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MAMMALIAN HIPPOCAMPAL NEURONAL PLASTICITY UNDER NORMAL AND PATHOLOGICAL CONDITIONS

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INTRODUCTION

Neuroplasticity is a term that includes all the functional and structural changes within a neural circuit in response to external or internal events, changes at synaptic level, in the morphology, or in the number of cells. These changes are related with functional modifications and have great relevance under physiological conditions and in neuropathology.

The malleability of the nervous system has a central role in shaping the brain during the prenatal and early postnatal development, in the childhood, but also in the adulthood, supporting vital functions, such as learning and memory. Therefore, the first aim of my PhD itinerary was focused on the expansion of the knowledge about the mechanism of physiological plasticity in the hippocampus in relation with network activation induced by common every-day experiences, such as physical activity. Hippocampus, indeed, attracts great attention in the neuroscience research field because it takes part to certain types of learning and memory but also because of its extraordinary degree of neuronal plasticity. In this structure, much of the attention is mainly focused on neuronal plasticity phenomena, such as synaptic Long Term Potentiation (LTP) and adult neurogenesis: this last phenomenon represents a fascinating example of plasticity occurring in a specific hippocampal area called Dentate Gyrus (DG). Here, new granule cells are daily generated and incorporated in the existing network. In the hippocampus, stem/progenitor cell proliferation and newly-generated granule cell integration are affected by numerous stimulus both physiological and pathological. In keeping with this statement, physical exercise represents a pro-neurogenic activity. Our previous findings highlighted that a brief physical activity, and in particular voluntary running, produces short-term [1] effects in very immature newborn granule cells of adult DG. The attention is therefore shifted in the research for possible long-lasting effects of the voluntary running on newly-generated granule cells, evaluating morphological and possible functional implications related with this activity, with the purpose of removing part of the shadows upon the possible mechanism of cognitive enhancement widely reported in association with physical exercise.

Additionally, since abnormal plastic adaptation underlies many neural diseases, a second aim of my PhD project has considered two pathologies, depression and epilepsy, in order to uncover and highlight possible treatments able to influence, or prevent, the aberrant plastic support to these neuropathologies.

Depression, a chronic and recurrent disease linked to significant dysfunction of neural plasticity has been studied in collaboration with Prof. Kjell Fuxe's group of the Karolinska Institutet in Stockholm. In particular, this project placed the focus on the study of Fibroblast Growth Factor Receptor 1 – 5-hydroxytryptamine 1A (FGFR1-5HT1A) heteroreceptor complex role in depression, which is a receptor-receptor (R-R) interaction of extreme interest since it represents the meeting point between two theories of depression, the serotoninergic and the neurotrophic factor hypotheses. The

FGFR1-5HT1A heteroreceptor complex is reported to exist in hippocampus [2] and midbrain raphe [3]. In addition, combined agonist treatment influences cellular throphism and morphology, suggesting that activation of FGFR1-5HT1A heteroreceptor complex might be related with antidepressant effect of serotonin in the brain and, combined activation of both receptors might result in more rapid and stronger antidepressant action than found with Selective Serotonin Reuptake Inhibitors (SSRIs). Indeed, an important clinical pursuit in the depression field is the research for fast-acting treatments or molecules able to speed up the effects of the canonical anti-depressive drugs, since commonly available treatments exert their therapeutic action after a delay that last from weeks to months [4]. Thus, this part of the PhD project has been focused on a first evaluation about the therapeutic potential of combined FGFR1 and 5HT1A agonists treatment, which has been firstly tested on Sprague Dawley (SD) rats, using electrophysiological, molecular and behavioural approaches. Afterward, to evaluate if disturbances of the FGFR1-5HT1A heteroreceptor complex might exist in depression and if the combined treatment with the agonists of the FRGR1 and 5-HT1A could exert antidepressant effects, the attention was moved on Flinders Sensitive Line Rats (FSL), a well-known model of depression [5]. Actually, the potential existence of disturbances in depression at FGFR1-5HT1A heteroreceptor complex level could represent an exciting finding since it might confirm these complexes as valid targets for future therapeutic treatments with possible fast-acting properties.

The other pathology concerning the second aim pursued in my PhD project is the mesial temporal lobe epilepsy (MTLE), the most common form of localization-related epilepsy, which is characterised by progressive plastic rearrangements that lead to the chronicization of the disease and the aberrant remodelling of the hippocampal network. Treatment able to counteract the chronicization of epilepsy represents an unmet clinical need. Previous findings from our laboratory of physiology suggested a potential and promising role of Vitamin E (as α-tocopherol) as antiepileptogenic treatment [6, 7], which might act through different mechanisms than anti-oxidant one. To validate this assumption, using the kainate rat model of epilepsy, the excitability of hippocampus circuitry, the neuroinflammation markers, neuron cell death and microRNA (miRNAs) expression, have been investigated in adult rat after 15-days of α-tocopherol treatment.

PLASTICITY OF THE CENTRAL NERVOUS SYSTEM

Plasticity is a term that has been adopted in neuroscience for over a century referring to the malleability of the nervous system, namely the capability to adapt through functional and structural modifications in response to events that organisms face during their life and to injury of its own integrity. Besides, it is firmly believed that plasticity is the substrate for learning and memory.

The term plasticity has been addressed for the first time by William James in his *Principles of Psychology* (1890) referring to possibility of changes in behavioural habits through modifications, after repeated use, in specific brain path [8]. He wrote: “Organic matter, especially nervous tissue, seems endowed with a very ordinary degree of plasticity […]: so that we may without hesitation lay down as our first proposition the following, that the phenomena of habit in living beings are due to the plasticity of the organic materials of which their bodies are composed.”[9]. Nevertheless, the first hypothesis that connect associative memories and practice-dependent motor skills with a localised facilitation of synaptic plasticity transmission was introduced by Eugenio Tanzi in 1893, and expanded by Ernesto Lugaro few years later through the relation of this plastic changes with the intuition about the chemical nature of synaptic transmission in the central nervous system (CNS) [8]. Concurrently, Ramón y Cajal completed Tanzi’s hypothesis with his own hypothesis of plasticity as the result of the formation of new connections between cortical neurons [10]. Indeed, an important contribution of Cajal, little known within the scientific community, is his application of the Neuron Doctrine to explain the relationship between brain plasticity and mental processes from a structural point of view, and his theories regarding the influence of the environment on brain development and function. Therefore, through Cajal's own work and his astute interpretation of the studies of others, the architecture of the cerebral cortex began to be considered as plastic and connections in this structure susceptible to change either in response to normal neuronal activity or to injury [10].

After the initial enthusiasm however, plasticity and the synaptic theory of learning came quickly under attack and some of the proposed connections between mental factors and neuronal activities were strongly criticized [8]. This trend was inverted in 1948 when Konorski attributed two fundamental properties to the central nervous system: reactivity and plasticity [11]. A year later, in 1949, Hebb published *The Organization of Behaviour* and the synaptic plasticity theory of learning was finally rehabilitated. He introduced the so called Hebbian plasticity, a form of synaptic plasticity that describes the increased synaptic strength that occurs if the presynaptic and postsynaptic element spike in a brief interval of time. In his words: “When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A’s efficiency, as one of the cells firing B, is increased” [12]. Hebb’s work returned to the topic of plasticity frequently during his carrier and researchers often refers to modifiable neuronal circuits

as "Hebbian" in honour of his theoretical contribute; moreover, synapses that change as a consequence of simultaneous firing are often referred to as "Hebbian Synapses" [8]. Nowadays the topic of plasticity is highly thriving and great interest is directed in the study of plasticity in healthy brain and, considering the impact of this topic on human health, in disease.

Mechanisms supporting brain plasticity

The mechanism behind neuronal plasticity could involve changes in synaptic strength, in the number of synapses or even in the number of neurons within circuits as pointed out by the more recent findings (1960s) about neurogenesis in the adult brain. Neuronal changes of plasticity are usually referred to as functional, as opposed to structural. It is nowadays clear that the dichotomy between functional and structural plasticity is arbitrary, as many of the changes that was previously been considered functional are accompanied by changes in number or shape of dendritic spines, or by the formation or apoptotic removal of neurons.

Long Term Potentiation (LTP) and Long Term Depression (LTD)

One of the most attractive cellular mechanisms sub-serving plasticity is synaptic plasticity, because it endows each neuron with the capacity to adapt dynamically the functional weight of specific inputs that it integrates. Long term changes in synaptic strength, such as LTP or LTD are believed to critical underline experience-induced neural adaptations in the brain [13]. These form of synaptic plasticity typically occur in the time scale of hours and can be expressed postsynaptically as a change in postsynaptic receptor number or function, or presynaptically as a change in neurotransmitter release.
Long term changes in synaptic strength was first discovered in the mammalian CNS by Bliss and Lømo in 1973, studying excitatory synapses response in hippocampal dentate gyrus after the application of a brief, 1-second bursts of high frequency stimulation (100Hz, called "tetanic") [14]. Using this protocol, they were able to elicit a long-lasting increase in the strength of these synapses that could persist for many days. They also discovered an increased probability of the postsynaptic neurons to fire an action potential (AP) in response to a constant level of presynaptic stimulation. Taken together, they named this phenomenon LTP and Hebbian plasticity was documented to exist in the mammalian CNS. As a matter of facts, LTP undergoes the definition of Hebbian plasticity because it has the properties of "cooperativity" and "associativity": a weak input, where only few excitatory synapses are tetanized, failed to induce LTP whereas a strong input able to activate many synapses, induces the potentiation (cooperativity); in addition, the simultaneous activation of two separate inputs, one of which is weak and fails to undergo LTP on its own, exhibits a robust LTP when tetanized together with

a strong input (associativity). It is nonetheless true that non-Hebbian LTP are documented to exist [15].

The complementary process of LTP is referred to as long-term depression and consist in a reduction of the efficacy of synaptic transmission. LTD was first discovered few years after LTP by Ito and Kano studying rabbit cerebellum and applying a low frequency stimulation of 4Hz for 30-120 seconds [16]. LTD is often observed after the induction of LTP, in which case has been referred to as "depotentiation"; in some cases however, LTD can be observed from baseline conditions and this has been termed "*de novo* LTD" [17].

