tauopathy, being also characterized, as we mentioned before, by the aggregation of abnormally phosphorylated Tau protein (Grundke-Iqbal et al., 1986). Tau is a microtubules-associated protein.

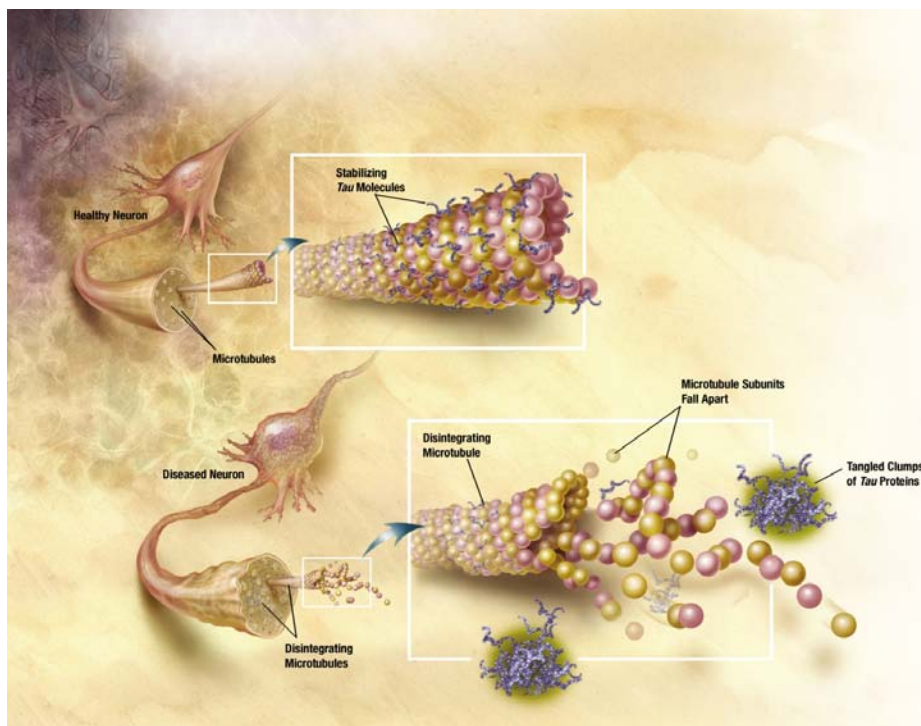


Figure 6.

Schematic representation of tau involvement in the stabilization of microtubules and the consequence of its detachment from microtubules after hyperphosphorylation in pathological conditions.

Its primary role, by interacting with microtubules, is to stabilize the neuronal cytoskeleton. It is coded by a gene located on chromosome 17 and it undergoes alternative splicing. Alternative splicing generates six isoforms. Tau undergoes phosphorylation at different serines and threonines, and phosphorylation decreases the affinity of Tau for microtubules, by preventing its binding and destabilizing neuronal cytoskeleton. Cytoskeleton destabilization causes the disruption of many cellular functions, including axonal growth, vesicle and organelle transport, nervous signal propagation. Tau hyperphosphorylation results from an unbalance between the action of phosphatases and kinases: the inhibition of phosphatase PP2A, which has been shown to be decreased by 50% in AD brains, or the over activation of the kinase GSK3 β .

Although Tau undergoes many different post translational modifications, such as glycosilation, glycation, prolyl-isomerization, nitration, polyamination; phosphorylation is the most commonly described. In AD, Tau hyperphosphorilation causes its detachment from the microtubules and its oligomerization in strucutres named neurofibrillary tangles, which strongly impair synaptic function. Exogenous expression of pseudo hyperphosphorylated Tau mimics structural and functional abnormalities typical of AD. However, Tau hyperphosphorylation seems to be required but not sufficient *per se* to induce aggregation, suggesting that other post translational modifications may be required (Martin et al., 2011).

2.4 Actual approaches in the treatment of AD.

Unfortunately until now effective treatments for AD are still missing. Indeed, the actual known treatments mainly aim to a stabilization of the patient's conditions, but they are not able to revert the disease progression.

Indeed, in neurodegenerative diseases the symptoms are manifest only when the neurodegeneration is largely diffused, and dead neurons cannot be replaced. Over the last

decades many risk factors for neurodegenerative diseases have been identified. However, laboratory biomarkers are important to specifically confirm a diagnosis and guide an appropriate medical therapy. So far, the combination of Tau and A β levels in the cerebro-spinal fluid (CSF) gave a good predictive value, better than the two individual biomarkers. However, although these results are promising, it is clear that this “strategy” needs to be optimized to be used as a screening for a specific treatment. For example, many studies report an increase of Tau levels in the CSF, and a decrease of A β in the CSF of AD patients. The decreased A β presence in the CSF has been explained as a consequence of a major accumulation of A β in the brain. If this is really the explanation to this phenomenon, it means that when AD is diagnosed by A β and Tau levels in the CSF, the disease is already in a very advanced stage.

Moreover, magnetic resonance imaging (MRI) and positron emission tomography (PET) imaging with amyloid ligands (such as PIB: Pittsburgh compound B), are now largely used in clinics. MRI measurements of hippocampal atrophy are also widely used, although they can only diagnose a disease already strongly manifested (Mattsson, 2011).

In the last decade the scientific community is pointing on vaccines for the treatment of AD. Using the immune system to attack one of the body's protein, A β , could seem a little risky approach, but also very clever and intriguing. The first vaccine, directed against A β 1-42, which is the A β isoform most prone to aggregation, seemed successful in mice, where it dissolved amyloid plaques and inhibited formation of new ones, but the trial in humans had bad outcomes, causing often meningoencephalitis (a severe brain inflammation). A new approach seemed to be more successful. It was based on the intravenous injection of anti-A β antibodies to AD patients. The levels of these antibodies are indeed lower in AD patients compared to healthy subjects of the same age. The results were surprisingly very good, with improvement of cognitive scores and a stabilization for all the treatment duration. Although these studies seem really encouraging and a large scale trial will be performed before the end of 2012, it encountered against some

problems due to the high cost of the treatment, and to the availability of these antibodies, whose production capacity by blood of the donors is limited. Moreover, unexpectedly, while the treatment was successful in some patients, it almost caused a worsening in patients who started the treatment later in the disease course (Schnabel, 2011). Thus, the possibility of diagnosing the disease in the early phases of the neurodegenerative process would give the possibility of interfering with the disease progression before the damage is so diffused. This would give the possibility of a more effective treatment.

2.5 Retina as a mirror of a pathological state in AD.

Although the brain has always been considered the exordium site of neurodegeneration in AD, increasing evidence shows that the retina is also affected in the earliest stages of this disorder. It is well documented that in many AD patients the pathological state shows up first as problems with vision rather than memory or other cognitive deficits. Although historically the visual impairments have been linked to neurodegeneration of the cerebral cortex, in particular in the visual association cortex, more recent studies revealed that these are also due to disease of the optic nerve and retina (Berisha et al., 2007; Iseri et al., 2006). Thus AD, which is the most common form of dementia, shows among other neurodegenerative events, severe retinal abnormalities, resulting in visual function impairment due to changes in retinal structure and function, such as reduction of retina fiber layer, where axons of RGCs are located, retinal thinning, astrocytosis and activation of immune competent cells like microglia and Muller cells. Analysis of pattern electroretinogram in AD patients showed severe abnormalities, consistent with harsh dysfunction of RGCs (Berisha et al., 2007). Moreover, recent studies conducted on transgenic AD mouse models have shown A β deposits in the retina, associated with RGCs apoptosis and visual functional impairment, further supporting the hypothesis of a retinal degeneration in AD pathology (Normando et al., 2009). Finally, more recent studies revealed the

presence of A β plaques in postmortem eyes from AD patients and in suspected early stage subjects, consistent with brain pathology and clinical reports, while A β deposits were undetectable in age-matched non-AD individuals (Koronyo-Hamaoui et al., 2011). RGCs degeneration is preceded by several cellular mechanisms, including a chronic state of oxidative stress, a dysregulation of electrolyte homeostasis and modulation of several proteins and peptides (Berisha et al., 2007;Iseri et al., 2006).

In light of these data, it is interesting to highlight that, being unprotected by the barrier constituted by the skull, the retina is the most exposed part of the CNS. Therefore, the retina is much more accessible to direct observation and to pharmacological treatments. We can thus consider the retina as a mirror of the pathological state of the brain, as an open window on the brain to directly inspect the neurodegenerative process.

Chapter 3

The mammalian retina.

Retina is the part of the eye that is responsible for the transmission of the visual signals to the central nervous system (CNS). It is the most exposed and thus the most accessible part of the CNS.

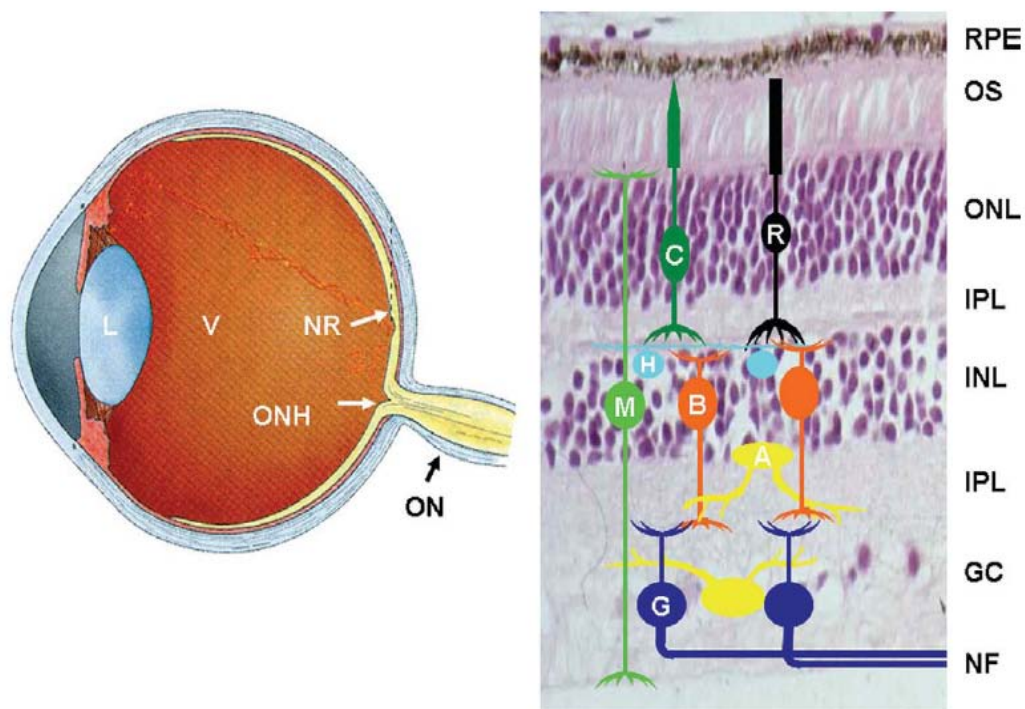


Figure 1

On the left, schematic representation of the vertebrate eye. L, lens; V, vitreous; NR, neural retina; ONH, optic nerve head; ON, optic nerve. On the right, histological image and diagram showing the different retina layers of an adult mammalian retina. A, amacrine cells; B, bipolar neurons; C, cone photoreceptors; G, ganglion cell; GC, ganglion cell layer; H, horizontal neurons; INL, inner nuclear layer; IPL, inner plexiform layers; M, Muller cell; NF, nerve fiber layer; NR, neural retina; ONL, outer nuclear layer; OS, photoreceptors outer segments; R, rod photoreceptors; RPE, retinal pigment epithelium. Adapted from Wallace et al., *Stem Cells*, 2011.

3.1 Structure and physiology of the mammalian retina.

The adult retina is a laminated structure. It is delimited on the apical side by the retinal pigmented epithelium (RPE), a single layer of epithelial cells, while on the basal side is bordered by the vitreous, which is the fluid filled cavity separating the retina from the lens. The nuclei in the retina are strictly organized in three main layers, separated by the synaptic layers, which are called "plexiform" layers. The light passes through the lens and the whole thickness of the retina and stimulates the photoreceptors. Photoreceptors can be divided in two types: cones, responsible for light vision, and rods, responsible for the dark vision. Their cell bodies are located in the outer nuclear layer, adjacent to the RPE. Photoreceptors make synapses in the outer plexiform layer with bipolar neurons, whose cell bodies are located in the inner nuclear layer (INL). In the inner plexiform layer (IPL) lay synapses between bipolar neurons and retinal ganglion cells (RGCs). The latter ones are located in the ganglionic layer. The axons of RGCs are gathered together forming the optic nerve that exits through the optic disc and transmits the information to the visual processing centers in the brain (Wallace, 2011).

Photoreceptors are the cell type responsible for light perception. They translate the light signal into chemical signal. The first step in phototranslation is the light absorption by the photopigments located in the outer segment of photoreceptors. Rods have only one type of pigment, while the cones have three different types: each pigment is characterized by an absorbance peak, thus resulting more sensitive to a specific wavelength. Each pigment molecule is made up by two different parts: a component called "retinal" common to all the pigments, and a second component named "opsin" that determines the sensitivity to the different wavelengths. The rod's pigment is located in the membrane of "discs". Discs are specific structures located in the outer part of the photoreceptor, like small invaginations that increase the total surface of the cell. Inside the discs there is Transducin, which is a G-protein, and the enzyme phosphodiesterase that catalyzes the degradation of cyclic GMP (cGMP). In the dark cGMP

levels in the photoreceptor are higher causing the opening of sodium channels. Sodium inflow determines a membrane depolarization that in turn opens calcium channels in the inner segment of the photoreceptor. The following increased calcium concentration causes the endocytic release of neurotransmitters that bind to receptors on the plasma membrane of bipolar cells.

One of the pigments expressed by rods is Rhodopsin. After light exposure, the conformational change of the retinal causes the detachment of the opsin which in turn activates Transducin. Successively Transducin activates the phosphodiesterase resulting in decreased levels of cGMP. Thus, the resultant closure of sodium channels determines a membrane hyperpolarization. As a consequence, less glutamate molecules are released from the photoreceptor: the light presence is thus signaled. Cones work in a very similar way, but due to the different sensitivity of the photopigments, they are sensitive only to very restricted wavelengths (Germann, 2008).

Once depolarized by the binding of glutamate released by photoreceptors, bipolar cells release the neurotransmitter that interacts with receptors present on the plasma membrane of RGCs. At the periphery of the retina, where only rods are present, many of them converge on each bipolar cell. This allows a major light sensitivity. However, in fovea and macula, where cones predominate over rods, synaptic convergence is reduced and only few photoreceptors converge on the same bipolar cell. In this region retina is much more sensitive to the details of the images (Dacey, 1999).

Bipolar cells are the neurons responsible to convey signals from photoreceptors to retinal ganglion cells. They can be categorized into two main classes: “diffuse” bipolar cells which have connections to multiple cones; and “midget” bipolar cells that have a single line connection to a single cone axon terminal (Wallace, 2011).

The light collected by photoreceptors activates a signaling that propagates throughout the retina circuits, through bipolar neurons, to modulate the spike firing in RGCs. RGCs axons make

up the optic nerve that exits the eye through the optic disc and send the information to the brain. Most of the axons of RGCs project to the dorsal lateral geniculate nucleus (dLGN), and from there to the visual cortex. Some of their axons project to brain centers other information, for other visual functions such as circadian photoentrainment. According to the original idea, all the light signals involved rods and cones at first. However, further studies have demonstrated that a small subset of RGCs express melanopsin, a photopigment able to capture the light and activate the translational pathway. Indeed, mice with degenerated rods and cones change their circadian rhythm according to the external light-dark cycle, although they do it with a different spectral sensitivity from wild type mice. This is due to the presence of melanopsin-expressing RGCs in rodless and coneless mice.

In binocular species some axons project ipsilaterally (crossing the midline), and some project contralaterally. Axons from the nasal retina project contralaterally, while axons from temporal retina project ipsilaterally. Animals that have their eyes near the front of their head have a higher degree of binocular overlap (thus binocular vision) and thus a higher number of ipsilaterally projecting RGCs. Humans are highly binocular, with almost 40% of uncrossed (ipsilateral) RGCs axons. On the other hand, mice are a poor binocular specie, with around 3-5% of uncrossed axons. Retrograde staining of RGCs in mammalian retina allowed the identification of many different types of RGCs, classifying them according to their morphology and their electrophysiological properties. Although almost 19 different types of RGCs have been identified, they have been classified into 4 main groups, according to the size of their cell bodies and the extensiveness of their dendritic tree (Dacey, 1999;Sun et al., 2002).

Horizontal cells are the lateral interneurons of the outer retina. Their dendritic processes innervate the axon terminals of the photoreceptors. Horizontal cells provide a negative feedback to photoreceptors, by modulating their synaptic contacts with bipolar neurons. They are

electrically coupled by gap junctions and form a sort of an “electrical syncytium”. (Wallace, 2011).

While horizontal cells are mainly responsible to modulate the synapses between photoreceptors and bipolar cells, amacrine cells modulate the synapses between bipolar cells and RGCs. Amacrine cells’ bodies are located in the INL together with bipolar neurons. A subpopulation of amacrine cells are the cholinergic amacrine cells, which are commonly called “starbucks” cells due to their typical morphology: they have four to five primary dendrites, each of them branching into secondary and tertiary processes. The dendritic arborization has a perfect symmetry around the soma, giving them their typical morphology. Due to the release of neurotransmitter such as acetylcholine, GABA and glutamate, they have a main role in modulating electrical signals coming from photoreceptors (Kolb, 1997;Lagnado, 1998).

The retina also contains Muller cells, which are the main glial element in the vertebrate retina, providing structural and trophic support to neurons. They are disposed radially, spanning the whole thickness of the retina from the inner to the outer limiting membranes. The cell bodies of Muller cells are located in the INL. Their apical processes form adherens junctions with photoreceptors, while their endfeet are in contact with the basal lamina at the inner limiting membrane, near the vitreal surface of the retina. Muller cells are the last subpopulation cell in the retina to differentiate (Wallace, 2011). Muller cells activity occurs in many retinal diseases. Muller cells gliosis is characterized by both specific response such as increased expression of glutamine synthetase (GS) for example after photoreceptor degeneration; and non specific responses, such as glial fibrillar acidic protein (GFAP) expression as a “universal” marker of retinal injury (Bringmann and Reichenbach, 2001). Muller cell gliosis may involve a de-differentiation and proliferation. The de-differentiation of Muller cells can be detrimental due to the impairment of neurotransmitter removal and unbalanced ion and water homeostasis that may

establish after the down regulation of potassium channels that accompanies Muller cells de-differentiation (Bringmann et al., 2002).

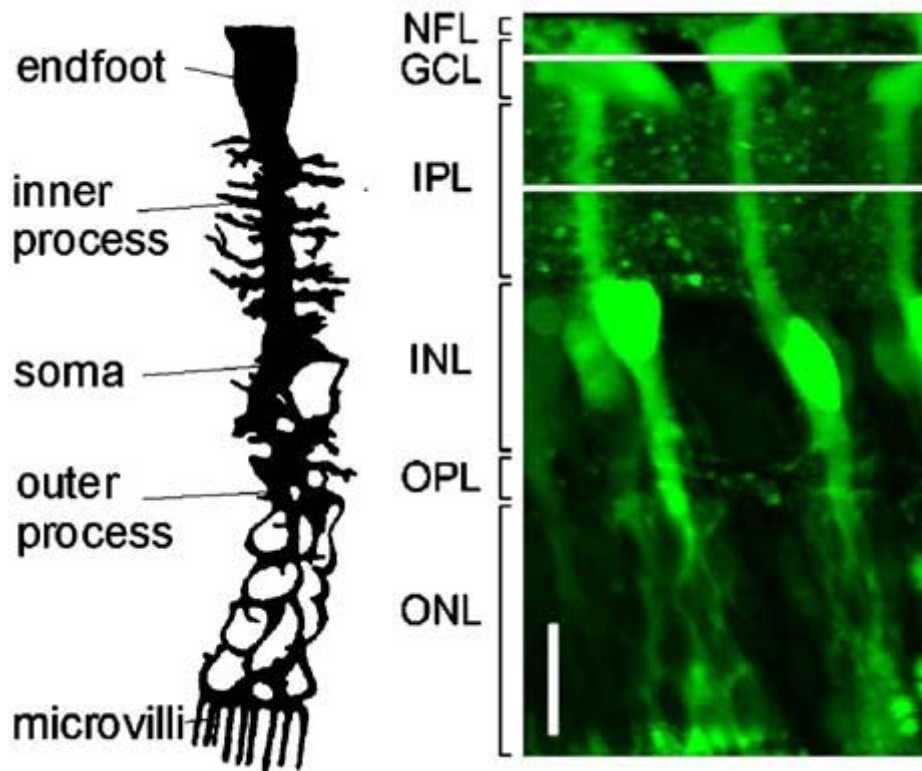


Figure 2

On the left, schematic representation of a Muller cell. On the right, immunostaining of a Muller cell in a section from guinea pig retina. Adapted from Bringmann et al., Progress in Retinal and Eye Research, 2006.

