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Roles of different glial populations in neuronal maturation: implications in Rett Syndrome and Alzheimer's disease

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Table of contents

Abstract

It is well established that in healthy brain, the interplay between neurons and glia play a very important role in the maintenance of a correct physiological activity. Recent studies have shown the existence of an active involvement of glia in both the formation and function of the synapse (Araque et al. 1999; Parpura et al. 2012). These findings have led to reconsider the role of glia also in the case of pathological situations. Indeed, beyond its fundamental implication in healthy brain, glia has been found to play a critical role in several neurological disorders.

It is now clear (Nguyen at al. 2012, Maezawa and Jin, 2010) that glial cells are involved not only in neurodegenerative diseases such as Alzheimer's disease (Angelova PR at al. 2014), but also in diseases characterized by intellectual disability. In this regard, recent evidence has shown the involvement of glial cells in Rett Syndrome (Ballas et al., 2009, Maezawa et al., 2009, Derecki et al., 2012 Okabe 2012).

The purpose of my thesis is to investigate the role of different populations of astrocytes in the development and survival of neurons using *in vitro* models of both neurodegenerative and neurodevelopmental disorders. To this aim, in the first part of my project I participated in the setting up of a microfluidic system, an experimental model aim at studying the interconnections between different brain cells, focusing my attention on the contribution of glial cells derived from different brain areas in these pathological states. Secondly, through the use of the microfluidic system, we have shown that astrocytes derived from distinct brain areas have different effects on neurons if exposed to several harmful stimuli. Specifically I have evaluated both the development and viability of hippocampal neurons co-cultured with either cortical or hippocampal astrocytes in a neuroinflammatory context represented by the stimulation of beta amyloid peptide (Aß) together with several proinflammatory cytokines. Taking advantage of the microfluidic system, we showed that hippocampal and cortical astrocytes exposed to these stimuli were able to significantly increase neuronal cell death. Interestingly, hippocampal astrocytes induced an elevated neuronal cell death if compared to cortical astrocytes. (Bianco F, N Tonna, Lovchik RD, **Mastrangelo R**, Morini R, Ruiz A, E Delamarche, Matteoli M. Anal. Chem. 2012). To assess whether neuronal death was due to soluble factors released by astrocytes, we monitored the neuronal calcium responsiveness upon the exposure of astrocytes to Aß and IL 1ß. We found that both cortical and hippocampal astrocytes elicited a specific calcium transient in neurons upon stimulation, however, the calcium transients were higher in neurons exposed to stimulated hippocampal rather than cortical astrocytes. Such calcium transient were blocked by the specific NMDA receptor antagonist APV, thus highlighting that the calcium

transients were mediated by the activation of these receptors following the release of astrocytic glutamate. More importantly, hippocampal astrocytes exposed to neuroinflammatory environment induced higher cell death and neuronal calcium transient compared to cortical astrocytes (Bianco F, N Tonna, Lovchik RD, **Mastrangelo R**, Morini R, Ruiz A, E Delamarche, Matteoli M. Anal. Chem. 2012)

We also used the microfluidic system to perform a pharmacological study in an experimental model of Alzheimer's disease. In particular, we evaluated the effectiveness of the drug FTY720 (Fingolimod, normally used for the treatment of multiple sclerosis) to prevent cell death in neurons cultured alone or co-cultured with microglia upon the stimulation with either fibrillar or oligomeric Aß form. The results showed that the drug prevented cell death in neurons exposed to Aß oligomers, both in the presence and absence of microglia. (Ruiz A, Joshi P, **R Mastrangelo**, Francolini M, Verderio C, Matteoli M Lab Chip. 2014).

In the second part of my PhD thesis, I have focused my attention on the role of different populations of astrocytes in supporting neuronal development in a model of Rett Syndrome. Observations made by Prof. Tongiorgi's lab, showed a different neuronal atrophy at the level of cortex and hippocampus in mouse model of Rett Syndrome, thus prompting us to investigate the effects of glial cells derived from these two different brain areas on neurons. Neurons were grown in conditioned medium obtained from hippocampal or cortical astrocytes established from WT or KO mouse, and the growth in terms of neurites length was evaluated. The morphological analysis showed that neurons grown in conditioned medium derived from hippocampal astrocytes displayed longer neuritic processes than those grown in cortical astrocytes-derived medium. In contrast, the growth of hippocampal neurons in conditioned medium from wt cortical astrocytes, hippocampal and cortical astrocytes Rett not significantly different compared to neurons cultured alone. This suggests that hippocampal astrocytes have a higher trophic effect on neuronal development than cortical astrocytes. In light of these results, we can assume that the atrophy observed in cortical region of Rett Syndrome mouse model may be due to a lower trophic capability of cortical astrocytes compared to hippocampal in supporting neuronal development.

Taken together these data suggest that astrocytic populations belonging to distinct brain areas have different capability in supporting neuronal growth, thus opening the possibility that region-specific atrophy observed in RETT mouse model may stem from a different release of trophic factors from glial cells.

1. Introduction

Dendrite morphogenesis is a complex but well-orchestrated process which includes the development of dendritic branches, the formation of dendrite arbors and the maturation of spines, thus allowing neurons to communicate with each other in the brain. An alteration in dendritic morphogenesis has been found to be associated with many neurological and cognitive disorders, including Alzheimer disease and Rett Syndrome (Kaufmann and Moser, 2000; Pfeiffer and Huber, 2007). Thus, the clarification of the molecular mechanisms underlying the proper formation of dendrite morphology is essential for the understanding of brain function in both healthy and pathological states.

Moreover, it is well established that the interconnections between neuron and glia are very important for the maintenance of the physiological activities of the brain. Indeed, recent studies demonstrated the existence of the tripartite synapse, in which not only pre and post synaptic compartments, but also astrocytes, participate in the proper transfer of information between neurons. Furthermore, beside their role in synaptic function, glial cells play an important role in several aspects of neuronal development, including dendritic growth and spine formation. In line with such evidence, alterations in dendrite morphology or defects in neuronal development contribute to several neurological and neurodevelopmental disorders, such as Alzheimer's disease (AD) and Rett Syndrome (Kaufmann and Moser, 2000).

1.1 Rett Syndrome

1.1.1 Historical overview

Andreas Rett, a neurodevelopmental pediatrician in Vienna, described Rett Syndrome (RTT) for the first time. In his waiting room he noticed two girls, seated next to each other on the lap of their respective mothers, who displayed exactly the same behaviour. He recognized that these girls' hand movements were different from the stereotypic behaviours among other disabled children. He and his nurse were able to recall and examine other girls in their care who demonstrated these same characteristics. One year later, he reported 22 girls with these abnormal clinical features. His publication written in German in the "Wiener Medizinische Wochenschrift" in 1966, however, remained largely unnoticed (Rett, 1966). In 1983, Dr. Bengt Hagberg, a Swedish neurologist, and his colleagues reported the clinical description of 35 cases with similar features described by Rett (Hagberg et al., 1983). He recognized that his patients showed overlapping phenotype with Dr. Rett's and called the disease as Rett Syndrome to honor the first description by Dr. Rett. In the years that followed, informations about RTT spread rapidly. Today we know that RTT is a neurodevelopmental disorder due to mutation in the gene encoding for the methyl-CpG binding protein 2 (MeCP2), a finding first demonstrated by researchers from Professor Zoghbi's laboratory at the Baylor College of Medicine in Houston, Texas (Amir et al., 1999).

RTT is mainly found in females, and it is due to the X-linked nature. The prevalence of this disease is 1/10,000 to 1/15,000 females (Hagberg and Hagberg et al., 1997). Most (99%) of the RTT cases are sporadic. In up to 97% of classic RTT the disease is associated with mutations in the X-linked methyl-CpG-binding protein 2 (*MeCP2*) gene (Amir et al., 1999) suggesting that RTT is caused by mutations in this gene and being the leading cause of mental retardation in females (Jedele et al., 2007; Shahbazian and Zoghbi et al., 2002). In rare cases, RTT also affects males that manifest a range of symptoms including severe encephalopathy, mild mental retardation and dystonia apraxia (Jan et al. 1999).

1.1.2 Clinical Features of classic RTT

Patients with classic RTT appear to reach developmental milestones seemingly normally until about 6–18 months after birth when signs and symptoms of the disease that typically includes severe mental retardation, stereotypic hand movements, motor coordination deficits, epileptic seizures, language and learning disabilities, and mild to severe cognitive impairments, begin to appear (Hagberg et al., 1983; Chahrour and Zoghbi, 2007).

In particular, the cascade of clinical symptoms is described in four stages (Fig. 1):

I) the early-onset stagnation,
II) the rapid developmental regression,
III) a pseudo-stationary stage
IV) a late motor deterioration (Hagberg & Witt Engerström, 1986).

During Stage I (6-18 months), RTT females cease to acquire new skills, the head growth undergoes deceleration that usually leads to microcephaly. During Stage II (1-4 years), girls lose the ability to speak and the purposeful use of the hands accompanied by a reduction in interpersonal contact and the appearance of autistic features. Also, involuntary movements and the classic stereotypic hand activities (tortuous hand wringing, hand washing, clapping, patting) become evident, as well as electroencephalogram (EEG) abnormalities and microcephaly. In Stage III (4-7 years), girls become more alert and interested both in people and their surroundings; however inability to speak, hand apraxia and the stereotypic hand activities persist. Other somatic and neurological handicaps, such as severe scoliosis, reduced somatic growth and epilepsy, become evident. Stage IV (5-15 years and older) is characterized by further somatic and neurological deterioration resulting in end-stage spastic quadriparesis.

In addition to the typical characteristics present in RTT a wide variety of features are present in affected individuals, such as:

- **Movement abnormalities**. Affected individuals are hypotonic at birth and early in life, but develop dystonia especially in the ankles and lower extremities. Choreiform movements of the limbs and oromotor dyskinesias with tongue thrusting can be present. Some individuals have truncal rocking, titubation, and/or tremor. Teeth grinding (bruxism) is a common problem (Neul et al., 2012)
- **Seizures and nonepileptic spells.** The majority of affected people have seizures during their lives, but commonly have also nonepileptic paroxysmal events (Neul et al., 2012).

- **Gastrointestinal problem**. Motility and coordination are disrupted throughout the entire gastrointestinal tract, leading to chewing and swallowing problems, gastroesophageal reflux, delayed stomach emptying, bloating, and constipation (Neul et al., 2012).
- **Cardiac abnormalities**. Approximately 20% of people with RTT have prolonged QT intervals (Neul et al., 2012).

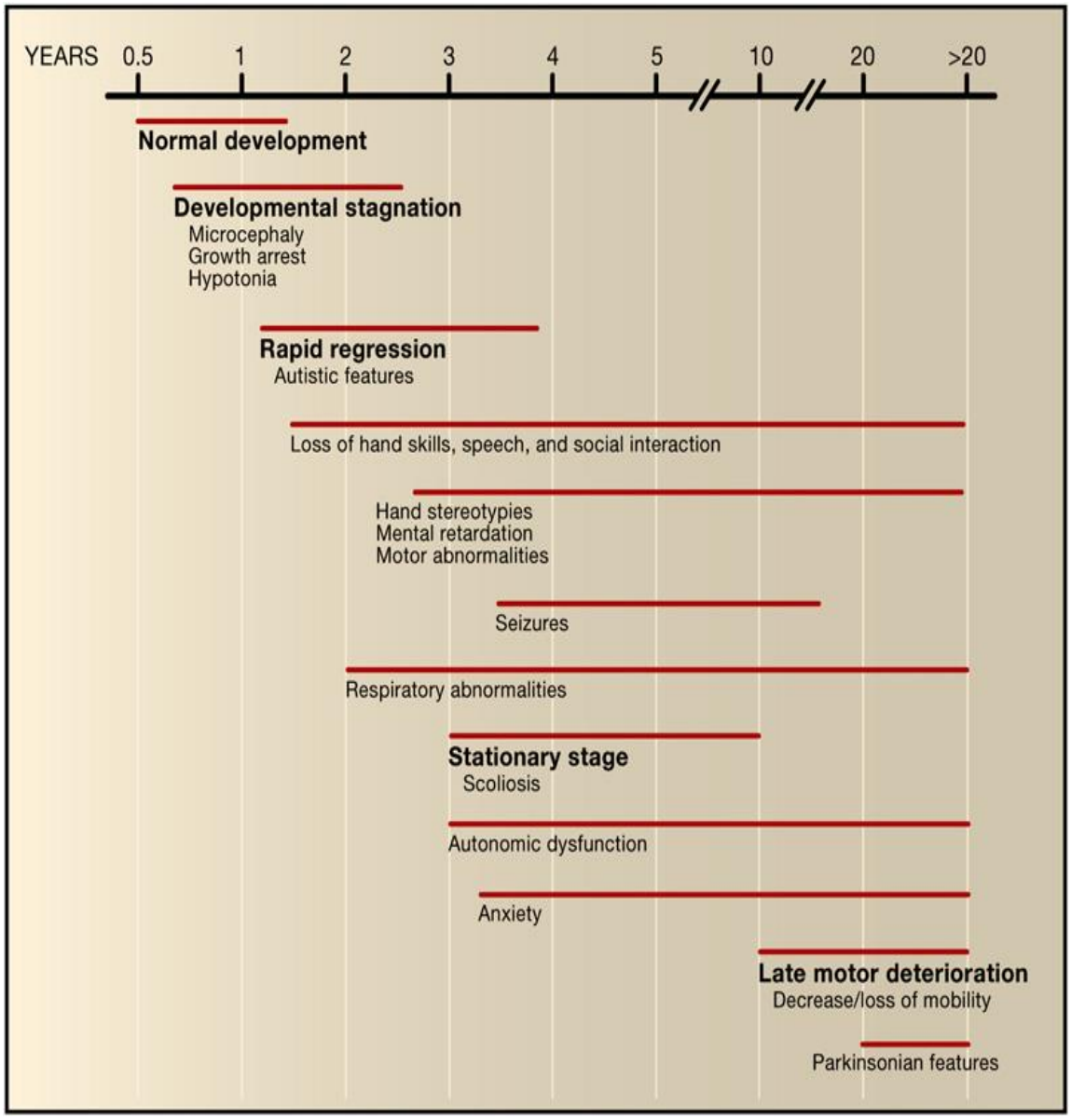

Figure 1. Onset and Progression of RTT Clinical Phenotypes.

After a period of normal development, a healthy-looking baby girl falls into developmental stagnation, followed by rapid deterioration, loss of acquired speech, and the replacement of purposeful use of the hands with incessant stereotypies, a characteristic of the Syndrome. Patients also develop social behavior abnormalities and are often misdiagnosed as having autism. The condition worsens with loss of motor skills and profound cognitive impairment. addition, patients suffer from anxiety, seizures, and a host of autonomic abnormalitie.

- **Breathing abnormalities.** Commonly hyperventilation and/or apnea can appear. The hyperventilation can be significant enough to cause hypocapnea. The apneic events can cause a decrease in blood oxygen and occasionally causes loss of consciousness. These breathing abnormalities are significantly increased during wakefulness and may be exaggerated by anxiety, but can be observed also during sleep (Neul et al., 2012).
- **Autistic features and other behavioral problems.** Autistic features such as social withdrawal and avoidance of eye gaze occurs in some people with RTT, often during the period of active regression (Stage II). In fact, some people eventually diagnosed with RTT are initially diagnosed with autism.(Neul, 2012).

The criteria, contrary, that no defined RTT patients are (Hagberg et al., 2002):

- organomegaly or other evidence of storage disorder
- cataract, retinopathy or optic atrophy
- history of perinatal or postnatal brain damage
- identifiable inborn error of metabolism or neurodegenerative disorder
- acquired neurological disorder due to severe infection or head trauma
- evidence of prenatal onset of growth retardation or microcephaly.

1.1.2.1 Variant forms of Rett Syndrome

In addition to the classic form of RTT, five distinct variants have been delineated on the bases of clinical criteria, they show some, but not all diagnostic features of classic RTT and can be milder or more severe:

1) The preserved speech variant (PSV) or variant of Zappella (Z-RTT), described for the first time since Zappella in 1992, is the most common variant. It has a more favorable clinical course and are characterized by milder severity and more regained spoken language after regression. (Zappella M. et al. 1994). The patients, unlike the Classic RTT, generally have a normal head circumference, kyphoscoliosis is more mild and somatic ipoevolutismo is reduced, sometimes with a tendency to overweight. During stage III, the patients again acquire some skills previously lost as verbal language. Some patients are able to speak only a few words while others are able to communicate well with complex sentences. An improvement is also noted in the use of the hands, although considerable dyspraxia and all of the classics stereotyped movements persists. Their motor skills

improved to the point that some girls are also can go up and down stairs independently. Nearly all people identified with PSV have mutations in *MeCP2*.