To date, several forms of long-lasting synaptic plasticity have been observed in the mammalian central nervous system. Many, but not all, forms of LTP and LTD are dependent on the activation of glutamate receptors that characterise most excitatory synapses in the mammalian brain. In particular, glutamate activates the ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazole receptors (AMPARs) whose number could determine the efficiency of synaptic transmission. Although there is still debate over the mechanism involved in synaptic plasticity, one of the most common forms of LTP depends on postsynaptic activation of ionotropic *N*-methyl-D-aspartate receptors (NMDARs). NMDA receptors are tetramers of various subunits (GluN subunits) and are cationic channels (allowing the passage of Na^{+}, K^{+} and Ca^{2+}) that open when their blockage by Mg^{2+} ions is removed by depolarization of the postsynaptic cell as that obtained after a strong activation of AMPARs. Calcium entering the postsynaptic neuron is a crucial signal triggering LTP or LTD. Indeed, LTP seems triggered by a fast and large increase in postsynaptic Ca^{2+}, whereas LTD results from a slow, and less intense, influx [18, 19]; besides, it should be considered that another source of Ca^{2+} necessary to trigger LTP or LTD is via voltage-gated Ca^{2+} channels (VGCCs). A great flux of this second messenger through NMDARs can activate kinases such as calcium/calmodulin-dependent protein kinase II (CaMKII), protein kinase C (PKC) and protein kinase A (PKA). These kinases then lead to LTP either by trafficking new AMPARs to activated synapses or by acting on the biophysical properties of postsynaptic membrane-localised AMPARs, via post-translational modifications. On the contrary, a smaller Ca^{2+} influx through NMDARs triggered by weak synaptic activation will recruit phosphatases, as Phosphatase 2B (PP2B) and Protein Phosphatase 1 (PP1), that can lead to LTD via the opposite mechanism. Additional studies demonstrated that norepinephrine, dopamine, and acetylcholine-mimicking compounds, as well as the Brain Derived Neurotrophic Factor (BDNF), can all modulate the likelihood of induction of LTP at various central synapses, merging Hebbian plasticity with neuromodulation [20]. Considering synaptic potentiation more in detail, contemporary mechanistic models divide this phenomenon in short-term potentiation (STP) and long-term potentiation, divided again into at least two phases, "early" and "late", based on additional studies probing the biochemistry of LTP. STP consist in the initially large potentiation of the evoked response after tetanic stimulation, fading after about 10 minutes in a more relaxed response that defines early LTP [21]. Such STD is synapse-specific and is largely dependent on NMDARs. The

mechanism of STP is not completely understood but considering that can develop within seconds after stimulation [22], it would seem likely that this potentiation might be produced by phosphorylation of AMPA receptors already held in the cellular membrane. Early LTP (E-LTP) is subserved by persistently activated protein kinases activated by Ca^{2+} entry through NMDARs, starts at around 30 minutes or less post-tetanus, and is over after about 2-3 hours [20]. The potentiation observed in this phase is mainly due to the increased number of AMPARs within the synapse. Induction of late LTP (L-LTP) is dependent on changes in gene expression driven by mitogen-activated protein kinases (MAPKs) and lasts many hours. L-LTP is mechanistically different from E-LTP, and involves enlargement of the synapse itself [23], explaining the protein synthesis requirement for late LTP. During L-LTP not only post-synaptic density enlarges but also presynaptic bouton also enlarges [23]. Importantly, L-LTP it is now called neoHebbian because it involves not only pre- and postsynaptic terminals (Hebbian) but also a third element [24]. In CA1 hippocampal area, the third element is represented by dopamine [25]. The existence of this third factor makes the transition between E-LTP to L-LTP conditional on properties such as novelty, prominence, or reward value of the stimulus [26].

As aforementioned, also the presynaptic component may contribute to synaptic plasticity under some conditions [27]. The induction mechanisms of presynaptic plasticity are diverse and may involve repetitive activity of the presynaptic cell, a retrograde messenger released from the postsynaptic cell (nitric oxide, arachidonic acid or endocannabinoids), or some signal arising from adjacent synapses or astrocytes [28]. The entrance of Ca^{2+} ions via VGCCs or ligand-gated presynaptic receptors, triggers a downstream cascade involving kinases and phosphatase activation. A well-known possible mechanism involves cAMP and PKA signalling. In particular, an increase in cAMP level could induce presynaptic LTP in many regions of the brain [28], while a presynaptic inhibition of the cAMP pathway via $G_{i/o}$ – coupled receptors could result in presynaptic LTD, which is also a widespread phenomenon [29]. Changes in the amount of neurotransmitter released have influence on the synaptic strength and support the expression of presynaptic LTP/LTD but the precise mechanism by which neurotransmitter release remains long-lastingly altered is largely unknown due to the difficulty of visualizing and manipulating axons and presynaptic terminals. Some possible mechanisms have been suggested, such as modification in Ca^{2+} influx through VGCCs and changes of the release machinery [28]. Besides, presynaptic terminals undergo long-term experience and activity-dependent structural plasticity in the adult mammalian brain [30]. These structural changes could underline functional alterations of presynaptic strength, for example, via changes in the size or number of active zones, the number of vesicles recruited or docked, or through changes in the distance between synaptic vesicles and presynaptic VGCCs [31].

The back-propagating action potential

The active control of dendritic membrane potential by voltage-gated channels, such as sodium channels, was a paradigm shift from the previous assumption that active propagation of membrane depolarization was assumed to be limited to the axon. The discovery of active propagation of action potentials originating in the axon initial segment and soma into dendrites, a phenomenon called back-propagation of action potentials, opened new ways of thinking about dendrites and their role in neuronal information processing.

Back-propagating action potential plays a role in controlling depolarization envelope of the postsynaptic terminal regulating NMDA gating and influencing LTP and LTD [32]. Action potential back-propagation is sustained by dendritic voltage dependent Na^+ and activates Ca^{2+} channels. Dendritic K^+ channels can modulate the amplitude and extent of back-propagation. The extent of back-propagation is therefore dependent on the densities of Na^+ and K^+ voltage-gated ion channels in soma and dendrites. Johnston's group first reported that there was a high density of transient A-type potassium channels in dendrites of hippocampal CA1 pyramidal neurons. These channels prevent initiation of action potentials in the dendrites, limit back-propagation of action potentials into the dendritic area, and reduce excitatory synaptic events [33]. The A-type K^+ channel, which mediates I_A current, is a low threshold, rapidly-inactivating potassium channel that opens at subthreshold membrane potential (-50mV) and influences repolarization and propagation of action potentials. The unique rapidly activating and inactivating properties of transient A-type K^+ channels and their distribution allow them to exert a profound impact on the coordination of synaptic responses with neuronal activities and the regulation of synaptic plasticity through attenuation of action potential propagation. This group further reported that dendritic attenuation of action potentials was reduced by theta-like simulation (a typical sinusoidal oscillation of the hippocampal electroencephalography critical for mnemonic process) protocol in CA1 pyramidal neurons [34]. The decreased dendritic attenuation facilitated the back-propagation of action potentials and the induction of LTP. Besides, membrane potential is influenced by postsynaptic potentials (PSPs) which reflect the temporal and spatial summation of excitatory and inhibitory synaptic input. When an EPSP is produced at a dendritic spine by presynaptic release of glutamate and propagates to the axo-somatic AP initiation region, it will be attenuated along the dendrite due to electrotonic attenuation, inhibitory shunt, and activation of dendritic A-type K^+ channels. Considering that the intensity of I_A is diminished significantly as a result of simultaneous induction of LTP, the decrease of I_A during the induction of LTP help to fulfil the function of synaptic and intrinsic plasticity securing the induction of these plasticity by facilitating AP backpropagation, influx of Ca^{2+}, and amplification of synaptic inputs, and finally, reducing the induction threshold of LTP. A-type potassium channels could nevertheless be influenced by many neuromodulators such as dopamine, serotonin, acetylcholine and others, which indirectly modulate NMDA activity and could influence membrane

potential. These types of mechanisms have the capacity to confer the necessary molecular/biophysical mechanisms for multi-contingency precision timing of the induction of neuronal plasticity in triggering behavioural change.

Dendritic spine turnover

Accumulating evidence over the past decades indicates that the connectivity of the synaptic network is remodelled during life, through the mechanism of synapse formation, stabilization and elimination. Dendritic spines are primary sites for structural modifications during memory formation: spines are highly dynamic as they grow, shrink and change form during lifetime [35]. Indeed, the morphology and stability of excitatory and inhibitory synapses change over time and are constantly regulated by synaptic activity [23] and consequently strictly related to LTP and LTD processes [36]. This phenomenon is regulated by activity, and the size of spine heads correlates with synaptic strength, presynaptic properties, and the long term stability of the synapse. Electron microscopy studies indeed provide evidence that the induction of synaptic plasticity could affect the size and shape of dendritic spines [37]. In addition, two-photon glutamate uncaging and imaging experiments demonstrated a close association between increased synaptic strength and an enlargement of spine head [38], enlargement that could account for increased synaptic strength at many synapses. A small but significant fraction of synapses undergo a continuous turnover in the adult brain, probably allowing a constant adaptation of the neural circuit to experience [39]. Despite the magnitude of this process is decreased in the adult brain, a certain capacity of circuit remodelling is maintained and can be reactivated by lesions [40]. Activity and sensory experience are able to regulate synapse turnover, acting not only through the formation of new synapses but also destabilizing the existing ones [39]. An interesting feature of the activity-mediated spine turnover is that some evidence suggest that plasticity induction is facilitated in the vicinity of potentiated spines and that new spines preferentially form close to activated ones [36, 41]. Indeed, using repetitive motor learning, it has been shown that new spine formed during learning born clustered near spines formed in the training sessions for the same task, resulting also in a higher persistence over the time in comparison to non-clustered ones [42]. Depending on the protocol used, dendritic spine formation/loss could be shifted through an increase in the total number of spines or could result in a balanced change, without marked alteration in spine density [43, 44]. Taken together, all these observations suggest that dendritic spine turnover and rewiring of the network are important structural correlates of learning.

The molecular mechanisms that control spine turnover are not completely clear. Nonetheless, several mechanisms that can modify spine number and dynamics, have been reported. Firstly, the mechanisms that control long-term modifications of synapse (LTP/LTD), have influence on activity-mediated spine enlargement and stabilization [39], thus indicating a close relation linking induction and expression of plasticity, and

synapse stability. Phosphorylation processes induced by LTP involves CaMKII and PKC, which in turn have influence respectively on enlargement of spine heads and synapses stabilization [43, 45]. Moreover, BDNF has been reported to exert effects on spine formation and destabilization in the cortex and hippocampus [46, 47]: the effects on spinogenesis could be probably related to the activation of MAPK pathway and PI3K pathway, which interact with AKT and have functional links with mTOR signalling [48], thus influencing protein synthesis. An additional mechanism affecting spine growth is realized by the action and modulation of proteins implicated in cytoskeleton remodelling, such as Rho GTPases and their regulatory proteins [49]. The peculiarity of some of this factor is the possibility to diffuse in the cytoplasm. For instance, the protein RAS, which is activated during LTP induction, it is demonstrated to diffuse locally promoting plasticity in neighbouring spines [50].

Adult Neurogenesis

The adult mammalian brain continuously generates new neurons that become integrated in the pre-existing networks. Under physiological conditions adult neurogenesis is mainly restricted to the olfactory bulb and dentate gyrus of the hippocampus. These areas produce very specific subsets of new neurons. The olfactory bulb incorporates new granule cells and periglomerular cells, both of which are inhibitory interneurons and release c-aminobutyric acid (GABA) [51]. The dentate gyrus generates dentate granule cells (DGCs), principal neurons which release glutamate [52]. The contribution of adult-born DGCs to hippocampal function is a central question in the field of adult neurogenesis and brain plasticity. Different approaches developed over the past 20 years have contributed to the concept that adult neurogenesis is necessary for hippocampal function. Nonetheless, what makes adult-born neurons relevant to the dentate gyrus network remains a puzzle. The continuous addition of neurons represents a remarkable degree of circuit plasticity and will be treated more in detail below, in the section of “Part I: plasticity in healthy brain”.

Homeostatic Plasticity

In spite of the numerous changes that nervous system has to face during development and learning, such as modifications in synaptic strength or number, the brain has the capability to maintain the stability of its functions. Plasticity indeed, might be conceptualized as the balanced interplay of mechanisms promoting change and those promoting stability.

First Claude Bernard, and then Walter Cannon, suggest that complex physiological systems tend to promote stability, or “homeostasis”, making adjustments of physiological parameters to bring them at values near to the reference value, called set-

point. Nowadays it has become clear that neuronal activity is itself a parameter subject to homeostatic regulation.