On the other hand, duplication may be detrimental by filling the space left by dying neurons, for example by dying photoreceptors in the retina detachment, establishing the so called “glial scar”. In the formation of a glial scar Muller cells processes form a fibrotic layer that completely inhibits regeneration of outer photoreceptor segments. Glial scars also involve the expression of inhibitory molecules that inhibit regular tissue repair and neuronal regeneration (Bringmann et al., 2006).

Retinal astrocytes form a dense network on the vitreal surface of the retina and are closely associated with the vasculature. Retinal microglia is a very meager cell subpopulation, and is “spread” throughout the layers, mainly scattered in the INL and OPL. However, its number increases in case of retinopathy that leads to neuronal degeneration (Huxlin et al., 1992).

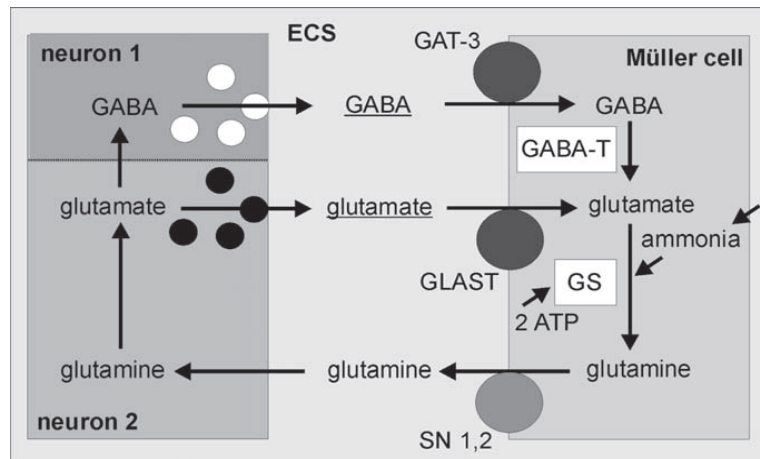


Figure 3

*Schematic diagram of neurotransmitter recycling in the retina. A GABAergic (neuron 1) and a glutamatergic neuron (neuron 2) release GABA and glutamate, respectively, into the extracellular space (ECS) from where they rapidly are taken up by the Müller cell. Thus, Müller cell expresses GAT-3 (GABA transporters) and GLAST (glutamate transporters). Once GABA has entered the cytoplasm, it is converted into glutamate by GABA transaminase (GABA-T). Then, glutamate is transformed into glutamine by the glia-specific enzyme, glutamine synthetase (GS). The GS reaction is the only pathway of ammonia detoxification in the retina. Glutamine is then exported by the glutamine transporters SN 1 and 2 into the ECS from where the neurons may take it up, and use it for the re-synthesis of glutamate and GABA. Adapted from Bringmann et al., *Progress in Retinal and Eye Research*, 2006.*

3.2 Retina development.

In the beginning of retinal development the neuroepithelium is entirely consisting of progenitor cells, which are proliferating cells. The appearance of the first post mitotic neurons, the RGCs, which marks the onset of neurogenesis, starts at embryonic day 11 (E11) in mouse (Young, 1985). In all the vertebrates studied, RGCs are the first neurons to differentiate in the

retina, followed by all the remaining neuronal subpopulations. The temporal differentiation of all the retinal neurons is strictly regulated by a precise genetic program. Soon after RGCs, cones, horizontal cells and half of the amacrine cells develop. Muller glia, bipolar cells and the remaining amacrine cells develop mostly in the postnatal period. Rods start to be generated in embryonic life, and continue throughout the first two weeks of post natal life (Wallace, 2011).

Retinal neurogenesis is not only temporally regulated, but also spatially regulated. It starts in the central retina and “spreads out” towards the periphery. So, from a developmental point of view the central retina, where the axons of RGCs are gathered together forming the optic nerve and exit the retina through the optic disc, is more advanced than the peripheral retina. The proliferation phase of retina is followed by a period of morphogenesis that includes synapse formation, remodeling and morphological maturation. The morphogenesis period starts around postnatal day 7 and continues until P21 (Wallace, 2011).

So the whole sequential production of retinal cell types takes place over approximately two weeks in rodents.

A mechanism that precisely regulates the balance between proliferation and differentiation of progenitor cells is important to ensure the appropriate number and proportion between all the different cell subpopulations. This is controlled by a variety of transcription factors temporally expressed by the progenitor cells, such as positive regulators of progenitor cells proliferation like Rax, Six6 and Pax6, Sox2 as a regulator of neurogenic competence of progenitor cells, or Vsx as an inhibitor of retinal pigmented epithelium cells differentiation. Figure 4 summarizes the transcription factors involved in the specification of each single cell type. The activation of the different transcription factors depends on a combination of different intrinsic and extrinsic factors. For instance, it has been demonstrated that progenitor cells differentiate in specific ages independently of the environment in which they develop, demonstrating the importance of intrinsic signals in this mechanism.

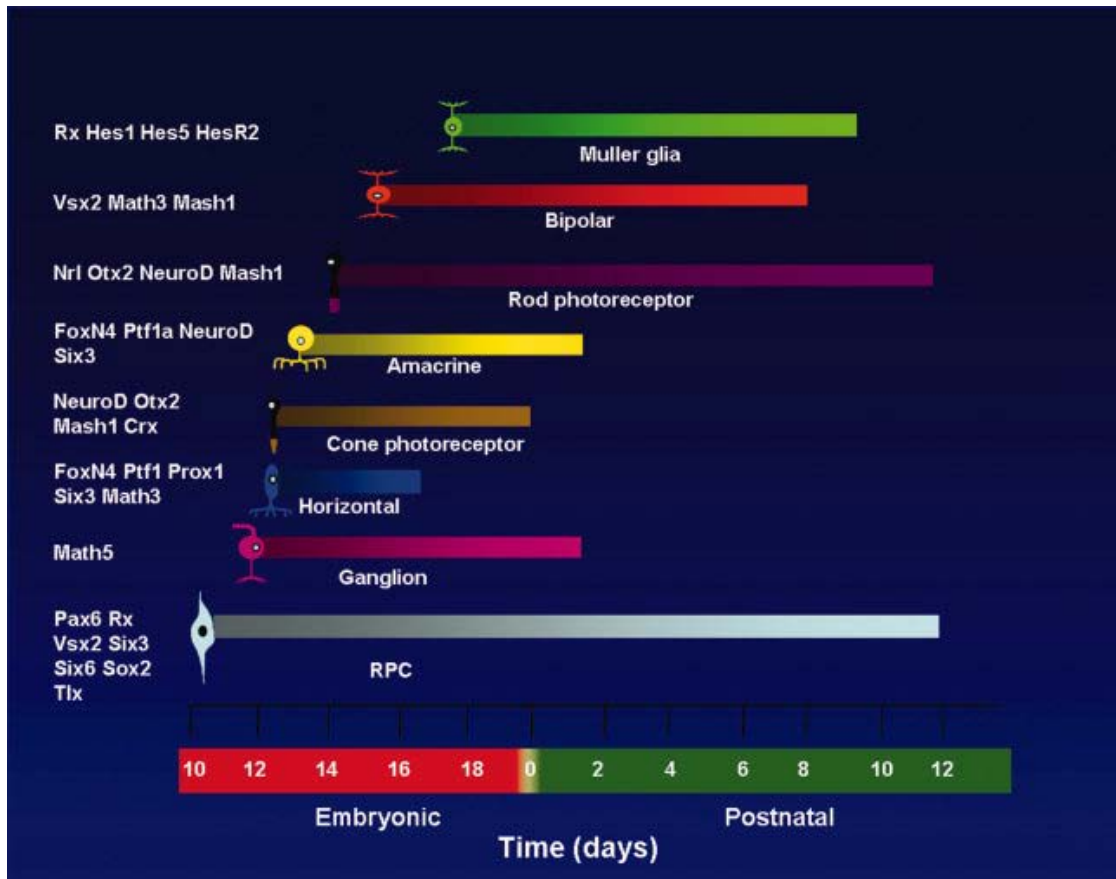


Figure 4

Temporal order of generation of the different cell types in mammalian retina, and regulation of retinal cell fate and development by transcription factors. Adapted from Wallace et al., Stem cells, 2011.

However, the differences between the differentiation process *in vivo* compared to explants suggests that factors coming from the environment have sure a role in the appropriate temporal differentiation of progenitor cells in the different retinal subpopulations. The *Notch-Delta* signaling is one of the best characterized pathways regulating neurogenesis in retina. Inhibition of *Notch* signaling determines a precocious differentiation, while increased *Notch* activity delays differentiation, promoting the progenitor state. The interaction of Notch ligands, like *Delta*, with *Notch* receptors causes the translocation of the cytoplasmic domain of *Notch* to the cell nucleus where it interacts with its specific transcriptional co-activator altering gene expression. Micro RNAs also seem to be implicated in such processes (Wallace, 2011).

Interestingly, the plane and symmetry of cell division also is implicated in a specific differentiation fate. Indeed, it has been demonstrated that by altering the spindle orientation of dividing progenitor cells, probably by altering the concentration of some proteins, can alter the fate of dividing cells. The orientation of the division plane is modulated by the retinal pigmented epithelium, although the specific factors involved are yet to be identified (Wallace, 2011).

The activation of a specific differentiation fate is then followed by a “maturation” stage that culminates into the formation of the complex retinal circuitry. For neurons this process involves the elongation of neurites and the differentiation into axons or dendrites. In the next chapter we will analyze this facet in more detail (Polleux and Snider, 2010).

At the end of the neurogenic process and the formation of the adult laminated retina structure, Muller glia still contains progenitor genes and is able to re-enter the cell cycle. These ability of Muller glia has always attracted the attention of many scientists, seeing a potential therapeutic use of these cells following neurodegeneration in eye diseases such as glaucoma, retinitis pigmentosa, age related macular degeneration. In fish, Muller glia are stem cells and retain the ability to regenerate neurons. However, in mammalian retina, although Muller glia proliferation can be reactivated by *Notch*, the neurogenic potential is very limited, at least *in vivo*. However, they can be induced to proliferate and differentiate in retinal neurons *in vitro* (Bringmann et al., 2006).

Many attempts of cell replacements in order to restore vision after neurodegeneration induced by different factors have been performed, made possible by the relative accessibility of the retina to external manipulations. For example, diseases primarily caused by retinal pigmented epithelium dysfunction, like age-related macular degeneration, are now in clinical trials, by the transplantation of new healthy retinal pigmented epithelium cells in order to restore vision (MacLaren et al., 2007). Of course, replacement of degenerated neurons is far more complicated,

due to the difficulty of the neurons to form new synaptic contacts and correctly integrate in the complex retina circuits.

Mature RGCs are typical CNS neurons and thus they possess only weak intrinsic potential to regrow injured axons following an optic nerve lesion. However, RGCs are switched into a robust regenerative state when β - and γ -crystalline proteins are released from an injured lens. In this state mature RGCs extend axons in culture at higher growth rates, and regenerate lengthy axons into an injured optic nerve *in vivo* (Ying et al., 2008).

Chapter 4

Actin and Tubulin cytoskeleton in neuronal maturation.

A crucial process in neuronal maturation, during its integration into the retina circuitry towards the formation of an adult fully functional retina, is neuronal morphogenesis. During this process the cell becoming a neuron expresses specific neuronal markers. Moreover, the ion channel composition and the expression of transporters changes abundantly during this period (Zolessi et al., 2006).

A key step in neuronal morphogenesis is polarization, when neurites differentiate either into axons or dendrites. Once exited the cell cycle, the cells designated to become neurons migrate towards their final destination, and most neurons undergo their polarization during this migration. After the determination of a neurite fate into axons, the elongation process takes place.

4.1 Neuronal polarization in retinal ganglion cells.

It has been demonstrated that *in vitro* hippocampal neurons go through different stages. Just after plating, the cells send out many thin and flat structures named lamellipodia. In stage 2, lamellipodia grow into minor neurites. Stage 2 is followed by stage 3 in which lamellipodia elongate. One of them starts to grow preferentially becoming the axon, while the others differentiate into dendrites in stage 4. In stage 5 neuron undergoes the final step of maturation. *In vivo* the scenario is a little different and far more complex, due to the necessity of the cell to integrate signals belonging to surrounding cells (Polleux and Snider, 2010).

However, the mechanism of polarization is quite different amongst the different neuronal types, and retinal ganglion cells (RGCs) work in a slight different way. Polarization of RGCs is prevalently inherited by the initial polarity of progenitor cells. Once the cells exit the cell cycle, they already have a proper apico-basal polarity. During migration from the retinal pigmented epithelium (RPE) to their final destination, signals belonging to the basal lamina cause the emergence of a leading process from the basal side, that starts growing in the direction of migration, and a tailing (“retracting”) process on the apical side that keeps in contact with the apical surface.

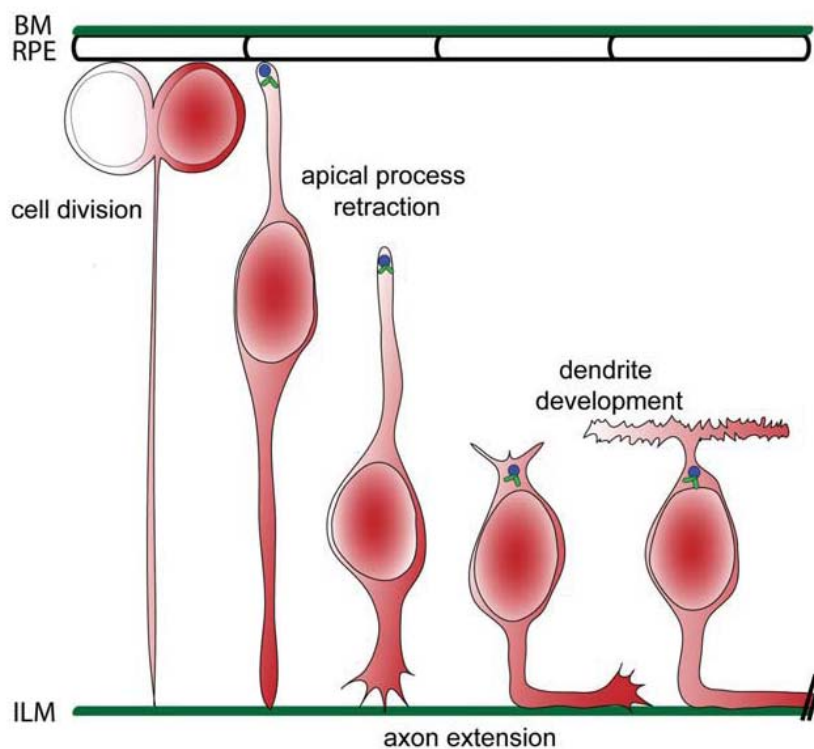


Figure 1

*Schematic representation of in vivo RGCs maturation. RGCs go through different maturation stages and migrate from the basal lamina (BL) towards the inner limiting membrane (ILM), in response to factors released by the retinal pigmented epithelium (RPE). Adapted from Randlett et al., *Developmental Neurobiology*,2010.*

During the migration of the cell body towards the vitreal surface, the apical process starts to retract, along with the basal neurite elongation, differentiating into axon. While the first one will

become the axon, the opposite will differentiate into dendrite. Axon differentiation usually precedes the retraction of dendrites on the opposite side, even though the leading process elongation is independent from the retraction of the apical process (Barnes and Polleux, 2009).

In vivo studies have demonstrated the role of extracellular cues from the basal lamina in neuronal polarization of RGCs (Randlett et al., 2011). Moreover, the destruction of the inner limiting membrane causes an abnormal axon outgrowth, further demonstrating the importance of this structure in neuronal polarization.

Although the importance of external cues in the polarization process of RGCs has been established, there are also many intrinsic signals guiding neuronal polarization. Indeed, *in vitro*, RGCs maturation appears quite similar to what happens in hippocampal neurons: during the differentiating process the cell undergoes a multipolar stage: all the neurites are identical and only successively one of them starts to grow faster, becoming the axon.

4.2 Intrinsic signals regulating neuritis specification.

Different intrinsic mechanisms are involved in axon specification and axon outgrowth, including local protein degradation, by regulating spatial protein expression. In dorsal root ganglion (DRG) cells the protein kinase AKT degradation has been shown to be involved in axon specification. The inactive form of AKT is degraded, resulting in an increased pool of active AKT over the inactive form (Randlett et al., 2010).

Local translation is also a key mechanism in axon specification. The translation machinery is indeed present in growing axons. Rossol and colleagues demonstrated the importance of local translation of actin mRNA in growing axons (Rossoll et al., 2003).

cGMP levels are also involved in branching. In particular, the balance between cAMP and cGMP is important in neuronal polarization and neurite differentiation into axon and dendrites. A shifted balance in the direction of cGMP over cAMP promotes dendrites differentiation over axons (Shelly et al., 2010).

cAMP levels that drive axon differentiation are regulated by different agents, including brain-derived neurotrophic factor (BDNF). Indeed, BDNF stimulation directs axon identity of neurites over dendrites (Goldberg et al., 2002).

cAMP is a wide second messenger involved in many cell functions (Gabellini, 2004; Pandey, 2004; West et al., 2001). Its increased concentration modulates several different ion channels (Lolicato et al., 2011; Meyer et al., 2002; Wang et al., 2001; West et al., 2001). It is known that Forskolin, by activating the adenylate cyclase, increases cAMP levels and results in cell depolarization (Goldberg et al., 2002). The cAMP pathway is also physiologically activated by the pituitary adenylate cyclase activating polypeptide (PACAP). PACAP is widely diffused in different mammalian tissues, including retina. It exerts its specific response by binding to specific receptors. In retina there are two types of PACAP receptors: PAC1R and VPAC1 and 2. The first one is more specific. Both types activate the adenylate cyclase, resulting in increased cAMP concentration that in turn results in CREB (cAMP responsive element binding) phosphorylation and subsequent activation. CREB activation, that in neonatal retinas happens already after 5 minutes of stimulation, translocates to the cell nucleus activating a specific genetic program (Atlasz et al., 2010; Vaudry et al., 2009).

In vitro studies, showed that other factors are involved in regulating axon specification. For example, the coating has a key role in this process. It has been shown that laminin is a key component in neuronal polarization (Barnes and Polleux, 2009; Shelly et al., 2010). Many studies demonstrated that the interaction with the extracellular matrix (ECM) is crucial in many cellular processes such as migration, proliferation or differentiation. The main receptors involved in the

transduction of the “signal” induced by the ECM are integrins, that translate the biochemical signal, across the plasma membrane, to the intracellular pathway. However, ECM may affect RGCs neuritogenesis and survival by inducing different signaling pathways during the different developmental stages. Indeed, while laminin coating promotes neuritis elongation at the beginning of the development stages, it results in axon outgrowth inhibition in rat retinas deriving from 2 weeks old animals, most likely by modulating the action of protein kinase C (PKC). Laminin is one of the main component of the ECM in the visual system, and its opposite effects on axon outgrowth in the different development ages, further support the importance of the basal lamina in the polarization process (de Ary-Pires and Linden, 2000;Goldberg et al., 2002;Randlett et al., 2011).

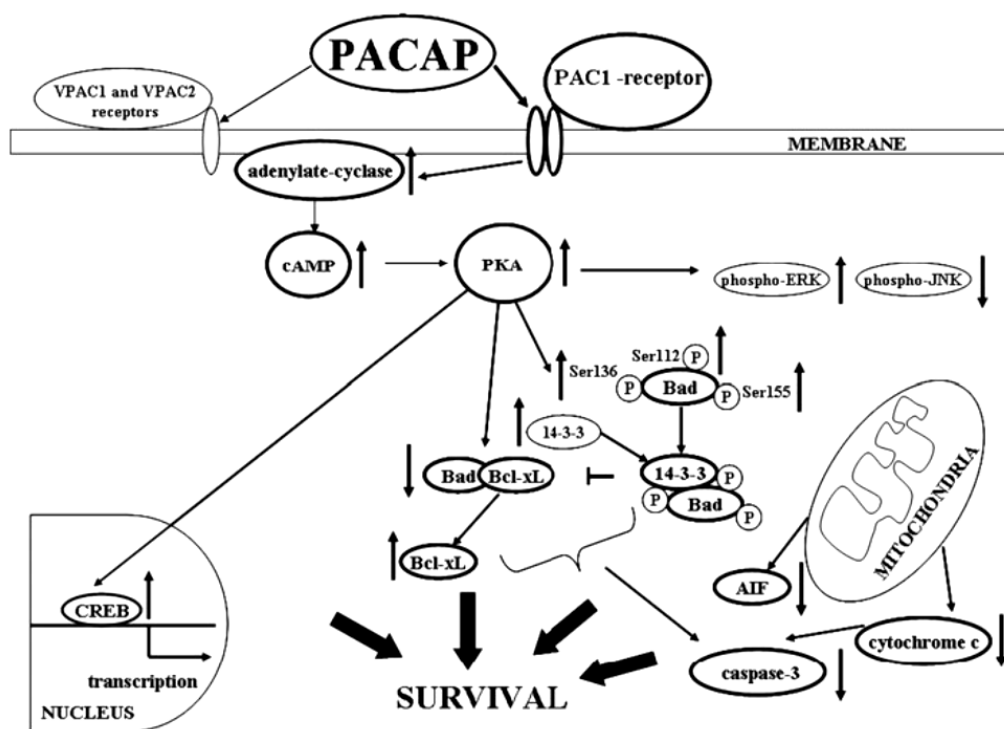


Figure 2

Schematic drawing of PACAP activated signaling pathway in retina. PKC, protein kinase C; PKA, protein kinase A; cAMP, cyclic 3,5-adenosine monophosphate; PAC1, VPAC1, VPAC2, PACAP receptors; PLC, phospholipase C; JNK, caspase-3, AIF (apoptosis inducing factor), proapoptotic factors; ERK, Bad, Bcl-xL, antiapoptotic factors; CREB, cAMP response element binding protein.