2) The infantile seizure onset variant described for the first time Hanefeld in 1985 and characterized by seizure onset before the regression. Notably, very few people with early seizure variant have been found to have mutations in Cyclin-dependent kinase like-5 (CDKL5), (Hanefeld F. 1985). The initial period is masked by the onset of seizures, usually in the form of spasms in flexion. The appearance of convulsions is accompanied by a delayed psychomotor development. Only later the girls develop the characteristics typical RTT as stereos manuals, particularly of "hand mouth." Furthermore, the head circumference, weight and height are normal in most cases.
3) The "forme fruste" with a milder, incomplete and protracted clinical course (Hagberg B et al.1986). This variations RTT do not have the typical characteristics the disease and typically the stage I appears later (1-3 years). The girls show milder initial symptoms and have a more protracted clinical deterioration; usually they retain some form of language and developmental abnormalities are very less obvious. The classic stereotypes of the hands may be atypical or absent altogether and the clinical picture becomes more like the RTT when these children reach adolescence and adulthood.
4) The congenital variant, lacking the normal perinatal period. Recent work has identified mutation in *FOXG1* in some people with the congenital variant (Rolando S et al. 1985). In this variant, psychomotor retardation is evident from the first months of life, often with hypotonia and early EEG alterations. In the following months different stages previously described in the classical Syndrome appear, as well as generalized convulsions.
5) The late regression variant, which is rare and still controversial (Gillberg C et al.1989). In this variant, stage I is more protracted and regression may arise during elementary schools. Until the development of stage II patients have a simple delay mental medium grade and in subsequent months appear different stages already described in classical Syndrome.

Table1 shows in brief the different mutations involved in the variant forms of Rett Syndrome.

Table I. Mutation detection rate in known genes

	MECP2 (%)	CDKL5 (%)	*FOXG1* (%)	Unknown (%)
Classic form	90	0	0	10
Preserved speech variant	50	0	0	50
Forme fruste	10	0	0	90
Congenital variant	10	0	90	0
Late regression variant	?	?	?	?
Early onset variant	0	50	0	50

Table1:Until recently, RTT was considered a monogenic disorder caused by mutations in the MeCP2 gene. Although new data demonstrate a greater genetic complexity and heterogeneity in RTT, MeCP2 is likely to be the gene primarily responsible for classic RTT cases. By contrast, a high percentage of patients with variants of RTT do not have MeCP2 mutations, leaving open the question of what is the molecular defect in these patients (Mari et al. 2005).

1.1.3 Causes of classic RTT

1.1.3.1 MeCP2 gene

The MeCP2 is a protein of the Methyl-CpG Binding Protein (MBD) family able to bind DNA sequences methylated at cytosine 5'CpG. This methylation is one of the most important epigenetic modification in mammalian genomes and is very important for normal development in mammals. Most CpG sites are methylated at a frequency of 60%-90% (Bird, 1980). However, distinct regions with a very high CpG content (known as CpG islands), which are found in promoters of highly expressed genes are not methylated (Bird AP, 2002). In human and in mouse, MeCP2 gene is an X-linked gene that spans 76 kb in the long arm of the X chromosome (Xq28) (Fig. 2). The gene comprises four major exons (exon 1–4) and three introns (intron 1–3) (Figs. 2b-c). The protein structure is composed of five major domains, N-terminal Domain (NTD), Methyl Binding Domain (MBD), InterDomain (ID), Transcription Repression Domain (TRD) and C-terminal Domain (CTD) and is approximately 53 kDa in size (Fig. 2d), but is usually detected at 75 kDa by probably due to its posttranslational modifications. However, the majority (60%) of MeCP2 protein is unstructured, while MBD is the only domain that has a

definite secondary structure (Vichithra R. B. Liyanage et al. 2014). The MeCP2 has two isoforms that differ in the N-terminus that are generated by alternative splicing of exon 2. The first identified isoform, MeCP2A, uses a translational start site within exon 2, whereas the other isoform, MeCP2B drives from an mRNA in which the exon 2 is excluded and a new in-frame ATG located within exon 1 is used (Fig. 2c).

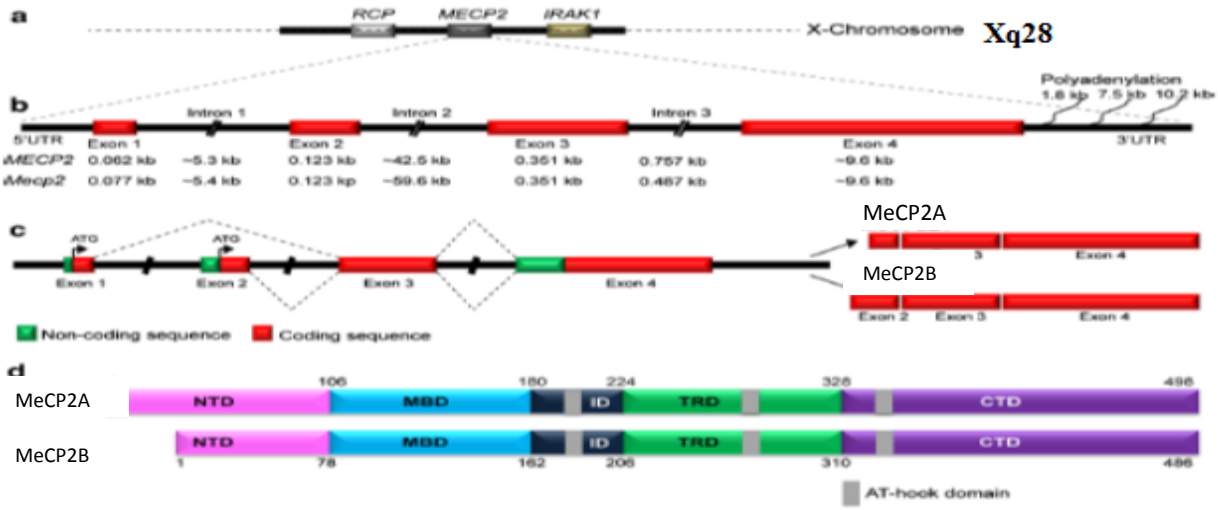

Figure 2. The structure of the MeCP2 gene and protein. a) The MeCP2 gene is locates in X-chromosome (Xq28), between RPC and IRAK genes. b) The composition of the MeCP2 gene. c) Two isoform of the MeCP2. d) The protein structure of MeCP2.

1.1.3.2 Role of MeCP2

MeCP2 was first identified as a transcriptional factor that inhibits gene expression through the involvement of two epigenetic markers, DNA methylation and histone acetylation. The discovery of this cooperative action among MeCP2, HDACs and DNA methylation suggested a mechanistic link between chromatin modifications and DNA methylation resulting in less active gene transcription. MeCP2-mediated gene silencing could occur through chromatin modification mediated by MeCP2 interaction with Sin3A/HDACI or Ski/NcoR/HDACII repression complexes (Jones et al., 1998; Nan et al., 1998; Kokura et al., 2001). These enzymes remodel chromatin, which then becomes inaccessible to the transcriptional machinery (Nan et al., 1998).

More precisely, the transcriptional repression domain (TRD) is involved in the repression of downstream genes by recruiting histone deacetylases HDAC1 and HDAC2 and corepressor factors (e.g. Sin3A, c-Ski, and N-CoR) (Jones et al., 1998; Nan et al., 1998; Kokura et al., 2001), or components of chromatin remodelling complexes (Harikrishnan et al., 2005; Nan et al., 2007).

The C-terminal domain probably facilitates binding to the naked DNA and to the nucleosomal core (Chandler et al., 1999).

It also contains evolutionary conserved poly-proline motifs that can bind to group II WW domain splicing factors (Buschdorf and Stratling, 2004). The original model of MeCP2 function involves deacetylation of core histones by HDAC resulting in the compaction of the chromatin and making it inaccessible to components of the transcriptional machinery (Jones et al., 1998; Nan et al., 1998) (Fig. 3). Interactions between TRD and various cofactors demonstrate that MeCP2 protein is capable to interpret methylated CpGs differently and, depending on the context, mediate multiple downstream responses.

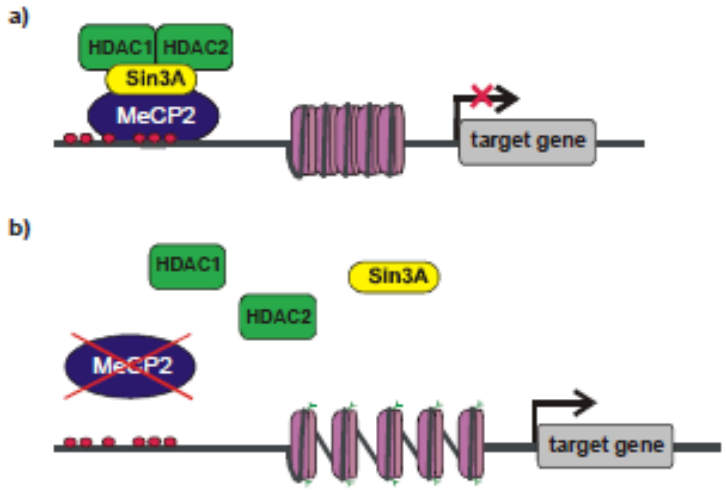

Figure 3. Model of MeCP2 function.
a) MeCP2 binds to methylated CpGs (red circles) and recruits corepressorproteins such as HDAC1, HDAC 2,and Sin3A. Chromatin remains in condensed and transcriptionallyinactive state.
b) Non-functional MeCP2 does not create the repressor complex and histones remain acetylated (blue Ys).Chromatin is in decondesed state, which allows transcription factors to bind to promoter and initiatetranscription

The function of MeCP2 is actually more complex than initially anticipated. MeCP2- directed transcriptional repression is also performed in HDAC-independent manner (Yu et al., 2000), through a direct interaction between the C-terminal domain and chromatin (Nikitina et al., 2007), or through an interaction with a component of the basal transcriptional machinery, transcription factor IIB (Kaludov and Wolffe, 2000). MeCP2 also appears to be an architectural chromatin protein, capable to establish silent domains by promoting chromatin looping without DNA methylation, ATP, and other cofactors (Georgel et al., 2003) (Fig. 4).

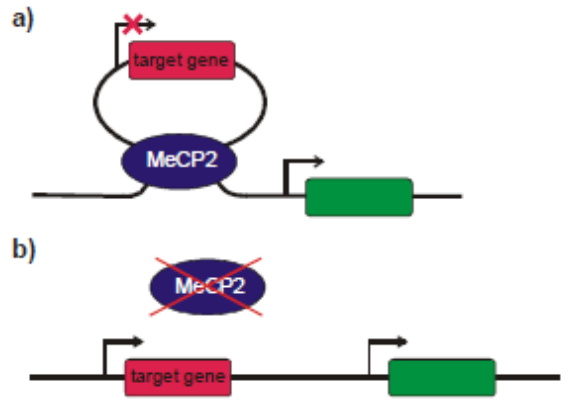

Fig. 4. Gene silencing by establishing of secondary chromatin structures.
a) MeCP2 binds to binding sites surrounding its target gene (red) forming a loop. A repressive environment is established within the loop while genes outside the loop (green) remain transcriptionally active. The loop can encompass one or more target genes.
b) Absent or non-functional MeCP2 does not create the loop and the expression of the genes is not affected.

So, MeCP2 can act both as repressor and as activator of gene transcription. Surprisingly, approximately 400 genes are identified as repressed targets and approximately 2,000 genes are identified as activated targets. Some of the target genes are involved in processes important for brain function (Chahrour et al., 2008). These target genes are highly interesting as their encoded proteins function in pathways that may be targeted by pharmacological means to improve RTT phenotypes.

The most extensively studied target genes are shown in Table. 2

Table 2: Some of MeCP2 target genes (Chahrour and Zoghbi, 2007)

Gene	Function
BDNF	neuronal development and survival, neural plasticity, learning and memory
DLX5	neuronal transcription factor (probably involved in control of GABAergic differenciation)
SGK1	hormone signaling (regulation of renal functions and blood pressure)
FKBP5	hormone signaling (regulation of glucocorticoid receptor sensitivity)
UQCRC1	member of mitochondrial respiratory chain
ID1, ID2, ID3	transcription factors (involved in cell growth, senescence, differentiation, angiogenesis)
FXYD1	ion channel regulator
IGFBP3	hormone signaling (regulation of cell proliferation and apoptosis)
CRH	neuropeptide (regulation of neuroendocrine stress response)
UBE3A	member of ubiquitin proteasome pathway, transcriptional coactivator
GABRB3	neurotransmission (GABA-A receptor)

The first gene shown to be repressed by MeCP2 was the gene encoding for brain-derived neurotrophic factor (BDNF), a protein that has essential functions for neuronal plasticity, learning and memory. In basal conditions, BDNF expression is repressed by MeCP2 bound to its promoter; In MeCP2-deficient neurons, the basal levels of BDNF are twofold higher than those of wild-type neurons. Recently, Horike et al.2005, reported loss of DLX5 in Rett patients. MeCP2 was shown to be essential for the formation of a silent chromatin structure at the Dlx5 locus by histone methylation and through the formation of a chromatin loop. Deregulation of a single gene, Dlx5, can provide a unique mechanism for some clinical manifestations of the disease. Dlx5 regulates GABA neurotransmission and osteogenesis; therefore, alterations in Dlx5 expression can account for epilepsy, osteoporosis and somatic hypoevolutism observed in RTT girls. Other genes that were subsequently found to be regulated by MeCP2 in brain tissues are the ubiquitin protein ligase 3A (UBE3A) and the glucocorticoid inducible genes, Sgk1 and Fkbp5 (Makedonski et al2005, Nuber, U..et al 2005). In summary, these findings demonstrate a role of MeCP2 in the regulation of a small subset of genes and support the current hypothesis that RTT onset is due to deregulation of genes that have crucial importance for the nervous system.

1.1.3.4 Mutations in MeCP2 cause Rett Syndrome

The MeCP2 mutations, including missense, nonsense, insertions, deletions, and splice site variations have been detected throughout the gene. Most of these mutations occur *de novo* and are predicted to result in a loss of function of the MeCP2 protein (Matijevic et al., 2009). The exceptions are familial cases where the mutation has been inherited from a healthy or mildly affected mother. Although mutations are dispersed throughout the gene, a clustering of missense mutations occurs in the MBD. A recent analysis (Mari et al.2005) revealed the existence of another cluster of missense mutations localized at the C-terminal extremity of the TRD. On the basis of the effect on the protein, mutations can be classified as: (i) early truncating mutations in the MBD and TRD domains leading to complete loss of MeCP2 function; (ii) late truncating mutations in the C-terminal domain leaving the MBD and TRD domains intact; and (iii) missense mutations usually clustered in the MBD and TRD domains (Mari et al.2005).

All these mutations in MeCP2 gene have been shown to cause the neurodevelopmental disorder Rett Syndrome (RTT), symptoms of which are primarily neurological (Amir et al., 1999).

The discovery of the localization of MeCP2 helped to explain the female prevalence of RTT (Quaderi et al., 1994). Females that are heterozygous for the mutated MeCP2 allele are able to survive with this debilitating disorder due to X chromosome inactivation (Amir et al., 2000), while males that are hemizygous for MeCP2 mutations have a drastically shortened lifespan of approximately 2 years and typically develop congenital encephalopathy (Ravn et al., 2003; Villard et al., 2000). Furthermore, mutations in MeCP2 not only result in classic forms of RTT, but also cause a range of related neuropsychiatric disorders. For example, patients diagnosed with Angelman Syndrome (Watson et al., 2001), non-syndromic mental retardation (Miltenberger-Miltenyi and Laccone, 2003), Prader–Willi Syndrome (Samaco et al., 2004), and some forms of autism (Shibayama et al., 2004) have mutations in the MeCP2 gene that significantly reduce MeCP2 protein expression levels. These data indicate that the effects of MeCP2 mutations are not necessarily consistent and that understanding the developmental trajectory of MeCP2 expression, and the coupling of developmental stage-specific expression with neuronal function, may provide important insight into the etiology of RTT and other MeCP2-associated disorders. However, some people with RTT do not have mutations in MeCP2, and interestingly people identified with MeCP2 mutations do not have the clinical features of RTT.

1.1.3.4.1 MeCP2 Mutations in People Without Rett Syndrome

Almost 70 % of all sporadic mutations found in typical RTT arise from mutation in MeCP2, but despite this strong correlation between mutations in MeCP2 and RTT, mutations in MeCP2 are neither necessary nor sufficient for the diagnosis of RTT. Recent studies show that people who clearly do not satisfy the criteria for diagnosis of typical or atypical RTT but have common RTT-causing mutations in MeCP2 (Suter et al. 2014) have neither classical nor atypical RTT, but rather carry clinical diagnoses of autism, PDD NOS, ADHD, and OCD. Thus, as mutations in the MeCP2 gene are not found in all clinical cases of RTT, it appears that a mutation in the MeCP2 gene is not necessary to develop RTT. Finally, the prevalence of MeCP2 mutations in neurologically normal populations is currently unknown because MeCP2 mutations do not necessarily lead to clinical features (Shevell et al. 2003).