Many of the changes that involve neural circuit work to destabilize its activity. Plasticity mechanisms such as LTP indeed, according with theoreticians' opinion, generate a powerful destabilizing force because increase the probability that a neuron undergoes further LTPs, leading to unconstrained synaptic strengthening [53]. If synapses are able to be potentiated and the resultant increase in synaptic strength is very long-lasting, over time the total synapses of a neuron could be driven towards high levels of synaptic strength, potentially overwhelming the cellular metabolic capacity. Homeostatic plasticity is an additional non-Hebbian form of neuronal plasticity that is a critical regulator and stabilizer of behavioural change. To be considered homeostatic, a plasticity mechanism should regulate a key parameter around a set-point value. This concept implies that a neuron must sense the value of the parameter (firing rate for instance) and eventually generate an error signal when this deviates from the set point. Homeostatic forms of neuronal plasticity regulate all the synapses of a neuron in unison, in an orchestrated fashion, thus differing from Hebbian forms of plasticity such as LTP and LTD. Currently many phenomena have been described as form of homeostatic plasticity. These include: the activity dependent regulation of intrinsic neuronal firing [54]; pre- and post-synaptic forms of excitatory synaptic plasticity, as synaptic scaling, able to move all of a neuron excitatory synapses up or down, in order to stabilize firing [55]; the balancing of excitation and inhibition inside neural networks [56]; compensatory changes in synapse number [57]; mechanisms that regulate the probability to induce LTP or LTD [58]; homeostatic regulation of intrinsic excitability [59].

At present, the best understood form of homeostatic plasticity at the central excitatory synapses is synaptic scaling (Fig. 1). Synaptic scaling tends to modify synaptic strength in a compensatory way once brain network undergoes a perturbation of its activity, restoring average firing rates at baseline value [60]. Neurons of the CNS indeed, seem to be able to maintain average firing rates around a homeostatic set point [61]. Modulating network activity induces uniform increases or decreases of miniature excitatory postsynaptic currents (mEPSC) of a single neuron [60], so that the average firing rate is maintained but the relative strength of each single synapse is adapted. Perturbations in network activity could be sensed by individual neurons as changes in receptor activation, or changes in secreted factors, and induce modifications that are strongly suggested to be cell-related and resulting in a modification of neuron's own firing. Thus, selectively blocking postsynaptic neuronal firing with Tetrodotoxin (TTX - a Na^{+} channel blocker), scales up synaptic strengths with an intensity degree comparable to the blockade concentration [62]. Synaptic scaling involves postsynaptic changes in receptor accumulation. Blocking postsynaptic firing in neocortical neurons scales synapses up through the reduction of CaMKIV activation and transcription as a result of somatic calcium drop; this leads to AMPAR accumulation in postsynaptic membrane at all excitatory synapses [62]. Like scaling up, scaling down in response to elevated

network activity is dependent on calcium flux and in particular involves enhanced calcium influx, gene transcription, the CaMKK/CaMKIV signalling pathway, and targets the GluA2 subunit of AMPAR [63]. Scaling down is likewise realized through the activation of Plk2 (polo-like kinase 2) that after a CDK5-dependent recruitment to SPAR, induces its degradation activating a pathway necessary for the reduction in synaptic AMPAR accumulation triggered by elevated activity [64].

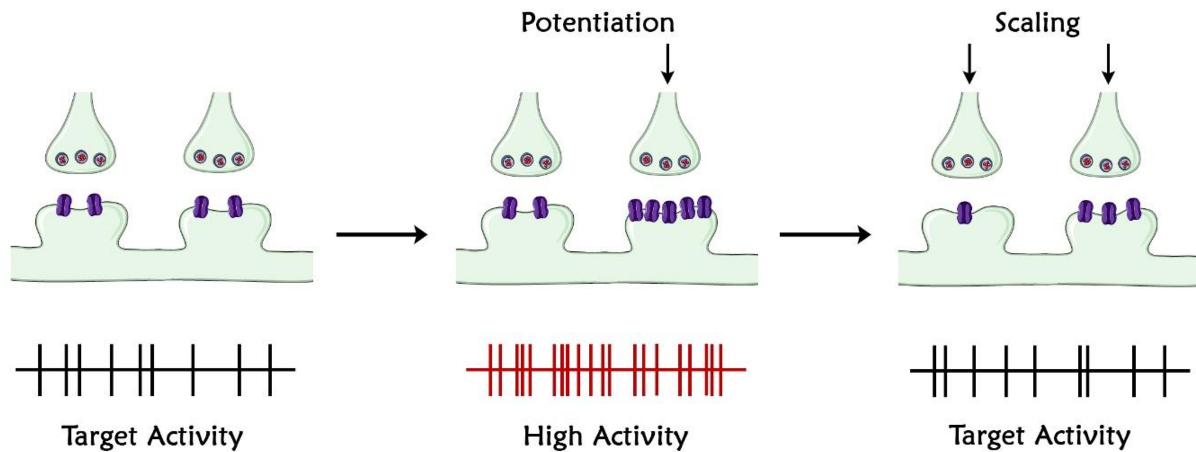

Figure 1. Illustration of synaptic scaling. When activity is perturbed (illustrated here as the potentiation of some inputs through Hebbian mechanisms) this triggers synaptic scaling, which produces a proportional reduction in strength at all synapses of the right magnitude to return firing to baseline levels. Note that, because this mechanism scales synaptic strength up or down proportionally, the relative difference in synaptic strengths induced by Hebbian mechanisms is preserved. Figure adapted from “Homeostatic Synaptic Plasticity: Local and Global Mechanisms for Stabilizing Neuronal Function” [61].

Homeostatic plasticity mechanisms could also exist at the network level operating through an activity-dependent release of secreted factors which modulate excitation/inhibition balance. In neocortical neurons, the activity-dependent release of BDNF seems able to mediate synaptic scaling inasmuch as blocking BDNF signalling mimics the effects of activity blockade on excitatory mEPSP while exogenous BDNF application does the opposite [65]. Another secreted factor suggested to contribute to homeostatic plasticity is the Tumour Necrosis Factor α (TNFα), a cytokine that is part of the inflammatory response to pathological states. Prolonged activity blockade with TTX stimulates glial release of TNFα which in turn acts on neurons increasing AMPAR insertion and scaling up mEPSC amplitude [66]. It seems that TNFα play an important role in the maintaining of scaling during prolonged (>24h) activity blockade, while it is not necessary for the induction of early scaling (4-6h) [66]. Considering that neural circuits are composed of excitatory and inhibitory synapses, it is expected that synaptic scaling would affect both type of synapses but, taking into account the homeostatic concept, in a different and maybe opposite manner. Indeed, it has been shown that inhibitory synapses onto pyramidal neurons are modulated in the opposite way in response to a drop in activity [67, 68]. Nevertheless, it has been shown that after activity blockade in hippocampus, under some conditions, inhibition and excitation change in

the same direction [69] and that interestingly, not all excitatory neurons express synaptic scaling since CA1 neurons scale synapses in response to activity blockade while CA3 neurons do not [70].

Synaptic scaling is induced in a global manner as a function of postsynaptic firing. Nevertheless, local or quasi-local changes in synaptic signalling can induce homeostatic modifications in synaptic strength. A truly local form of homeostatic plasticity would involve a single synapse in relation with changes in presynaptic transmitter release or postsynaptic receptor activation. Despite evidence for the existence of a synaptic-specific form of homeostatic compensation (realized through the accumulation of GluA1) at postsynaptic sites in response to reduced presynaptic neurotransmitter release [71], contrasting evidence make this topic still controversial [62]. Moreover, on a theoretical level, the meaning of truly local homeostatic plasticity is not clear considering that such a mechanism would counteract memory storage considering that potentiating a synapse through LTP would induce a homeostatic depotentiation (and the opposite after LTD). On the other hand, quasi-local forms of homeostatic plasticity act on groups of nearby synapses and would exert a useful normalization function without markedly affecting Hebbian plasticity [72, 73]. On the postsynaptic side, the global blockage of neuronal firing with TTX together with local glutamate receptor block with AP5 ((2R)-amino-5-phosphonovaleric acid) enhance GluA1–containing/GluA2–lacking AMPAR accumulation in the blocked synapses resulting in a substantial change in synaptic AMPA receptors composition [74]: this effect has recently been attributed to retinoic acid (RA) production and RA receptor (RARα) activation [75]. This is in contrast with the mechanism behind global synaptic scaling since blocking neuronal firing alone leads to the enhancement of mEPSC increasing the number of synaptic GluA2-containing AMPAR. On the presynaptic side, enhanced synaptic activity was observed to reduce the release probability (P_r) through a mechanism that was local to particular dendrites [76]: considering that synapses that contact the same dendritic branch have the same P_r and the less the number of synapses the more the P_r is, it is suggested that this regulation happens in a quasi-local way in function of dendritic hyperpolarization. This form of homeostatic plasticity has been suggested to prevent synaptic saturation and excessive depolarization onto a dendrite [76] but the mechanism for the induction remains elusive. Interestingly, postsynaptic BDNF release has recently been suggested to locally regulate the P_r of neurotransmitters [77], highlighting the role of activity-dependent factors on homeostatic plasticity.

PART I: PLASTICITY IN HEALTHY BRAIN

The adult brain has been often considered similar to a circuit board, and thus reliant on a fixed and precise connectivity. However, neural network undergoes an important and constant remodelling process throughout the lifetime. Brain plasticity is seen as a nature's stratagem to adapt rapidly to a changing environment, thus overcoming genetic limitations, which has slower occurrence [78]. Plasticity represents an intrinsic property of the nervous system retained throughout life that enables modification of function and structure in response to environmental demands via the strengthening, weakening, pruning, or adding of synaptic connections and by promoting neurogenesis.

During the early childhood, there is a considerable capacity for cross-modal plasticity [79], i.e. the adaptive redeployment of neurons to integrate the function of different sensory systems. The observed brain changes in all conditions related to alterations of one of the sensory fields highlight the important physiological role of adaptative neuronal plasticity. For instance, tactile acuity is significantly superior in blind subjects compared to controls [80], speech processing and auditory localization activate the visual cortex in congenitally blind humans [81, 82] and the sensorimotor representation of the reading finger is expanded in blind Braille readers [83]. However, brain plasticity has a pivotal role also in more common and less extreme conditions. Indeed, an intriguing model for neuroplasticity studies are musicians. Exposure to musical training in early life shapes the brain. The anterior corpus callosum, consisting of nerve fibers connecting prefrontal regions crucial for coordination of bimanual motor activities and frontal motor-related regions, is larger in musicians that started their training before the age of 7 than in musicians without an early start, or controls [84]. Moreover, the cortical representation of the left hand fingers in string players is increased and is correlated with the age at which the person had begun to play [85].

Even though the developing brain is far more plastic than the adult brain, memory process involves brain plasticity and even in the adulthood the mature brain undergoes a continuous remodelling of the existing connections by experience of the everyday life and by performance of specific movements during motor and cognitive learning [86]. Plastic short-term modulations are important in the acquisition of new skills and can lead to structural changes in the cortical network as the skill become more established and automatic [87].

A fascinating example of plasticity in the adult brain regard London taxi drivers, who have a training period of 2 years before qualifying as drivers. The volume of grey matter in their right posterior hippocampus, a crucial region for spatial representation of the environment, is greater than controls subjects and increases together with the amount of time spent practicing as taxi driver [88].