Many studies have also demonstrated the importance of electrical activity not only in neurons survival, but also in neuronal maturation. It has been demonstrated the potentiating effect of depolarization on BDNF-induced axon outgrowth. RGCs are the only neuronal subpopulation in the retina able to produce burst of action potentials, and stimulating their intrinsic electrical activity favors their development. RGCs are indeed already electrically active far before the photoreception starts. Thus, the stimulation of electrical activity in RGCs, by inducing the firing, promotes axon outgrowth (Goldberg et al., 2002).

In other neuronal cell types, such as cerebellar neurons, voltage gated sodium channel tetrodotoxin- (TTX) sensitive are involved in axon outgrowth. Indeed, the inhibition of sodium channels impairs axon outgrowth by reducing cell excitability (Brackenbury et al., 2010).

Other ion channels, other than TTX-sensitive sodium channels, are involved in axon outgrowth. Clapham's lab demonstrated a role of TRPC channels in neurite outgrowth. Due to their specific localization at the growth cones they control growth cones morphology (Greka et al., 2003). On the other hand, Jablonka and colleagues showed the role of calcium channels in neurites growth of motor neurons. Neuronal growth cones, indeed, undergo multiple intracellular calcium elevations every hour and these calcium waves have been inversely correlated to neurite elongation. Indeed, culturing cells in calcium free medium causes an increase of axon length, while restoring the physiological extracellular calcium concentration, by reducing growth cones adhesion to the substrate and growth cone dynamics, overall reduces axon outgrowth. *In vivo* axon length is increased when spontaneous calcium waves are reduced (Jablonka et al., 2007).

Regarding chloride channels involvement in this process, many studies reveal the key role of ligand-gated chloride channels in neuronal maturation. However, not much is known about the role of voltage gated anionic current in this process. It has been demonstrated the role of GABA and glycine receptors in this process in cerebellar granule neurons, in hippocampal neurons and cortical neurons. Due to the higher intracellular chloride concentration in immature neurons,

their activation causes a chloride-mediated depolarization. This results in increased calcium concentration by regulating voltage gated calcium channels (Sernagor et al., 2010). Recent studies have shown the involvement of chloride channels in differentiation and neurite elongation of PC12 cells induced by NGF treatment. NGF treatment elicits a chloride current that in turn determines the phosphorylation of TrkA receptor and the activation of the ERK pathway. The treatment with chloride channels inhibitors such as DIDS, SITS and NPPB, that are widely used blockers of volume-regulated chloride current, greatly reduced neurite length of NGF treated PC12 cells and TrkA activation (Kim et al., 2010). Moreover, it is known the role of chloride, and in particular of GABA receptors, in dendrite maturation of cortical neurons (Sernagor et al., 2010).

4.3 Tubulin cytoskeleton in neuronal polarization.

Many studies have demonstrated the importance of cytoskeletal dynamics in axon specification. Actin and tubulin are the main constituent of the neuronal cytoskeleton. Actin filaments regulate the shape of the growth cone and the direction of axon growth, whereas microtubules give structure to the axon and are essential for axon extension.

Tubulin is a 54 KDa protein and is a widely diffused protein in vertebrates. Polymerization of microtubules proceeds through the heterodimerization of α - and β -tubulin into protofilaments that associate into a polar structure. This polarity is responsible for different polarization rates at the two microtubule ends: the fastest polymerization rate at the plus end (“capped” by β subunits) and the slowest polymerization rate at the minus end. There are six different genes for α -tubulin and seven genes coding for β -tubulin in mammals. β III-tubulin is normally expressed only in neurons, while the β II-isoform is usually predominantly expressed in elongating neuronal processes. However, microtubules are usually heterogeneous polymers composed by different

combinations of the different tubulin isoforms. The different tubulin isotypes confer different properties to the microtubules; for example β III-tubulin-microtubules are more stable than those assembled almost exclusively by β II-tubulin. Moreover the different tubulin isotypes composition alters the microtubules dynamics. Different proteins are associated with microtubules contributing to neuronal stabilization (Poulain and Sobel, 2010).

Microtubule cytoskeleton dynamics is important in axon outgrowth. APC protein is involved in microtubule dynamics, by binding to plus end of microtubules. APC is a target of GSK3 β kinase. Phosphorylation of APC by GSK3 β reduces its binding to microtubules. GSK3 β regulates by phosphorylation also two other microtubules binding proteins, such as MAP1 β and Tau. Both protein bind to microtubules and are regulated by phosphorylation. It is known that Tau hyperphosphorylation causes its detachment from microtubules impairing axonal transport and causing abnormal Tau aggregates common in Alzheimer Disease and other tauopathies.

The opposite role of PTEN phosphatase and PI3K on the PIP2 pathway, are also involved in axon outgrowth. PTEN is a protein- and lipids-phosphatase, and its activity ensures, among others, the correct lipid composition of the growth cones membranes during neuronal polarization (Polleux and Snider, 2010).

Other signaling pathways like the ERK/MEK, or the JNK pathway are involved in axon elongation. Moreover, it has been demonstrated the involvement of atypical PKC (aPKC) in this process, acting on Par-3 and Par-6, LKB1, Ras- and Rho-family small GTPases (Polleux and Snider, 2010).

4.4 The actin cytoskeleton in axon outgrowth: the growth cones.

Monomeric actin (G-actin) is 42 KDa protein, binding either ATP or ADP. *In vitro*, ATP-actin rapidly polymerizes into polarized filamentous structures: the F-actin. F-actin is composed

by two twisted helices. Once F-actin starts nucleating, the polymerization process proceeds quickly by adding monomers at the plus end, and proceeds more slowly at the minus end. Capping proteins associate at the plus or the minus end, resulting in a tight regulation of actin polymerization, either preventing filament elongation or inhibiting depolymerization. Monomer binding proteins control the availability of actin subunits, while the so called severing proteins such as the members of the Cofilin family regulate disassembly (Nurnberg et al., 2011).

Actin cytoskeleton results to be highly enriched at the tips of growing axons, in structures called growth cones, that guide axon outgrowth (Geraldo and Gordon-Weeks, 2009).

During neurite development, growth cones pass through different environments and integrate several cues encountered along their pathway.

Growth cones are the most distal part of growing axons. Growth cones are highly dynamic structures, with a typical fan-shaped morphology made up of a central tubulin-rich area, surrounded by an actin-rich area, as shown in figures 3 and 4. Thus, it is likely that subtle alterations of the cytoskeletal dynamics are tightly linked to axon outgrowth, and the fine structure of the growth cone is one of the first affected. Growth cones probe the environment searching for specific cues, by protruding and retracting flat protrusions called lamellipodia and finger-like projections named filopodia (Dent et al., 2011). The dynamics of the cytoskeleton at the growth cone is crucial for axon outgrowth. The balance between cytoskeleton polymerization and depolymerization sets the neurite extension. The pioneering studies of Bradke and Dotti in 1997 showed that increasing the actin cytoskeleton dynamics by local perfusion of actin-depolymerizing agents, such as Cytochalasin or Latrunculin, might be sufficient to induce the axon-determination. Indeed, axons usually grow ten times more and faster than dendrites, and favoring actin depolymerization is sufficient to increase actin dynamics and thus induce a faster neurite elongation and axon differentiation (Bradke and Dotti, 1997).

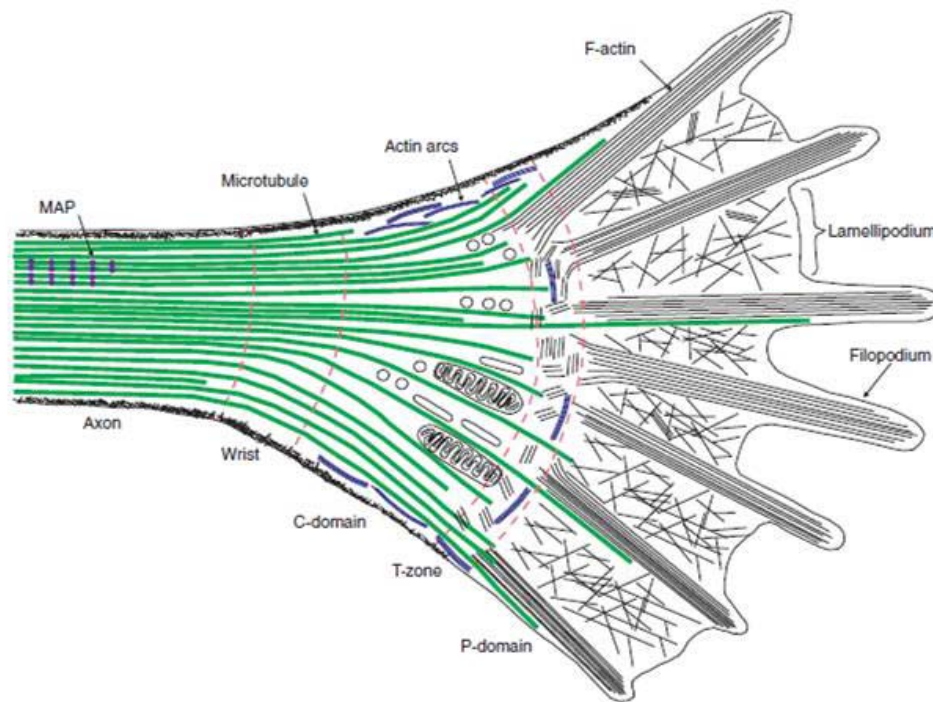


Figure 3

Schematic representation of the morphological regions constituting a growth cone cytoskeleton. The microtubules in the axon shaft (green lines) are maintained parallel to each others through MAPs (microtubules associated proteins). They extend individually in the C-domain and in the T-zone. The P-domain is dominated by parallel filament of F-actin. Adapted from (Geraldo and Gordon-Weeks, 2009).

Many accessory proteins are involved in the tight control of the balance between polymerization and depolymerization, shaping the growth cone. One of the main capping proteins is Cofilin. Cofilin can bind both to G-actin and F-actin. When bound to F-actin it causes filament twisting leading to actin depolymerization, while bound to G-actin prevents F-actin polymerization. Thus Cofilin contributes in controlling growth cones morphology and axon elongation.

Actin dynamics is also important in growth cones guidance towards specific cues. Depolymerizing agents, indeed, do not impair axon outgrowth, but neurons treated with these specific agents are no more able to turn respectively towards or away from specific guidance

cues. The mechanism is not common to all the neuronal types, indeed, for example, the severing protein Cofilin is important for turning of hippocampal axons, but not for cerebellar neurons (Dent et al., 2011;Gehler et al., 2004).

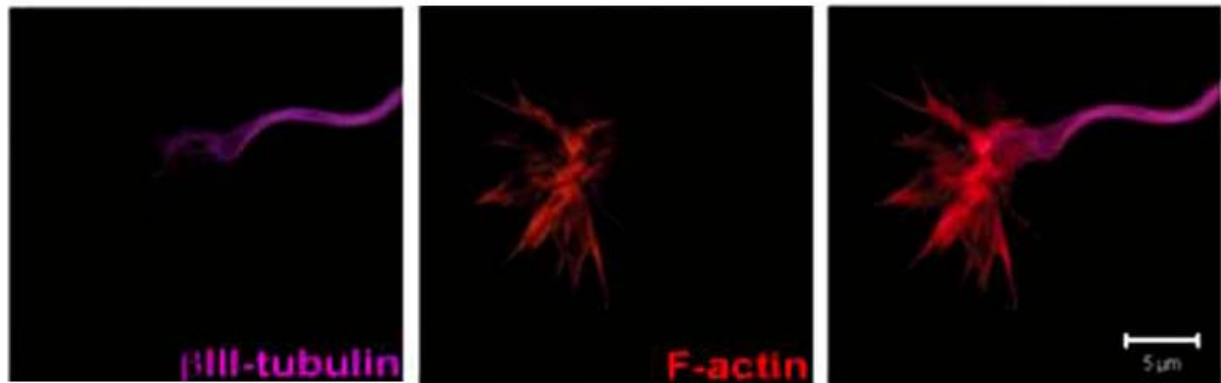


Figure 4

Representative image of a growth cone, with the tubulin rich area (magenta) surrounded by the fan-shape of F-actin (red).

The importance of actin at the growth cones for axon elongation is supported by the studies showing that a local translation of actin mRNA at the growth cones help in the neurite growth, although the molecular mechanisms involved in this process are not fully understood (Rossoll et al., 2003).

Impairment of axon outgrowth is usually accompanied by altered morphology of growth cones. Davis and colleagues demonstrated that knock out mice for the β -subunit of the CP protein have altered morphology of the growth cone, with a collapse of the structure, and an increased co-localization of actin and tubulin, accompanied by a reduced axon length (Davis et al., 2009).

AIM OF THE WORK

Due to the broad expression of CLIC1 throughout the central nervous system (CNS), and more importantly to the crucial role of CLIC1 in the CNS physiopathology, as a result of its peculiar property to respond to cellular oxidation by moving from the cytosol to the plasma membrane, we hypothesized the involvement of CLIC1 in other oxidation-based mechanisms in the CNS.

Recent studies showed severe retinal dysfunctions in Alzheimer disease (AD) patients, due to the loss of retinal ganglion cells (RGCs), much before other cognitive symptoms. RGCs are the cells whose axons gather together making up the optic nerve, and many studies have demonstrated that RGCs degeneration is linked to oxidative stress. Thus, we hypothesized a role of CLIC1 in this mechanism. Interestingly, not being protected by the skull, retina is the most exposed and thus the most accessible part of the CNS. Therefore, we focused on the retina in the search for an early diagnosis of the neurodegenerative process in AD.

Moreover, due to the broad expression of CLIC1 in mouse retina and in cultured RGCs, we investigated on the role of CLIC1 also in RGCs physiology and in particular in the development process of the optic nerve.

Chapter 5

Methods

Confocal microscopy. Confocal microscopy was pioneered by Marvin Minsky in 1955 while he was a junior fellow at Harvard. Confocal microscope brings up considering improvements over other conventional fluorescence microscopes: *in primis*, the possibility of collecting light from a single focal plane of the sample, by a point-by-point illumination of the specimen. In this way it is possible to exclude most of the scattered light belonging to the excitation of other focal planes of the sample, rendering the image sharper and the details more defined: the image results less blurred and represents a thin section of the sample. Thus not only images are more defined, but it is also possible to assemble a series of thin slices along the vertical axis re-creating a 3D image.

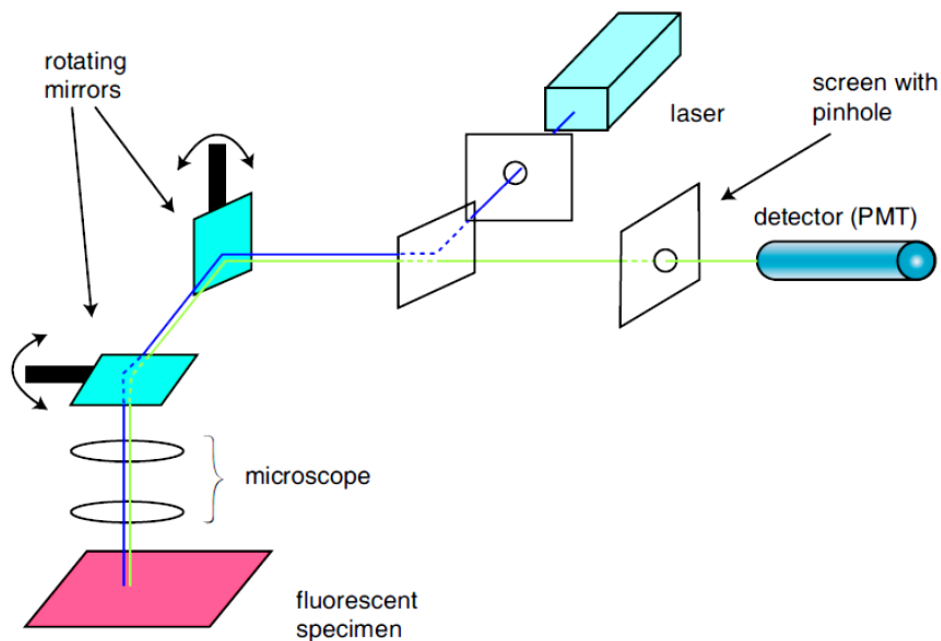


Figure 1.

Schematic representation of a confocal microscope.

In order to collect light from a single point of the sample at the time, a high intensity source of light is needed. In confocal microscopes this is provided by illuminating the sample with a laser.

In Figure 1 is shown a schematic representation of a confocal microscope. The fluorescent specimen is excited by the blue laser light with the help of a dichroic mirror. Two rotating mirrors that direct the laser light through the sample allow to scan the whole specimen. The green light emitted by the specimen is collected through the same rotating mirrors, through the dichroic mirror, to the pinhole. The pinhole allows to define the size of the point excited by the laser light. In the end the signal is detected with a photomultiplier (PMT). Given that only one point at the time is excited every instant, there is never a complete image, that is rebuilt only on a computer, one pixel at the time. The image created by the microscope is a thin region of the specimen, which we use to refer to as an optical section: out-of-plane unfocused light has been rejected.

In order to visualize these images, a fluorescent sample is needed. Specific proteins in cells and tissues are labeled by specific primary antibodies. Primary antibodies are recognized by specific secondary antibodies that are conjugated to fluorescent molecules. This allow to visualize under a fluorescence microscopes the labeled molecules.

In this case all the images were acquired with a Leica SP5 microscope, excited with either 405 or 488, or 546, or 647 laser.

5.1 Extraction of retinas from rat and mice. Adult mice were anesthetized with isoflurane and enucleated, then sacrificed by dislocation. The eyes were kept overnight at 4°C in 4% paraformaldehyde (PFA). A small hole in the cornea helped the penetration of PFA into the internal tissue. After 24 hours the eyes were washed several times in PBS in order to eliminate any residual PFA, and left overnight (ON) in 30% sucrose, in order to dehydrate the tissue that

otherwise would result damaged once frozen. The last day the eyes were included in OCT and frozen in dry ice. After one day, 20 μm slices were made at the microtome.

5.2 Immunohistochemistry. Retina slices were washed twice in PBS and blocked for 2 hours at room temperature (RT) in 5% BSA and 0.5% Triton X-100. Then the excess was removed and the slices were incubated ON at 4°C in PBS with 1% BSA, 0.2% triton X-100 and primary antibody. Anti-CLIC antibody (custom made, kindly provided by Dr Mark Berryman at Ohio University College of Osteopathic Medicine (Athens, OH) was diluted 1:400; anti-tubulin (Sigma Aldrich) 1:300. After 3 washes with PBS they were incubated with their specific secondary antibody diluted 1:1000. For CLIC1, anti rabbit 546; for tubulin, anti mouse-660. The slices were then washed with PBS and incubated for 10 minutes in PBS containing Hoechst (1:400), and mounted with Fluoromount G (Southern Biotech).

5.3 Mice. TgCRND8 mice and their control littermates (non transgenic mice with C3H/B6 background) were used. TgCRND8 encode a double-mutant form of amyloid precursor protein (APP) 695 (KM670/671NL+V717F) under the control of the Prion Protein gene promoter (Chishti et al., 2001). 3xTg mice (carrying three mutations respectively in the APP gene, in Tau gene and in Presenilin gene) and their control littermates (carrying only the PS1 mutation) were used (Oddo et al., 2003). C56BL6 mouse pups from Charles River were used to isolate RGCs.

5.4 Brain slices preparation. Mice were anesthetized and perfused with saline, then brains were removed, bisected in the mid-sagittal plane and fixed in 10% neutral buffered formalin for a minimum of 48 h. The specimens were then processed on an automatic tissue processor overnight with vacuum to aid penetration of the fixing solution. Paraffin sections were cut at 5 μm . Sections were stained with DAPI, and Lectin-488 in order to visualize activated microglia cells.