1.1.3.5 MeCP2 in the brain

Although MeCP2 is widely expressed in many organs, the brain-specific localization of such gene has been the focus of several studies because within the brain, both the distribution and levels of MeCP2 expression have been shown to be highly variable during neuronal development. During embryogenesis the neuronal MeCP2 level is relatively low but increases progressively during the postnatal period of neuronal maturation (Kishi and Macklis, 2004; Skene et al., 2010). Expression of MeCP2 develops progressively from deep cortical layers to superficial layers as development progresses. For instance, The first detection of MeCP2 expression happens in the spinal cord and brainstem around day E12, with mRNA being detected in subcortical regions at the beginning stages of embryonic neurogenesis (Jung et al., 2003; Shahbazian et al., 2002b). The expression of MeCP2 in the cerebral cortex occurs at embryonic day 14 (E14), where MeCP2 expression is initially limited to deeper cortical layers before eventually spreading out to more superficial layers by E18. The thalamus, caudate putamen, cerebellum, hypothalamus, and hippocampus are immunoreactive for MeCP2 beginning at days E14–16. Thus, there is a clear parallel between neuronal maturation and MeCP2 expression onset (Shahbazian et al., 2002; Kishi and Macklis, 2004). The loss of MeCP2 expression within this time window may be responsible for the observed decrease in neuronal and overall brain size in RTT patients. Disruptions in MeCP2 function might therefore interfere with neuronal maturation and synaptogenesis, culminating in abnormal development of the CNS.

Beside such variations during brain development, several studies reported a different pattern of MeCp2 expression among distinct brain regions. Analysis of whole cell extracts isolated from different brain regions indicated the highest MeCP2 expression in the cortex and cerebellum (Zachariah et al. 2012). Given the importance of brain region-specific MeCP2 expression in Rett Syndrome pathogenesis, the groups had study the expression levels of MeCP2 in different mouse brain regions in correlation with impaired behavioral phenotypes in a RTT mouse model (Wither et al. 2013). Indeed, several RTT mouse models have been generated by deleting the MeCP2 expression in specific brain regions and/or specific cell types within the brain regions showing different degrees of RTT phenotypes. For example, loss of MeCP2 expression in the neurons in basolateral amygdala causes increased anxiety-like behavior and impaired cue-dependent fear learning (Adachi et al. 2009; Wu and Camarena 2009). Several others studies have shown abnormal social behaviors, anxiety and also autistic features in RTT mouse models lacking MeCP2 expression in the neurons of forebrain (Gemelli et al. 2006; Chen et al. 2001; Chao et al. 2010). Moreover, MeCP2 deletion from hypothalamic neurons resulted in abnormal physiological stress response, hyperaggressiveness and obesity (Fyffe et al. 2008)

At cellular level, MeCP2 expression is predominantly detected in neurons (Shahbazian,2002, Zachariah et al. 2012). However, recent studies have also demonstrated the MeCP2 expression non neuronal cell types, including astrocytes, oligodendrocytes and microglia (Zachariah et al. 2012; Ballas et al. 2009; Rastegar et al. 2009; Derecki et al. 2012; Liyanage et al. 2013; Olson et al. 2014), even though neurons show the highest MeCP2 expression (Zachariah et al. 2012; Ballas et al. 2009). Proper MeCP2 expression in neurons is required for neuronal maturation (Kishi and Macklis 2004; Singleton et al. 2011; Thatcher and LaSalle 2006; Shahbazian et al. 2002b) and proper neuronal functions (Nguyen et al. 2012; Shahbazian and Zoghbi 2002). At the same time a proper expression of MeCP2 in astrocytes has been also shown to be critical for normal neuronal function. For example, abnormal neuronal morphology in primary neurons can be caused by MeCP2-deficiency in the neighboring astrocytes (Ballas et al. 2009; Maezawa et al. 2009). Furthermore, loss of MeCP2 in astrocytes has been shown to cause abnormalities in neurodevelopment and thereby contributing to RTT progression (Maezawa et al. 2009 Lioy et al. 2011). A recent study reported the importance of a specific set of MeCP2 target genes in astrocytes which might be relevant in RTT pathology which include Apoc2, Cdon, Csrp and Nrep genes, which are involved in proper astrocytic functions (Yasui et al. 2013). Similar to astrocytes, MeCP2 expression in microglia cells was shown to be involved in the modulation of neuronal morphology through secreting soluble factors. Indeed, MeCP2-deficient microglia secrete higher levels of glutamate, leading to abnormal neuronal morphology, highlighting the

potential role of MeCP2 in microglia that impacts RTT progression (Maezawa and Jin 2010). Furthermore, the re-expression of MeCP2 in microglia of MeCP2-null mouse model has been shown to restore several RTT phenotypes (Derecki et al. 2012). MeCP2 is expressed also in oligodendrocytes. MeCP2-deficiency in the cortex and hippocampus of a RTT mouse model has been shown to cause reduced expression of a specific marker for oligodendrocytes (Wu et al. 2012). The role of MeCP2 in regulating myelinrelated genes including myelin basic protein, proteolipid protein and myelin-associated glycoprotein has also been shown in a RTT mouse model (Vora et al. 2010).

1.1.3.6 Morphological features of Rett Syndrome

Morphologically RTT patients exhibit small brains with no evidence of atrophy, a phenotype consistent with a lack of proper development rather than ongoing degeneration. Neurons in RTT patients display decreased dendritic arborization, fewer dendritic spines, and increased packing density. Armstrong and other colleagues analyzing post-mortem cortical and hippocampal dendrites of Rett patients observed a reduced dendritic arborization of the layer II and III pyramidal neurons belonging to frontal, motor and inferior temporal regions as well as in hippocampal neurons confined in layers II and IV of the subiculum (Armstrong et al. 1995; Kaufmann and Moser 2000; Belichenko et al. 1997). Other abnormalities documented in Rett patients' brain include decreased spine number (Belichenko et al .1994), hypo- and hyperactivity of neuronal circuits in specific brain regions such as cortex, hippocampus, basal ganglia and enthorinal cortex (Leontovich et al. 1999) and disorganized axonal boundles (Belichenko et al. 2004).

Study regarding the impact of MeCP2 mutations on dendritic arbors development was published in 2008 by Schule et al. (Schule et al. 2008). In particular, they analyzed post-mortem tissue from temporal and frontal lobes of male patient carrying a MECP2 null mutation, comparing the neuronal morphology with that of a normal age-matched male brain processed using the rapid Golgi technique. Following a Sholl analysis they observed a significant decrease of the dendritic arborization in both the brain areas when compared with the control, in total agreement with what was previously described for RTT female patients (Schule et al.2008).

1.2 Alzheimer's Disease

2.1.1 Historical overview

The disease takes its name from Alois Alzheimer, who first described the clinical and neuropathological features of the disease. Alois Alzheimer participated to a meeting in Monaco, where he described the case of a woman of 51 years old, Auguste D., who had serious cognitive impairment and progressive loss of memory. Thanks to the silver staining technique, he noted the two major brain lesions in Alzheimer's disease: senile plaques and neurofibrillary tangles.

Later, in the 1960s, the advent of electron microscopy made possible to identify for the first time the ultrastructures of the two brain lesions described by Alzheimer's. Furthermore, in the following years was observed the degeneration of cholinergic neurons, associated with a decreased activity of the acetylcholine synthesis and degradation enzymes: cholineacetyltransferase and acetylcholinesterase. For this reason, the first pharmacological studies were aimed to stimulate and increase the levels of acetylcholine, and led to two drugs still used for the treatment of AD: tetrahydroaminoacridine and donepezil.

Subsequent studies allowed understanding that the neuronal degeneration typical of AD is very heterogeneous and involves different classes of neurons (as explanation of the limited effectiveness of cholinergic drugs).

Since then, the researchers were mainly interested to the understanding of the mechanisms leading to the formation of the two neuropathological lesions typical of AD, on which Alzheimer had focused the attention from the beginning (Selkoe, 2001). It's the most common cause of age-related cognitive impairment and dementia and its prevalence increases exponentially with age. Indeed, about 13% of people aged over 65 and 45% of people over 85 years are affected by AD.

The disease is also known as Alzheimer's dementia, because it represents 65-75% of all dementia cases. In addition, as the other forms of dementia, the development of AD is characterized by a continuous progression of the disease. Around 90-95% of patients is affected by a sporadic form of the disease, which occurs in the old age and has a multifactorial etiology.

1.2.2 Clinical Features of AD

Alzheimer's disease (AD) is a chronic, progressive and neurodegenerative disorder that affect more than 35 million of individuals' worldwide (Parkinson et al., 2013). The classic form is sporadic late onset form.

This pathology leads progressively to (Salloway et al., 2008):

- Loss of memory
- Loss of disorientation and misinterpreting spatial relationships
- Loss of the ability to read and write
- Loss to make judgments and decisions
- Loss of the ability of Planning and performing familiar tasks
- Change in personality and behavior

The pathophysiological processes of Alzheimer's disease come first of the onset of clinical symptoms, but can begin many years before the diagnosis (Mattsson et al., 2009).

The pathology of AD is a complex disease that affects many areas of the brain (Fig. 5): entorhinal cortex, hippocampus, parahippocampal gyrus, amygdala, frontal, temporal, parietal and occipital association cortices (Selkoe, 2001). The neurological damage usually begins in temporal and parietal lobes of the cerebral cortex and progresses to reach hippocampus and amygdala (Block and Hong, 2005).

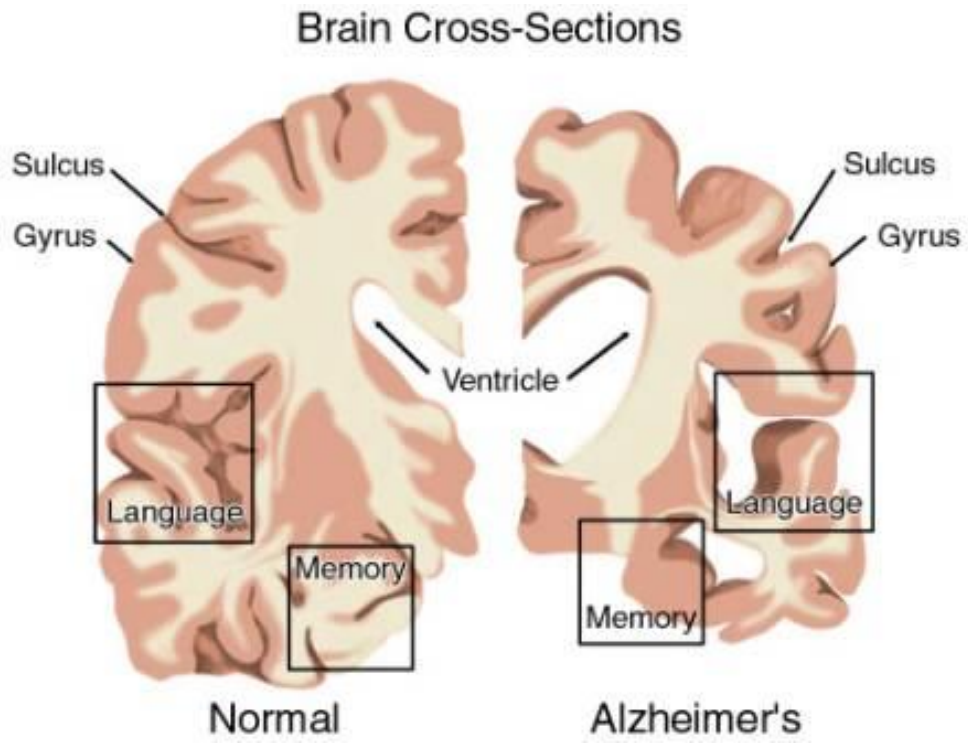

Figure 5. The figure shows on the left a healthy brain, on the right a brain affected by Alzheimer's disease. The figures draws attention to the main areas of the brain interested by the pathology: entorhinal cortex, hippocampus, parahippocampal gyrus, amygdala, frontal, temporal, parietal and occipital association cortices.

There are also many physiological processes involved in the development of AD: tau and β-amyloid deposition, abnormalities of cholesterol metabolism, inflammation, oxidative damage and lysosomal dysfunction. The results of these pathological processes are neuronal death and brain atrophy (McGhee et al., 2014)

1.2.2.1 Variants of AD

There are several known types of Alzheimer's disease. Familial Alzheimer disease (FAD) is an autosomal dominant disorder and is extremely rare, accounting for less than 10% of all cases of Alzheimer's disease. This is a form of Alzheimer's disease that is known to be entirely inherited. The FAD comprises:

- Early-onset familial AD is disorder with an incidence of 5-10%. Typically, the disease occurs before 65 years old and is associated with mutations in the APP and presenilin genes. The clinical manifestations of the disease are very similar to those with sporadic onset, although some families may show distinctive clinical signs, such as myoclonus, seizures, early and prominent extrapyramidal signs (Liu et al., 2013).
- Late-onset familiar AD, whose the main genetic risk factor is the ε4 allele of the apolipoprotein E (APOE) gene and the disease occurs after 65 years old. The gene is involved in the regulation of lipid homeostasis, the metabolism of Aβ and in modulating the activity of the γ-secretase. It mediates the transport of lipids from one tissue to another. In particular, in peripheral tissues, where it is produced by the liver and macrophages, regulates the cholesterol metabolism through a mechanism dependent from the isoform involved (Liu et al., 2013).

1.2.3 Morphological microscope and macroscope features of AD

The main microscope alteration of AD are two: the neurofibrillary tangles and the senile plaques. The Neurofibrillary Tangles are abnormal bundles of fibers that occupy the perinuclear cytoplasm of neurons of the regions typically affected by AD. In general, the tangles are composed of the microtubule-associated protein tau, which during the development of the disease becomes hyperphosphorylated, adopts an altered conformation and is localized in the axonal or somatodendritic compartment. Instead, in homeostatic conditions tau is involved in

microtubules modeling, signal transduction, organization of the actin cytoskeleton and intracellular transport of vesicles (Gotz et al., 2004; Selkoe, 2001).

Neuritic plaques (NPs) are extracellular deposits with a core of insoluble fibrillar β-amyloid protein (Aß) associated with axonal and dendritic damage present mainly in the cortex of the brain. The fibrillar structure of Aß which is more common in the plaques is $A\beta_{42}$ (aminoacid 42) that is the most hydrophobic form. Furthermore, co-localized with the $A\beta_{42}$ there is the $A\beta_{40}$, which is the most abundant fibrillar form in the brain. Plaques form slowly over the years and they have characteristic very variable between them: they present a diameter that varies greatly, from 10 to 120μm, but also the density and the degree of compaction of the fibrillar protein appear to be variable.

The formation of plaques in the brains of AD subjects is one of the first events that occur in the cascade process that leads to the appearance of cognitive symptoms (Gu and Guo, 2013; Selkoe, 2001).

1.2.4 Causes

1.2.4.1 The β-Amyloid protein

The β Amyloid is a protein that derived from the proteolytic cleavage of its precursor protein, called *Amyloid Precursor Protein* (APP). The APP is a single type 1 transmembrane polypeptide present in all the cells of the body with three major isoforms, arising from alternative splicing: APP695, APP751 and APP770. In particular, in neurons is present the APP695 isoform. This protein is involved in growth of neurons, synaptogenesis, calcium metabolism, cell adhesion and neuronal protein trafficking.

The protein is synthesized in the endoplasmic reticulum and subsequently, is trafficked through the secretory pathway to the cell membrane. During the trafficking is post translational modified, with the addition of N- and O- linked sugars, sulfation and phosphorylation. Once at the cell-surface, the APP undergoes a series of proteolytic cuts by the secretases, leading to the release of some protein fragments in the extracellular space. The first cut is made by the α secretase (ADAM10), which cuts in correspondence of the amino acid (aa) 12 to the N-terminal, within the Aß domain, and leads to the formation of a fragment of 83 amino acid residues, the α-APPs (Fig. 6). The peptide generated plays an important role in neuronal survival and is protective against the excitotoxicity. Some APP isoforms however are cut by the β-secretase (BACE),

which cuts at the residues 16 at the amino terminal end, generating a smaller fragment, the β-APPs (Fig. 6). Two isoforms of β-secretase were observed: BACE1, a membrane-bound aspartyl protease that is present only in the brain and that is considered the major β secretase, and BACE2, which is distributed throughout the body. The inhibition of BACE1 prevents the formation of Aβ deposits and is correlated to a rescue of cholinergic dysfunction, which is why this enzyme is a promising therapeutic strategy for AD (Harada et al. 2006; Selkoe, 2001; Zhang et al., 2011).