Brain plasticity however has not the same efficiency in each subject: individual differences are likely quite large [78]. Important factors that contribute to differences in

mechanisms of plasticity include genetic and epigenetic mechanisms, such as polymorphisms or genetic expression, hormonal factors, such as gender or phases of menstrual cycle, impact of morbidities, such as diabetes or cancer, and lifetime experiences, such as brain injury stress, sleep deprivation, substance abuse or poor nutrition [78]. In keeping with this assertion, a number of genetic factors have been identified to regulate human brain plasticity [89]. The BDNF for example, has an important role in neuronal plasticity. A polymorphism of BDNF gene, which consists in a substitution of valine to methionine (Val66Met), leads to reduced levels of mature BDNF and differentially modulate human cortical plasticity and the response to training, brain stimulation and motor learning [90-92]. Another common mutation that highlights the influence of genetic factors on brain plasticity is the Apolipoprotein E (APOE) gene, and its ε4 allele, strictly linked with the risk of Alzheimer's Disease [93], and able to influence brain network plasticity and the extent of plasticity throughout the lifespan [94]. Environmental factors contribute to genetic expression interacting with genes but also via epigenetic modifications, influencing plasticity mechanism across the lifespan [78]. These factors include educational experience, family upbringing and other social interactions, hormones, stress or physical activity.

Plasticity in hippocampus: adult neurogenesis

In recent years, neural plasticity has been a field which met an explosion in scientific research. It is now clear that environmental influences, including specific experiences, have a profound effect on adult brain structure and function [95]. In keeping with these evidence, much attention has focused on hippocampus, both because of its important role in certain types of learning and memory, in particular episodic and spatial memory, and its impressive degree of structural plasticity.

The hippocampus is localised in the medial temporal lobe (MTL), is considered a three-layer cortex and is made up by the Dentate Gyrus (DG) and *Cornu Ammonis* (CA), which is in turn divided in three main areas, CA1, CA2 and CA3 (Fig. 2). The information processing in the hippocampus follows a main pathway through its constituting areas forming the so-called "tri-synaptic circuit": in particular, the information from the Entorhinal Cortex (EC) reaches the DG and then, in the order, CA3, CA2 and CA1, from which it returns to EC passing through the subiculum [96](Fig. 2).

In line with computational models, the hippocampus appears to rapidly learn associations between arbitrary events - *one-shot learning* -, form distinct representation from overlapping neocortical input - *pattern separation* - and, retrieve a complete representation in the presence of ambiguous or partial neocortical input at retrieval of memories - *pattern completion* - [97]. The hippocampal pattern separation function is thought to be primarily supported by DG, thanks to its large number of neurons (relative to its principal input, the EC) and sparse coding, which might lead to the production of distinct non-overlapping representations from similar overlapping input. Moreover,

despite many numerous questions still remain, vast literature suggests that the DG function is also strictly linked with a peculiar phenomenon which takes place in this hippocampal field: neurogenesis.

Neurogenesis in the dentate gyrus

Adult neurogenesis in the DG has been demonstrated in a wide range of mammalian species, including humans [98, 99]. The dentate gyrus has a typical arrowhead shape (ventral and dorsal leaflets linked by the crest) (Fig. 2), and is considered a three-layer cortex. The molecular layer (ML), is the external one, is relatively poor in cells and contain the dendritic trees of granule cells and the fibers constituting the perforant pathway, which originate from neurons in the EC. The principal layer of the DG is the Granule Cell Layer (GCL) made up of 4-8 lines of granule cells which represent the principal neurons of DG; beneath the GCL is localised the Subgranular Zone (SGZ), where neural stem cells could be found and which therefore represents one of the only two widely acknowledged regions to date that retains neurogenesis under physiological conditions in adulthood [52, 100].

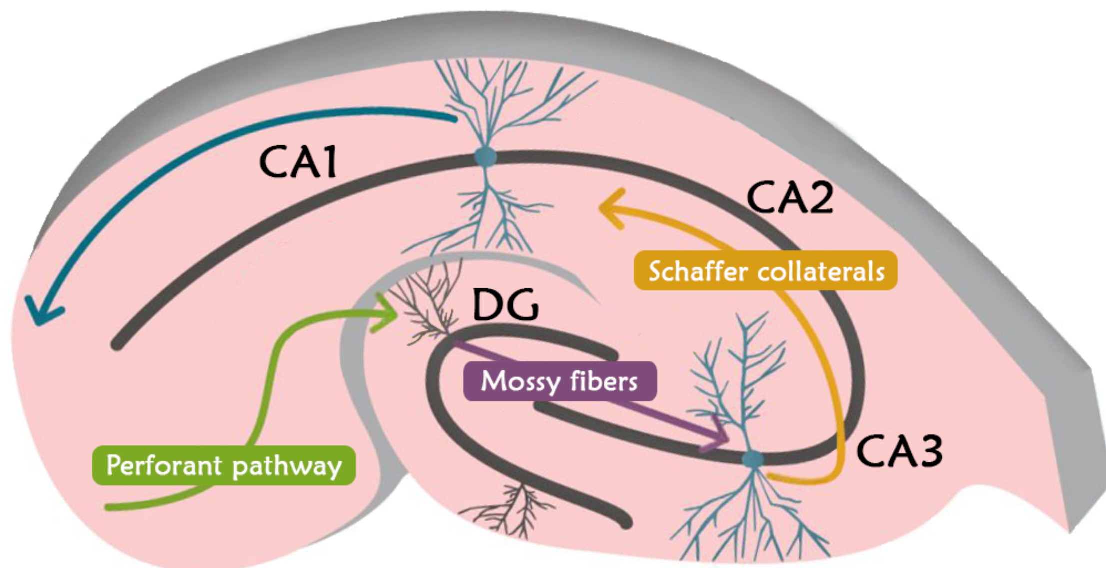

Figure 2. Hippocampus structure. The principal constituting areas are reported. DG: dentate gyrus; CA3: *Cornu Ammonis* 3; CA2: *Cornu Ammonis* 2; CA1: *Cornu Ammonis* 1. The tri-synaptic circuit is also reported. In particular, the stellate cells located in the entorhinal cortex represent the principal input onto granule cells and their axons constitute the perforant pathway. Granule cell axons, the mossy fibers, make synapses onto CA3 pyramidal cells which, in turn, contact CA1 pyramidal cells. The information is subsequently brought to external areas.

Lastly, the third layer is the polymorphic one, which is placed between the dorsal and the ventral leaflet of GCL, constituting the hilus, where many different types of interneurons could be found and where the DGCs' axons, the mossy fibers, pass until they reach and contact the CA3 pyramidal neurons.

In adult rodents, there are several thousand new neurons generated every day in the DG, modifying approximately 6% of the total DGC population per month [101]. Although most of these newborn DG cells (60–80%) undergo apoptosis within about one month following birth [101, 102], a remarkable number of new neurons survive and functionally integrate into the existing neural circuits [103] (Fig. 3). As new granules survive and integrate into the existing neural circuit, they form new connections with afferent projections and efferent targets within the neural circuit. Therefore, continuous addition of new DGCs in the DG introduces structural plasticity throughout the adulthood.

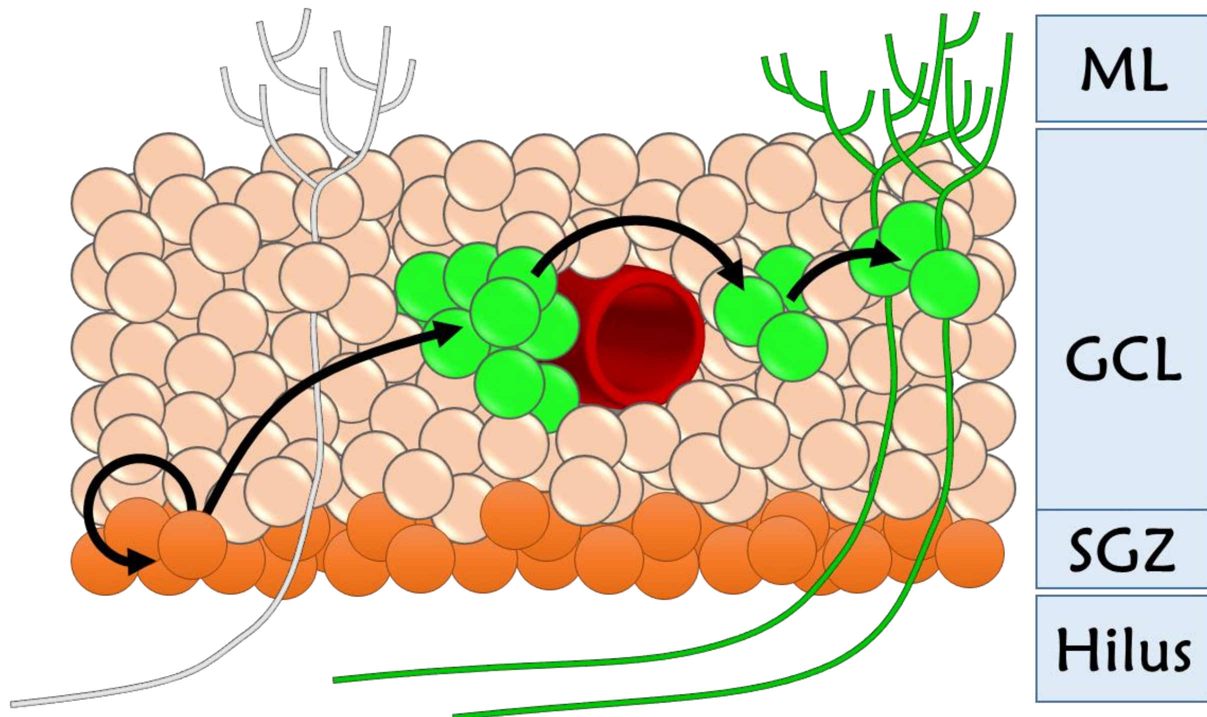

Figure 3. Hippocampal Neurogenesis. In the hippocampal dentate gyrus new granule cells are daily generated along the proliferative zone called subgranular zone. Progenitor cells (green cells - often localised around blood vessels) migrate along the granule cell layer, differentiate and integrate into the hippocampal network (green mature cells represented with dendrites and axons). ML: Molecular Layer; GCL: Granule Cell Layer; SGZ: Subgranular Zone.

Following what happens in the embryonic development, newborn DGCs in the adult brain follow a precise sequence of neuronal development and synaptic connectivity before they become fully mature [104-106]. During the first week, newborn DGCs have limited processes, crossing the granule cell layer toward molecular layer. All cellular properties resemble those of typical immature neurons of the developing brain, as they start to express neuronal sodium channels and fire immature action potentials [107]. After 2 weeks, new neurons have begun to migrate into the granule cell layer and to display typical granule cell morphology, with more numerous and elaborate dendrites traversing the molecular layer. However, no dendritic spines are observed at this stage [107]. Membrane properties become more mature but the characteristics of immature neurons still remain. At 4 weeks, newborn DGCs display the morphology of

mature granule neurons, including spiny dendrites that reach the outer border of the ML and axons that project to the CA3 region. Basic physiological properties mimic mature neurons at this stage, exhibiting mature action potentials and all known types of DGC synaptic connections. Nonetheless, the integration into functional circuit, the electrophysiological maturation and the plasticity seems to continue to evolve for at least three months [108]. During the development of newly-generated DGCs, GABA has been shown to play crucial roles [109]. Lacking synaptic inputs in the first week, newborn DGCs in the adult brain are tonically activated by ambient GABA. Functional GABAergic synapses that receive phasic GABAergic inputs from local interneurons start 8 days after birth [110]. Physiologically, these GABAergic inputs to adult-born DGCs share the same characteristics of those found on mature DGCs born in embryonic and early postnatal stages, and have similar functional properties [111]. GABA, as opposed to what happens in mature DGCs, has an excitatory action on immature granules owing to the high cytoplasmic chloride ion content of newborn DGCs in the first 2–3 weeks, and plays crucial role in regulating migration, development, and synaptic integration of newborn neurons [109]. Tonic GABA activation depolarizes newborn DGCs, and more importantly, it constitutes the majority of GABA-induced activation during the initial integration process when phasic GABA activation either does not exist or is weaker than tonic activation.