5.5 Immunohistochemistry on brain slices. Brain sections were rinsed 3 times and then placed in blocking solution (PBS, pH 7.4 with 0.3% Triton X-100, 2% BSA and 5% Goat Serum), for 30 min at RT. Biotinylated *Lycopersicon esculentum agglutinin* (LEA) (1:2000; Vector Laboratories) was diluted in fresh blocking solution, applied for 1 h at 4°C and revealed by streptavidin-Alexa 488 (1:1000, Invitrogen). Following 3 additional rinses, sections were finally coverslipped using Vectashield water-based mounting medium with DAPI (Vector Laboratories, Burlingame, CA).

5.6 Dissociation of retinal ganglion cells. Retinal ganglion cells were isolated by immunopanning from P1 mouse retinas, as previously described (Goldberg et al., 2002). Briefly, mouse pups were enucleated and the eyes were put in neurobasal medium (Invitrogen). A small hole on the cornea was made in order to decrease the pressure and allow an easier manipulation of the eye. The retinas were isolated and put in fresh pre-warmed neurobasal. The tissue was then digested at 37°C for 30 minutes in a digestion solution containing 16 U/ml papain (Roche), 0.04 mg/ml DNase (Roche), and 1.3 mg/ml L-cysteine (Sigma Aldrich) added only 10 minutes before the digestion in order to activate papain. Successively, digestion solution was removed and the tissue was carefully washed three times with ovomucoid solution containing trypsin inhibitor (Roche) 15 mg/ml, BSA and 0.04 mg/ml DNase, letting the pieces to settle after each wash. Then the digested tissue was resuspended in 5 ml ovomucoid solution and gently subjected to a mechanical digestion. The cells were counted and after centrifugation at 400g they were resuspended in 360 µl of a pre-warmed separation buffer containing 0.5 mg/ml BSA and 2mM EDTA, in PBS. The cells were then incubated for 10 minutes at 4°C with 1:100 anti Thy1.1 antibody conjugated to magnetic microbeads (Milteny). Thy 1.1 is a membrane protein specifically expressed by RGCs in the retina. The antibody binding to Thy1.1 allowed to isolate, by a magnet, all the cells expressing Thy1.1 in the retina (specifically bound by the antibody). Thus, we were able to isolate RGCs over the other retinal subpopulations. After 10 minutes

incubation, the antibody in excess was removed by washing the cells with separation buffer, and centrifuged at 300g for 10 minutes at RT. The cells were successively resuspended in 3 ml of separation buffer. The cell suspension was added to the affinity column placed in a strong magnetic field. Only Thy1.1-labeled cells were thus kept in the column, while all the remaining cell subpopulations flew through it. After washing the column with 2 ml of separation buffer to eliminate the cells unspecifically bound to the column, the column was removed from the magnetic field and the cells were eluted with 5 ml of separation buffer. Eluted RGCs were counted, centrifuged at RT for 10 minutes at 300g, and resuspended in culture medium. The cells were plated at a density of 55000 cells per each well. The culture medium contains: 100 µg/ml Transferrin (Sigma aldrich), 100 µg/ml BSA (Sigma aldrich), 60 ng/ml Progesterone (Sigma aldrich), 16 µg/ml Putrescine (Sigma Aldrich), 40 ng/ml sodium selenite (Sigma aldrich), 2X serum-free B27 supplement (Invitrogen), 40 ng/ml T3 (Sigma Aldrich), 1 mM sodium pyruvate (Sigma), 2 mM glutamine (Sigma aldrich), 50 ng/ml Insulin, 10 µM Forskolin, 50 ng/ml BDNF, 100 ng/ml CNTF (Invitrogen). The cells were kept at 37° and 5% CO₂. The slides were pre-coated with 10 µg/ml poly-D-lysine for 30 minutes and with 10 µg/ml Laminin overnight.

5.7 Electrophysiology recordings. 7 DIV RGCs were bathed in a physiological solution containing (in mM) 140 NaCl, 5 KCl, 2.5 CaCl₂, 1 MgCl₂, 10 Hepes, 10 Glucose. Membrane potential recordings were performed in gramicidin perforated patch. 2.5 µg/ml gramicidin was dissolved in pipette solution containing (in mM) 140 KCl, 10 Hepes, 10 Glucose.

5.8 Amyloid beta preparation. Aβ powder (Bachem) was resuspended in water-free DMSO at 1 mg/mL concentration, by shaking it for 90 minutes. Then it was placed at -80°C for 45 minutes. It was placed still frozen in a lyophilizator overnight at -20°C under centrifugation. The fluffy pellet was stored at -20°C until use. Just before use, Aβ was dissolved in DMSO,

quantified by using BCA kit (Pierce) according to the manufacturer instructions, and dissolved in bath solution.

5.9 RGCs treatment. All the treatments were performed by diluting the compounds in the culture medium. IAA94 (dissolved in ethanol) was dissolved in the culture medium at a final concentration of 50 μ M. DIDS (dissolved in DMSO) was diluted at a final concentration of 200 μ M. Anti N-ter CLIC1 antibody (kindly provided by Dr Sam Breit, University of Sidney) was diluted 1:100 in culture medium. For axon length quantification the cells were cultured for 24 hours, while for growth cones analysis they were cultured and treated for 3 days.

5.10 Immunocytochemistry on isolated retinal ganglion cells. RGCs were fixed for 45 minutes in pre-warmed 4% paraformaldehyde (PFA), than washed 3 times with PBS and incubated 2 hours at RT with blocking solution containing PBS with 5% BSA and 0.1% Triton X-100. They were successively incubated overnight (ON) with anti-CLIC1 antibody (kindly provided by Dr Mark Berryman at Ohio University College of Osteopathic Medicine (Athens, OH)) diluted 1:400 in antibody incubation solution (PAIS) containing PBS with 1% BSA and 0.1% triton X-100. The cells were then washed twice in PBS and incubated with the appropriate secondary antibody, anti rabbit-546 diluted 1:1000 in PAIS. After 2 washings in PBS, anti-beta tubulin III (Millipore) diluted 1:300 in PAIS was added ON at 4°C. Then the cells were washed and incubated with secondary anti-chicken antibody alexa-660 diluted 1:1000 in PAIS, for 2 hours at RT. After 3 washings the cells were incubated in a solution containing PBS, 1% BSA and phalloidin-488 (Invitrogen) for 30 minutes at 37°C. During the last 3 washings the cells were incubated with Hoechst diluted 1:400 in PBS and mounted with Fluoromount G (Southern Biotech).

5.11 Axon length quantification. Tubulin-labeled RGCs were visualized with a Olympus BX51 Neurolucida microscope, using a dry 20X objective. The cells were excited with a fluorescence

lamp, and excited and acquired by using the appropriate filters. Only neurites longer than the cell body diameter were considered, and the longest axon of each neuron was measured and averaged with the axons of other cells.

5.12 Co-localization analysis. Co-localization between actin and CLIC1, and between tubulin and CLIC1 at the growth cones was performed by using the ImageJ software. In order to define the area of analysis, a “mask” was created based on the phalloidin labeling, and the number of co-localized pixels was counted and expressed as a percentage of the total mask area (considered as 100%).

5.13 Quantification of altered growth cone morphology. The area of the mask created on the phalloidin (actin covered surface, AcS) and on the tubulin labeling (tubulin covered surface, TcS) was measured and the “absolute value” was considered for the ratio.

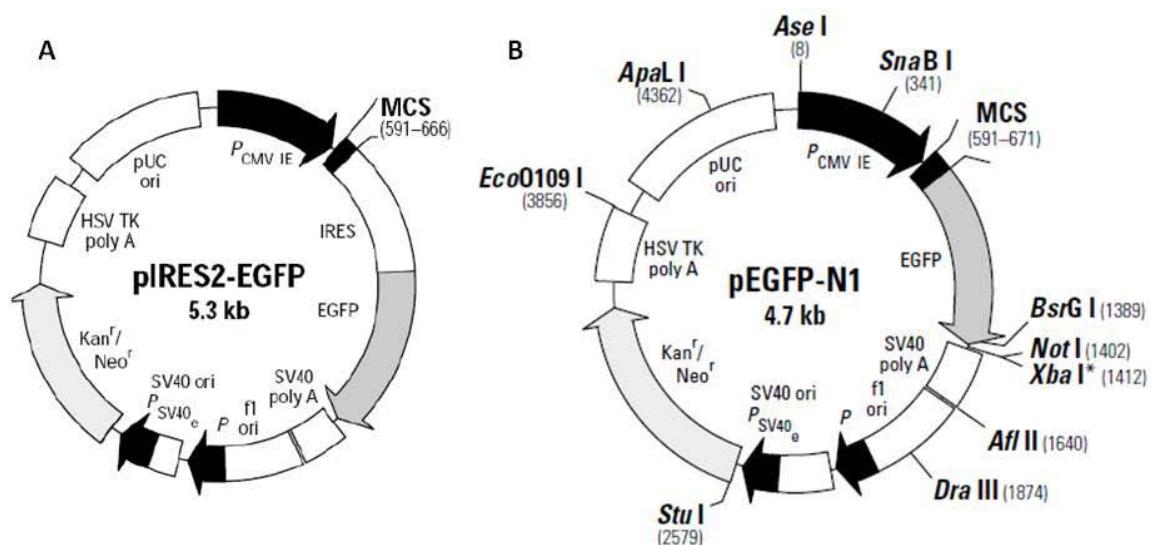


Figure 2.

Map of pIRES2-EGFP plasmid in A, and of the pEGFP-N1 in B.

5.14 Cell cultures. N2a (ATCC CCI-131) and HEK293T (human embryonic kidney) (ATCC CRL-11268) were kept in Advanceded D-mem (Invitrogen) containing 10 % Fetal bovine serum

(FBS), 2 mM glutamine, 1X Pen strep, at 37°C and 5% CO₂. The cells were split every 3 days by incubating for 5 minutes at 37°C with Trypsin-EDTA (Invitrogen) and re-plated at 1:10 dilution.

5.15 DNA and cell transfection. CLIC1 gene was inserted in the pIRES2-EGFP plasmid (see map in figure 2) into the BglII-BamHI sites; and in the BglII-BamHI sites also into the pEGFP-N1. 60% confluence N2a cells were transfected with a mixture of Lipofectamine : DNA = 3 µl : 1 µg, in serum-free medium. After 5 hours post transfection an equal volume of medium containing 20% FBS was added in order to restore the final concentration of 10% FBS. All the experiments were performed 48 hours after transfection.

5.16 Western blot analysis. Cells were lysed for 30 minutes in ice with 150 µl Lysis buffer containing 50 mM Tris pH 7.4, 1% Triton X-100, 50 mM NaCl, 1 mM EDTA, protease inhibitor cocktail. After 30 minutes the cells were detached with a scraper and each sample was sonicated for 1 minute. The sample was briefly centrifuged at 4000g in order to roughly eliminate the membrane fraction, and the supernatant containing the cytoplasmic fraction was loaded on a 10% acrylamide gel and run at 130 V for 2 hours. Then it was blotted on a 0.2 µm nitrocellulose membrane for 2 hours at 4°C at constant 200 mA current applied. The blot was then blocked for 1 hour with TBS containing 5% milk and 0.2% tween-20, by shaking it at RT. After blocking, it was incubated with anti-CLIC1 antibody (kindly provided by Dr Sam Breit, University of Sidney) diluted 1:800 in TBS with 1% BSA and 0.2% Tween-20, ON at 4°C. After 3 washings with TBS (5 minutes each), it was incubated with the appropriate secondary antibody (Alexa goat anti sheep-488), 1:1000 for 2 hours at RT in the dark, in shaking. After 3 washings in large volumes of TBS the membrane was left to dry up for 30 minutes and then the signal was detected at the typhoon, by exciting with 488 laser. Anti-actin antibody (Sigma) was diluted 1:500 in the same solution and detected with anti-rabbit-546 secondary antibody.

5.17 Cytochalasin treatment of N2a cells. N2a cells were treated with the vehicle and with 10 μ M cytochalasin B (Sigma Aldrich) for 60, 90 and 120 minutes respectively. The cells were then lysed for 30 minutes in ice in 150 μ L lysis buffer containing: 50 mM NaCl, 50 mM Tris pH 7.8, 1 mM EDTA, 0.1% Triton X-100, protease inhibitor cocktail. Differently from the previously mentioned lysis buffer, here the percentage of Triton X-100 was much lower in order to keep the eventual interaction between Actin and CLIC1. The cells were then homogenized in a Dounce and centrifuged for 45 minutes at 4°C and 100000g, in order to separate the soluble fraction from the insoluble fraction. The insoluble fraction was then resuspended in the same lysis buffer containing also 0.1% SDS. The samples were quantified with BCA kit according to the manufacturer instructions and run on a acrylamide gel as described above.

5.18 Statistical analysis. Data were presented as mean values \pm standard error of the mean (s.e.m.). The significance was calculated with two sample student-T-test by using Origin software, with 0.05% significance.

Chapter 6

Results

6.1 CLIC1 as an early marker of neurodegeneration in AD.

Visual deficits often accompany and, most importantly, precede cognitive impairment in Alzheimer disease (AD) patients, due to the loss of Retinal ganglion cells (RGCs) (Berisha et al., 2007). Being unprotected by the skull, retina is the most accessible part of the central nervous system (CNS). Previous studies have documented the expression of CLIC1 throughout the CNS. (Milton et al., 2008;Novarino et al., 2004). Thus, in the search for an early marker of AD that could represent an useful tool for an easy diagnosis of the disease, necessary for an appropriate medical therapy, we investigated CLIC1 expression in the mammalian retina.

Figure 1 depicts an image of a mouse retina section. DAPI labeling, in blue (upper left panel), allows to visualize the different retina layers; as indicated in the figure: the photoreceptor layer, the outer nuclear layer (ONL), the outer plexiform layer (OPL), the inner nuclear layer (INL), the inner plexiform layer (IPL) and the gangliar layer (G). In green (upper right panel), the tubulin labeling displays the fiber layer (FL), where axons of retinal ganglion cells (RGCs) are located, parallel to the gangliar layer, and RGCs dendrites in the IPL. CLIC1 staining, in red (bottom left), showed that CLIC1 was expressed in the mouse retina, and it was mainly expressed in the cell bodies of the RGCs, in the gangliar layer, which is more evident in the inset, and in the IPL and OPL, where dendrites of RGCs and neurites of bipolar neurons and photoreceptors lay, respectively. On the bottom right panel is shown the merge.

Once we assessed CLIC1 expression in mammalian retina, we asked whether its expression could be affected in the neurodegenerative process affecting RGCs in AD patients.

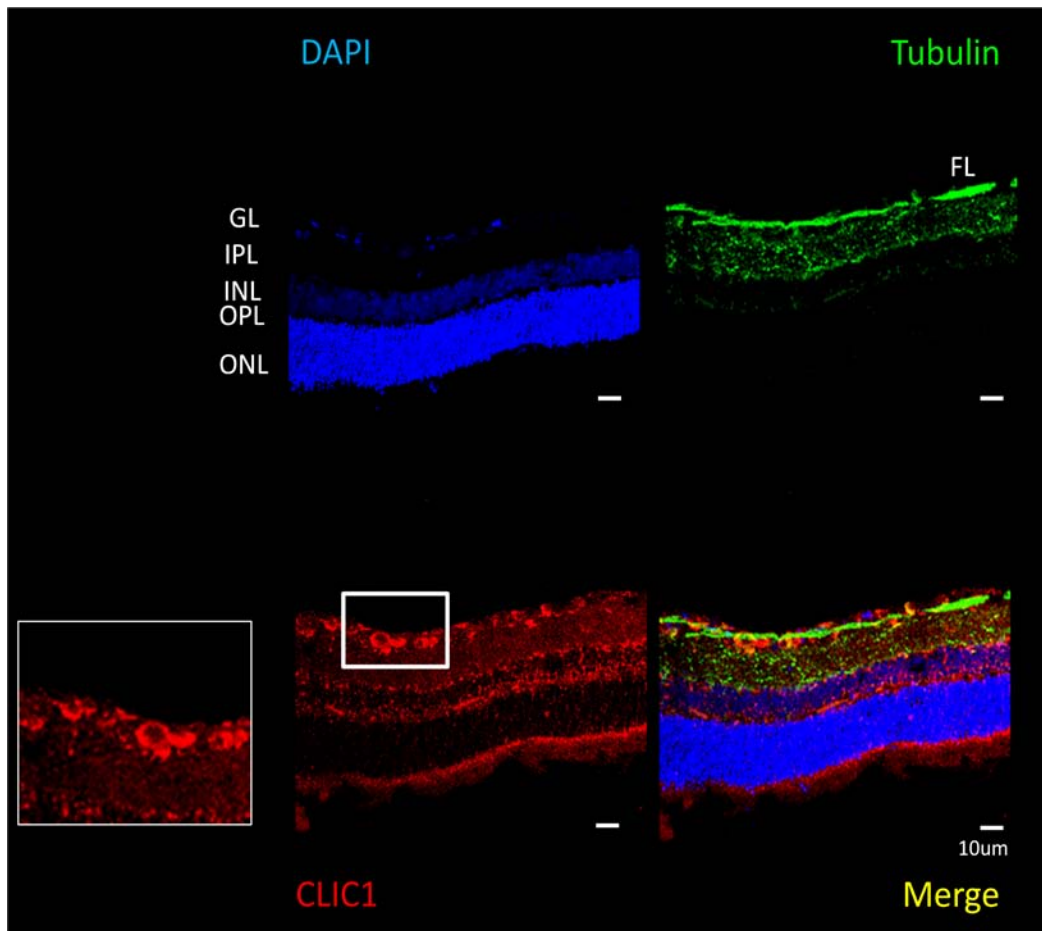


Figure 1.

Immunohistochemistry of a mouse retina. In the upper left panel nuclei labeling with DAPI (blue) identifies the gangliar layer (GL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), and outer nuclear layer (ONL). In the upper right panel the tubulin staining (green); in the bottom left panel in red is shown CLIC1. The inset depicts an enlargement of CLIC1 staining in the GL. The bottom right panel shows the merge of all panels.

Our hypothesis was also reinforced by previous studies linking CLIC1 functional expression with neurodegeneration in the CNS, in AD pathogenesis (Milton et al., 2008;Novarino et al., 2004). Thus, we analyzed CLIC1 expression in the retina tissue of AD mouse models. AD mouse models have been widely used in biomedical research. Thus, many different genetically engineered mice have been developed. TgCRND8 encode a double-mutant form of amyloid

precursor protein (APP) under the control of the Prion Protein gene promoter (Chishti et al. 2001).

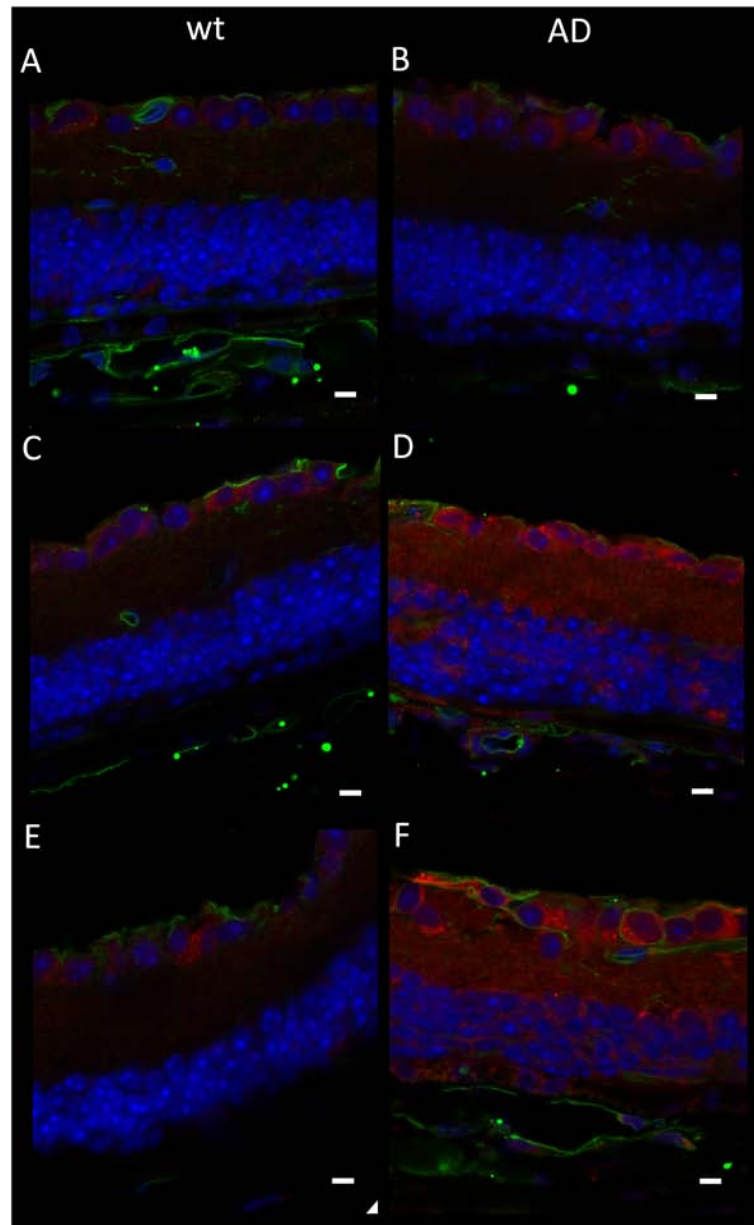


Figure 2.