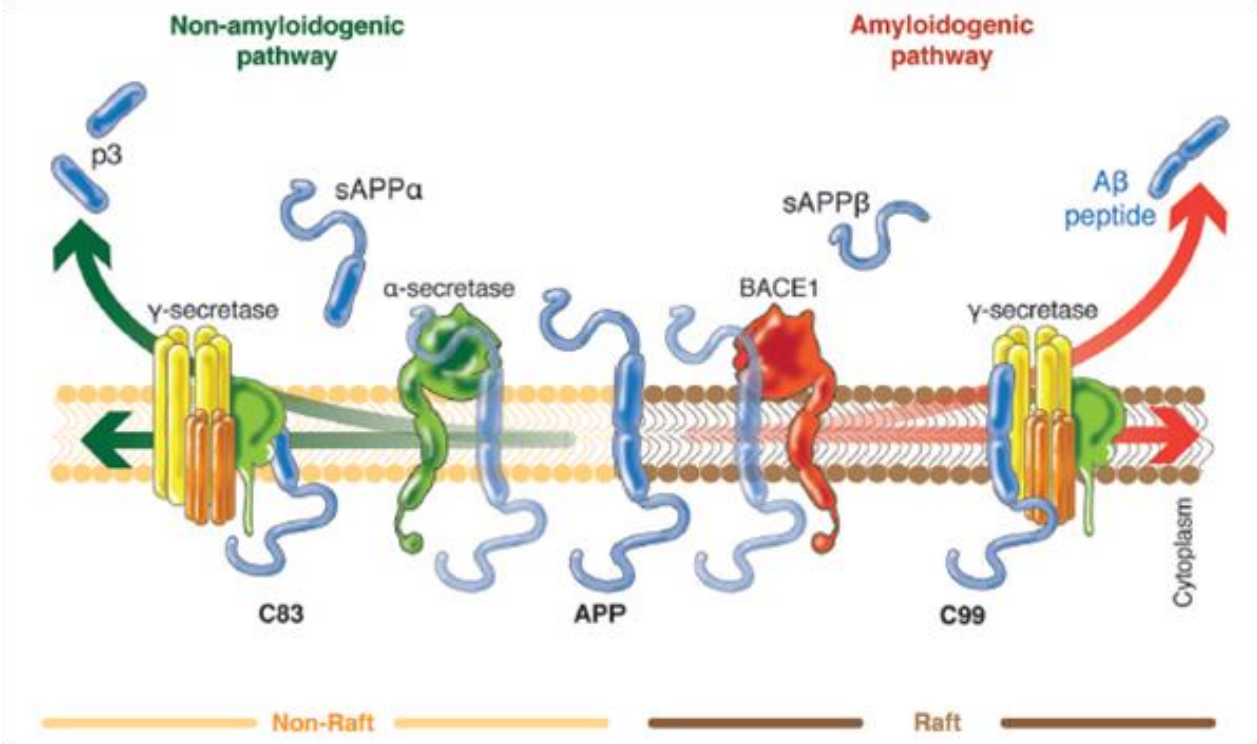

Figure 6. The figure shows the two processing pathways of APP: the non amyloidogenic and the amyloidogenic pathway. The fist leads to the release of p3 and α-sAPP by the clivage of α and γ-secretases. The last indeed, leads to the formation of β-sAPP and Aβ, by the clivage of β and γ-secretases (from Zolezzi et al., 2014).

After the first cleavages performed by α secretase and β-secretase, APP is subsequently processed through further cuts by the γ-secretase complex, composed of presenilin 1 and 2 (PS1 and PS2), anterior pharynx-defective-1 (APH- 1), nicastrin and presenilin enhancer-2 (PEN-2), at the carboxyl terminal end (Fig. 6). In physiological conditions, low levels of Aß are normally produced and released outside the cell where degraded and removed by degradative enzymes. The APP cuts by α and γ secretases forms the p3 peptide as a consequence of normal metabolic event precluding the formation of Aß plaques,

1.2.4.2The Aß protein causes AD

Until few years ago, the main theory regarding the pathogenesis of AD was the amyloid hypothesis (also called the amyloid cascade), according to which there was a close relationship between the onset of the disease and the accumulation of senile plaques in the brain. In particular, the cut of APP by a sequential cleavage of β and γ-secretases form $A\beta_{40}$ or $A\beta_{42}$ peptides that increase the accumulation and deposition of intracellular Aβ (Parkinson et al., 2013). This theory, which emerged in the '80s, affirms that the accumulation of fibrillar Aß in the brain was closely related to an alteration in neuronal circuits and synaptic dysfunction (Ferreira and Klein et al., 2011).

Furthermore, the presence of fibrillar Aß appears to be related to tau phosphorylation and then, causes the neurofibrillary degeneration. Indeed it has been proposed that the presence of Aß facilitates the tau phosphorylation, facilitating the activation of GSK3, a PDP kinase. This hypothesis is supported by several evidence including the fact that the Aß causes an increased formation of aberrant insoluble tau aggregates. Indeed, more detailed studies have shown that tau phosphorylation in dystrophic neurites of senile plaques is related to the accumulation of amyloid, but not to the tau phosphorylation in neurofibrillary tangles (Pérez et al., 2005).

In recent years, some studies have suggested the hypothesis that soluble Aß oligomers, but not the fibrillar Aß deposits, are responsible for triggering the synaptic dysfunction and the deterioration of cognitive functions,. Indeed, some recent studies, including that of Mucke and colleagues, have shown that the loss of synaptic function is not due to the presence of deposits of fibrillar Aß, but to soluble Aß oligomers, that causes memory impairment and neuronal death. The Aß oligomers inhibit the long-term potentiation (LTP) and the synaptic function in mature neurons. Also, they alter the trafficking of the ionotropic and metabotropic glutamate receptors. The reason for which the oligomers accumulate themselves remains still unknown. However, is known that the clearance of Aß in the AD pathology is reduced by 30% compared to healthy subjects, then an alteration of this mechanism could be involve in the accumulation of toxic oligomers. Is still unknown which of the different Aß assembly states is responsible for the neurodegeneration (Bao et al., 2012; Ferreira and Klein, 2011).

In this regard, a 2009 study shows that in a mouse model the Aß dimers are highly neurotoxic and cause impairment of the synaptic activity. On the contrary, the Aß monomers have a protective effect, designed to support the neuronal activity by activating the phosphatidylinositol-3-kinase (PI3K) pathway (Giuffrida et al., 2009).

1.2.4.3 The β-Amyloid protein in the brain

Aβ deposits were observed in the vessels of the cerebral cortex. Despite the presence of microvascular amyloid deposits, most of the subjects affected by AD have no presence of cerebral hemorrhages. In the brain APP protein is present not only in neurons, but also in astrocytes, microglia, endothelial and smooth muscle cells, contributing thus, to the Aβ that accumulates itself and forms the extracellular deposits. Furthermore, the Aß produced in the peripheral tissues and circulating in the blood may exceed the BBB by receptor-mediated endocytosis and contribute to the accumulation of toxic protein in the brain (Selkoe, 2001).

1.2.5 The neuroinflammation in Alzheimer's Disease

Many evidences exist demonstrating the involvement of inflammation in the development of AD, such as the elevated levels of pro-inflammatory cytokines in serum and brain tissue of AD patients and the co-localization of Aß to T cells, microglia, astrocytes and dystrophic neurites produced from the degeneration of neuronal processes. The accumulation of the Aß peptide in the parenchyma and the blood vessels induces the migration of microglia and promotes the beginning of a chronic inflammatory response directed against senile plaques, with the production of pro-inflammatory cytokines, NO, ROS and PGE2, that cause neuronal death.
These findings suggest that the neurological damage characteristic of AD is not only due to the accumulation of senile plaques and neurofibrillary tangles, but it might be the result of a strong chronic inflammatory response, whose main actors are astrocytes and microglia (Varnum and Ikezu, 2012).
In response to the pathological changes that occur during the AD, the astrocytes, which normally play a protective role in the brain, can be activated through Toll-like receptors (TLRs) and express high levels of glial fibrillary acidic protein (GFAP), vimentin, nestin and receptors that bind the Aß, such as receptor for advanced glycation endproducts (RAGE), receptor-like density lipoproteins, proteoglycans and scavenger receptors. RAGE is a receptor belonging to the family of the immunoglobulins, which binds several ligands, including products of non-enzymatic glycoxidation (AGEs), Aß and S100. Its expression is normally low in the CNS, but greatly increases during the disease. In particular, this receptor seems to be involved in the Aß-induced microglia migration, in the cytokine induction and in the impairment of the mechanism of Aβ clearence. (Fang et al., 2010). Then, the cells begin to phagocyte and degrade the amyloid

peptide, contributing to the removal of Aß deposits in the brain. These changes result in an impairment of normal neurological functions of the astrocytes such as the maintenance of the glutamate concentration in the extracellular space and the expression of inflammation-associated factors, such as S100β, a cytokine that promotes excessive neurite growth and that is correlated with the number of dystrophic neurites in AD patients. As a result, there is a local neuron depolarization, which causes the citotoxic damage. Moreover, the hyperactivation of the astrocytes, also called astrogliosis, which is characterized by an increased in numbers, size and mobility of cells, accelerates the progression of the disease and exacerbates the neuronal damage. So, the behavior of astrocytes seems to be closely related to the amount of Aß accumulated in the brain (Fig. 7) (Meraz Rios et al., 2013).

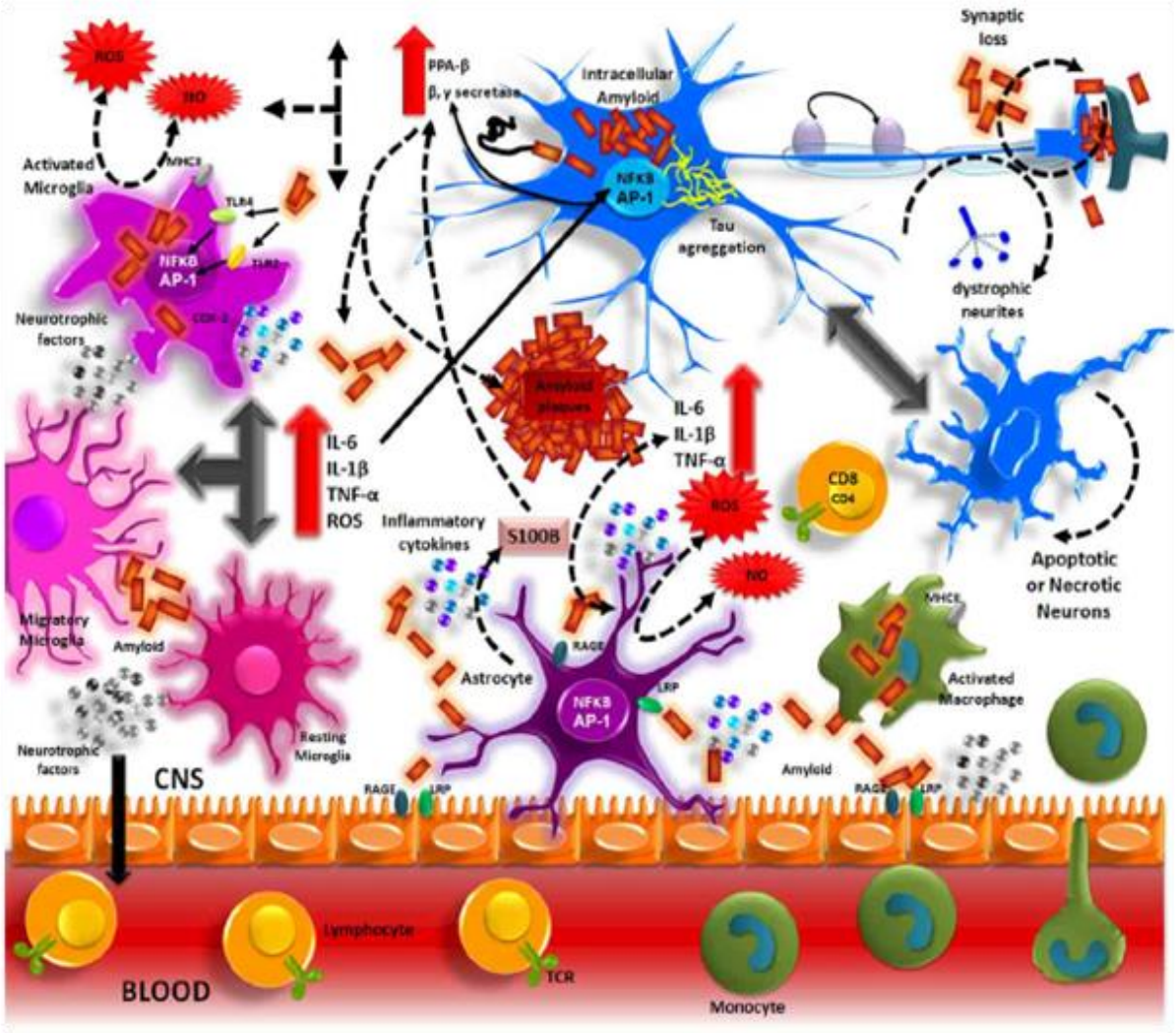

Figure 7. The figure shows the inflammation process of the Alzheimer's disease. Microglia is activated by the Aβ peptide through TLRs and RAGE receptors. Once activated, the cells produce pro-inflammatory cytokines, such as IL6, IL1β and TNFα, and the reactive oxygen species (ROS). These inflammatory factors stimulate the activation of the astrocytes, which amplify the inflammatory process and directly act on neurons (from Meraz Rios et al., 2013).

Assuming that also the microglial cells play an active role (positive or negative) in the development of AD, it is important to understand the factors that activate and direct them to the deposits of Aß. The interaction between Aß and microglial cells seems to involve different receptors, such as the CD14 and the β1-integrin receptor, the class B scavenger receptor type 2 (also called CD36), α6-β1 integrin and CD47 (integrin-associated protein). The latter receptor is not only involved in the Aβ internalization through a non-traditional pathway, but also in the production of superoxide by the microglia (Block and Hong, 2005).
Negative factor activating and directing the microglial cells to the deposits of Aß include RAGE, which stimulates the immune response exacerbating the neuronal damage, and the formyl peptide receptor 1 (FPRL-1). On the other side the positive factors such as TLRs, the CD36 and A scavenger receptors, the fractalkine, the receptor for the vascular endothelial growth factor 1 (VEGFR-1) and the proteolytic enzymes, such as the insulin-degrading enzyme (IDE), the neprilysin (NEP), matrix metalloproteinase 9 (MMP9) and plasminogen stimulate the phagocytic activity of microglia (Hickman et al., 2008; Mosher and Wyss-Coray, 2014).

1.2.6 The role of microglia in the pathogenesis of AD

The AD was one of the first neurodegenerative disorders to be associated with the activation of microglia in the neurotoxic sense. In 1987 McGeer and colleagues proposed a further explanation of the neuronal death typical of AD: the microglial cells. Indeed, they identified in post-mortem tissues of patients affected by AD, a considerable presence of microglial cells activated in correspondence of the Aß deposits. Over the years it has been confirmed its neurotoxic role in the pathogenesis of the disease. (Nayak et al., 2014).
The idea that plaque-associated microglia plays a role in the immunological pathogenesis dates back to 1989 when Sue Griffin showed that in a subject affected by AD these cells secrete IL1. Concurrently, it was published a work in which Goldgaber showed that the same cytokine modulates the APP synthesis. Therefore, this work suggested the importance of the immunomodulatory role of microglia in the development of neuritic plaques.
The microglial cells in pathological conditions are activated through TLRs and RAGE-dependent pathways and undergo a phenotypic change that seems to be connected to the cortical and hippocampal atrophy that is observed in the brains of patients with AD. Numerous studies report that during the progression of the disease the microglial cells switch from M2 (anti-inflammatory phenotype) to M1(inflammatory phenotype) phenotype and start to secrete pro-inflammatory

cytokines, stimulating the accumulation of the amyloid peptide and the disruption of neurogenesis, mitochondrial damage and the decrease in neural differentiation, causing a serious impairment of cognitive functions. Studies on the microglia's phenotype showed that the anti-inflammatory M2 phenotype has a protective effect in AD: cells with the M2a phenotype secrete IL10, which suppresses the inflammatory response of astrocytes and microglia and promotes the restoration of spatial learning and neurogenesis; cells with M2c phenotype secrete IL4 and also act on the Aß deposits reducing them and stimulating the neural differentiation. Therefore, an interesting therapeutic strategy would to favor again the switch of the microglia phenotype from M1 to M2 (Meraz Rios et al., 2013; Varnum and Ikezu, 2012).

However, the involvement of microglia seems to have a different basis, linked not only to the Aβ pathology, but also to taupathy and cognitive deficits. Indeed, microglial cells are distributed not only in proximity of the senile plaques, but also of the neurofibrillary tangles. The overproduction of IL1 by activated microglia, especially when the cells is located near the neuritic plaques, cause excessive tau phosphorylation and is related to the tangle development (Mrak and Griffin, 2005; Varnum and Ikezu, 2012).

The Alzheimer's disease is characterized by numerous susceptibility genes, some of which are also expressed by microglia, confirming that these cells play a role in the pathogenesis of the disease. One of these is CD33, which if altered prevents the ability of microglia to phagocyte the Aß: it is well known indeed, that the AD in humans is also associated with a reduced clearance of Aß. This indicates therefore, that the phagocytic system of the CNS is compromised or is overwhelmed by the continuous deposition of Aß (Nayak et al., 2014).

Furthermore, recent studies have shown that also TREM2 is a genetic risk factor for AD, in particular for the LOAD, and it appears to be up-regulated in the plaque-associated microglia. So, mutations in this gene would cause an impairment of microglia clearance abilities (Mosher and Wyss-Coray, 2014).