Following the formation of GABAergic synapses, glutamatergic inputs from the entorhinal cortex initiate synaptic connections onto the growing dendrites of adult-born DGCs 10 days after the birth [110]. By 4–8 weeks, adult-born DGCs display functional glutamatergic synaptic inputs similar to mature neurons [105]. Glutamatergic inputs also regulate neurogenesis in the adult hippocampus, presumably by modulating neuronal integration and survival during development. Some studies have shown that AMPA receptor potentiation increases adult neurogenesis [112], while loss of NMDA receptors activity decreases newborn neuron survival [113]. Seemingly, in contradiction, application of NMDA or AMPA receptors antagonists increase adult neurogenesis in the DG mainly by the regulation of cell proliferation [114-116]. These results suggest that the regulation of adult neurogenesis by glutamatergic activity is complex, possibly through different downstream signalling pathways, or sensitive to environment or behavioural changes following treatment.

As newborn DGCs extend dendrites into the molecular layer, they extend axons rapidly toward the CA3 region. One week after birth, newborn axons pass through the hilus and reach the proximal CA3 region; from the second week, they begin to form *en passant* expansions [106, 117, 118]. Axons continue to grow along the CA3 within 3–4 weeks while their expansions grow into larger, mossy fiber buttons [117]. Newborn DGC axons do not extend beyond CA3, so they ultimately share the same trajectory as pre-existing mature mossy fibers. The earliest output synaptic contacts form on the dendritic shafts of target neurons starting from the second week. It takes 8–16 weeks for these new mossy fiber buttons to reach full maturity, with multiple invading dendritic spines and a stable number of synaptic contacts [117]. Recently, thanks to the optogenetic

approach, it has been found that mature adult-born DGCs establish functional synapses with hilar interneurons, mossy cells, and CA3 pyramidal cells and release glutamate as their main neurotransmitter, as mature DGCs [119]. However, the complete process of axonal integration and maturation remains unclear.

Newborn granule cells' "critical period" and their potential role in hippocampal functions

Newborn neurons are continuously produced and incorporated into the existing hippocampal circuits throughout the adulthood. Therefore, a fundamental question is: do they contribute to hippocampal functions? Several studies using different approaches have shown the involvement of adult-born DGCs in hippocampal-dependent behaviours [120, 121]. The enhancement of neurogenesis, is usually associated with elevated synaptic plasticity in the DG and/or improved hippocampal-dependent learning and memory [122, 123]. Recent data also demonstrated that genetically increased DG neurogenesis through the specific inhibition of newborn cell death is able to improve hippocampal-dependent pattern separation [124]. In keeping with the hypothesis that neurogenesis exerts a positive effect on learning and memory, decreased neurogenesis in either transgenic mouse lines, such as Methyl-CpG binding protein 1 knockout (MBD1-/-) mice, or ablation of neurogenesis by irradiation results in decreased synaptic plasticity in the DG and/or deficits in some forms of hippocampal-dependent learning and memory [125, 126]. Besides, Drapeau and colleagues observed a direct connection between water maze performance and the number of newborn neurons in the hippocampus of aged animals, in which animals that retained spatial memory exhibited a higher level of cell proliferation and a higher number of new neurons in comparison to those with spatial memory impairments [127].
Additionally, the removal after learning of integrated, adult-born neurons, using a diphtheria toxin-based strategy without affecting ongoing neurogenesis, degraded existing hippocampal-dependent contextual fear and water maze memories, suggesting that adult-born neurons form a critical and enduring component of hippocampal memory traces [128]. Taken together, these studies suggest that adult-born DGCs are involved in hippocampal functions.

During their maturation, the functional properties of newborn granule cells face changes which give rise to periods of development possibly related with specific roles in hippocampal functions. Hubel and Wiesel established the term ''critical period'' to describe a particular time window in which neuronal properties are particularly susceptible to modification by experience, together with extensive anatomical changes that become irreversible after the closure of this period [129, 130]. This time window is characterised by enhanced morphological and synaptic plasticity, and is now considered a central mechanism for establishing fine-tuned neuronal circuits in the developing brain [131]. Thus, knowledge of neuroplasticity within critical periods emerged primarily from research on sensory systems such as the visual system. Nonetheless, even newborn

granules, during the integration in hippocampal circuit, when they start to receive experience-driven inputs from existing neural network, go through a phase of enhanced plasticity. Synaptic plasticity such as LTP has been thought to be the primary cellular basis of hippocampus-dependent learning and memory. As demonstrated by Ge et al., young adult-born DGCs display enhanced LTP with decreased induction threshold at the age of 4–6 weeks that rapidly drops by 8 weeks of age [132], indicating a critical period with enhanced synaptic plasticity. These data confirm previous findings [133, 134] and are consistent with other studies showing adult-born neurons display a high level of anatomical plasticity during this period which decreases thereafter, such as spine motility [106], suggesting that the newborn DGCs undergo a short period of fine-tuning while integrating into existing circuits. How are new DGCs more plastic during this period? Immature adult-born granules display distinct active and passive membrane properties such as high input resistance (IR) [105, 107]. Besides, in young neurons, high levels of T-type Ca^{2+} channels can generate isolated calcium spikes and enhance fast Na^{+} APs, contributing to the induction of synaptic plasticity [134]. Another key mediator of plasticity is the NMDA type of glutamate receptors. During adult neurogenesis, NMDARs are expressed early, starting from immature neuronal stages [107, 135, 136]. It is known that during early postnatal neuronal development, switching of NMDARs subtypes from NR2B to NR2A changes the direction and degree of synaptic plasticity [137-139]. NMDARs containing NR2B subunit are expressed early during postnatal development and appear to be associated with enhanced synaptic plasticity during the critical period [137, 140], while NMDARs containing NR2A, which are expressed and dominant later, mediate dramatically decreased LTP after the critical period [137]. Using field potential recordings, Snyder et al. revealed that LTP in the DG with intact GABAergic inhibition appears to be largely dependent on young adult-born neurons, and could be specifically blocked by NR2B antagonist ifenprodil [125]; in contrast, mature DGCs display much less LTP in the same condition [133], or have higher threshold for LTP induction [134]. Moreover, by specifically targeting adult-born DGCs, Ge et al. showed ifenprodil completely abolished LTP on DGCs of 4-weeks old, but not 8-weeks old or mature DGCs, providing a temporal correlation between synaptic expression of NR2B subtypes and critical period plasticity [132]. They also found that the plasticity of newborn DGCs within the critical period relies significantly more on NR2B-containing NMDARs than on pan-NMDARs, suggesting that NR2B, which is the major NMDARs subtype expressed during the critical period, plays an instructive role in the enhanced synaptic plasticity of adult-born DGCs within this time window [132]. These studies suggest that adult-born neurons in the critical period undergo molecular mechanisms similar to neurons in the early postnatal critical period.

Adult-born DGCs integrate into existing hippocampal circuits and express an improved plasticity during the critical period in relation to events other than mature neurons that have passed the critical period [132]. It is therefore natural to ask if they make unique contributions to hippocampal function. As suggested by numerous emerging evidence, adult-born DGCs might be preferentially recruited into hippocampal

circuits related to spatial information processing, contextual fear conditioning, novelty recognition and memory formation [103, 120, 141]. This preferential recruitment is consistent with the critical period of the adult-born DGCs and appears at 4–6 weeks after birth [120, 141]. In relation to their high excitability [105, 142] and the critical period of enhanced plasticity, adult-born DGCs of the critical period are more readily recruited into the hippocampal circuit for the encoding of novel information. As hypothesised by Aimone and his colleague, the special properties of young adult-born neurons are required for the formation of temporal clusters which associate individual elements of long-term episodic memories, a function called "pattern integration" [143]. Indeed, newborn neurons are preferentially activated in the critical period of enhanced plasticity and then, after the maturation, become less excitable but part of mnemonic traces: the progressive maturation of these granule cells create a temporal link between the different input that reach the DG. Overall, the available evidence strongly indicates that young adult-born neurons play an important role in participating in certain types of hippocampus-related behaviours, particularly learning and memory. However, the specific role of adult-born neurons which mature cells couldn't achieve still remains under investigation.

Adult-neurogenesis as substrate for experience-dependent change

Adult neurogenesis is dynamic and highly dependent on the activity of the neural network. As DG receives various innervations from multiple brain regions, adult-born neuron development at distinct stages is regulated by numerous factors related to global and local neuronal activities. Actually, a growing body of literature indicates that adult neurogenesis is strongly influenced by the environment [95, 144]. Findings for overall literature on experimental modulation of adult neurogenesis seem to converge on a basic model: rewarding experiences, such as physical activity or mating, tend to increase the production of new neurons, whereas more aversive experiences, such as social defeat or predator smell exposure, tend to decrease the production of new neurons, [95, 144].

Hippocampal neurogenesis and physical activity

Voluntary physical exercise is one of the most studied activity able to positively influence adult neurogenesis. Neurogenesis improvement induced by physical activity is considered able to maintain the brain in fit: indeed, a large body of literature bring evidence about the cognitive performance improvement after physical and mental training. Physical activity is able to significantly increase the number of newly-born granule cells [123, 145]. The effect of physical exercise on precursor cell proliferation is related to many possible factors such as BDNF [146, 147], serotonin (5-Hdroxytriptamin – 5HT) [148, 149], Vascular Endothelial Growth Factor (VEGF) [150-153] or Insulin-like Growth Factor-1 (IGF-1).

BDNF seems to be the principal factor in mediating the effects of physical activity on neurogenesis, and its mRNA and protein levels increase in hippocampus after exercise [154]. Thus, the blockage of BDNF signalling lead to impairments in the learning improvement and neurogenesis promotion induced by physical activity [155]. Serotonin depletion in the brain of Tryptophan Hydroxylase 2 knockout mice (Trp2 -/-) affects the neurogenesis induced by physical activity, blocking the increase in granule number: it is therefore suggested that 5-HT play a role in stimulating neurogenesis after physical exercise [156]. Since Selective Serotonin Reuptake Inhibitors (SSRI) increase BDNF levels in the DG, the effect of serotonin might be linked to the BDNF action [157]. VEGF expression during exercise is also related to the increased neurogenesis but in a similar way to serotonin action, also VEGF probably act in concert with other factors, such as BDNF or IGF-1 [158]. Another factor which levels are found increased after exercise is IGF-1, which in turn increases angiogenesis and neurogenesis, in cooperation with VEGF action [159, 160]. Indeed, physical activity stimulates GH-IGF-1 axis, increasing IGF-1 blood level and the uptake of this factor not only in the muscle [161] but also in the brain [152]. Moreover, the increased levels of BDNF in the hippocampus after exercise seem to be triggered by the increasing in IGF-1 levels [152]. Physical activity is able not only to improve neurogenesis through enhanced proliferation but also to increase the number of surviving newly-generated granule cells, thus promoting survival [162].