Immunohistochemistry on retina slices from TgCRND8 mice (B, D, F) and their control littermates (A, C, E) at 1 month (A, B), 5 months (C, D) and 10 months (E, F) of age. CLIC1 staining (red) increased with age mainly in TgCRND8 mouse model. DAPI (in blue) labeled nuclei allowing to distinguish the ganglionic layer and the inner nuclear layer, while lectin (in green) labels blood vessels. Scale bar 10 μ m.

These mice show A β deposits, and the first cognitive symptoms resulting in impaired performances at the water maze test, at 3 months of age, even though they do not show neurofibrillary tangles. Left panels of figure 2 depict immunostaining of retina slices from control non transgenic mice with the C3H/B6 background, while on the right there are images of retinas from the TgCRND8 mice, at the different ages indicated: from the top to the bottom at 1, 5 and 10 months of age respectively. We can clearly see that in AD retinas there is a slight increase of CLIC1 expression (red) in the retinal ganglion cells layer already at 1 month of age, which means much before the manifestation of the first disease symptoms. At 5 and 10 months of age CLIC1 expression is visibly increased also in the IPL. The lectin staining, in green, highlights the absence of activated microglia, but labels the blood vessels.

In order to confirm these results, we analyzed CLIC1 expression in a different AD mouse model, the 3xTg. This model was developed by LaFerla and colleagues few years ago. This model carries three mutations in the APP gene, in Tau gene and in Presenilin gene. The three mutations cause the deposition of amyloid beta (A β) aggregates in the CNS, neurofibrillary tangles, and defects in the mechanism of long term potentiation (LTP) (Oddo et al., 2003).

Differently from the TgCRND8 model, these mice develop the first memory deficits, and show amyloid plaques around 6 months of age. Figure 3 depicts an image of mouse retinas from 3xTg mice (on the right) and their age-matched control littermates (carrying the mutation in the PS1 gene, on the left), stained for CLIC1 (red). From the top to the bottom there are retinas from animals at 5, 10 and 17 months of age. We can clearly see that in AD models there was an increased CLIC1 (red) expression compared to their control littermates, that was already visible in 5 months old mice and increased with aging. Although there was a slight increase of CLIC1 expression also in control mice, the enhancement was higher in 3xTg mice. There was a broad increase of CLIC1 expression in the whole retina tissue, even though we can notice that this was more pronounced in the gangliar layer, as indicated by the white arrows. As we have mentioned

above, the onset of the first symptoms in these mice is around 6 months of age, so CLIC1 expression increases before the manifestation of the first symptoms.

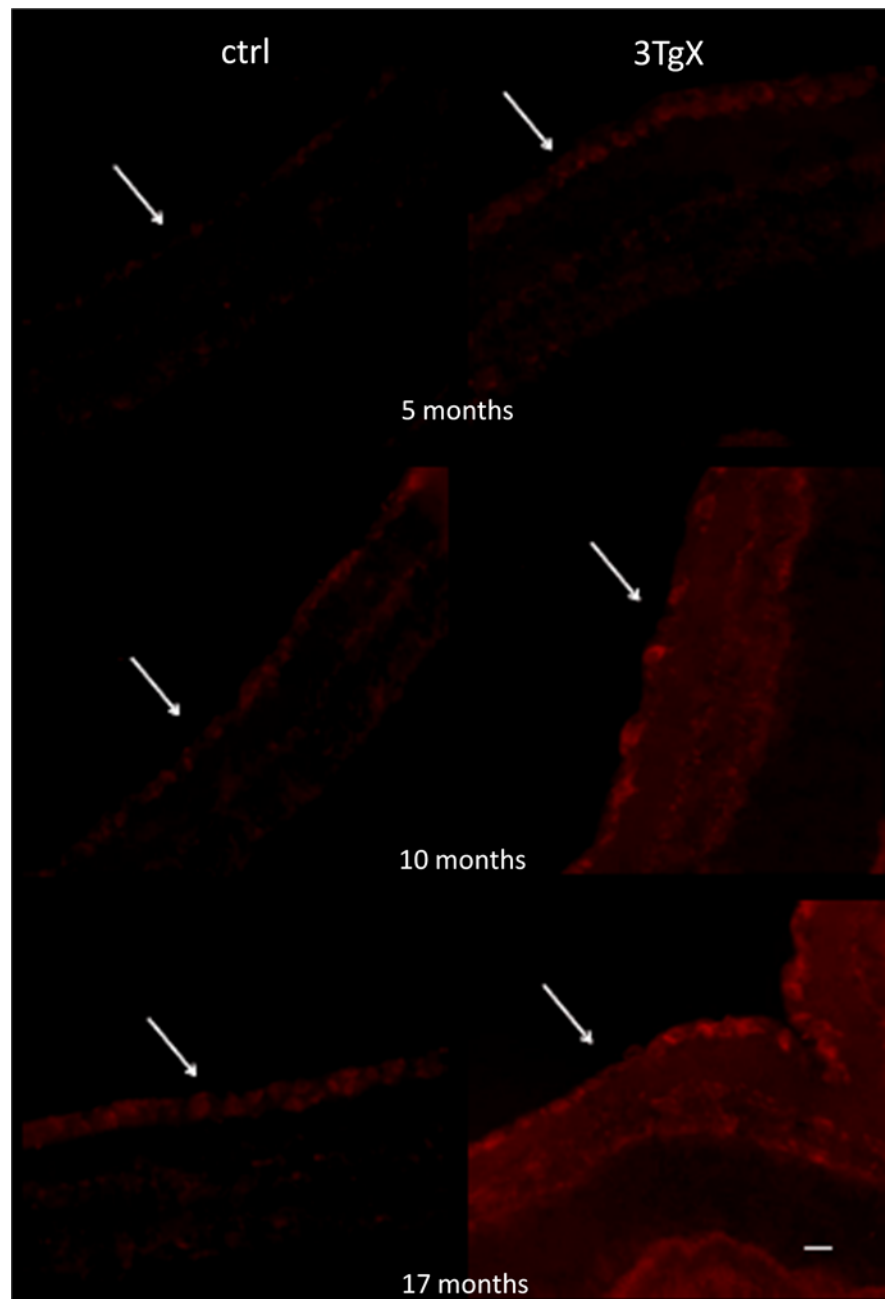


Figure 3.

Immunohistochemistry on retina slices from 3xTg mice (right) and their control littermates (left panels) at 5, 10 and 17 months. CLIC1 staining increased in the retinal ganglion layer of tg3x mice compared to wt, (white arrows). Scale bars 20um.

Fiorentini and colleagues (ref) have previously demonstrated that TgCRND8 mice show an AD phenotype already at three months of age, with cognitive deficits accompanying an increased inflammatory state both in the hippocampus and in the cortex, underlined by microglia and astrocytes activation (Fiorentini et al., 2010). Thus, in order to verify whether the increased CLIC1 expression in the retina was actually linked to the pathological state of AD brain, we compared our results obtained from immunohistochemistry of TgCRND8 retina slices with those obtained from the hippocampus. In the upper panel of Figure 4 it is shown the staining of brain slices from 5 months old TgCRND8 mice with lectin (green), to label activated microglia, while the bottom panel depicts CLIC1 staining (red) in the retinas belonging to the same animals. We can see an increased number of activated microglia in TgCRND8 mouse brain (upper right) compared to their control wt littermates (upper left), in parallel with the increased CLIC1 expression in the AD mouse retina (bottom right) compared to the control retinas (bottom left). These results further suggest that CLIC1 increase in retina is actually parallel to a pathological brain condition.

To test the hypothesis of a “direct” effect of A β on RGCs as a causative factor of the increased CLIC1 expression, we stimulated dissociated RGCs cultures with A β .

It has been previously demonstrated that A β alters the electrophysiological properties of neuronal membranes, affecting membrane potential and evoked action potentials (Hartley et al., 1999).

Thus, we performed whole cell patch clamp experiments and we measured membrane potential of these cells upon A β treatment. In the upper panel of figure 5 is shown one example of a membrane potential recording of a 7 DIV (days in vitro) RGC upon A β stimulation. Average resting membrane potential of these cells after 7 DIV is around -60 mV. We added growing A β

concentrations every 5 minutes, and after 20 minutes of A β stimulation we can clearly see a membrane depolarization. Membrane potential slowly changed and reached -20/-10 mV.

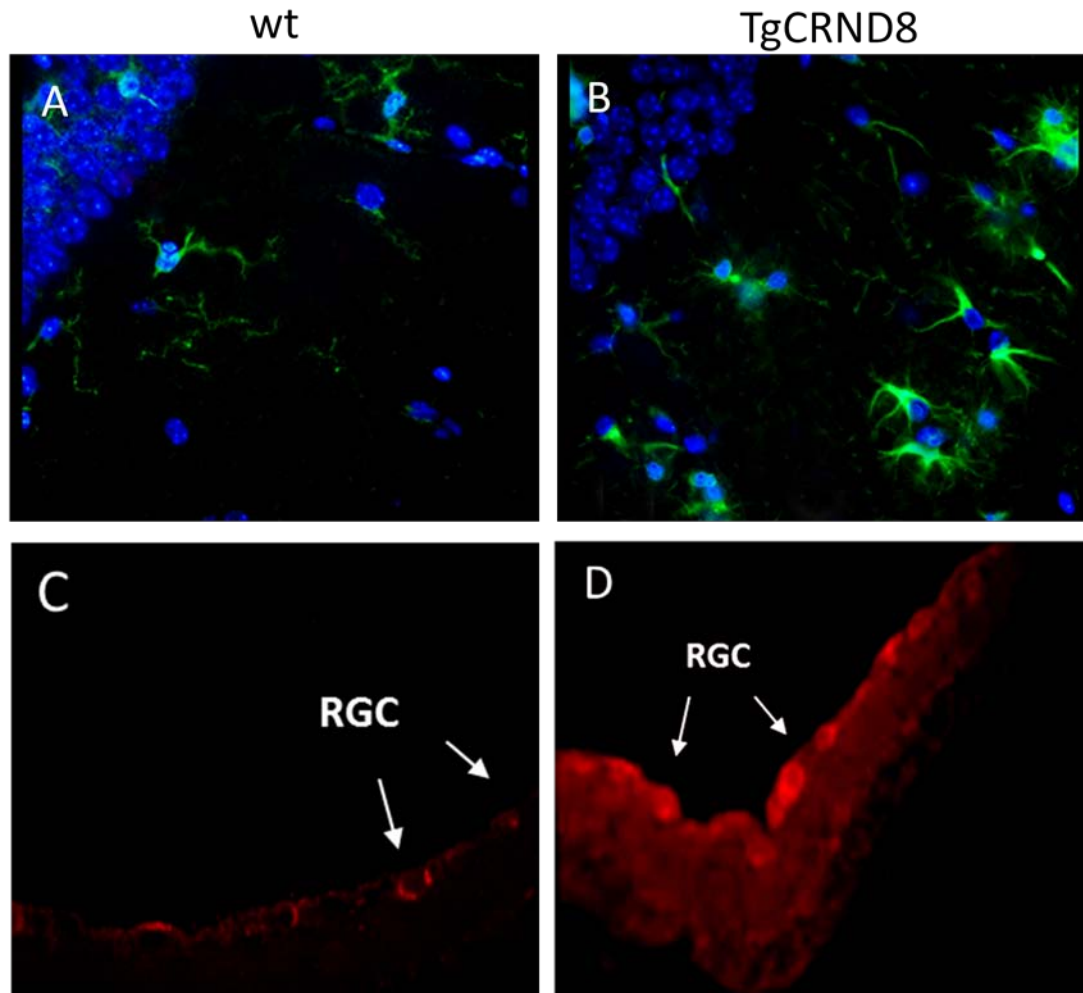


Figure 4.

Microglia (green) activation in the hippocampus CA1 layer is parallel with increased CLIC1 expression in the retina of 5 months old TgCRND8 mice. Microglia cells increase in number (upper panel). CLIC1 expression (red) increases in retina of 5 months old TgCRND8 mice.

To check for the optimal concentration of A β able to exert a response in RGCs, we made a dose-response curve of A β concentration versus membrane potential, shown on the bottom panel of figure 5. In a first set of experiments, we acutely stimulated RGCs with increasing

concentrations of A β ranging from 1 nM up to 100 nM while continuously measuring the membrane potential.

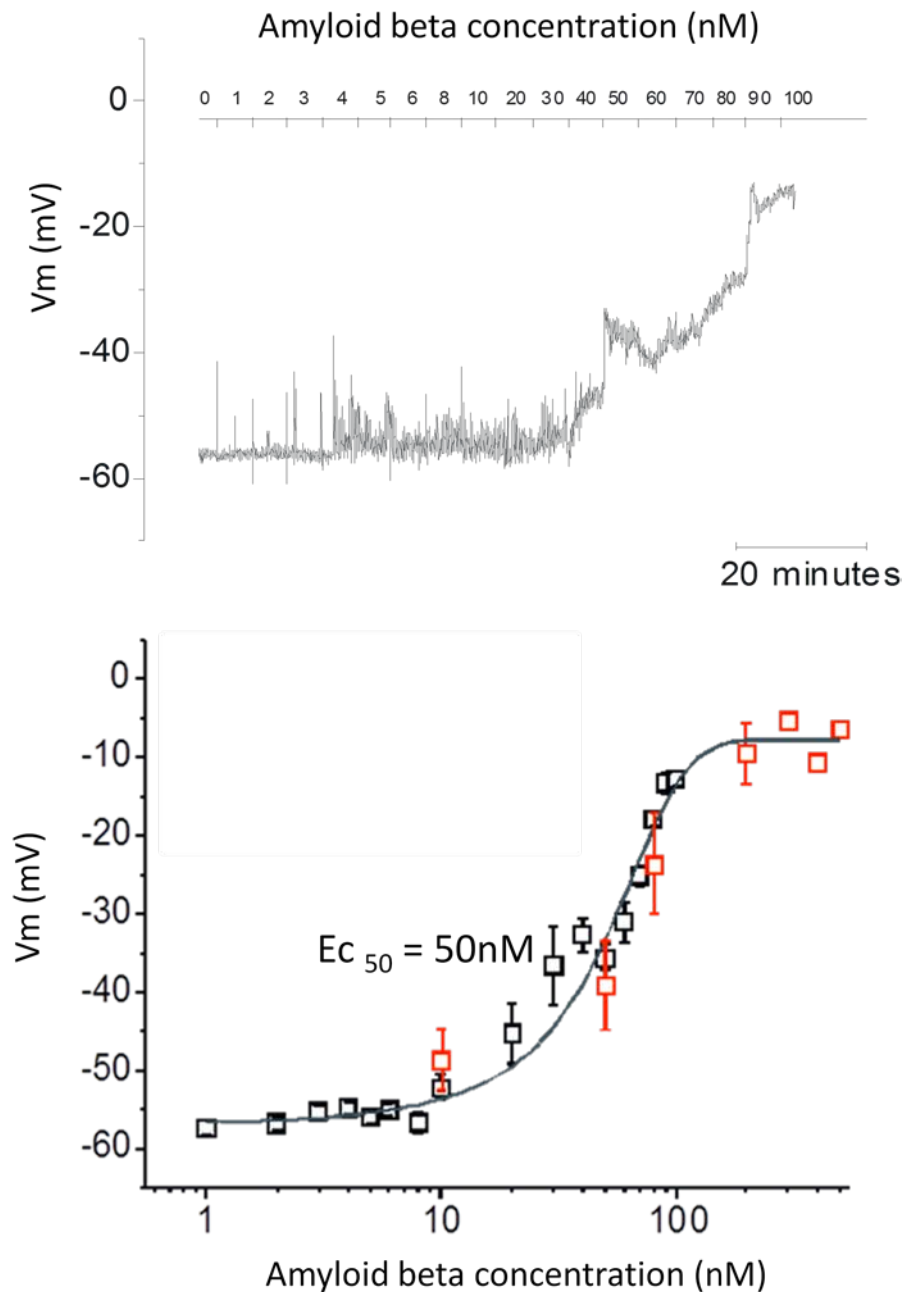


Figure 5.

In the upper panel the membrane potential recordings of a 7 DIV RGC upon sequential stimulation with growing concentrations of A β , from 1nM up to 100nM, as indicated in the x-axis. In the bottom panel the dose response curve of membrane potential obtained by sequentially applying different A β concentrations (black squares). The red circles refer to the average resting membrane potential of 5 cells per each point, incubated for 24 hours with the different A β concentrations indicated.

The membrane potential (on the y-axis) recorded at the corresponding A β concentration (on the x-axis) exerting a depolarization is reported as the black squares.

In a different set of experiments, we incubated RGCs for 24 hours with different A β concentrations (reported on the x-axis) and measured the membrane potential of 5 cells in the dish. The average values of 5 cells chronically treated with A β is reported in the graph as red squares. From the comparison between the values obtained in the acute treatment and in the chronic treatment, we can see a comparable effect of A β stimulation in the two experimental conditions. However, while 100 nM is the maximum concentration tolerated by the cell in the acute treatment, after which the seal is lost, in chronic treatment we were able to stimulate the cells with greater A β concentrations, up to 400 nM. However, we did not see further changes in the membrane potential.

Once we had checked for A β direct effect on isolated RGCs upon A β stimulation, in an environment that is free of glial contribution, we investigated A β stimulation effect on CLIC1 in isolated RGCs, in the same conditions in which we measured membrane potential changes. Figure 5 shows CLIC1 staining in dissociated mouse RGCs. In the bottom panel the staining shows CLIC1 expression after 24 hours stimulation with low concentrations of A β (100 nM), while on the upper panel we see CLIC1 expression (red) in vehicle-treated cells. However, differently from what we saw in whole retinas, in these conditions the increased CLIC1 expression is not as clear as we saw in the whole retina tissue.

The observed differences were quite unexpected. However, we need to consider two main facets: first of all that the effects of A β on CLIC1 expression in RGCs in the whole retina can be the result of both the direct effect of A β on RGCs and the additional effect of A β -activated glia.

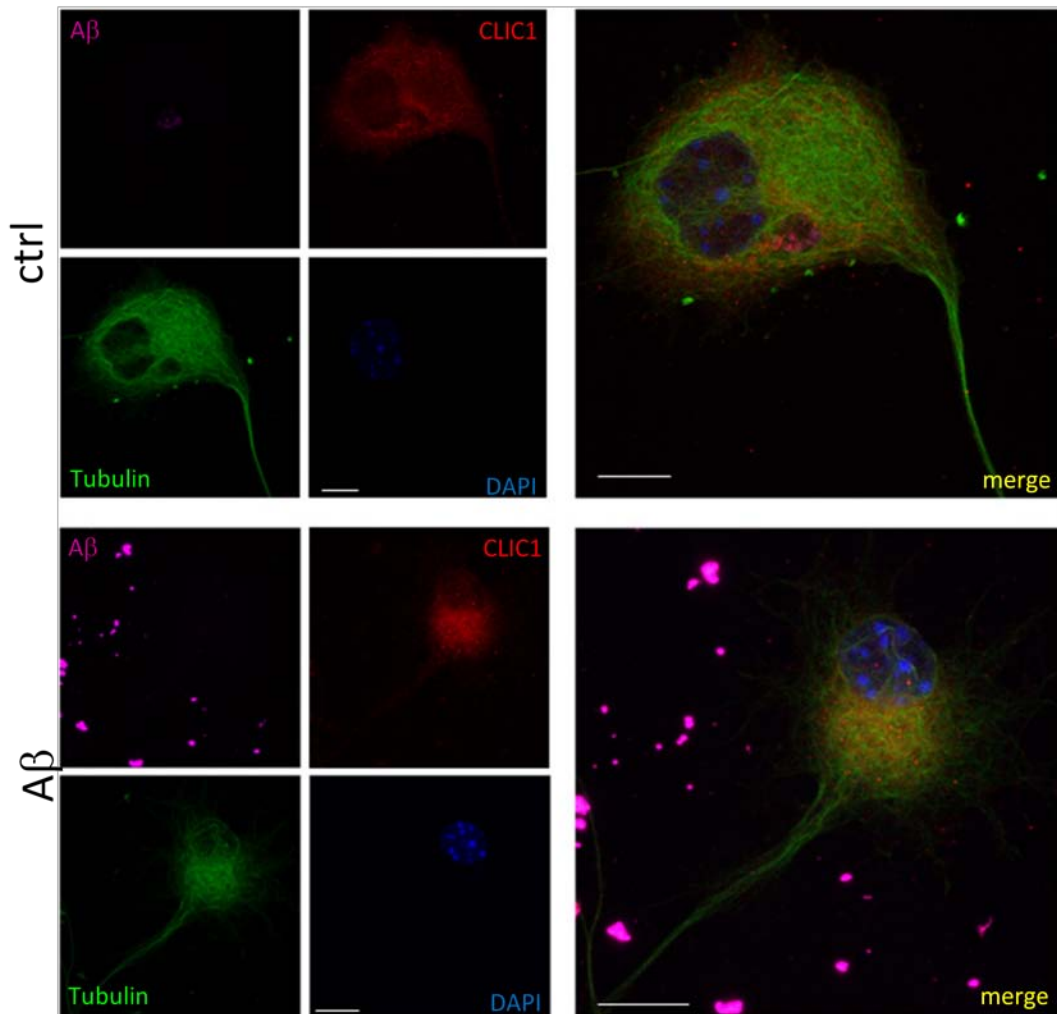


Figure 6.