Based on the involvement of receptors as TLR2, TLR4 and CD36, it becomes increasingly clear that the degenerative diseases as the AD trigger the immune system through the release of pro-inflammatory signals (Nayak et al., 2014).

For this reason, there are several ongoing clinical studies designed to investigate the efficacy of anti-inflammatory therapies in Alzheimer's disease. It has been seen that the inhibition of the activation of microglia reduces the neuronal toxicity. In particular, a study on a mouse model of AD notes that the non-steroidal anti-inflammatory drug (NSAID) treatment induces the reduction of Aß deposits, stimulates the non-amyloidogenic APP-processing pathway, inhibits the activation of microglia and consequently, produces the neuroprotection. Unfortunately, these

drugs have serious adverse effects that undermine the effectiveness and the safety of the treatment.

However, these studies show, once again, that microglia is involved in the pathogenesis of AD (Block and Hong, 2005).

However, the precise role of microglia in the pathogenesis of AD remains controversial. Indeed, studies in a mouse model of AD show that the selective ablation of microglia has no effect on the formation of plaques. Data on animal models of AD show also that the phagocytic activity of microglia decreases over time, especially during the course of a degenerative disease.

Therefore, it's still unclear to what extent the microglial cells is involved, via phagocytosis, in opposing the progression of AD. It's plausible, however, think that they plays an important role in the early stages of the disease, in which they slow the Aβ toxic effects, while they seems to be less involved in the late stages of the disease, in which their phagocytic function decreases. In the final stages of the disease instead, they plays a pro-inflammatory role, characterized by the production of cytokines that going to exacerbate the inflammation and promote the cognitive decline.

Interestingly, the locus coeruleus, which is damaged in the majority of AD patients, is able to decrease the phagocytic ability of microglia through the reduced production of norepinephrine and to promote the chronic release of proinflammatory cytokines.

2. Aims of the thesis

The ability of cells to communicate with the surrounding environment is a fundamental property for the normal tissue homeostasis, and defects in such crosstalk might be at the basis of the many pathological states. In the brain, neurons are able to communicate one with each other through specialized cellular junctions called synapse, composed by a presynaptic terminal and a post synaptic compartment. Although neurons represent the essential functional unit of the brain, recent studies have demonstrated that astrocytes actively participate in the modulation of synapse properties, thus rising the concept of a tripartite synapse in which astrocytic cells tightly cooperate with pre and post synaptic terminal. Beside their role in controlling synaptic function, astrocytes play a fundamental role in neuronal development and survival, as demonstrated by the evidences that several neurological disorders are characterized by altered functions of astrocytes in supporting a correct neuronal growth. All these findings draw a very complex picture of the interplay between different brain cells types, in which neurons and astrocytes give rise to a fine network of interconnections fundamental for both physiological and pathological states. Given the large number of possible combinatorial interactions between these cells and since cellular responses can be modulated by spatial and temporal signals from the surroundings, there is a critical need for the identification of new strategies enabling cell-to-cell interactions to be addressed while manipulating cellular microenvironments.

The aims of my PhD project are the following:

1. development of a standardized screening test on primary cell cultures based on microfluidic system in order to recreate complex environments, thus dissecting the interactions between different cell types. Taking advantage of such microscale device, to perform a pharmacological study in an experimental model of Alzheimer's disease
2. to investigate the differential effects of astrocytes derived from distinct brain areas on neuronal development in RETT Syndrome.

The results from this study will provide new information about the molecular mechanisms underlying the glial modulation of neuronal function both in healthy and pathological conditions.

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3. Materials and methods

3.1 Culture of primary neurons

The primary cultures of hippocampal neurons of mouse embryos was obtained from the eighteenth day of gestation (E18). Primary cultures are prepared according to the method described by Banker and Cowan (1977) and Bartlett and Bunker (1984). After sacrificing the animals, you extract the brains and dissect the cerebral hemispheres. The next step is the removal of the meninges and subsequently separate seahorses from the barks. Are subjected to two washes in a solution of Hanks balanced salts, HBSS (Invitrogen), at 4 °C. Thereafter, the hippocampi isolated are subjected to digestion 0.25% trypsin in HBSS for 10 minutes at 37 ° C. Hippocampi are then washed 3 times (washes for 5 minutes) and mechanically dissociated in HBSS. The dissociated cells are plated on coverslips previously treated with poly-L-lysine, in Neurobasal medium (Invitrogen) supplemented of B27, 0.5 mM glutamine, and 12.5 μM glutamate (Brewer et al., 1993). The neuronal cultures are maintained in an incubator at 37 ° C in the presence of 5% CO2. These cells require a change of means of nourishment 3 days after plating.

3.2 Culture of primary astrocyte

The cortical and hippocampal astrocytes are isolated from the cerebral cortex and hippocampus of neonatal rats 2 days (P2) in accordance with the method described by Booher and Sensenbrenner (1972), subsequently modified by Kimelberg et al. (1978). Once sacrificed animals by incising the head, are extracted brains and kept in saline HBSS [(Hank's Balanced Salt Solution), physiological saline without Ca2 + and Mg2 +, containing HEPES 10 mM, a mixture of the antibiotics streptomycin 100 mg / ml and penicillin 100 U / ml (Gibco)] cold. With the aid of an optical microscope with a scalpel and the brains are cut, so as to eliminate the cerebellum and separate the two hemispheres. We then proceed to the step of removing the meninges and the cerebral cortices of isolation which are then shredded with a razor blade. The material obtained is incubated at 37 ° C for 15 min in HBSS containing DNAse (0.001% w / v) and trypsin (0,25% w / v) to digest the tissue. If the fabric is not completely digested this operation is repeated twice. The two supernatants are recovered and, after inactivation of trypsin

with serum, the whole is filtered with a nylon membrane with pores of 72 uM in diameter, to remove the tissue that would otherwise remain undissociated. The suspension containing the cells, is centrifuged for 10 min at 800 rpm at room temperature and the pellet is resuspended in glial MEDIUM [Minimum Essenzial Medium (MEM, Gibco), 20% (v / v) calf serum (FCS, Invitrogen), 0.6% glucose (w / v), and a mixture of antibiotics penicillin (100 U / ml) and streptomycin (100 U / ml)].

The cells are dissociated mechanically by pasteur pipette glass and plated in sterile flasks; then are maintained in a humidified atmosphere, at a constant temperature of 37 ° C and with 5% CO2 until 60-70% confluence, which typically is achieved in about ten days. These cells require the change of the ground, at least once a week.

3.3 Culture of primary microglial cells

Primary cultures of microglial cells were prepared from brains of postnatal 1–2 days old C57BL/6 mice. The brain was extracted and maintained in cold HBSS [BSS (Gibco®), supplemented with Hepes 0.3M (Sigma Aldrich), 100µg/ml streptomycin and 100 U/ml penicillin (EuroClone). Later, tissues were cut and the homogenate was collected in HBSS in a 50ml Falcon tube with digestion buffer: HBSS supplemented with 0.3% Trypsin (Sigma Aldrich) and 0.25% DNAse (Roche, Mannheim, Germany). The homogenate was incubated twice 15 min at 37°C in digestion buffer. The digestion was blocked with Eagle's minimal essential medium (MEM, Gibco®) supplemented with 10% Fetal Bovine Serum (FBS, EuroClone®), 33mM Glucose, 2Mm L-ultra glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin. Cell suspensions obtained from the digested brains were filtred on 80 µm nitex membrane and centrifuged 10 min at 800x g and 20°C. In the end, the cell pellet was resuspended in medium of growth and the cells were seeded T75 flasks.

After seeding, the cells were left grow without change the medium. In this time, microglial cells grow on the astrocytes monolayer. After 10 days microglial cells were harvested by shaking the flasks at 230 rpm for 45 min at room temperature (RT)on the shaker: the medium in excess from each flask (were left 5ml of medium for flask) were collect in a 50ml falcon and left in the incubator for the shaking time. Then, the flasks were submitted to shaking. After the shaking, the medium from each flask was collected in a falcon and centrifuged at 800g for 10 min. The pellet obtained by centrifugation was resuspended in glial medium and cells seeded about 150000

cells/ml on the appropriate plate, previously coated with 0.05 mg/ml poly-ornithine (Sigma®) diluted in water.

3.4 Fabrication of microdevices

3.4.1 oMFNs fabrication and Setup

oMFNs were fabricated by casting PDMS (Sylgard 184, Dow Corning, Midland, MI) onto microstructured 4 inch Si wafers (Siltronix, Geneva, Switzerland) used as molds. These Si molds were made using standard photolithography (64 000 dpi polymer masks, Zitzmann GmbH, Germany) and deep reactive ion etching (STS ICP, Surface Technology Systems, U.K.). The Si molds were coated with a fluorinated material (1H,1H,2H,2H-perfluorodecyltrichlorosilane from ABCR GmbH, Karlsruhe). The PDMS oMFNs were separated using a scalpel and stored in a plastic dish until used. The Si lids were fabricated using similar lithography. Nano part assemblies were mounted to the backside of the lids, in alignment with the ports for liquid connection. Prior to the seeding of cells, the oMFNs were treated with an oxygen-based plasma for 30 s (Technics Plasma 100-E, Florence, KY) at 200 W of coil power and then coated with a 0.5 mg mL solution of poly-L-lysine. The oMFNs were incubated with the poly-L-lysine overnight at room temperature. After a washing step with PBS and water, the oMFNs were dried under a stream of N_2, and cell suspensions were added onto the chambers. This was done by placing ~750 cells mm−2 in each chamber of an oMFN. The oMFN was then placed in a Petri dish with a few milliliters of water next to it to prevent evaporation of the medium in the cell chambers. The Petri dish with the oMFN was then kept in an incubator for up to 10 days. If necessary, a replacement of growth medium during incubation was done using a pipet. The tubing and fittings needed for microfluidic experiments were purchased from Upchurch Scientific. Active pumping of liquids was done using high-precision syringe pumps (Cetoni GmbH, Korbussen, Germany), which were equipped with 50µL glass syringes (Hamilton, Bonaduz, Switzerland). A custom- made aluminum holder facilitated the assembly of the oMFN with the lid. During this procedure, the lid was normally connected to prefilled tubing to prevent air bubble formation in closed oMFNs.

3.4.2 MFN fabrication

Photolithography based on SU-8 photoresist was used to fabricate silicon moulds with depth features of 100 μm, which will be the depth of the microfluidic channels. To fabricate the masters for replica molding, the SU-8 photoresist was spin coated (500 rpm for 13 s and 2000 rpm for 30 s) on silicon wafers and pre-baked at 65 °C for 5 min and at 95 °C for 30 min. The resist was exposed to 260 mJ cm−2 UV light through ink-printed masks and post-baked at 65 °C for 5 min and at 95 °C for 12 min. After development, the wafers were washed thoroughly in isopropanol and dried under N_2 gas. To facilitate peeling-off during the replication process, the silicon masters were coated with a Teflon-like layer, CFx, deposited by plasma polymerization. The patterns created in SU-8 were transferred to PDMS by replica molding. A mixture of polymer precursors was made by stirring vigorously 1 : 10 parts of base prepolymer : curing agent (Sylgard 184). The viscous mixture was poured on the masters, degassed under a vacuum at room temperature for 1 h and cured at 65 °C for 4 h. Then, the PDMS wafer was cut to release the individual microchips, which were punched to open up the cell chambers and the inlets–outlets for microfluidic flowing. Finally, the chips were covalently bonded to glass coverslips by applying oxygen plasma treatment (30 W, 30 s, 5 sccm O2). Before cell seeding, the MFN were sterilized with ethanol for 10 min, followed by UV treatment for 40 min.

3.5 Cell death assay

Neuronal cell death has been evaluated through in vivo labeling with propidium iodide (PI) and DAPI. Cell cultures were incubated for 10 minutes at 37 ° C with PI (20μg/ml) diluted in PBS, then after a quick wash with PBS neurons were imaged using the fluorescence microscope (Leica Ctr4000). The percentage of cell death was calculated as the percentage of PI-positive dead cells respect to the total number of cells stained with DAPI. Images were analyzed by ImageJ software.

3.6 Calcium imaging

To monitor changes of intracellular calcium at single cell level, we have used the ratiometric calcium sensitive dye Fura-2 (Molecular Probes). The spectral characteristics of the Fura-2

represent an important tool for the quantitative analysis of intracellular calcium concentrations. The fluorescent indicator is capable of translating the variations of calcium changes in fluorescence intensity.

The Fura-2 possesses two different emission peaks after excitation at a wavelength of 340nm and 380nm. Upon binding of Fura-2 with the calcium ion occurs a change in intensity of emission in the two peaks. In particular high concentrations of intracellular calcium fluorochrome emits the maximum intensity of fluorescence when excited at the wavelength of 340 nm and the minimum intensity to 380nm.

The measurement of changes in cytoplasmic calcium is obtained by following the ratio between the fluorescence emission at a wavelength of 340nm to 380nm compared to the release. This allows monitoring the levels of calcium inside the cell surface excluding the signal variation due to the different volumes of cells. The indicator Fura-2 may be present in two forms: one form the ester present in the extracellular level and form a de-esterified at the intracellular level. The de-esterification of the tracer, once penetrated the cell membrane, involves the maintenance of Fura-2 so as to enable analysis of changes in the ion concentration inside the cell. To be able to analyze the variations of intracellular calcium, the cells are loaded for 45-60 min at 37 ° C with the ester form, permeable to membranes, the indicator for calcium Fura-2 at a concentration of 1 mM in solution Krebs- Ringer-Hepes (KRH: 150 mM NaCl, 5 mM KCl, 1.2 mM MgSO 4, 2 mM CaCl2, 10 mM glucose, 10 mM Hepes / NaOH, pH 7.4) containing 1% BSA. After washing the cells, they are transferred to a recording chamber mounted on an inverted microscope (Leica CTR6000) equipped with a unit of calcium imaging. The recordings were performed using a Polychrome V (Till Photonics) lighting system. The fluorescence images were acquired, digitized and integrated in real time by the software package Imagin Workbench 6, able to provide in pseudocolor the ratio between the images at 340/380 nm. The experiments were conducted at room temperature (20-25 ° C).

3.7 Transfection of Cultured Neurons

Neurons were transfected with GFP plasmids at 7 and 11 using Lipofectamine ™ 2000 (Invitrogen) and OptiMEM (Gibco). The transfection mix was prepared in two parts of equal volume: one part containing the DNA, the second part containing Lipofectamine (total volume 100 µl for each glass cover slip). Each component was gently mixed, incubated at room temperature for 5 min before being combined, mixed again, and incubated at room temperature

for further 20 min. Cells were incubated with the transfection mix for 30 min at 37° C in a 5% CO2-humified incubator. Following incubation the medium of culture (NeuroBasal + B27) was added and cells incubated at 37°C, until immunofluorescence experiments were performed.

3.8 Morphological analysis of neurons transfected

The morphological analysis has been performed with ImageJ software (NIH, Bethesda, USA). Total number and length of neurites were determined using neuronJ plugin by quantifying all the neuritic processes of GFP-expressing neurons at 8 and 12 DIV. Neurites were classified as primary neurites, defined as those neurites originating from the cell body, and secondary neurites, defined as those neurites originating from primary neurites. The starting point of a neurite was defined as the point of the dendrite that intersected the curvature of the soma. We also performed Sholl analysis to evaluate the dendritic branching complexity. This analysis was conducted by means of Sholl analysis plugin, counting the number of dendrite intersections for concentric circles centered at the centroid of the cell body.

3.9 Oligomeric and fibrillar Aβ preparations

To prepare the oligomeric and fibrillar Aβ, Aβ1–42 (AnaSpec) was first monomerized by dissolution in pure hexafluoroisopropanol (Sigma) to obtain a 1 mM solution and then aliquoted in sterile microcentrifuge tubes. Hexafluoroisopropanol was removed under vacuum using a SpeedVac concentrator and the peptide film was stored in a dessicator at −80 °C. For each experiment, an aliquot of the dried HFIP peptide film was freshly resuspended in 100% DMSO to 5 mM and further diluted to 100 μM in F12 medium for preparing the oligomeric Aβ or in 10 mM HCl for the fibrillar form. Oligomers were incubated for 24 h at 4 °C and then centrifuged at 14 000 g for 10 min at 4 °C. The oligomers were collected from the supernatant after centrifugation. Fibrils were vortexed for 15 sec immediately after dilution in HCl and incubated for 24 h at 37 °C or incubating freshly solubilized peptides at 50 μM in sterile water for 5 days at 37 °C

4. *Results*

4.1 Glia-neuron interaction: use of oMFN to study cells interactions in AD contest.

4.1.1 Capabilities of oMFN and functional analysis of cells

To investigate possible differences in neuronal growth mediated by glia (different brain area derived) in Alzheimer disease I used microfluidic system. Here, we use overflow microfluidic networks (oMFNs). Such microfluidic chips permit depositing and culturing cells for days and afterward closing the chambers with a lid for circulating precise amounts of chemicals/solutions throughout the chambers. Here, we specifically combine microfluidic-based experiments (i.e., culture and stimulation/stress of cells) with characterization methods such as immunocytochemical staining, quantitative intracellular calcium imaging.