Hippocampal neurogenesis and Enriched Environment

Donald Hebb, after the discovery of enhanced cognitive performance of pets over laboratory animals kept in cage condition, introduced for the first time the concept that an Enriched Environment (EE) could be able to improve the quality of life in animals [163]. After this first statement, Rosenzweig and colleagues developed the enriched environment model as we know it today [164]. Enriched environment consists in breading conditions able to offer a higher amount of sensory, motor and cognitive stimuli compared to standard laboratory conditions, which is considered a deprived environment [165, 166]. Often, the EE include social interaction since many animals are housed together. Many EE protocols consist in placing 8-12 rats in large cages containing many different objects, daily replaced: this condition provides novelty elements which assure cognitive stimuli, spatial representations and social interactions [167]. The items generally used in EE are characterised by different colours and shape, to provide visual stimuli, wood-items with different textures, to stimulate sensorial perception, tunnels with different shapes, for spatial navigation, and wheels for voluntary running: the number of items placed in an EE positively correlates with the number of immature granules in DG [168]. However, EE is able to affect neurogenesis only until the animal doesn't familiarize with the environment; once the EE lost its novelty, neurogenesis in the DG is increased no more [169].

The effect of EE on neurogenesis consists in increasing cellular survival rather than enhancing neural proliferation [123, 169]; however, Kempermann & Gage [169]

revealed the ability of EE to increase proliferation as well. The effect of EE on proliferation seems to be mediated by GABA signalling, which could act on silent synapses characterised by only Mg^{2+}-blocked NMDAR, promoting AMPAR incorporation and the synaptic integration [170]. Since NMDAR is crucial for immature neuron survival and integration [113], the increase of synapses with activated NMDAR might promote neuronal survival.

Hippocampal neurogenesis and Learning

Learning is able to positively influence neurogenesis. Nonetheless, only certain types of learning can promote neurogenesis. For instance, a hippocampal-dependent learning such as the Morris Water Maze test, where the animal is tested for the detection of a hidden platform in a pool, is able to promote granule survival [171, 172], whereas learning to localise a visible platform (hippocampal-independent learning) has no effect [171].

Hippocampal-dependent task promotes neurogenesis when the task is quite tricky and the more the attempts to complete the test are, the more the effect on neurogenesis is high; in addition, the effect on neurogenesis is obtained only if the test is successfully ended [173]. These findings suggest that mental effort and network activity are crucial to rescue newly-born dentate granules, and suggests that a bigger mental effort induces better effects. In keeping with this assertion, comparing a demanding task such as Tool Use test and a less challenging task, such as the Radial Arm Maze test, Kumazawa-Manita and colleagues [174] clearly showed that Tool Use test produces a higher number of newborn granule cells than the Radial Arm Maze.

Spatial learning promotes neuronal survival and is influenced by the complexity of the task, the species employed in the test, sex differences, and the age of immature cells during learning [175]. Spatial learning promotes the survival of 6-10 days-old granule cells in rat, but seems to induce the opposite effects on older cells [107]. The survived cells, rescued by spatial learning, are reactivated when the animal is exposed to the test again, suggesting that these cells could be part of memory traces [176].

Hippocampus-dependent tasks make precocious the first GABAergic synaptic contact onto newborn granule cells, which are detectable 4-6 days after birth; this effect could represent the basis of increased survival induced by learning processes in newly-born granule [110]. It is therefore suggested that a higher number of GABAergic synapses could avoid an excessive activation of T-type Ca^{2+} channels, which might lead to cytotoxic effect related to prolonged and elevated levels of this ion in the cytoplasm. Indeed, $GABA_AR$ activation tends to stabilize the membrane at the Cl^- equilibrium potential that, in immature neurons, is depolarized with respect to the RMP. On the other hand, fast changes of membrane potential, such as that observed in immature neurons [110], are more efficacious than a constantly depolarized potential in opening T-type Ca^{2+} channels, which are highly expressed in these cells [107]. Therefore, the

formation of GABAergic synapses might prevent dramatic, possibly dangerous, Ca^{2+} transients.

It's worth mentioning that another training protocol called physical skill learning can promote DGC survival and the more the exercise is complex, the more the survival is increased. Comparing a difficult task such as accelerated rotarod training with a simpler one, such as slow continue rotational wheel training, Curlik and colleagues demonstrated a positive correlation between the difficulty of the training and the number or survived cells in DG [177]. This kind of learning is hippocampus-independent and highlights that also tasks that don't rely on this structure for their learning affect hippocampal neurogenesis. Though, the hippocampus independence is not absolute, and hippocampus lesions could affect physical skill learning, highlighting the role of this structure on motor sequence consolidation [178].

Hippocampal neurogenesis and Stress

Many research groups have been analysed the effects of stress on neurogenesis varying the intensity and type of stressor event. Principally, based on the duration of the stressor event, stress can be considered acute or chronic.

Studies on acute stress, that is the stress provoked by a single stressor event, report divergent results regarding the effects on hippocampal neurogenesis. Social defeat provoked by the presence of a dominant specimen of the same animal species, seems to decrease neural proliferation in mouse [179] and, predator's smells, such as fox, is able to reduce cell proliferation in the DG of rats [180]. Acute Restraint Stress gives rise to different contrasting results, which might be linked to different protocols in time, stress intensity or species belonging. Thus, on one hand restraint stress of 2-6 hours has no significant effect on proliferation rate in DG of rats [181], while on the other, 3 hours are able to reduce proliferation in hippocampus of rats [182]. Moreover, Brain et al. reported that the same protocol they applied on rats exerts the opposite effect on mice, increasing the number of newly-generated granule cells [182].

Chronic stress, daily experienced for many days or weeks, leads to the reduction of proliferation rate in hippocampus. Stress caused by chronic social restriction, in mouse, reduces the level of cellular differentiation in newly-born neurons [183]. Physical Restraint, besides acting on proliferation, is also able to reduce the survival of newborn granule cells in rat [181]. Moreover, if learning becomes a stressing event, it seems able no more to exert its beneficial effects on neurogenesis, thus affecting negatively the proliferation rate [184]. In addition, age and aging have effect on survival and cellular differentiation. Indeed, as emerged on research on tree shrews, psychosocial stress induces a higher degree of cell proliferation decrease in aged animals compared to young animal [185].

Stress is linked with hypothalamus-pituitary-adrenal (HPA) axis activity, which leads to the release of glucocorticoids in blood stream that, in turn, seem to inhibit neurogenesis in DG. Thus, exogenous glucocorticoids administration alters neural

proliferation in the same way as the stress does. Moreover, stress-related effects induced on proliferation by predator odour, can be blocked preventing the increase in glucocorticoids level [186]. However, it remains to establish if these effects might be mediated by the direct action of these steroids on progenitor cells or might be involved a still unknown factor. Certain types of stress are able to increase the cytokines levels, such as Interleukin (IL) -1 (IL-1), both at peripheral level and central level [187]. IL-1 is able to increase the HPA sensitivity to future stressing events [188]. It is therefore possible that stressor events could inhibit neurogenesis acting on the release of glucocorticoids enhancing IL-1 level. Moreover, considering that progenitor cells have the receptors for IL-1, whose activation is able to reduce proliferation [189], it is possible that this cytokine might act directly on neurogenesis. However, it has been not currently clear if all these different stress types are capable of increasing IL-1 levels.

Uncovering the effect of physical exercise on neurogenesis in the dentate gyrus

Physical exercise, as afore mentioned, was found to be a neurogenic stimulus, promoting neuron progenitor proliferation [123] and affecting newborn cell survival [190]. Besides, it seems to have beneficial effects on mental health and brain activity, enhancing memory function and hippocampal plasticity [122, 155, 191].

Previously, the section of Physiology of the Urbino University pointed out that a brief period of three days of physical activity in a very precocious period of adult-generated granule cells life, is able to antedate the appearance of the first GABAergic synaptic contacts [1, 110]. Indeed, they demonstrated that after voluntary physical activity on a running wheel, about 26% of 7-days old granule cells clearly display a GABAergic contact, which is normally not seen at this point of development. In addition, this very protocol is also capable of increasing the number of 7-days old immature granule showing T-type Ca^{2+} channels [1]. These data are of particular interest because could be correlated with the increased survival probability of newly generated granules seen in association with physical activity [190] and, considering the role of GABA and Ca^{2+} in fostering neuronal maturation and development, could have important implication in granule cell maturation [110, 192]. From a morphological perspective, they bring evidence that the exercise protocol applied induces the protrusion of a significantly higher number of primary dendrites without changing the total length and complexity degree of the dendritic trees. In addition to these data, a contribution of neuronal-activity-induced BDNF release in mediating the effects of the protocol they applied has been shown. This correlation is of particular interest since the neurotrophin BDNF has been implicated in activity-dependent synaptic plasticity and network remodeling [193, 194] and it is able to regulate the extent of adult hippocampal neurogenesis [195], presumably via its specific TrkB receptors [196] which are expressed on proliferating neural progenitor cells in the dentate gyrus [197], suggesting a direct

influence of BDNF on neurogenesis. Thus, the TrkB agonist 7,8-dihydroxyflavone mimicked the effect of physical exercise in rats kept under control condition, while the TrkB antagonist ANA-12 counteract the effect of the three-days voluntary running [1].

Considering the promising results described above, during my first year of PhD we decided to go further and evaluate the long-term effects of the same protocol consisting of three days of voluntary running on a wheel. The focus of our study was therefore moved on 30-days old granule cells where we performed morphological and functional analysis in order to asses if long-term effects exist and what might be their functional consequences on hippocampal functions.

Experimental procedures

Animals

For these experiments, five-week-old Sprague-Dawley male rats (Charles River Laboratories, Italy) (n = 22) were used. The animals have been housed in standard cages with water and food *ad libitum*. The environment temperature has been maintained at 21±1 °C, the humidity was 50±5% and the light/dark cycle was 12-12 h (light on at 6.00 a.m.).

To *in vivo* label adult newly generated granule cells in hippocampal DG, the animals were anesthetized with sodium thiopental (45 mg/Kg body weight) and stereotaxically injected with Green Fluorescent Protein (GFP)-expressing retrovirus. Retrovirus carrying the GFP transgene was infused bilaterally (twice 30 min spaced) into the dentate gyrus (1 μL at 0.5 μL per min) (anteroposterior: 23 mm from bregma; lateral: 2 mm; ventral: 3.2 mm) DG. Retroviral GFP-expressing virions were prepared co-transfecting the ecotropic packaging Phoenix cell line (ORBIGEN), at 70% of confluency in 100 mm dishes, with 15 mg of GFP-expressing Pinco vector [198] and 7.5 mg of pCL-Eco packaging vector (IMGENEX). The transient transfection was conducted by calcium-phosphate/cloroquine method [198] and the retroviral-containing supernatant was collected after 48 h upon transfection, filtered and immediately frozen in aliquots at -80 °C. Infection of NIH-3T3 cells was performed to check the surpernatant containing viral titer (~10^7 CFU/mL).

On the fourth day after surgery, the animals were randomly assigned to the follows experimental groups: 1. voluntary running in a wheel cage (RUN; running for three days: 4°, 5° e 6° day after retroviral injection); Control rats (CTRL, n = 20) not exposed to any behavioural experience, nor treated with any drugs. All experiments were carried out in accordance with the Italian law on animal experimentation.