Immunocytochemistry on dissociated RGCs. A β oligomers (100nM; bottom panel) or vehicle (upper panel) were added to RGCs for 48h. CLIC1 is stained in red, A β in magenta, tubulin in green and nuclei in blue (DAPI). E and L represent the merge of images shown in the left small panels. Scale bars 10 μ m.

The second important point to consider is that while the increased CLIC1 expression was seen in whole adult retinas (3 months old mice and older), A β stimulation of cultured RGCs was performed on young (P1 mice-derived retinas) RGCs. The age of RGCs can “make the difference” in the sensitivity of RGCs to A β .

We hypothesized that the changes in CLIC1 expression that we noticed in adult RGCs of AD mice, might be linked to CLIC1 role in RGCs physiology, thus we proceeded in further investigating this mechanism in “young” isolated RGCs.

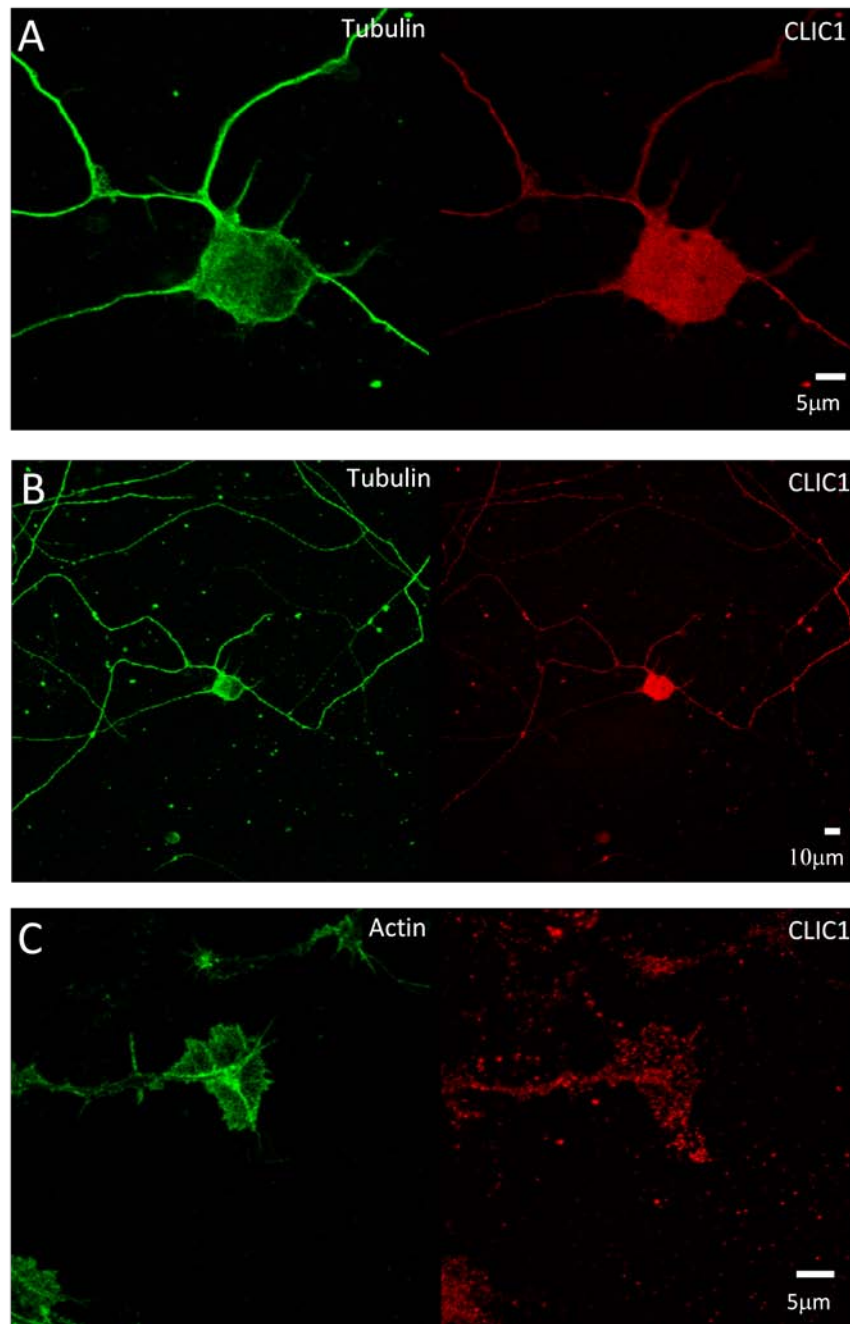


Figure 7.

CLIC1 expression in isolated RGCs. Tubulin (green) and CLIC1 (red) staining in cell body and neurites of RGCs (A and B). In C Actin (green) and CLIC1 (red) staining shows CLIC1 distribution throughout the growth cones of RGCs.

As we have shown in figure 6, CLIC1 is expressed in the cell body of isolated RGCs. We analyzed in more detail CLIC1 expression in cultured RGCs.

We isolated RGCs from retina tissue of postnatal day 1 (P1) mice. Figure 7 shows 3 (DIV) RGCs from P1 mice. The cell is labeled with tubulin (green, A and B) and the growth cones (C) are labeled with phalloidin (green), in order to visualize the complex structure of the F-actin cytoskeleton of the growth cone. CLIC1 staining, in red, localizes CLIC1 in the cell body, along the neurites and in the growth cones.

As we observed a strong expression of CLIC1 at the growth cones of RGCs, we wondered whether CLIC1 can be somehow involved in axonal outgrowth.

6.2 CLIC1 in axon outgrowth of retinal ganglion cells.

When progenitor cells in the retina stop duplicating and exit the cell cycle, they migrate towards their final destination site. During migration they undergo a maturation process and their neurites differentiate either into dendrites or axons. In response to factors released by the basal lamina, they send out a basal process that will drive the migration and in the end will become the axon, and an apical process that will become the dendrite. Even though factors from the basal lamina are very important in this mechanism, many other intrinsic factors are crucial, such as neuronal excitability, ion channels, and it has been demonstrated the key role of cytoskeleton dynamics at the growth cones in this process (Polleux and Snider, 2010).

Growth cones are rich in actin and previous studies have demonstrated the effect of F-actin on the activity of CLIC1 ion channel (Singh et al., 2007). Moreover, many studies have shown the interaction of other members of CLIC family with actin in kidney and brain (Berryman et al., 2004; Suginta et al., 2001).

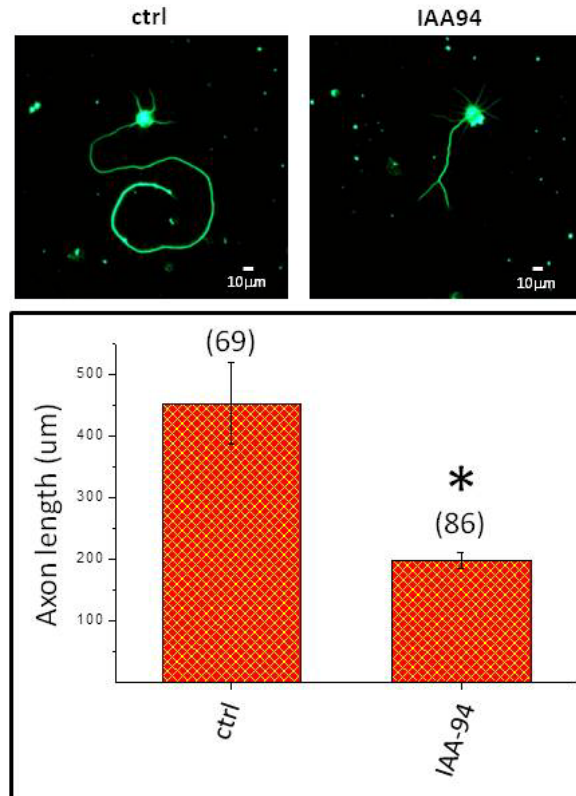


Figure 8.

Quantification of axon length in vehicle-treated (ctrl) and IAA94-treated cells. RGCs in control conditions (upper left panel) and upon IAA94 treatment (upper right panel). In the bottom panel is shown the histogram depicting the average axon length of RGCs neurites from 3 different experiments. The number of neurons analyzed is indicated on the top of each column. Data are presented as mean values \pm s.e.m. Student t-test $p < 0.05$.

We isolated RGCs from P1 mice and after 30 minutes from plating they were treated with 50 μ M IAA94, which is a widely used inhibitor of CLIC1-mediated current (Novarino et al., 2004). After 24 hours we measured the axon length. On the upper left panel of Figure 8 there is an example of a control cell after 24 hours from plating, while on the upper right panel is shown a cell after IAA94 treatment. From the images it is noticeable a marked reduction of the axonal length after IAA94 treatment. The histogram (bottom panel) depicts the axon length quantification of one representative experiment. We can see that upon IAA94 treatment there is a 60% reduction in axon length (t-student test, $p < 0.05$).

In order to investigate the specific involvement of CLIC1-mediated current, we incubated P1 mice-derived RGCs with an antibody specifically directed against the N-terminus domain of CLIC1. Previous studies have demonstrated that, once inserted into the plasma membrane, CLIC1 exposes its N-terminal domain on the extracellular side (Tonini et al., 2000). The binding of this antibody to CLIC1 causes a reduction of CLIC1-mediated current (Milton et al., 2008). As shown in figure 9, 24 hours incubation of RGCs with the N-terminus directed antibody (Ab) caused a significant reduction of axonal length compared to the control. This reduction was comparable to that seen upon IAA94 treatment.

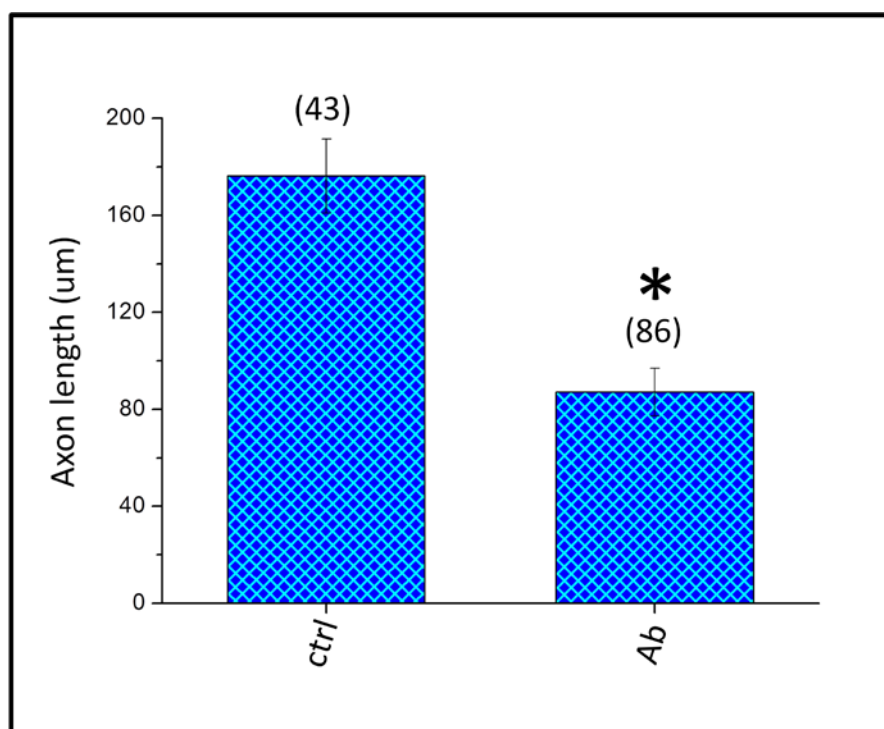


Figure 9.

Axon length quantification of N-terminus directed antibody (Ab) treated RGCs. The histogram depicts the average axon length of RGCs neurites from 3 different experiments. The number of axons analyzed is indicated on the top of each column. Data are presented as mean values \pm s.e.m. Student t-test $p < 0.05$. Student t-test $p < 0.05$.

These results suggest CLIC1 involvement in axon elongation of RGCs. Previous studies performed in microglia cells stimulated by A β demonstrated that CLIC1 mediated current is

strictly correlated to a DIDS-sensitive component (Milton et al., 2008;Novarino et al., 2004). DIDS is a potent inhibitor of chloride channels resident in membrane. The genetic identity of DIDS-sensitive channels is not clear. They are mostly identified for their electrophysiological properties, as a voltage dependent, outward rectifying current (Hwang et al., 2000;Jentsch et al., 2002). A β stimulation of microglia cells induces a IAA94-sensitive current, which is promptly followed by a DIDS-sensitive component. The activation of the DIDS-sensitive component seems to be triggered by the activation of the IAA94-sensitive component. Indeed, stimulation by A β of microglia cells transfected with RNA interference specifically directed against CLIC1, elicits an almost null IAA94 sensitive current, due to the remaining endogenous CLIC1, and a very small DIDS-sensitive component (Milton et al., 2008). Thus we tested the involvement of the DIDS sensitive current in axon elongation of RGCs.

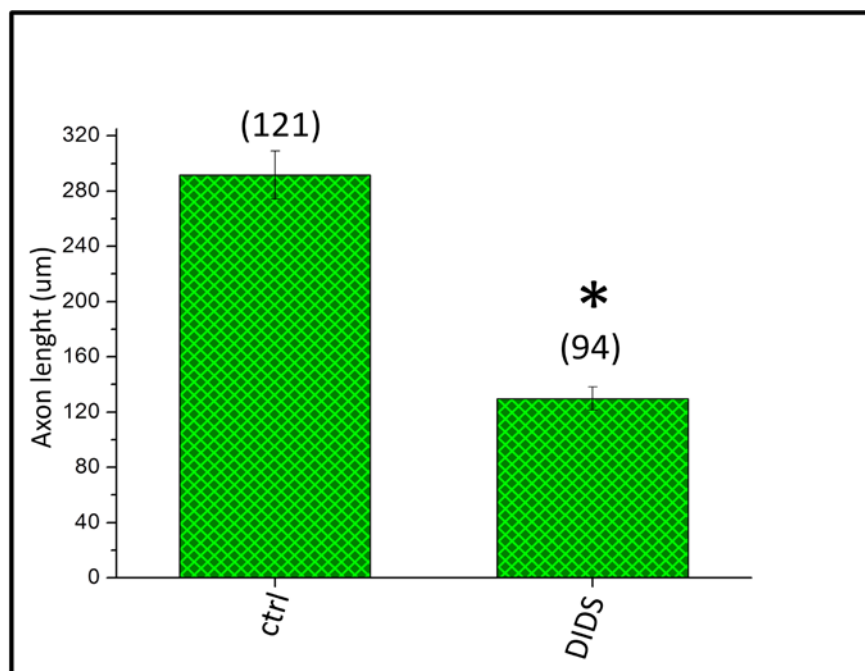


Figure 10.

Contribution of the DIDS-sensitive current in RGCs axon outgrowth. The histogram depicts the average axon length of RGCs neurites from 3 different experiments. The number of axons analyzed is indicated on the top of each column. Data are presented as mean values \pm s.e.m. Student t-test $p < 0.05$.

The treatment of our cells with DIDS caused a marked reduction in axon length after 24 hours of treatment, compared to the vehicle-treated (control) cells, as it is shown in figure 10. Although further experiments are needed in order to establish the precise link between the DIDS-sensitive and the IAA94-sensitive current, to demonstrate the effective link of these two components in axon outgrowth, it is reasonable to hypothesize a possible interaction of these two currents in axon elongation of RGCs, similarly to microglia (Milton et al., 2008).

As the growth cone is the most involved structure in axon elongation (Geraldo and Gordon-Weeks, 2009), we investigated whether any alteration of its morphology was associated with reduced axon outgrowth.

We incubated RGCs with 50 μ M IAA94 and after 72 hours we analyzed the growth cones morphology. Figure 11 shows an example of growth cone of a RGC. In control conditions (A), it is clearly visible the classical fan-like morphology of a growth cone, with the tubulin rich area (green) surrounded by the actin rich area

(magenta). However, upon IAA94 treatment (B), the growth cone structure appears strongly altered, actin seemed “collapsed” on tubulin. CLIC1 localization (red) seemed to follow the actin distribution.

Actin and tubulin occupy two completely different regions of the growth cone, thus usually there is a very low percentage of actin/tubulin co-localization. We thus quantified the degree of tubulin/actin co-localization in IAA94-treated cells compared to vehicle-treated cells. The histogram in figure 12 shows the quantification of actin/tubulin co-localization. In IAA94-treated cells actin/tubulin co-localization is much higher than in control conditions, indicating a strong altered growth cone morphology.

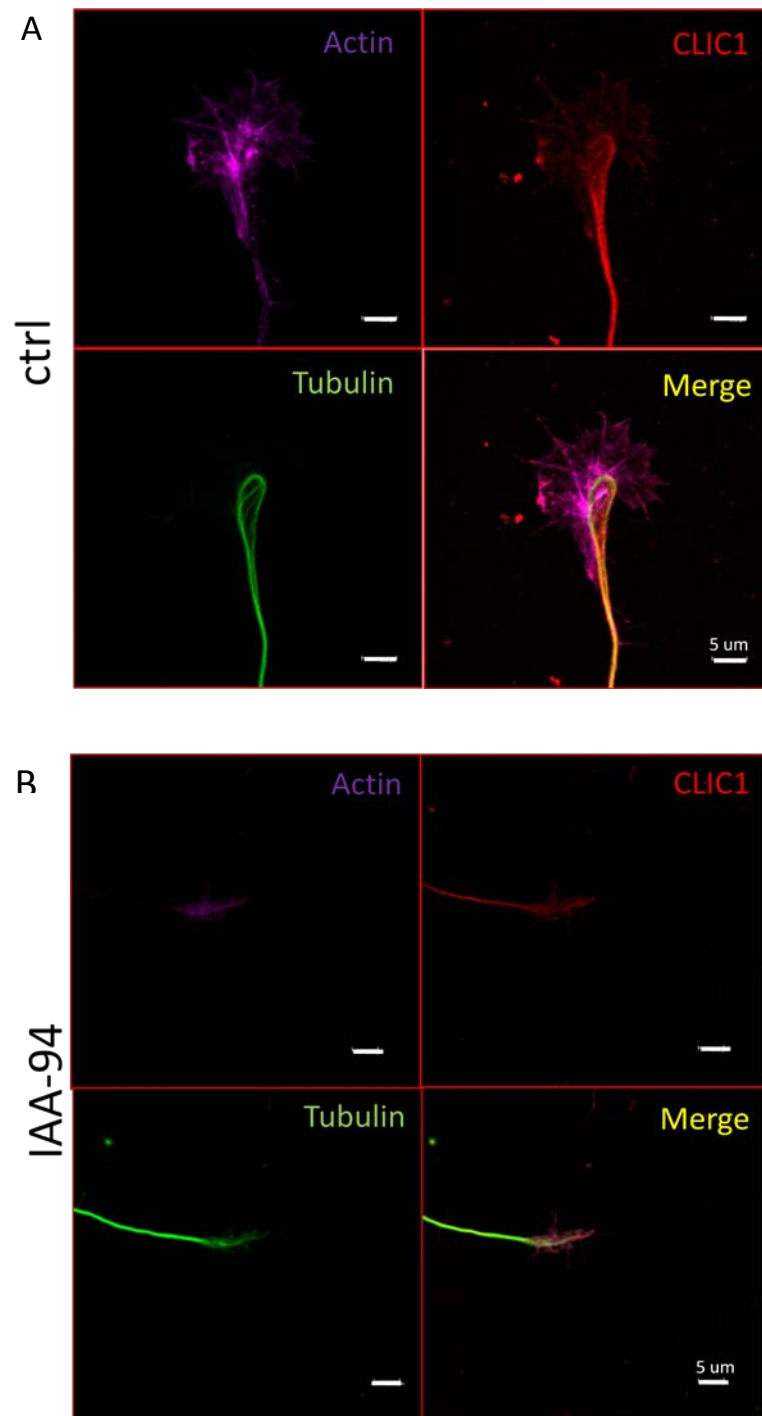


Figure 11.

Morphology of 4 DIV RGCs growth cones. Axon growth cones of RGCs in control conditions (A) and upon IAA94 treatment (B). Co-staining of Actin (magenta), CLIC1 (red), tubulin (green).