The oMFNs used for all experiments presented here are made in poly(dimethylsiloxane) (PDMS). The oMFNs have a footprint of 32 × 26 mm^2, and the small separation distance of 1.6 mm between the chambers (edge-to-edge distance) allows visualization of both chambers simultaneously using a 4× microscope objective. The layout of the channels and corresponding ports liquids to be drawn sequentially or independently, if needed, through the chambers. The oMFNs are briefly treated with an oxygen-based plasma and coated with poly-L-lysine before a cell suspension is placed on each cell chamber. The overflow zones around the cell chambers have numerous wettable microstructures “overflow zones”, which withdraw any excess liquid during the closing of the chip and ensure a sealing of the cells chambers. The figure 8a shows that different liquid droplets deposited on each cell chamber do not mix when the chip is closed. After sealing the oMFN with a Si lid, six ports are available for establishing specific culture or stimulating conditions by pumping media through the chambers with well-defined flow rates for the required time. For example, ports 1, 2, and 5 can be used to flush liquids through chamber A sequentially or in parallel so as to create a biochemical gradient in this chamber. For flushing a chemical in both chambers, port 2 can be used while keeping ports 1, 3, 5, and 6 closed and port 4 open. The volume of each chamber is approximately 0.5 μL, thus minimizing the amount of cells required. Moreover, by using high-precision pumps with flow rates ranging from 10 to 50 nL s^{-1} very small amounts of reagents or culture media can be drawn through the cell chambers. For the experiments described in this study, primary HNs from E18 rat embryos were seeded in a cell chamber and cultured for up to 10 days in vitro (DIV) before seeding primary astrocytes from P2 pups in the other chamber. Cell containing oMFNs can be placed in an incubator, and

the medium can conveniently be exchanged by pipetting as much and often as required before performing experiments. The figure 8b shows the different combinations of distinct cell populations plated in the chambers, the cell chamber connectivity, and the modalities of cell stimulation used in the present study. Cells can be grown in chambers either separately (||) or while being in microfluidic communication (↔). One cell population can be separately challenged with specific stimuli for a desired duration while the other cell population is kept perfused with culture medium (i.e., As||Bc) and then put into microfluidic communication for the required experimental time (i.e., As↔ Bc). Alternatively, cells can be challenged while in microfluidic communication, thus enabling analysis of functional effects produced by soluble factors and/or organelles released from cells cultured in chamber A on target cells in chamber B. Target cells can be regularly inspected and assessed in terms of morphological/functional properties using standard optical, fluorescence, and electrophysiological characterization methods. Primary HNs plated on oMFNs develop normally and form wide networks of cells connected by synaptic contacts, as revealed by immunocytochemical staining with βIII tubulin (Fig. 8b). The ability of neurons plated on oMFN to form synaptic contacts (sites of communication among neurons) is demonstrated by immunocytochemical staining for the synaptic vesicle glutamate transporter vGlut which labels presynaptic boutons (Fig. 8b, arrowheads). Quantitative evaluation of intracellular calcium concentrations (Fig. 8b) shows basal intracellular calcium levels comparable to neurons maintained under standard culturing conditions on poly-L- lysine-coated glass (F340/380; glass, 0.69 + 0.04; PDMS, 0.56 + 0.03; n = 20, p = 0.35, Student's t test) and a similar amplitude of the peak response following exposure to depolarizing stimuli with 50 mM KCl (F340/380; glass, 0.14+ 0.01; PDMS, 0.15 + 0.01; cells number = 15, three independent experiments, Student's t test). Furthermore, HNs grown on glass or oMFNs show comparable amplitude (pA; glass, 92.67 + 10.01; PDMS, 105.97 + 15.45; cells number = 10, three independent experiments, p = 0.26, Student's t test) and frequency (Hz; glass, 2.74 + 0.58; PDMS, 2.41 + 0.73; cells number = 10, three independent experiments, p = 0.97, Student's t test) of miniature excitatory postsynaptic currents (mEPSCs) recorded using whole-cell patch-clamp (Fig. 8). These data indicate that primary neuronal cultures from rat hippocampus grown on oMFNs exhibit morphological, functional, and electrophysiological properties similar to those of neurons grown in standard culturing conditions on glass coverslips; hence, growth of cultures in the cell chambers of the chip neither influences neuronal viability nor the overall cellular phenotype and development.

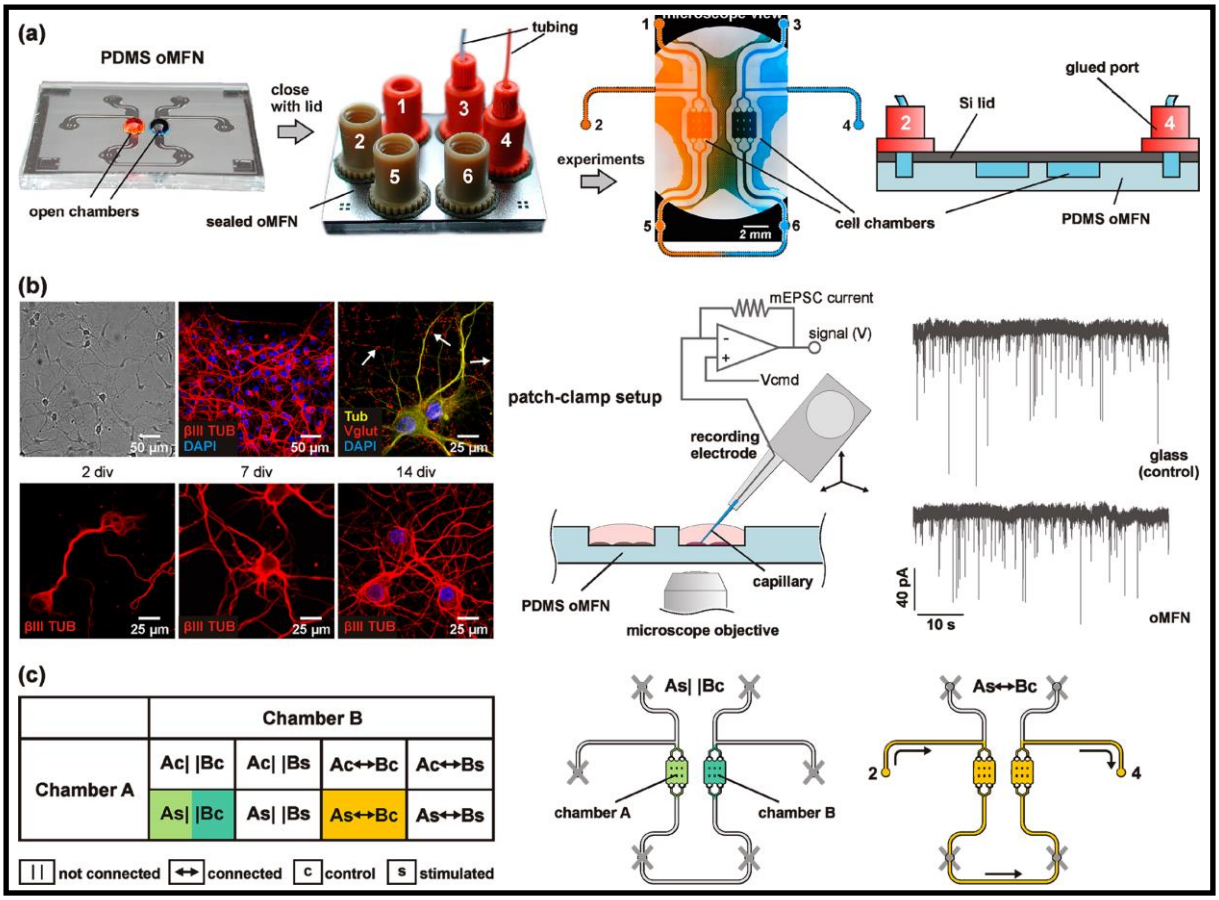


Figure 8. Illustration of an oMFN having two cell chambers, functional analysis of the cells in the chambers, and protocols for probing cells interactions. (a) Cell cultures can be grown independently, each with its own culturing medium, on the vicinal cell chambers of a oMFN made in PDMS. The oMFN can subsequently be closed using a lid at the appropriate culturing time. The wettable microstructures around the chambers wick away excess culture medium, guaranteeing perfect sealing and avoiding mixing of liquids. The six ports glued on the lid are in regard with microchannels servicing the chambers and can be left open, closed with a threaded plug, or connected to a computer-controlled syringe. For example, injecting a solution via port 2 while leaving port 4 open and the other ports closed results in perfusing both chambers one after the other. (b) Before closing the oMFN with a lid, cells can be extensively monitored as shown in these representative images of HNs grown on oMFNs. Cells differentiate in culture and express late-stage specific markers (i.e., Vglut), and staining with βIII tubulin shows development of the neuronal network. Moreover, representative traces of mEPSCs on HNs grown either under standard culturing conditions on glass or on oMFNs are shown. These traces exhibit comparable amplitude and frequency (see text for details). (c) Table and sketch showing experimental combinations for culturing and stimulating different cell populations plated in the chambers.

4.1.2. oMFN to study the role of hippocampal (HAs) and cortical (CAs) astrocytes on neuronal viability in the context of Aβ insult.

To evaluate the different effect on the neuronal viability exerted by astrocytes derived from different brain regions we employed oMFNs to dissect cell-specific contributions in neuroinflammatory context, namely, the exposure of brain cells to amyloid β fibrils. HNs were grown in chamber B, while either CAs or HAs were grown in chamber A (Fig. 9a) in open microfluidic setting. CAs and HAs in chamber A were independently exposed during 24 h to either Aβ alone or to Aβ + the proinflammatory cytokine IL-1β (Fig. 9a), washed, and put into closed microfluidic communication for 6 h with HNs (chamber B).

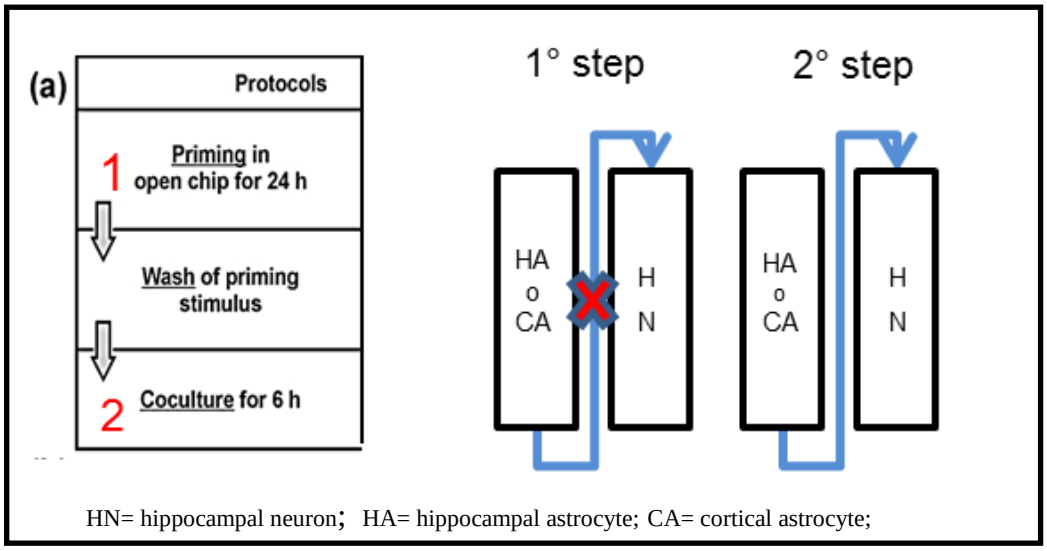


Figure 9a. Role of hippocampal (HAs) and cortical (CAs) astrocytes on neuronal viability in the context of Aβ insult. Protocols involved HNs that were cocultured with either CAs or HAs, previously primed with Aβ or with Aβ + IL-1β.

HAs, CAs, and HNs independently challenged with either Aβ or Aβ + IL-1β do not show significant cell death (HN = 2.65 + 1.72; HA = 6.71 + 1.33; CA = 5.97 + 1.12; cells number = 15, three independent experiments, post hoc Tukey's method). Also, HNs, put into closed microfluidic communication for 6 h with CAs and HAs, previously primed only with Aβ or IL-1β, show only a moderate increase in neuronal death, thus confirming the previously reported observation that IL-1β or Aβ fibrils alone do not heavily impact neuronal viability . However, when HNs were put into closed microfluidic communication with HAs previously challenged with Aβ + IL-1β, a significant increase in cell death was detected which was not observed in HNs cocultured with CAs under the same conditions (Fig. 9b-c, HN = 6.48 + 1.47; cells number

= 15; three independent experiments, p = 0.35, post hoc Tukey's method. HN = 16.20 + 2.44; cells number = 15; three independent experiments, p < 0.01, post hoc Dunnett's method).

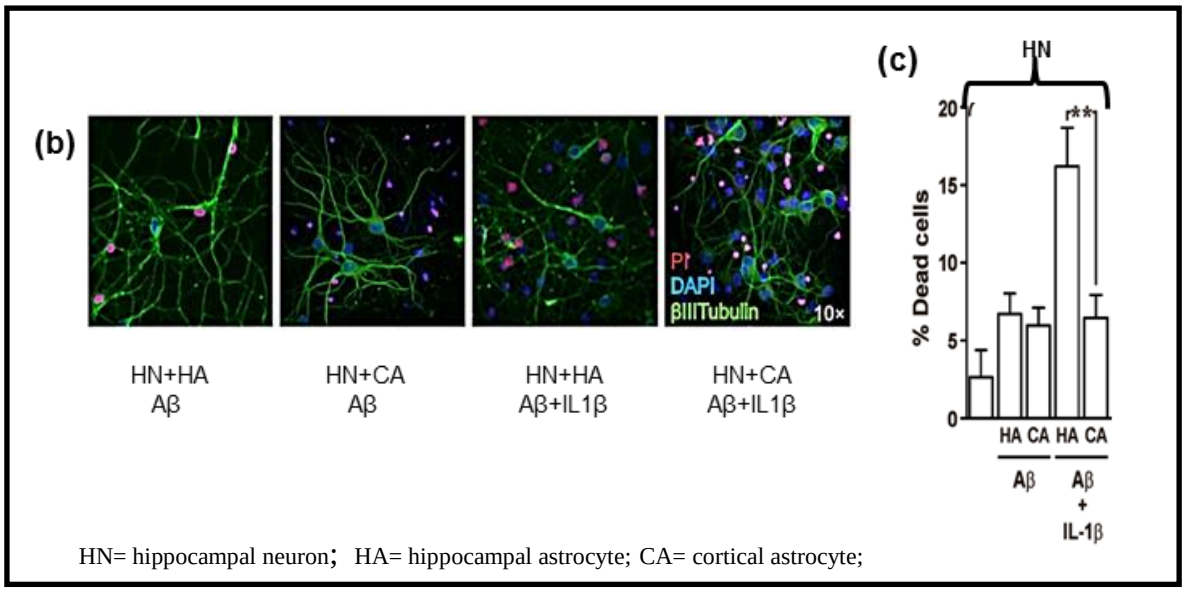


Figure 9b-c. Role of hippocampal (HAs) and cortical (CAs) astrocytes on neuronal viability in the context of Aβ insult. (b) Representative vitality images showing DAPI (blue)/PI (red) and βIII tubulin (green) staining of HNs from the four protocols described in panel a. (c) Comparison of death rates of HNs (unlabeled bar). HNs in microfluidic communication with HAs shown a significant increase of cell death. This is not observable when HNs are cocultured with CAs.

4.1.3. HNs grown in oMFN and challenged with HA postpriming medium (PPh) exhibit higher calcium transients as compared to HNs exposed to CA postpriming medium (PPc).

To address whether HN damage may result from the release of soluble mediators by HAs when exposed to Aβ + IL-1β, we took advantage of the possibility to close the oMFN lid and flush the cells in chamber B with the medium from chamber A, while monitoring cellular response using single-cell calcium imaging. Astroglial cells, either HAs or CAs, independently cultured in chamber A of open oMFNs, were then primed with Aβ + IL-1β for 24 h, washed, and further cultured for 6 h (Fig. 10a). Subsequently, the chambers were put into microfluidic communication using the closed microfluidic setting, and intracellular calcium dynamics of HNs in chamber 2 were acquired by time-lapse ratiometric single-cell calcium imaging acquisitions (Fig. 10b). A quantitative analysis of the response of HNs to either HA postpriming medium (PPh) or CA postpriming medium (PPc) was then carried out. The figure 10c shows that HNs

challenged with PPh exhibit higher calcium transients compared to HNs exposed to PPc. The respond similarly to 50 mM KCl stimulation exclude a different intrinsic neuronal responsiveness to depolarization (Fig. 10c). In both cases, the calcium elevation produced by the postpriming solution was blocked by the NMDA receptor blocker APV (Fig. 10b).