Slices preparation

Thirty days after the retroviral injection, rats were anesthetized with ketamine (65 mg/Kg b.w.) and killed by decapitation. Brains were quickly removed and incubated

in chilled oxygenated solution containing in millimolar: 110.0 choline Cl^-, 2.5 KCl, 1.3 NaH_2PO_4, 5.0 $NaHCO_3$, 0.5 $CaCl_2$, 7.0 $MgCl_2$, 20.0 dextrose, 1.3 Na^+ ascorbate, 0.6 Na^+ pyruvate, 5.5 kinurenic acid (pH: 7.4; 320 mOsm). Hippocampal transversal slices (400 μm thick) were obtained from each hemisphere by vibrating microtome (Campden Instruments) and allowed to recover in oxygenated Artificial Cerebrospinal Fluid (ACSF) containing in millimolar: 125.0 NaCl, 2.5 KCl, 1.3 NaH_2PO_4, 25.0 $NaHCO_3$, 2.0 $CaCl_2$, 1.3 $MgCl_2$, 1.3 Na^+ ascorbate, 0.6 Na^+ pyruvate, 10.0 dextrose (pH: 7.4; 320 mOsm). The slices were kept in this solution for at least 1 h at room temperature before electrophysiological recordings. Individual slices were then transferred into a recording chamber where they were held in place with nylon mesh and continuously superfused throughout the electrophysiological recordings with oxygenated ACSF at a rate of 3 mL/min.

Electrophysiological Recording

The influence of three days of voluntary running on synaptic plasticity was investigated evaluating the ability of DG granules to elicit LTP after the high frequency stimulation (HFS) of the Medial Perforant Pathway in CTRL (n = 6 rats) and RUN (n = 8 rats). To specifically asses the role of immature granule cells, which are affected by the training protocol applied [1], a particular stimulation protocol developed by Snyder and colleagues [125] and able to elicit LTP on the sole immature granule cells has been applied.

To this purpose, recording and bipolar stimulating electrodes were prepared and filled with ACSF: the first electrode was placed in the molecular layer, while the latter on the medial perforant pathway. Slices giving extracellular field excitatory postsynaptic potentials (fEPSPs) of at least 1 mV in amplitude were considered for recordings. The stimulation intensity that produced a half-maximal response was chosen for test pulse and tetanic stimulation. Low-frequency test pulses (at 30-seconds intervals) were applied to elicit baseline responses. Once obtained a stable baseline of approximately 20 minutes, the medial perforant pathway was simulated applying the LTP protocol consisting of 2 trains, 500 ms each, 100 Hz within the train, repeated every 20s. The fEPSP was then monitored by recordings for 40 min. Slope (between 10% and 80% of max) of the fEPSP was analysed and taken as measures of synaptic strength; values were normalized to the mean value obtained over the last 20 min of the baseline period and expressed as a percent of this baseline value. To confirm that the LTP elicited was due to the immature granule cells only, since NMDAR containing NR2B subunit are preferentially expressed on immature granule cells membranes [140], some slices underwent the LTP protocol in the presence of Ifenprodil (3 μM), an NMDAR antagonist that selectively inhibits receptors containing the NR2B subunit, in the perfusion ACSF.

Morphological Analysis

To verify if the exercise protocol exerted long-term modifications of newly-generated granule cells born during the training, a morphological study of 30-day old DGCs has been performed on slices from RUN group (n = 4 rats) and CTRL (n = 4 rats). The slices, obtained as described above, were immediately fixed in paraformaldehyde 4% in 0.1 M phosphate buffer saline (phosphate buffered saline - PBS -, pH = 7.4) and kept overnight at room temperature (RT). To reveal GFP, the slices were immunohistochemically processed by incubating free floating slices overnight at 4 °C with the primary antibody monoclonal anti-GFP made in mouse (1:200 in PBS; Sigma) followed by the secondary antibody FITC-conjugated horse anti-mouse IgG (1:50 in PBS; Vector, D.B.A.). Immunostained slices were observed using confocal microscope (Leica TCS-SL) through a x63 immersion oil objectives.

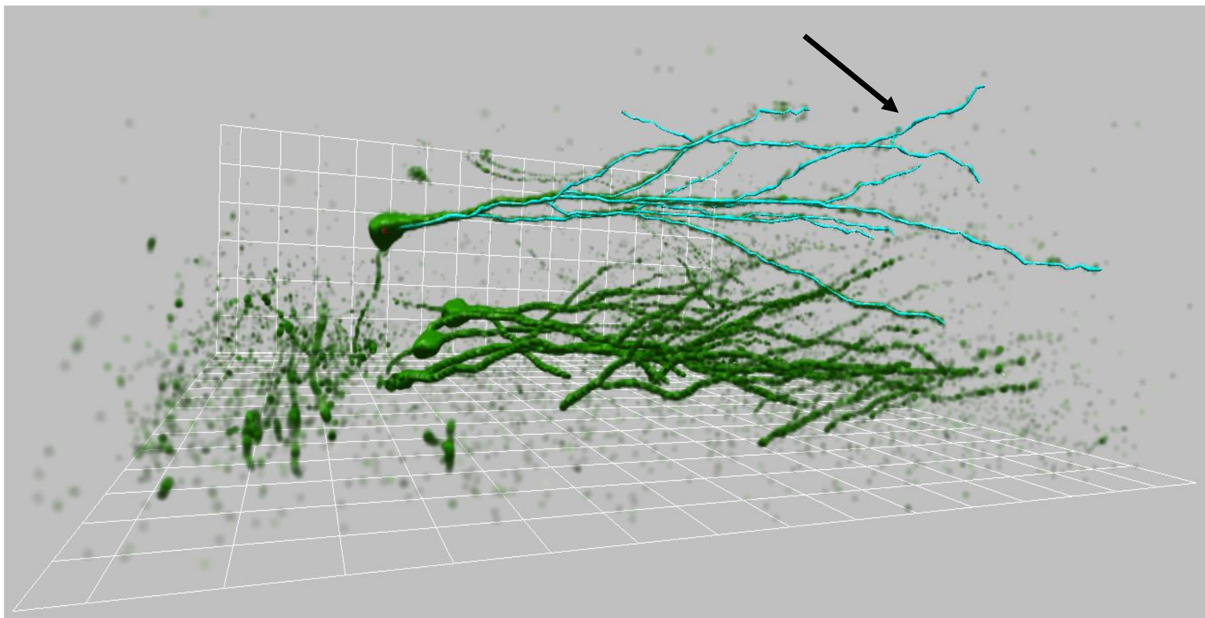

Figure 4. 3D reconstruction of granule cells. The three dimensional reconstruction of 29 day-old GFP-positive granule cells was performed using Neuronstudio softwere on confocal microscopy stacks. It is possible to notice the blue traces of dendrites (arrow) which spread through the three dimensions of the slice.

Morphological reconstruction of each GFP-positive cell was performed using a Leica TCS-SL confocal microscope equipped with Argon and He/Ne laser sources. Morphological analysis was carried out on a subset of reconstructed cells showing no clear dendritic cutting at the slice surface. The reconstruction of each granule cell has been performed using NeuronStudio software, following the dendritic arborization through the three dimensions of the slice thickness made of confocal stacks (Fig. 4). The images obtained were used to evaluate the total length of dendrites and the number of primary dendrites. To evaluate dendritic arborization, the images obtained in NeuronStudio were saved as ".TIFF" and analysed in NeuronJ. Sholl analysis was adopted to estimate dendrite arborization and was performed by Sholl Analysis Plugin:

(http://biology.ucsd.edu/labs/ghosh/software/ShollAnalysis_.class –Ghosh Lab Website), using an 8-μm interval between concentric circles. Moreover, considering that throughout the development stages, the newly-born DG granules moves from the SGZ through the granular zone, the distance of each GFP-positive granule cell from the hilus has been evaluated as index of neuronal migration.

Statistical Analysis

Data are expressed as the mean±SEM. Statistical analyses were performed appropriately, applying Student's t-test, one-way ANOVA with Tukey post-hoc test and two-way ANOVA with Sidak or Fisher LSD post hoc test. The significance threshold was established at p = 0.05.

Results

Results of the electrophysiological analysis

High frequency stimulation of the perforant pathway elicited a robust LTP in both groups, RUN (Mean total distance covered: 6.6±1.2 Km. n = 18) and CTRL. In slices from RUN group, the induced LTP was similar to CTRL group during the first 15 minutes but fEPSP in RUN animals reached significantly higher values as compared to controls, especially in the last minutes of recordings (Fig. 5).

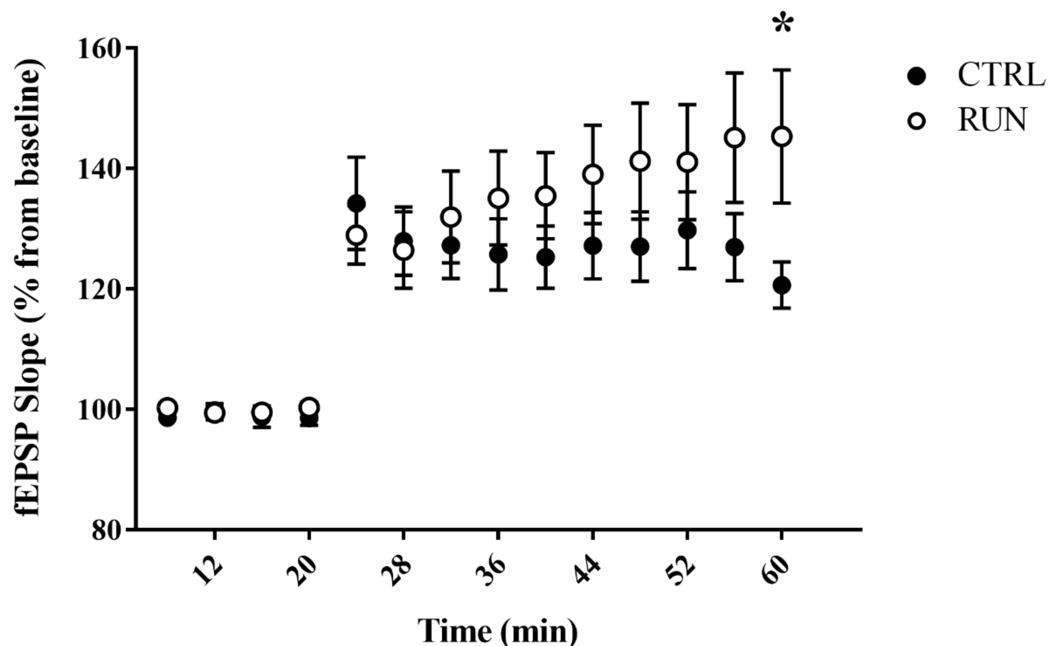

Figure 5. Long-term potentiation (LTP) in perforant path-granule cell synapses in CTRL and RUN group. Slope of the fEPSP was analysed as measures of synaptic strength; values were normalized to the mean value of the baseline and expressed as a percentage of this baseline value. Student's t-test * $p < 0.05$.