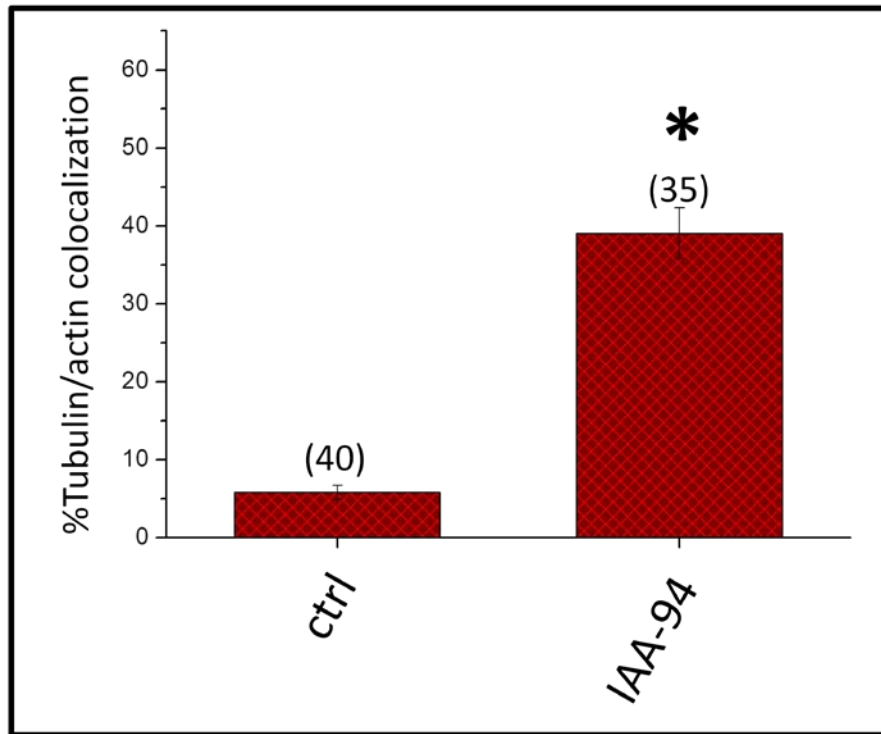


Figure 12.

Quantification of Tubulin/Actin co-localization. The histogram depicts the percentage of Tubulin/Actin co-localization in control conditions and upon IAA94 treatment. The number of growth cones analyzed is indicated on the top of each column. Data are presented as mean values \pm s.e.m. Student t-test $p < 0.05$.

Moreover, if we consider the surface covered by actin and the surface covered by tubulin at the growth cone and we make a ratio between the two values, in control condition the ratio actin-covered surface (AcS) and tubulin-covered surface (TcS) is higher than 1 (Figure 13) because AcS is larger than TcS. On the other hand, in IAA94 treated cells the ratio AcS/TcS is almost 1, supporting the idea of an altered growth cone morphology.

The different CLIC1 distribution throughout the growth cone between control and IAA94-treated cells, prompted us to analyze CLIC1/actin co-localization in vehicle-treated cells and in growth cones of IAA94-treated cells. IAA94 treatment caused a specific drastic reduction of CLIC1/actin co-localization (Figure 14) compared to the control, whereas, as it is shown on the right panel, IAA94 treatment did not affect CLIC1/tubulin co-localization.

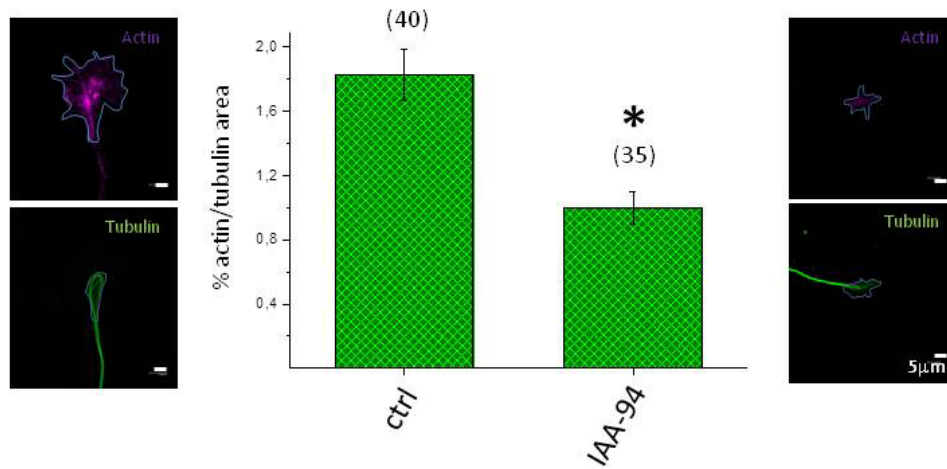


Figure 13.

Quantification of altered growth cones morphology. The histogram depicts the average ratio between AcS and TcS in control conditions and upon IAA94 treatment. The data are presented as mean values \pm s.e.m. Student t-test $p < 0.05$. The left and the right panel schematically show the analysis performed (see methods for details) on two growth cones, in control conditions (on the left) and upon IAA94 treatment (right). The number of growth cones analyzed is indicated on the top of each column.

The treatment of the cells with IAA94 not only impaired axonal outgrowth, resulting in a reduced axon length and altered growth cone morphology, but it also caused an alteration of CLIC1/actin co-localization.

In order to test the possibility of a direct physical interaction between CLIC1 and actin, we used a biochemical approach.

CLIC family members have a high degree of homology, so first of all we tested the specificity of our antibody, using two different cell lines: HEK293T (human embryonic kidney) and N2a cells. HEK 293T are a kidney-derived cell line, while N2a are a murine neuroblastoma cell line. Panel A of Figure 15 shows a western blot (WB) of HEK293T cells and N2a cells transfected with pIRES2-EGFP.

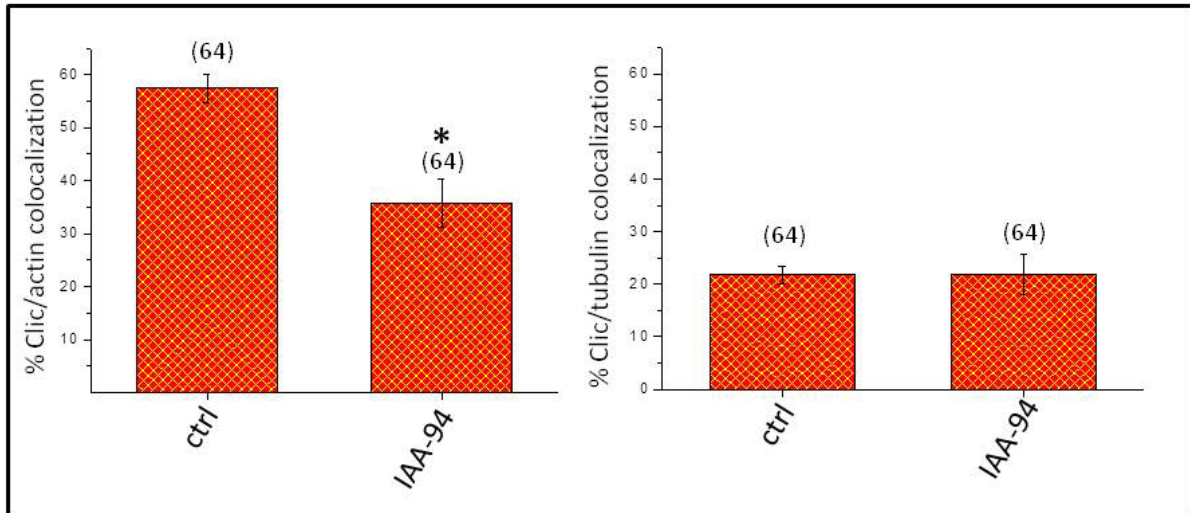


Figure 14.

Quantification of CLIC1/actin and CLIC1/tubulin co-localization. The percentage of CLIC1 co-localization with actin (left panel) and with tubulin (right panel) in control conditions and in IAA94 treated cells. The number of growth cones analyzed is indicated on the top of each column. The data are presented as mean values \pm s.e.m. Student t-test $p < 0.05$.

pIRES2-EGFP is a bicistronic plasmid that allows the translation of two separate proteins: EGFP and CLIC1 carrying a 8-aminoacid flag at the N-terminal domain. In cell lysates belonging to untransfected cells (either N2a or HEK293T cells) we can see a weak band around 31 KDa corresponding to the endogenous CLIC1, while in transfected cells we see a higher band, corresponding to the overexpressed CLIC1-flag protein. On the right panel we see HEK293T cells transfected with pEGFP-CLIC1 that allows to express a fusion protein CLIC1-EGFP. In transfected cells we see a band around 54 KDa, corresponding to the fusion protein CLIC1-EGFP, that is missing in untransfected cells.

We then performed a biochemical assay in order to check for CLIC1/actin interaction. N2a cells have been treated with cytochalasin B (CytoB). This compound has been shown to interact with the actin cytoskeleton, causing a depolymerization of F-actin and shifting the ratio G-/F-actin towards G-actin (Dubinsky et al., 1999).

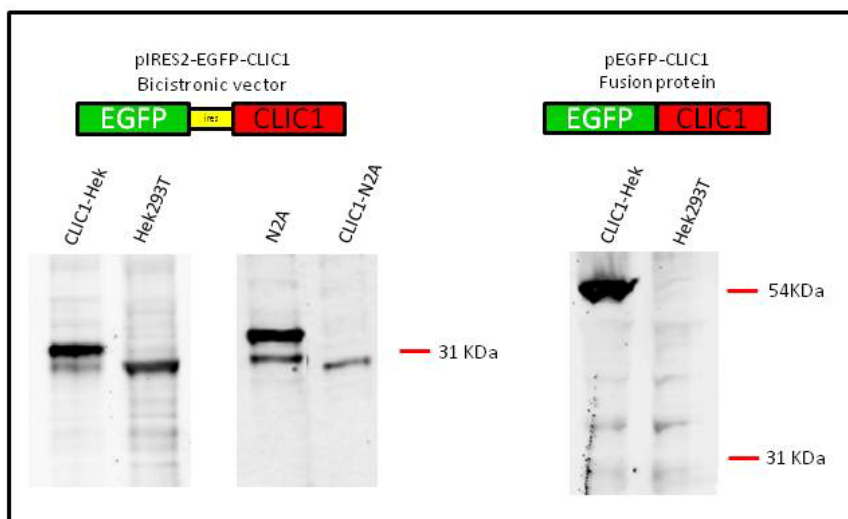


Figure 15.

Specificity of CLIC1 antibody. (A) WB of Hek293T (on the left) and N2a (on the right) cells transfected with piRES2-EGFP-CLIC1, immunodecorated with anti-CLIC1 antibody. (B) WB of Hek293T cells transfected with pEGFP-CLIC1, immunodecorated with anti-CLIC1 antibody.

As it is shown in figure 16, N2a cells have been treated with CytoB for 60, 90 and 120 minutes. After each incubation time, the soluble fraction was separated from the insoluble fraction. Soluble fraction is enriched in cytoplasmic proteins, including G-actin. On the other hand, the insoluble fraction contains F-actin and all the cytoplasmic proteins bound to F-actin. If CLIC1 interacts with F-actin we would find it in the insoluble fraction, together with F-actin. More importantly, in CytoB-treated cells where the F-/G-actin ratio is altered, we expected a different CLIC1 distribution between the two fractions compared to the control.

In the upper panel of the blot in figure 16, it is shown the distribution of actin between the two fractions in all the samples, as a control of the effectiveness of CytoB treatment. While in control cells the percentage of F- and G-actin was approximately the same, in CytoB-treated cells G-actin (in the soluble fraction) was much more abundant than F-actin (insoluble fraction).

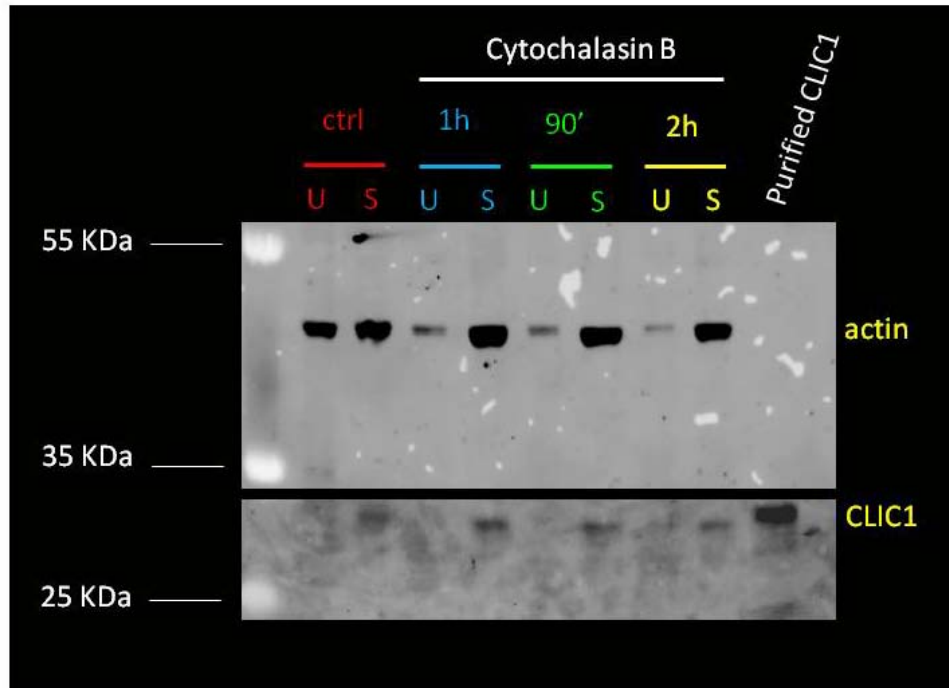


Figure 16.

WB analysis of cytochalasin treated N2a cells. Soluble (S) and insoluble (U) fractions of lysates from N2a cells in control conditions and after cytochalasin B treatment for the different times indicated. In the upper panel the blot is immunodecorated with anti-actin antibody while the bottom panel is immunodecorated with anti-CLIC1 antibody.

In the bottom panel, we see CLIC1 distribution in the different fractions. CLIC1 was not present in the insoluble fraction. Moreover, CLIC1 presence in the soluble fraction did not change significantly following the alteration of G-/F-actin ratio due to CytoB treatment.

Thus, even though immunocytochemistry experiments showed CLIC1 colocalization with F-actin, these results suggest that CLIC1 and F-actin may not directly interact with each others.

Membrane depolarization, by increasing neuronal excitability, has a key role in axon outgrowth. The depolarization that favors axon elongation is accompanied and caused itself by an increase of intracellular cyclic AMP (cAMP) (Goldberg et al., 2002).

Our cells are cultured in a Forskolin-containing medium (see methods for details). Forskolin enhances the axon outgrowth, by increasing cAMP and causing neuronal depolarization.

In order to verify a possible regulative role of cAMP on CLIC1-mediated axon elongation, we measured axon length in IAA94-treated cells cultured in the absence of Forskolin.

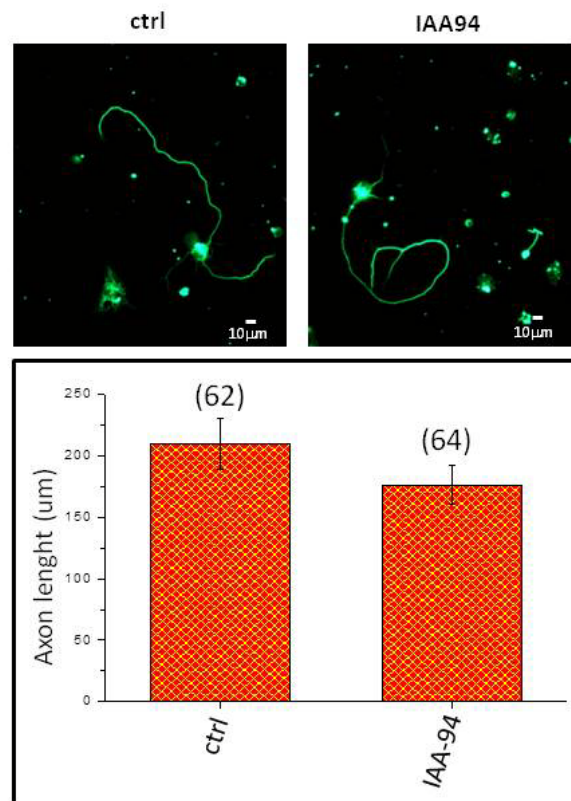


Figure 17.

Quantification of axon length in RGCs cultured in Forskolin-free medium. The histogram depicts the average axon length of RGCs neurites from 3 different experiments. The number of axons analyzed is indicated on the top of each column. Data are presented as mean values \pm s.e.m..

Figure 17 shows the results of this analysis. In this conditions, IAA94 treatment failed to induce any effect on axon outgrowth of RGCs. Although we can see a similar trend in the axon length reduction, the difference is not significant. These results suggest that probably either the depolarization or the direct effect of cAMP on the ion channel affects axon outgrowth.

Chapter 7

Discussion

In 1997 Breit and colleagues were able to successfully clone CLIC1 protein from a macrophage cell line (Valenzuela et al., 1997). Since then, different groups all over the world focused their attention on the biochemical and biophysical properties of this protein. CLIC1 unusual behavior as a soluble protein and as a membrane-inserted protein has attracted and fascinated many scientists (Averaimo et al., 2010).

Although many concerns have been raised on the real possibility of this small protein to actually constitute *per se* a functional ion channel once inserted into the plasma membrane, growing evidences support the “ion channel hypothesis” (Novarino et al., 2004;Singh and Ashley, 2006;Tonini et al., 2000;Tulk et al., 2002;Valenzuela et al., 1997;Valenzuela et al., 2000;Warton et al., 2002).

Even though many studies have been performed in order to unravel its biochemical and biophysical properties, its physiological role has been only recently elucidated.

CLIC1 regulation by oxidative conditions (Singh and Ashley, 2006;Tulk et al., 2002;Warton et al., 2002) has led to suppose its involvement in many different cellular processes involving oxidation, such as cell proliferation, differentiation and migration (Menon and Goswami, 2007). However, the most provocative physiological role has been postulated only recently, in A β -stimulated microglia cells in Alzheimer disease (AD), where it resulted to be modulated and in turn having a crucial role in reactive oxygen species (ROS) production by the NADPH oxidase (Milton et al., 2008;Novarino et al., 2004).

Microglia cells represent the immune system of the central nervous system (CNS) (Block et al., 2007). The retina is part of the CNS and, given that many studies report an inflammation of this tissue in AD (Berisha et al., 2007), we expected to find CLIC1 expressed and functional also in the immune system cells of the retina.

However, surprisingly, CLIC1 was not expressed in retinal microglia, whereas our staining of mouse retina sections showed its localization in the ganglionic layer, where cell bodies of retinal ganglion cells (RGCs) are localized (Figure 1). The images showed CLIC1 expression also in the inner plexiform layer (IPL), where dendrites of RGCs lay and make synapses with bipolar neurons, and in the outer plexiform layer (OPL) where we find the synapses between bipolar neurons and photoreceptors, suggesting that CLIC1 expression is not limited to the cell bodies, but it can be diffused throughout the cell, including the neurites. This hypothesis was confirmed by immunocytochemical analysis on dissociated RGCs, showing CLIC1 expression all along the neurites.

Even though immunohistochemistry experiments showed CLIC1 expression also in bipolar neurons, CLIC1 expression in the RGCs appeared us immediately quite interesting. RGCs are indeed the cells whose axons gather together making up the optic nerve, and many studies report that AD patients often complain visual deficits before the cognitive symptoms (Berisha et al., 2007). The visual impairment has been imputed to RGCs degeneration rather than brain neurodegeneration in the visual cortex (Berisha et al., 2007;Guo et al., 2010), resulting in the thinning of the nerve fiber layer. Furthermore, LaFerla and colleagues have demonstrated the presence of A β deposits throughout the retina layers in the transgenic AD mouse model (Normando et al., 2009), and we have shown a cell depolarization in A β -stimulated cultured RGCs (Figure 5), testifying the toxic effect of this peptide on neuronal physiology. Thus, due to the known effects of A β on CLIC1 physiology, we wondered if in retinal neurons A β might have an indirect effect on CLIC1, as it happened in brain microglia (Milton et al., 2008;Novarino et al., 2004).

Our experiments clearly showed an increased CLIC1 expression in AD mouse models, both in the 3xTg mice and in the TgCRND8 mice. It is important to keep in mind that TgCRND8 mice show alterations of the retina structure. Indeed, as it is noticeable in TgCRND8 mice retinas in figure 2, we could not see the photoreceptor layer. TgCRND8 mice, indeed, belong to the CRND8 strain, that often show photoreceptor degeneration (Garcia et al., 2004;Normando et al., 2009).

However, we can be quite confident of our results for two reasons: first of all due to their reproducibility in a different AD model, the 3xTg. Moreover, both mouse models analyzed have been widely used in the biomedical research on AD and have been carefully characterized, showing A β deposits both in the brain and in the eye, and evident cognitive AD symptoms, (Fiorentini et al., 2010;Normando et al., 2009).