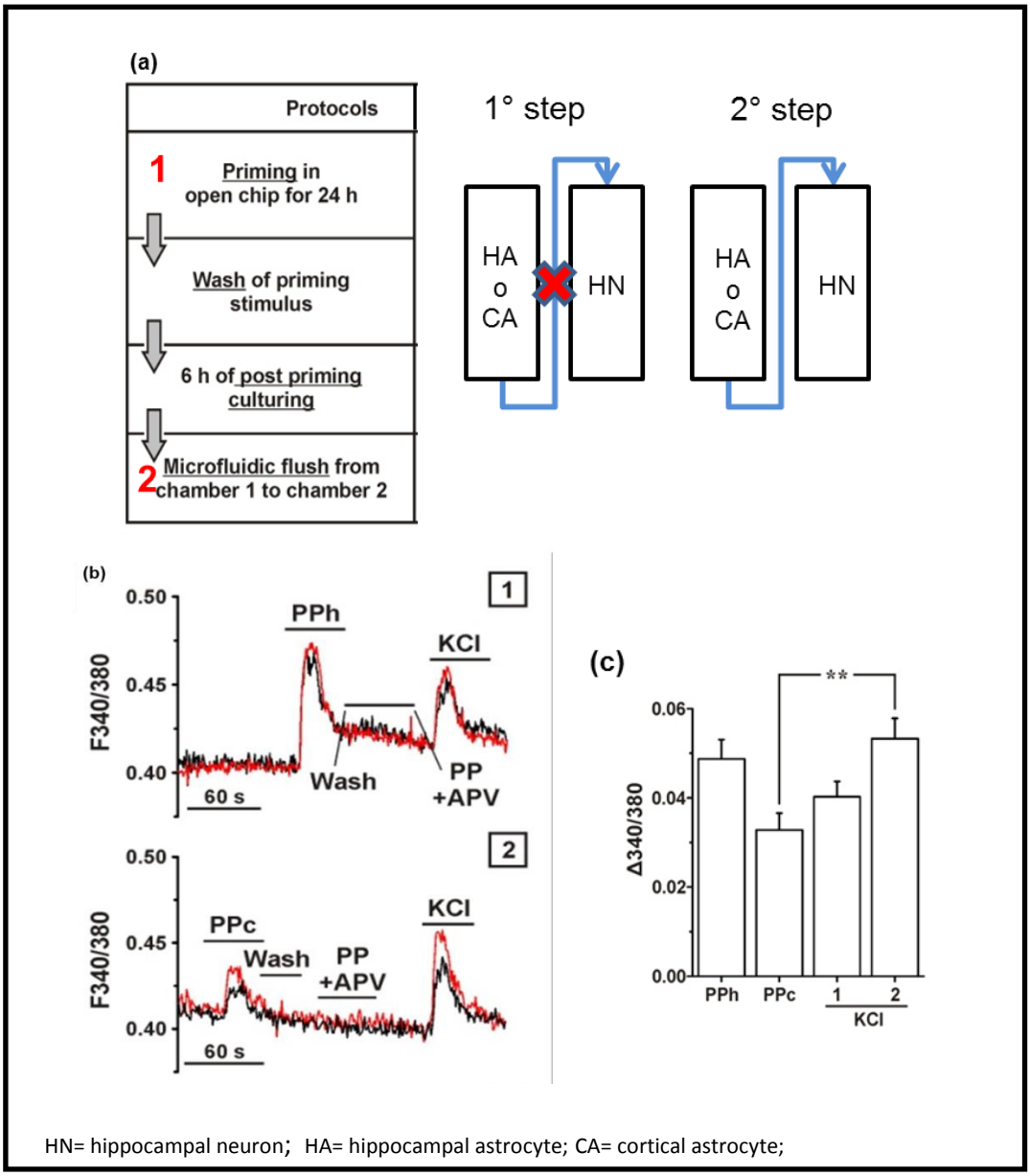


Figure 10. (a) Representation of the experiments in which hippocampal and cortical astrocytes are primed with Aβ + IL-1β and then either further cultured independently. (b) Two representative traces showing single-cell ratiometric calcium acquisitions of HNs in chamber B exposed to glial-conditioned medium from chamber A coming either from independent culturing (PPh = postpriming medium from HAs; PPc = postpriming medium from CAs; CMh = coculture medium from HAs; CMc = coculture medium from CAs). (c) Quantification of either cell calcium influx in HNs following exposure to glial-conditioned medium either from HAs (PPh) or CAs (PPc) with respect to biochemical depolarization by exposure to 50 mM KCl.

4.2. MFN devices to screen Aβ toxicity on different populations of CNS cells

The microsystems can also be used as method to screen the effect of toxic agent and drugs on different populations of CNS cells. Here, we use microfluidic networks (MFN) in order to analyze the neurotoxicity of different forms of AB when neurons are incubated alone or in the presence of glial cells. In this case, the microfluidic chips have been fabricated through replica moulding in PDMS of a master produced by SU-8 lithography, and are operated by gravity-induced flow. The figure 11 shows the schematic of the chip configuration and the principle of operation. By applying a difference of the liquid level between the inlet and the outlets, the substances flow from the inlet, through the cell chamber and towards the outlets. The surface tension in the open cell chambers avoids the spread of liquid outside the confined area providing microfluidic perfusion over the cell compartment when the difference in liquid height is applied. The chips consist of 4 chambers with an area of 4.6 mm^2, placed in a circular position and connected by 50 μm wide, 100 μm depth microchannels. Different cell combinations can be plated in each cell chamber depending on the cells required for the experiments. The PDMS chips are punched and then covalently bonded to standard glass coverslips (24 mm diameter), which allows plating the cells in direct contact with glass and ensures compatibility with high-resolution microscopy characterization. Stimuli, applied through one inlet, are divided in 4 parallel flows in 4 microchannels and driven into the cell culture chambers.

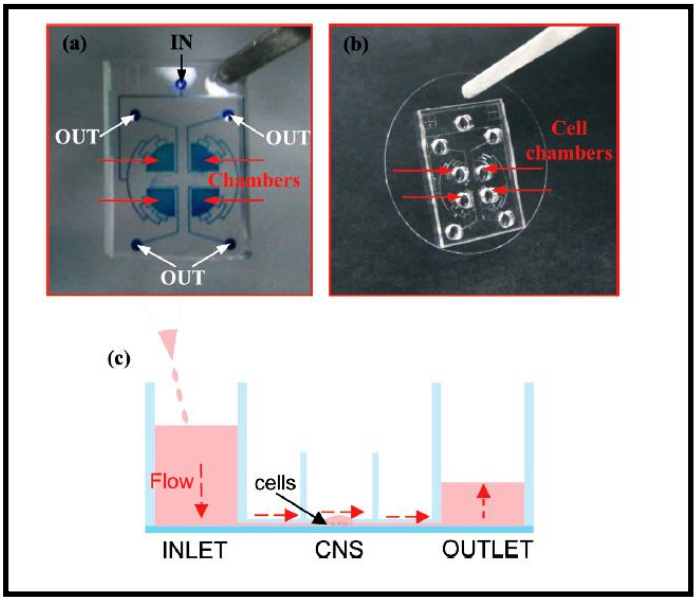


Figure 11. (a) PDMS chip before punching and mounting. The blue ink shows the path of the microfluidic channels from the inlet to the outlets; (b) final microfluidic chip mounted on a glass coverslip, and (c) illustration of the principle of operation via gravity-induced flow (CNS stands for Central Nervous System and indicates the chamber where primary cells are plated).

4.2.1. Analysis of the neuroprotective effect of FTY720 on Aβ-exposed neurons using MFN

We show the microfluidic chip as an efficient method to screen the effects of toxic agents and drugs on different populations of primary CNS cells. The use of these microfluidic chips in the context of Alzheimer's disease allowed to demonstrate the high toxicity of oligomeric Aβ compared to the fibrillar form, and a protective effect of the anti-inflammatory drug FTY720, when applied to neuronal cultures or microglia-enriched neuronal cultures.

Oligomeric and fibrillar Aβ preparations were diluted in conditioned neuronal medium at 2 μM concentration and loaded in the inlet port of chips hosting either neuronal cultures in all chambers or microglia-enriched neuronal cultures in all chambers. Neuronal loss was evaluated 96 h after Aβ exposure by calculating the ratio of PI positive cells to the total number of cells, given by Hoechst nuclei staining (Fig. 12). In neuronal cultures, the neuronal loss caused by 96 h exposure to oligomeric Aβ reached a 4-fold increase as compared to the control neuronal cultures, while the toxicity of the fibrillar form was much lower (below a 2-fold increase). Significantly, the neurotoxicity induced by oligomeric Aβ decreased when the neuronal cultures were enriched with microglia. This could be due to a major clearance activity of microglia triggered by small Aβ aggregates. Alternatively, the reduced number of damaged neurons in neuron–microglia co-cultures exposed to oligomeric Aβ may be due to the well-known capability of reactive microglia to phagocytose dead or dying cells, thus resulting in progressive loss of damaged neurons. After exposure to the fibrillar form, however, the levels of toxicity were similar to either neuronal or neuron– microglia co-cultures.

We then assessed the possible neuroprotective activity of the anti-inflammatory drug used in Multiple Sclerosis (MS) FTY720. FTY720 is a S1P receptor agonist whose immunomodulating action has been proved effective for limiting the infiltration of autoreactive lymphocytes in CNS, thus suppressing subsequent neuroinflammation. Recent studies lighted hope on the therapeutic power of FTY720 for other CNS disorders (Y. Hasegawaet al., 2010 Y. Doiet al., 2013)for example Alzheimer disease. Remarkably, recent studies also indicate that FTY720 has a beneficial effect in restoring memory loss in AD rats (F. Hemmati et al. 2013; L. Dargahi et al. 2013). In view of these promising features, we were interested in evaluating whether the neuroprotection of FTY720 against oligomer-induced neurotoxicity could be further amplified by a synergistic action on microglia, and which was the effect on the fibrils-induced neurotoxicity. In order to assess the long-term neurotoxicity of Aβ in the different cell combinations the amyloid-induced neurotoxicity was also evaluated 96 h after exposure to 2 μM oligomeric or fibrillar Aβ in the presence of 200 nM FTY720 (Fig. 12). Interestingly, the toxicity

of the oligomers was reverted to control levels upon FTY720 treatment, in both neuronal and neuron–microglia cultures. Conversely, the drug did not significantly affect the toxicity of fibrils. These data indicate that FTY720, a drug used for the treatment of MS targeted to glial cells, may play a neuroprotective role in the AD context, acting primarily on neurons. These results are in favor of a direct interaction of the drug with neuronal cells or with Aβ, rather than an indirect action on glial cells by which it could prevent inflammation and/or promote the clearance of extracellular Aβ aggregates.

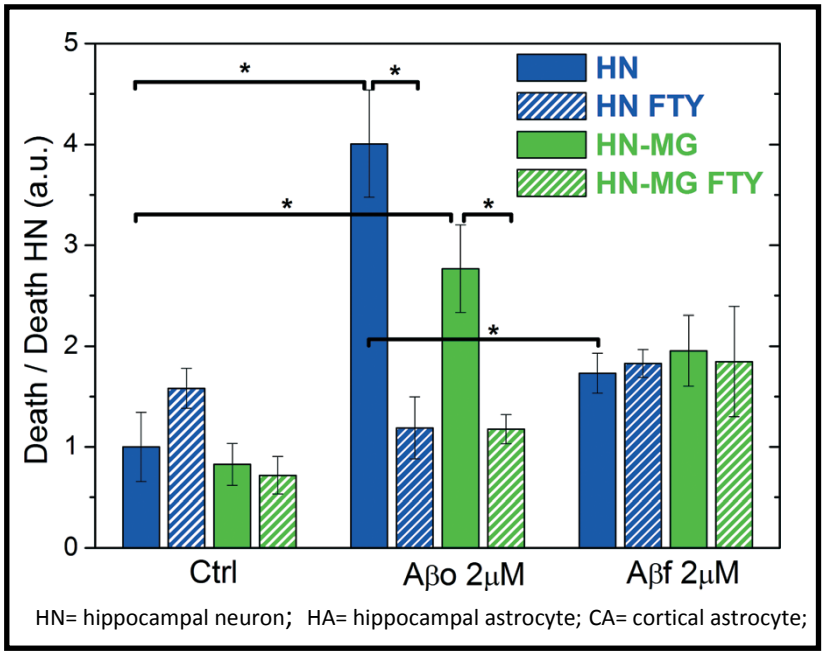


Figure 12. Neuronal death in 20 day-old neuronal cultures or in neuron– microglia co-cultures, 96 h after exposure to different Aβ1–42 species. Neuronal loss is observed more prominently after exposure to Aβ1–42 oligomers. Long-term Aβ toxicity and rescue by FTY720 in neuronal cultures or neuron–microglia co-cultures plated in the different chambers of microfluidic chips. (*one-way ANOVA, $p < 0.05$).

4.3 Glia-neuron interaction in the context of RTT Syndrome.

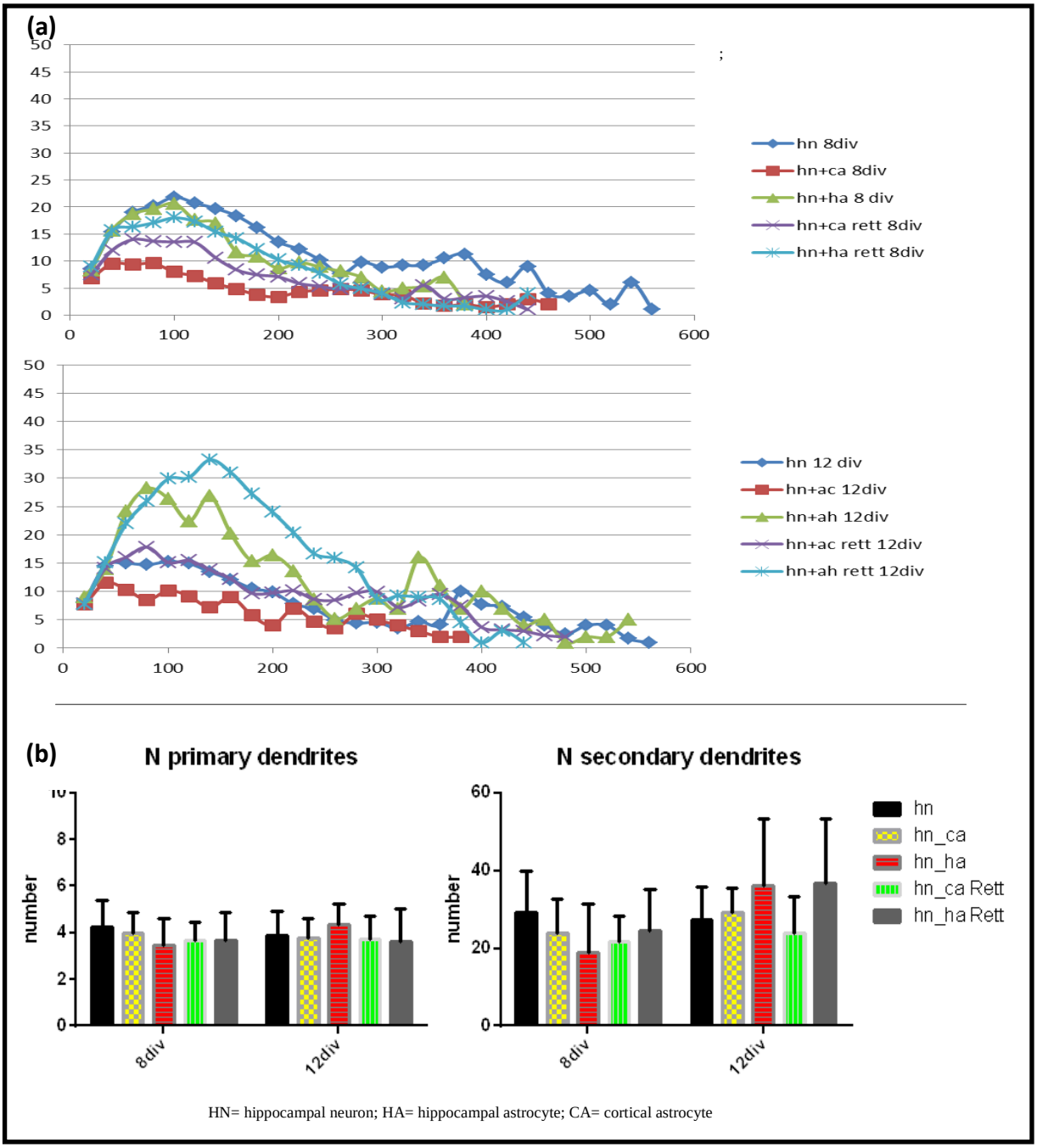
4.3.1. Glial cells from different brain area support in different way the neuronal development.