The most interesting facet to highlight is that the increased CLIC1 expression was clearly visible in both models before the first cognitive symptoms. In TgCRND8 mice the disease onset is around 3 months of age (Chishti et al., 2001;Fiorentini et al., 2010), and around 6 months in the 3xTg mice (Oddo et al., 2003). CLIC1 expression appeared slightly increased in the gangliar layer already in 1 month old mouse retinas in TgCRND8 and became spread throughout the IPL at 5 month of age, when there are the first symptoms of cognitive impairment. The lectin staining aiming at label microglia cells in the retina, failed to label activated microglia in the retina, suggesting that maybe the insult represented by A β deposits that have been shown in AD mouse retinas is not sufficient to activate microglia cells, most likely due to the main role of Muller cells as immune system cells in the retina. In 3xTg mice, CLIC1 expression increased already in 5 months old mice, and became more evident at 10 months, when it appeared also diffused throughout the other retina layers. In 3xTg mice there was a slight increase of CLIC1 expression also in wt mice, even though it was much more significant in AD mice. This might be due to a subtle inflammation linked to ageing, that is much stronger and much more prominent in AD mice compared to the wt mice.

Therefore, these experiments propose CLIC1 as an early marker of neurodegeneration in AD. We have a protein whose expression is increased in the retina, the most accessible part of the CNS, before the manifestation of cognitive impairment, which means before that the neurodegenerative process is spread enough to severely impair the basal brain functioning.

The research field in AD has recently shifted towards the identification of early markers of this disease. Indeed, one of the main problems in AD treatment is due to a late diagnosis that reduces the probabilities of a successful therapy. AD is usually diagnosed only after a profound cognitive deficit, when neuronal death is already largely diffused, rendering the actual treatments nearly ineffective and hampering any possibility to reverse the neurodegenerative process.

Cordeiro's lab recently developed a technique that allows the visualization of RGCs apoptosis *in vivo*, by visualizing fluorescently labeled dying cells (Cordeiro et al., 2010;Guo et al., 2010). This technique might be an useful tool to transiently visualize fluorescently labeled-CLIC1 in AD patients, in order to quantify its expression in AD retina and perform an easy and early screening.

Although we were able to clearly observe an increased expression of CLIC1 in AD mouse models, and we saw a direct effect of A β stimulation on the membrane potential of cultured RGCs, we were not able to detect a clear increased CLIC1 expression in isolated RGCs.

We might explain this effect by considering the possibility of an additional contribution of Muller cells activation, that several studies have demonstrated in rat retinas of AD mouse models (Howlett et al., 2011). Muller cells activation, by releasing neurotoxic factors, might exacerbate a pathological state induced by A β , causing increased CLIC1 expression and RGCs death. Of course, this effect cannot be seen in pure dissociated RGC cultures.

Furthermore, we need to consider that isolated RGCs belong to young pups, where the majority of RGCs are in the development stage (Wallace, 2011), while retina staining are performed on adult mice where RGCs are already completely mature and inserted in the complex retina circuitry.

Thus, the “cell age” can be a key point to explain the observed differences.

According to this idea, we made a hypothesis that led our successive studies. A recent report proposes a new hypothesis for neurodegeneration in AD. It has been reported that degenerating neurons have increased expression of proteins specifically linked to the cell cycle, such as cyclins. These data led to hypothesize that the “insult” represented by A β can force the neuron to re-enter the cell cycle. However, a highly differentiated cell could not be able to successfully accomplish a task normally attributable to immature cells, ending up in starting an apoptosis program (Copani et al., 1999).

Thus, if we consider the hypothesis of neurodegeneration induced by A β as a result of the abnormal activation in “mature” cells of a pathway that is typical of immature cells, we can speculate that CLIC1 can be part of this pathway, that in the end results in cell death in A β -stimulated “mature” neurons. However, the activation of this pathway would be physiological and “successful” in immature cells. Thus, we hypothesized a “double” role of CLIC1, not only in RGCs pathology, but also in RGCs physiology, and we proceeded by investigating its role in RGCs physiology.

Unfortunately, due to technical limitations we were not able to register CLIC1 mediated current in isolated RGCs. Indeed, after few hours after plating, RGCs send out neuritis that quickly become very long. Although this did not represent a problem for membrane potential recordings, it prevented us from measuring ion current, due to the incapability of successfully clamping the voltage in order to measure the current, on such a large surface (Armstrong and Gilly, 1992).

Thus, in order to analyze CLIC1 functional expression in RGCs, we took advantage of an *escamotage*, by inhibiting CLIC1-mediated current with IAA94 and analyzing the subsequent “macroscopic” effects on maturing cells.

We focused on axon elongation for two main reasons. First of all, while at the beginning CLIC1 was identified in transfected cells mainly in the nucleoplasm and in the nuclear membrane, later, it

has been demonstrated that its subcellular localization varies strongly amongst the different cell types. In non-polarized cells it appears diffused throughout the cytoplasm, whereas in polarized cells its localization results to be also polarized (Ulmasov et al., 2007). Like all neurons, RGCs are highly polarized cells. RGCs progenitors, in the early postnatal phases, already have an apico-basal orientation which is maintained during the neuronal maturation process. Once exited the cell cycle, they send out a leading process that guides the migration from the retinal pigmented epithelium towards the vitreal surface (Barnes and Polleux, 2009). The leading process responds to different external cues in the maturation process. Growth cones are the most distal part of the growing axon, belonging to the basal region of the progenitor cell. During migration they encounter many different environmental cues, they interact with different regions of the extracellular matrix, responding by undergoing deep changes in their structure (Dent et al., 2011). Thus, due to the specific CLIC1 subcellular localization in polarized cells, it was likely to hypothesize a role of CLIC1 in the axonal growth or axonal specification. Although it is worth to mention that in the cell line analyzed by Ulmasov and colleagues CLIC1 seems to be mostly expressed in the apical region of the cell, just underneath the plasma membrane, whereas the growth cones belong to the basal region of the progenitor cells differentiating into neurons, we need to keep in mind that Ulmasov analyzed a cell line belonging to the colorectal cancer and the different apical versus basal distribution of CLIC1 can vary amongst the different polarized cell types, according to a different function in the different cell types (Ulmasov et al., 2007).

The second reason concerns our staining on dissociated RGCs that showed a strong CLIC1 expression at the growth cones that, as we mentioned before, guide axon outgrowth, further supporting the idea of CLIC1 role in axon outgrowth of RGCs. Our hypotheses on CLIC1 role in axon outgrowth due to the strong expression in the growth cones is supported by strong additional considerations: growth cones are highly dynamic structures, with a complex and organized cytoskeletal structure, rich in actin (Geraldo and Gordon-Weeks, 2009), and many studies have

demonstrated the interaction of actin with other members of the CLIC family. Biochemical assays demonstrated that CLIC4 indirectly interacts with the actin cytoskeleton (Suginta et al., 2001), and CLIC5 was first identified as a protein associated with the actin cytoskeleton of placental microvilli (Berryman and Bretscher, 2000). Furthermore, *in vitro* studies have shown an interaction of actin with CLIC1, unraveling a contribution in the modulation of the ion channel activity. Indeed, Singh and colleagues showed that in artificial bilayer the F-actin cytoskeleton specifically reduces CLIC1 ion channel activity by interacting from the intracellular side, while the induction of actin depolymerization provides a complete recovery of ion channel functionality (Singh et al., 2007). Although we cannot rule out the possibility of an indirect effect of F-actin on CLIC1 due to membrane deformation, as so far nothing is known about the presence of actin-binding sites in CLIC1, it was likely to hypothesize a direct effect of actin on CLIC1.

Our immunocytochemistry experiments on dissociated RGCs showed that the most part of IAA94-treated growth cones have a deeply altered morphology: they result to be “collapsed”. Active growth cones have a well defined fan-shaped morphology, with a central tubulin-rich area surrounded by an actin-rich area (Geraldo and Gordon-Weeks, 2009). IAA94-treated cells have a high co-localization degree of tubulin and actin. This would be in line with previous data in literature linking a reduced axon outgrowth with collapsed growth cones (Davis et al., 2009). Bradke and Dotti in 1997 demonstrated that an high dynamics of the cytoskeleton at the growth cone is crucial for an efficient axon elongation. Indeed they showed that in hippocampal neurons an increased dynamics of the actin cytoskeleton by locally increasing actin depolymerization could *per se* be sufficient to promote axon differentiation over dendrite differentiation, leading to a fast axon elongation (Bradke and Dotti, 1997). Actin dynamics is important as well as local actin translation at the growth cones (Rossoll et al., 2003). It is possible that CLIC1-mediated current could somehow reduce the actin dynamics causing an aberrant “stabilization” of the actin cytoskeleton, thus resulting in collapsed growth cones and reduced axon outgrowth. Neuron excitability has been

demonstrated to be also very important in axon elongation (Goldberg et al., 2002), most likely resulting in changes in intracellular calcium concentration due to the activation of voltage gated calcium channels. Increased calcium concentration could alter actin filaments structure, by affecting the interaction amongst the different subunits, resulting into an altered actin dynamics and reduced axonal outgrowth. CLIC1-mediated current can be part of this mechanism, by affecting membrane potential and thus neuronal excitability.

The collapsed morphology of the growth cones in IAA94-treated cells is parallel with a significantly reduced co-localization between F-actin, specifically labeled with phalloidin, and CLIC1. This can be also explainable as a consequence of a reduction in F-actin compared to G-actin. The altered ratio between F-actin and G-actin might cause an altered growth cones morphology and can be parallel to a reduced axon growth. We cannot rule out the possibility of a reduced CLIC1/F-actin co-localization due to an increased interaction of CLIC1 with the monomeric G-actin. We can also speculate that an interaction between CLIC1 and G-actin could lead to a sequestration of G-actin, preventing the polymerization into F-actin filaments and thus resulting into reduced axon length due to altered dynamics of actin cytoskeleton at the growth cone.

Based on these results we postulated a direct interaction of CLIC1 with actin. However, our biochemical analysis suggested that the co-localization does not reflect into a physical interaction. Although we expected to see an increased CLIC1 localization in the soluble fraction upon F-actin depolymerization with cytochalasin, we were unable to see CLIC1 re-localization between the two fractions upon cytochalasin treatment. Moreover, we could not see CLIC1 in the insoluble fraction together with F-actin, neither in control conditions, where actin is all polymerized and thus CLIC1 is supposed to interact with it. These results strongly suggest that although there is a clear co-localization between the two proteins, there is not a physical interaction. However, we need to consider that due to technical limitations, our biochemical assay has been performed on N2a cells, which are a neuroblastoma cell line, very different from mature, or developing, neurons. Although

we hypothesized an interaction of CLIC1 and F-actin unrelated to the cell type, we cannot rule out that, mainly considering the unusual nature of CLIC1, this interaction can be transient and thus be present only in very specific times of the cell cycle or the cell differentiation, upon different stimuli. Thus in our specific conditions, CLIC1 may interact with F-actin specifically at the growth cones, maybe transiently, during axon elongation, when CLIC1 needs to be localized in the plasma membrane in order to exert its role as an ion channel and mediate a chloride flux crucial for the cell physiology.

Although we established a role of CLIC1 current in neuronal physiology, we cannot rule out the possibility of an additional role of CLIC1 as a cytoplasmic protein in RGCs physiology and axonal maturation. Our results clearly show that CLIC1 role in axonal elongation seems more strictly linked to its role as an ion channel. Indeed, both IAA94 and the N-terminus directed antibody inhibit the ion channel activity, by reducing CLIC1-mediated chloride current without affecting its possible role as a soluble protein. Nevertheless, other studies need to be performed in order to define an eventual additional “cytoplasmic” role of CLIC1 in this process, by specifically knocking down CLIC1 expression.

Previous studies performed in microglia cells showed a tight link between IAA94-sensitive current and the DIDS-sensitive current. A β stimulation of microglia cells is indeed strictly correlated to the presence of a CLIC1-mediated component. Indeed, in microglia cells in which CLIC1 expression was specifically knocked down by siRNA, A β stimulation not only elicits a very low IAA94-sensitive current, attributable to the low levels of endogenous protein, but it also fails to induce a DIDS-sensitive current (Milton et al., 2008). Thus, CLIC1-mediated current was considered a “trigger” for chloride current mediated by other membrane resident chloride channels, such as the ones mediating the DIDS-sensitive current. Indeed, Jentsch and colleagues showed that many chloride channels are activated by increasing intracellular chloride concentrations, maybe due to the interaction of chloride ions with aminoacidic residues in the ionic pathway (Jentsch et al.,

2002). Thus, the reduced axon outgrowth in DIDS-treated cells was not completely unexpected. However, additional studies are needed in order to define the precise link between these two currents.

Our results on IAA94 inhibition of axon outgrowth is in line with previous data in literature showing increased chloride concentration in immature neurons compared to mature adult neurons. In immature neurons, the low expression of the potassium chloride co-transporter KCC2 and the higher expression of NKCC1, causes a large accumulation of chloride inside the cell. Thus, intracellular chloride concentration results to be increased and, as a consequence, chloride current results to be depolarizing instead of hyperpolarizing, like is typical of mature neurons instead. During development KCC2 expression changes, leading to a reduction in intracellular chloride: chloride current results to be hyperpolarizing (Achilles et al., 2007). Many studies show that depolarization and increased neuronal excitability favor axon elongation (Goldberg et al., 2002). From our studies, and based on the current knowledge on chloride currents in developing neurons, we can argue that CLIC1 mediated current in our experimental conditions, is depolarizing, thus helping in axonal growth. The inhibition of CLIC1 mediated current by IAA94 treatment could cause a hyperpolarization, impairing axonal outgrowth. As we have previously seen in microglia cells, the IAA94-sensitive component could be promptly followed by a DIDS-sensitive component that can contribute in setting the membrane potential, and thus can contribute together with CLIC1 by increasing cell excitability and consequently axonal elongation.

The most intriguing observation derives from the comparison of the experiments performed with cells cultured in forskolin-free medium and in forskolin-containing medium. Indeed, it is well known the effect of forskolin, resulting in increased intracellular cAMP concentration (Seamon et al., 1981). These results suggest a possible regulation of CLIC1 by cAMP. IAA94-induced reduction of axon outgrowth is indeed only significant in Forskolin-stimulated cells, where intracellular cAMP concentration is increased.

Many other ion channels are modulated by cyclic nucleotides. For example, the cardiac HCN, responsible for the pacemaker “funny” current, has been shown to be regulated by cyclic nucleotides that shift their activation threshold respectively towards hyperpolarizing and depolarizing potentials (Wainger et al., 2001). Furthermore, many studies clearly show a regulation of the Ca-activated – K channels by cAMP. In these channels, cAMP increases the open probability of the ion channel (Meyer et al., 2002).

Recent studies have demonstrated that A β stimulation of neuroblastoma cells and neuronal cultures leads to the down regulation of the CREB (cAMP response element-binding protein) pathway, by decreasing CREB phosphorylation (Tong et al., 2001). Moreover, long term potentiation (LTP) deficits in A β -treated slices can be rescued by treating brain slices with inhibitors of cAMP degrading enzymes that results in increased cAMP concentrations (Puzzo et al., 2005). Furthermore, constitutive activation of CREB has been demonstrated in AD mouse models (Muller et al., 2011), suggesting an increased cAMP concentration. Thus, many evidences suggest the involvement of cAMP pathway in AD.

As we discussed previously, our studies have also shown changes in CLIC1 expression in AD mice. Thus, according and further supporting our initial hypothesis, we can suppose that CLIC1 has a role in both mechanisms, in A β -stimulated neurodegeneration and in axon outgrowth, and we can conjecture that this can happen through the same molecular pathway, driven by cAMP. We can speculate that during RGCs neurodegeneration in AD mice the cell initiates a signaling that includes the activation of the molecular pathway leading to axon elongation, by increasing cAMP levels and involving CLIC1, in the end leading to cell death. Increased cAMP, in turn, might cause CLIC1 increased expression and thus neurodegeneration. However, as we said, these are only speculations and these hypotheses need to be demonstrated.

The same mechanism, involving cAMP, that in young RGCs causes axonal elongation could cause death in adult RGCs.

Several observations lead us to consider an interaction and a possible modulation of CLIC1 by cAMP. First of all, Valenzuela and colleagues in 1997, by *in silico* studies on CLIC1 primary structure, suggested the presence of a putative cAMP phosphorylation site at threonine residue 49 (Valenzuela et al., 1997). Moreover, our data in axon outgrowth on cells cultured in forskolin-containing medium compared to the results obtained in cells cultured in forskolin-free medium, strongly suggest a mechanism of CLIC1 modulation by increased cAMP concentration elicited by forskolin stimulation.

Our results can be considered very promising with regards to possible future medical application. Adult RGCs have very low re-growth potential after optic nerve crush (Bischof et al., 2011). In light of our results we can assume that CLIC1 could represent an useful tool in this mechanism, favoring axon re-growth. Many different approaches have been used in the last decade in order to treat many different eye diseases, ranging from glaucoma, optic neuritis, vascular disruption or trauma. Gene therapy is now largely diffused in the treatment of different pathological conditions. For example, recombinant lentiviral, adenoviral or adenovirus-associated vectors have been used to specifically target cells in the inner retina, in particular RGCs. For example, intravitreal injection of viral vectors has been proved to be very effective to introduce growth promoting genes like ciliary neurotrophic factor (CNTF) and increase RGCs survival and regeneration after optic nerve crush (Hellstrom and Harvey, 2011). Although several experiments are needed in order to completely elucidate this mechanism, as we have demonstrated the role of CLIC1 functional expression in axon outgrowth, and assuming that this can be modulated by cAMP as our experiments suggest, we can speculate of a possible gene therapy approach to re-increase CLIC1 expression in these cells and stimulate them with cAMP in order to increase their functional expression.

Moreover, many studies have shown that the CREB pathway, activated by increased cAMP concentrations, is implicated in adult neurogenesis (Merz et al., 2011). This can lead us to speculate about a role of CLIC1 in this mechanism. Although for decades has been postulated that neurogenesis stopped during adulthood, it is now accepted and clearly demonstrated that adult neurogenesis continues throughout life in specific regions of the brain, such as the subventricular zone and the dentate gyrus (Ming and Song, 2011). One of the main problems in this process is that, although many newborn neurons are born every day, only few of them are actually able to make up the right connections and thus survive and correctly integrate into the brain circuitry. Axon outgrowth is a key step in this mechanism, and we can speculate of a possible implication of CLIC1, eventually providing a tool to modulate this process.

The very important point to highlight is that with these data we have shown a direct link between axon outgrowth, that is a crucial step in neuronal development, and CLIC1 functional expression that results easily controlled from the outside. Many ion channels have been used as pharmacological targets due to their accessibility. Indeed, voltage gated sodium channels have been used as pharmacological targets in ameliorating the symptoms of chronic pain (Liu and Wood, 2011). Moreover, specific drugs interacting with ryanodine receptors have been used in clinical trials against heart failure (Wehrens et al., 2005). Finally, more recently, the so called “correctors” of cystic fibrosis transmembrane receptors (CFTR) activity and surface expression have been used in order to interact with the cause of CF (Becq et al., 2011).

On this regard, it is also very important to emphasize that CLIC1 is not a membrane-resident ion channel, but its expression at the plasma membrane level is transient and stimulated by different factors. Several studies suggest an increased CLIC1 expression at the plasma membrane only in stress conditions (Averaimo et al., 2010). In particular we have previously demonstrated its expression at the plasma membrane following oxidative stress in microglia cells; and many other

studies support the hypothesis of CLIC1 insertion into the plasma membrane in proliferative conditions (Tonini et al., 2000).

Given that interfering with the functionality of membrane-resident ion channels might be dangerous for the side effects due to the knock down of physiological functions that can be key to the cellular physiology, we found very interesting considering the possibility of interfering with the function of a channel that is transiently expressed on the plasma membrane only during a specific process, without affecting the basal cell physiology. This seems a promising approach in order to reduce the side effects and to diminish the interference with main cellular processes.

Moreover, our studies strongly suggest that even though it is plausible the presence of an additional cytoplasmic role of CLIC1, the axon outgrowth is strictly linked to CLIC1 expression at the plasma membrane and its functional expression as a chloride-selective ion channel.

Although it would be sure interesting to explore the specific involvement of CLIC1-mediated current over other chloride currents in this process, exploring the link between the DIDS-sensitive component and the possible involvement of other ion channels, such as the NPPB-sensitive chloride current, many studies suggest the involvement of other ion channels in this process (Brackenbury et al., 2010; Greka et al., 2003; Jablonka et al., 2007; Sernagor et al., 2010). Indeed, an important process like axon elongation is sure finely modulated by different factors. However, again we highlight the involvement of CLIC1-mediated current in this mechanism, paying particular attention on the unique feature of being transiently expressed at the plasma membrane.

In light of these observations it would be interesting to analyze a possible direct regulation of cAMP on CLIC1 insertion into the plasma membrane or the direct regulation of CLIC1 electrophysiological properties by cAMP, like it happens in HCN channels, as a tool to modulate CLIC1 functional expression in this process.

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