The Tongiorgi lab revised a staging system for the development of cultured hippocampal neurons from rat, and created a new staging system for mouse hippocampal neurons (Baj et al. 2014) using the overall adopted Dotti's model (Dotti et al 1988). In particular, they have described the processes that lead to the achievement of hippocampal neurons morphology and function from DIV1 to DIV12, through the analysis of several different parameters, such as total

dendritic length, the amount of primary dendrites and of all the branches, the outgrowth of the apical dendrite and the synaptogenesis in terms of dendritic spines and synapses formation. The results proposed some intermediate phases that could complete or at least improve the current description of the entire process Their systematic description of each stage in hippocampal neuronal cultures using morphological and functional features allowed them to identify two breakpoints in the maturation of neurons from MeCP2 deficient mice, consisting in a delayed development of dendritic arborization and a reduction in spines formation.

Although it is thought that the primary molecular mechanisms leading to RTT is mostly driven by cell autonomous defects due to lack of functional MeCP2 in neurons, whether non-cell autonomous factors contribute to the disease, is unknown. Recent studies show that loss of MeCP2 occurs not only in neurons but also in glial cells of RTT brain. To verify the effects of astrocytes on neurons we used an *in vitro* co-culture system. We plated wt neuron together with either wt or MEPC2 deficient astrocytes from different brain areas and then we analyzed the morphology of neuron at different time point. In particular, we evaluated both length and complexity of the neuronal dendritic arborization. Taking advantage of Sholl analysis, which allows to evaluate dendritic length by calculating the number of processes intersecting a series of increasingly larger concentric rings centered on the soma, we provided a reliable assessment of dendritic branching. The analysis was performed both at 8 DIV and 12 DIV.

The morphological analysis performed at 8 DIV showed no significant differences among the treatments. However, at 12 DIV, we found that although primary neurons grown in conditioned medium from WT cortical astrocytes were morphologically similar to those grown alone, neurons in conditioned medium from WT hippocampal astrocytes exhibited an increase in dendritic branching complexity. Such differences were also observed with astrocytes established from a mouse model of RETT Syndrome in which medium conditioned by hippocampal (but not cortical) RETT astrocytes induced an increase in dendritic branching complexity compared to neurons alone (Fig. 13a). Furthermore, a more detailed analysis based on the number of different neuritic processes (primary vs secondary) revealed that although the overall number of primary neurites was equal in all conditions, the number of secondary neurites was higher in those neurons grown in conditioned medium obtained from hippocampal astrocytes established from both WT and RETT mouse (Fig. 13b).



HN= hippocampal neuron; HA= hippocampal astrocyte; CA= cortical astrocyte

Figure 13. (a) Sholl analysis showing dendritic branching complexity of the hippocampal neuron grown with conditioned medium of astrocyte from different brain areas at two time point, 8 div (upper graph) and 12 div (lower graph); shown N cells at 8 DIV HN=50; HN-AC=35; HN-AH=27; HN-AC RETT=20; HN-AH RETT=10. At 12 DIV HN=60; HN-AC=16; HN-AH=27; HN-AC RETT=29; HN-AH RETT=10. (b) Quantitative analysis of the number of primary (data shown as mean±sd. 8 DIV: HN 4,2 ±1.11; HN-AC 4,1± 0,8; HN-AH 3,5± 1,1; HN-AC RETT 3,7± 0,8; HN-AH RETT 3,7±1,1. 12DIV: HN 3,9±1,0; HN-AC 3,7±0,8; HN-AH 4,3±0,9; HN-AC RETT 3,7±0,9; HN-AH RETT 3,6±1,4) and secondary neuritis (data shown as mean±sd 8DIV: HN 29,2±10,5; HN-AC 23,9±08,9; HN-AH 18,9±12,5; HN-AC RETT 21,7±6,5; HN-AH RETT24,7±10,5. 12DIV: HN 27,3±8,5; HN-AC 29,4±6; HN-AH 36,2±17,0; HN AC RETT 24±9,1; HN-AH RETT 36,7±16,5) in neurons in the same conditions.

Subsequently, we have evaluated the total length of neuritic processes. Again, no significant differences were observed in the total length of neurons at 8 DIV, however, the analysis performed at 12 DIV showed that, in contrast to neurons grown in conditioned medium obtained from WT cortical astrocytes, those in the presence of medium obtained from WT hippocampal astrocytes exhibited longer neurites than neurons grown alone (Fig. 14). Interestingly, although a similar result was observed with astrocytes coming from RETT mouse model, RETT hippocampal astrocytes were found to be less effective in promoting neuritic elongation than WT hippocampal astrocytes. Taken together, these data suggest that distinct astrocytic populations from specific brain areas have different ability to support neuronal growth. In particular we found that hippocampal astrocytes are more effective in promoting both dendritic branching and neuritic elongation either WT or RETT. Moreover, although such different ability was preserved in a mouse model of RETT Syndrome, RETT hippocampal astrocytes are less supportive in promoting neurites elongation.

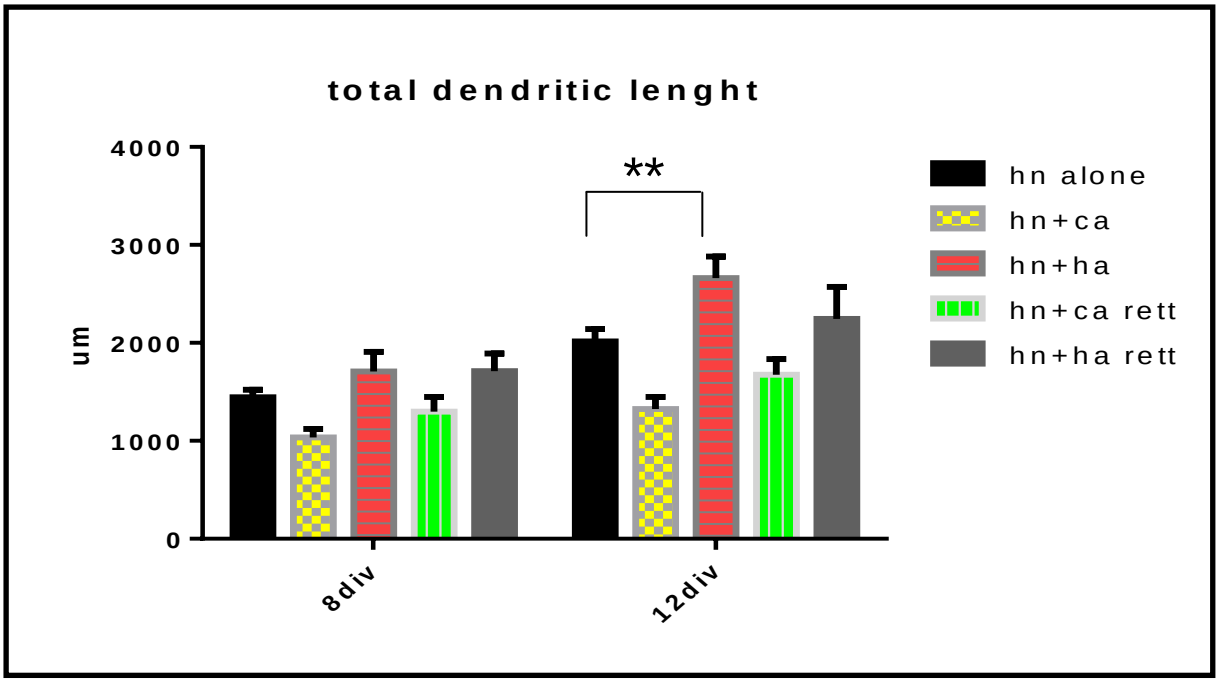


Figura 14. Quantitative analysis of total dendritic length in different conditions. (a) Note that primary neurons exhibit longer neurites in the presence of conditioned medium from hippocampal astrocyte at 12 DIV. Data are shown means±SEM 8DIV: HN 1446±411,2; HN-AC 1036±479,2 ; HN-AH 1707±868; HN-AC RETT 1298±338; HN-AH RETT 1710±575; 12DIV: HN 2016±615,8; HN-AC 1326±338 ; HN-AH 2661±913; HN AC RETT 1676± 617; HN-AH RETT 2245±525.

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5. Discussion and conclusion

5.1 Microfluidic Networks, in closed or in opened, to study the interaction between glia and neurons

Astrocytes are the principal house-keeping cells of the nervous system, playing multiple supportive tasks for neurons. Failure of any of these supportive functions of astrocytes can represent a threat for neuronal survival. Although astrocytes throughout the central nervous system share many common traits, a marked phenotypic diversity is detectable among astrocytes from different brain regions (Han et al., 2004). These phenotypic differences result in functional heterogeneity among cortical and hippocampal astrocytes which results in specific functional features, for example, in an increased glutamate uptake capability by cortical rather than hippocampal astrocytes following injury (Morga et al 1998; Kipp et al 2008). A primary role of astrocytes in control of neuronal viability has also been demonstrated in neurodegenerative disorders, such as Alzheimer's disease (AD), in which neuroinflammatory events are heavily involved. The main inflammatory players in AD are the glial cells which initiate the inflammatory response. Neuroinflammatory processes, in which reactive glial cells release several pro-inflammatory mediators, are a common protective response to brain damage. However, these reactive inflammatory responses can turn detrimental and contribute significantly to increase, rather than protect from, neuronal damage.

Leveraging microfluidic networks in closed we investigate the role played by astrocytes derived from different regions of the brain in controlling neuronal viability in the context of Aβ insult (Aβ or Aβ + IL-1β). Our data indicate that astrocytes from different brain regions differently affect neuronal viability upon exposure to inflammatory stimulus Aβ + IL-1β, with hippocampal astrocytes contributing more heavily to neuronal death than cortical astrocytes. It is observable a clear difference between hippocampal and cortical astrocytes, in fact the hippocampal astrocytes fail in supporting neurons after exposure to Aβ + IL-1β. Hippocampal astrocytes, cortical astrocytes and hippocampal neurons independently stimulated with Aβ do not show significant cell death. Hippocampal neurons show a significant increase in cell death when put into closed microfluidic communication with hippocampal astrocytes, but not cortical astrocytes, previously challenged with Aβ + IL-1β. Moreover, hippocampal neurons challenged with PPh (hippocampal astraocyte postpriming medium) exhibit higher calcium transients compared to hippocampal neurons exposed to PPc (cortical astrocyte postpriming medium). The harmful effect of hippocampal astrocytes could presumably be mediated by the higher levels of glutamate

accumulated in the hippocampal relative to cortical astrocyte medium upon stimulus challenge, given that neuronal death is prevented by the NMDA blocker APV. The combined action of morphological and functional analysis on primary cell cultures, either cultured independently or in microfluidic communication, has allowed us to distinguish brain-region-specific glial contribution *in vitro* models of neuroinflammation,. We suggest oMFNs and the method shown here to be a valuable, versatile, and flexible analytical solution to unravel how different cell types of the brain contribute to the crosstalk events leading to neurodegeneration.

Contrary, exploit of the microfluidic networks in opened we were used as method to screen the effect of toxic agent and drugs on different population of CNS cells. may help to accelerate drug development by providing alternative cell-based in vitro methods with enhanced predictability for initial screening, in order to prioritize treatments for further testing. Our work show on a simple of efficient method to screen the effects of toxic agents and drugs. In this case the microfluidic device, operated by gravity-induced flow, have been designed to divide the flow of stimuli into 4 parallel streams that reach 4 cell chambers that can host different CNS cells populations. The stimuli (e.g. Aβ species or drugs) are applied through a common inlet and distributed to the 4 chambers by perfusion achieved through a difference in the volume level between the inlet and the outlets. The perfusion system is very easy to operate and the open culture chambers are extremely accessible for loading primary cells. This enables a straightforward scalability for high-throughput implementation and allows a reproducible evaluation of the influence of different cell types on neuronal viability. The use of these devices in the context of Alzheimer's disease allowed demonstration of the high toxicity of oligomeric Aβ compared to the fibrillar form, and a protective effect of the anti-inflammatory drug FTY720, when applied to neuronal cultures or microglia-enriched neuronal cultures. FTY720 is a S1P receptor agonist whose immunomodulating action has been proved effective for limiting the infiltration of autoreactive lymphocytes in CNS, thus suppressing subsequent neuroinflammation. Recent studies lighted hope on the therapeutic power of FTY720 for other CNS disorders. The drug has been recently shown to prevent apoptosis after cerebral ischemia and proposed as a novel compound for the treatment of stroke. Likewise, it has been demonstrated that FTY720 downregulates the production of several pro-inflammatory cytokines in microglia and proposed as a potent therapeutic agent in neurological diseases associated to microglial activation. Moreover, FTY720 modulates the BDNF levels in brain cells, improving the symptoms of Rett Syndrome, promoting the neuroprotective effects of microglia, and attenuating the neurotoxicity of oligomeric Aβ. Remarkably, recent studies also indicate that FTY720 has a beneficial effect in restoring memory loss in AD rats. In view of these promising

features, we were interested in evaluating whether the neuroprotection of FTY720 against oligomer-induced neurotoxicity could be further amplified by a synergistic action on microglia, and which was the effect on the fibrils-induced neurotoxicity. In order to assess the long-term neurotoxicity of Aβ in the different cell combinations the amyloid-induced neurotoxicity was also evaluated 96 h after exposure to 2 μM oligomeric or fibrillar Aβ in the presence of 200 nM FTY720 Interestingly, the toxicity of the oligomers was reverted to control levels upon FTY720 treatment, in both neuronal and neuron–microglia cultures. Conversely, the drug did not significantly affect the toxicity of fibrils. These data indicate that FTY720, a drug used for the treatment of MS targeted to glial cells, may play a neuroprotective role in the AD context, acting primarily on neurons. These results are in favor of a direct interaction of the drug with neuronal cells or with Aβ, rather than an indirect action on glial cells by which it could prevent inflammation and/or promote the clearance of extracellular Aβ aggregates. Our demonstration of a predominant direct action on neurons, rather than a crucial contribution of microglia to the neuroprotective effect of FTY720 in Aβ-exposed neurons, suggests FTY720 as a promising candidate to directly prevent neuronal death in AD.

5.2 Glia-neuron interaction in the context of RTT Syndrome

The evidence of aberrant dendritic morphology in Rett Syndrome has been well documented in post mortem human brain samples as well as in several in vivo and in vitro studies. However, the mechanism leading to defective neuronal structure in absence of MeCP2 have not been understood yet. In particular, it is still unclear if the defective in neuronal morphology is the result of a deficit in development or maintenance of the neuronal phenotype or if both these two mechanisms are affected. Recently, Tongiorgi's lab created a new staging system for the study of the development of mouse hippocampal neurons that allowed them to identify two breakpoints in the maturation of neurons from MeCP2 deficient mice. In particular they found a delayed development of dendritic arborization that seems to occur early during *in vitro* neuronal maturation, probably before at 6 DIV and a reduction in established excitatory synapses that occur at 7 DIV. These results indicate that both developmental and maintenance setbacks affect the final morphology and function of neurons in RTT individuals.

It is well known that the extracellular environment is deeply involved in the process of neuronal maturation in both healthy and pathological states. In line with this idea, several studies have reconsidered the role of glial cells, showing that glia cells played a key role in disease

progression (Amiri et al. 2012;Nguyen at al. 2012 , (Ballas et al., 2009, Maezawa et al., 2009, Maezawa and Jin, 2010)Lioy et al., 2011, Derecki et al.). Although these evidences clearly confirm a critical role of glia cells in such disease, the underpinning molecular mechanisms are less understood. In parallel, several observations made in RETT mouse model highlighted that specific brain areas are particularly affected by the disease, for instance cortical region exhibited an higher level of neuronal atropy than other brain regions, including hippocampus,thus opening the possibility that different glial cell populations belonging to distinct brain areas might be responsible for the selective neuronal impairments observed in the disease. Thus, in order to address whether different glial populations differentially affect neuronal development, primary neurons were co-coltured with distinct populations of astrocytes established from both WT and RETT mouse model and several parameters related with neuronal development were evaluated. In particular we have studied the contribution of cortical and hippocampal astrocytes to dendritic branching complexity and neurites elongation, thus providing evidences of a different involvement of these two glial cell types in RETT disease. We found that hippocampal, but not cortical astrocytes enhanced neuronal growth regardless their genotype. This suggests that hippocampal astrocytes have higher trophic effects on neuronal development than cortical astrocytes. Interestingly, RETT hippocampal astrocytes were less effective in promoting neuronal growth than WT astrocytes, indicating that the loss of MeCP2 in these specific glial cell types might be important in neuronal development. Moreover, the selective atrophy observed in cortex of mouse brain model of Rett Syndrome may be due to a lower trophic capacity of cortical astrocytes than in hippocampus. Taken together, these data suggest that astrocyte populations from specific brain areas differentially affect normal neuronal development probably trough the release of region specific glia-derived soluble factors. It should be noted that our study was mainly based on WT primary neurons as control conditions. Thus, the real impact of different glial populations in RETT neurons is still completely unknown and further studies will help in elucidating this issue.

In conclusion, our study provides further evidences about a critical involvement of glial cells in several neurological disorders, including neurodegenerative diseases (i.e. alzheimers) and neurodevelopmental disorders (i.e. RETT Syndrome). The clarification of the basic mechanisms underlying the role played by astrocytes in brain development will provide fundamental knowledge about the pathogenesis of a wide spectrum of brain diseases.

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