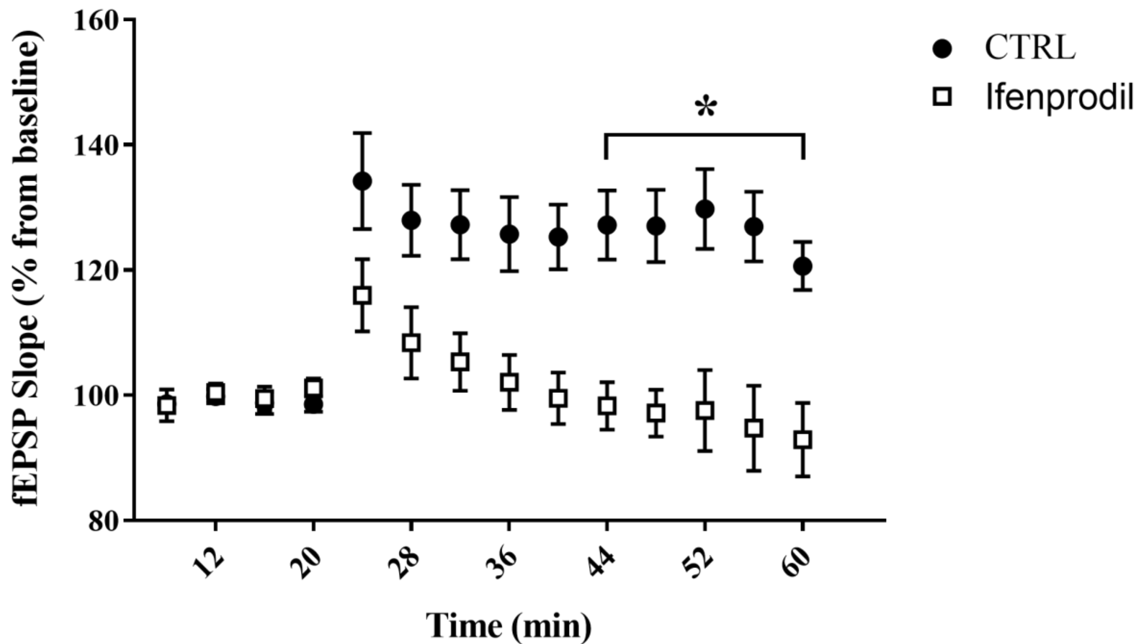

Figure 6. Effect of Ifenprodil (3 μM) on LTP elicited in perforant pathway-granule cell synapses. The application of Ifenprodil, an NMDAR antagonist that selectively inhibits receptors containing the NR2B subunit, in the perfusion ACSF, abolished the potentiation induced by the high frequency stimulation on perforant pathway, confirming the exclusive role of immature granule cells in the LTP observed with the stimulation protocol applied. Two-Way ANOVA $p < 0.05$; Sidak multiple comparison test * $p < 0.05$.

This finding indicates that immature granule cells, affected by three days of physical exercise in a precocious period of their development, contribute to the improved LTP maintenance observed after the high frequency stimulation of the perforant pathway, which induces a synaptic potentiation based only on immature cells. Consistent with this statement, Ifenprodil, which inhibit NR2B-containing NMDA receptors - almost exclusively expressed in immature cells - blocked the synaptic potentiation after the tetanic stimulation of the perforant pathway (Fig. 6).

Results of the morphological analysis

Marked cells from both groups, CRTL and RUN, showed one or more primary dendrites, which generally emerged from the top of the cellular soma and branched in higher order dendrites. The dendrites reached the molecular layer of DG and the dendritic spines were clearly noticeable. Total dendritic length was significantly higher in RUN group in comparison with CTRL (Fig. 7A) Moreover, in spite of the similar number of I order dendrites between the two groups, in RUN group a significant higher number of III and V order dendrites was observed (FIG. 7C), suggesting a higher dendritic complexity trees in the RUN group.

Another index of granular development considered was the distance of the cells from the hilar zone, consdering that these newly-generated granules, during their

development, migrate throughout the granular layer, moving away from the hilus. This analysis revealed that neurons of RUN group were generally farther from the hilus in comparison to cells of CTRL group (FIG. 7B).

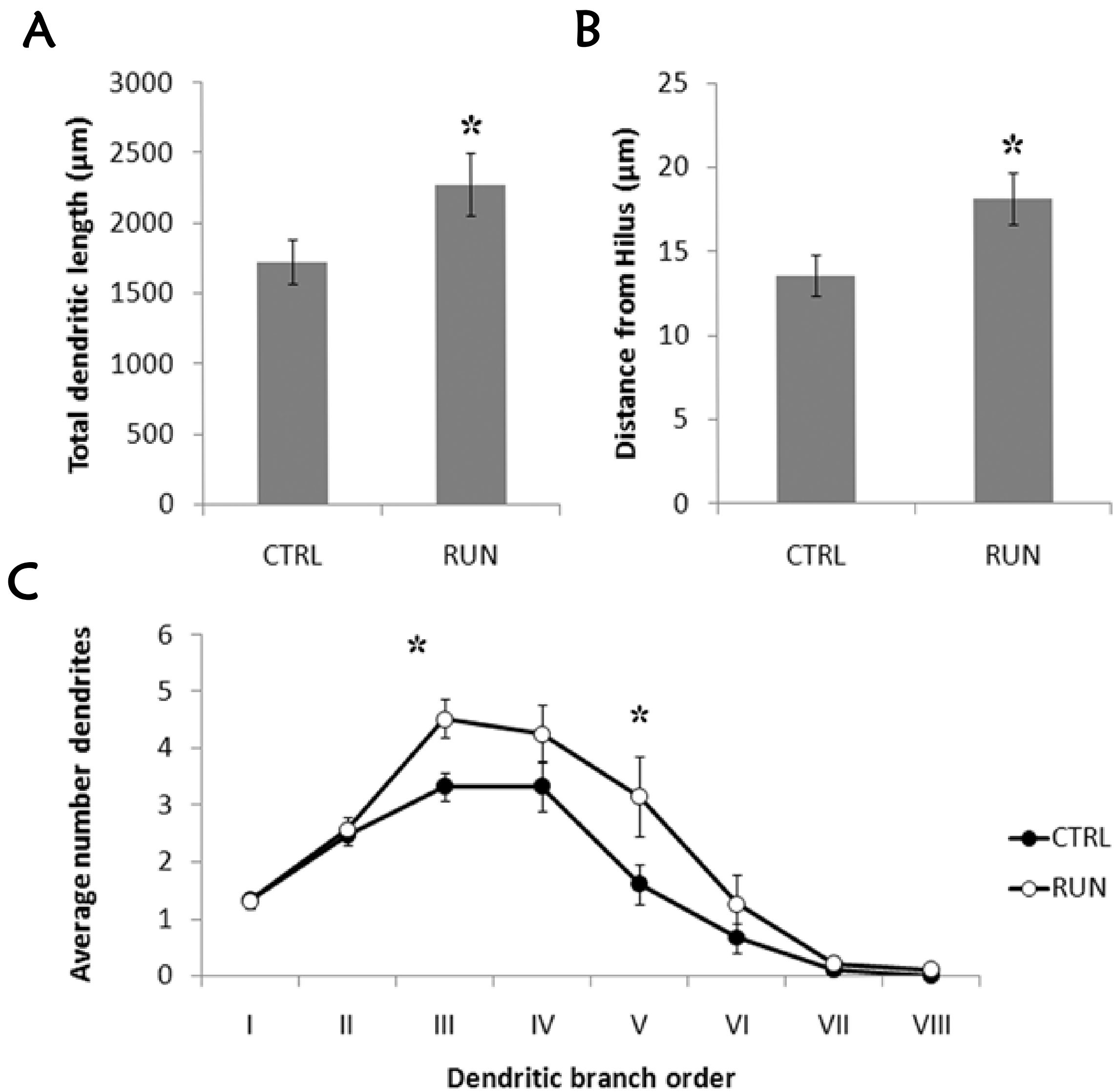

Figure 7. Morphological properties of 30-day-old granule cells. Comparison of total dendritic length (A) distance from hilus (B) and dendritic tree complexity (C) between GFP-positive neurons of rats belonging to CTRL group (n = 21 cells) and RUN group (n = 19 cells): Student's t-test * $p < 0.05$ (A, B). Two-way ANOVA $p < 0.01$; Fisher LSD post-hoc test * $p < 0.05$ (C).

Focusing our attention on RUN group cells, it is emerged that the differences between CTRL and RUN groups were principally due to a subpopulation of cells in this latter group. In particular, part of the granule cells in RUN (RUN1) group were not different in total dendritic length and distance from hilus when compared to CTRL cells; on the other hand, a subpopulation representing about the 26.6% of RUN cells (RUN2), was characterised by a greater total dendritic length and distance of migration (Fig. 8). In addition, data from Sholl analysis, which provides an estimate of dendrite arborization by evaluating the dendritic crossing along the Sholl rings, revealed a

significant greater degree of arborization in dendrites of RUN2 group if compared with control and RUN1 group (Fig. 9). These morphological findings are outlined in Fig. 10.

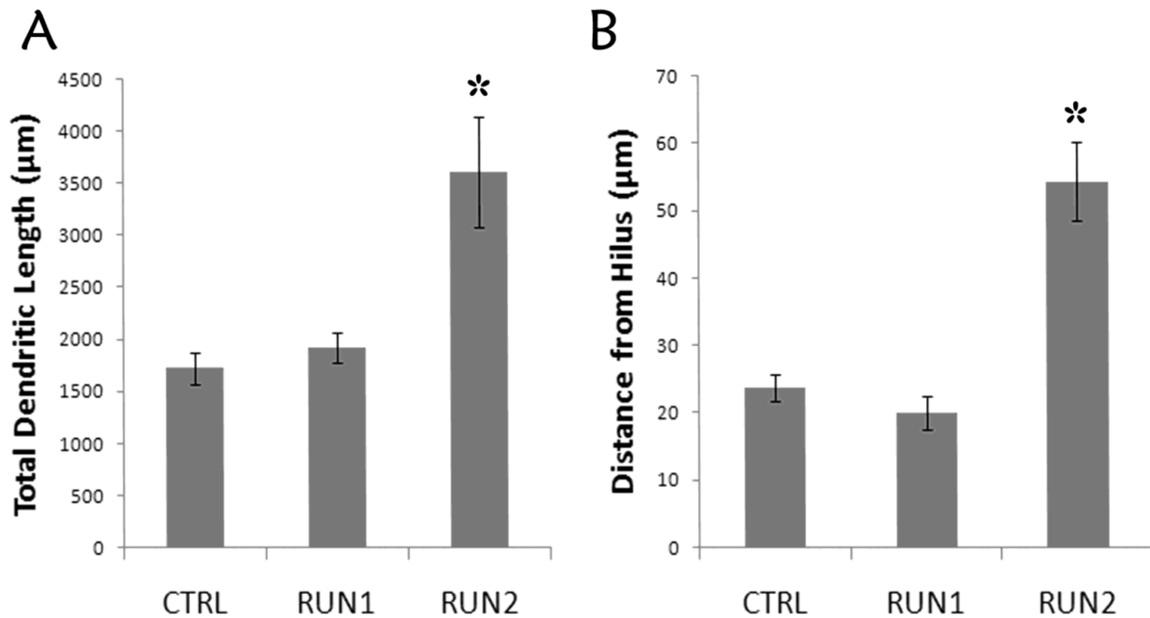

Figure 8. Morphological properties of CTRL, RUN 1 e RUN 2. (A) Total dendritic length of GFP-positive neurons in CTRL rats (n = 21 cells), RUN1 rats (n = 15 cells) and RUN2 rats (n = 4 cells): One-way ANOVA $p < 0.001$; Tukey HSD * $p < 0.01$ RUN2 vs CTRL and RUN1. (B) Distance from hilus of GFP-positive neurons of CTRL rats (n = 21 cells), RUN1 rats (n = 15 cells) and RUN2 rats (n = 4 cells): One-way ANOVA $p < 0.001$; Tukey HSD * $p < 0.01$ RUN 2 vs RUN 1 and CTRL.

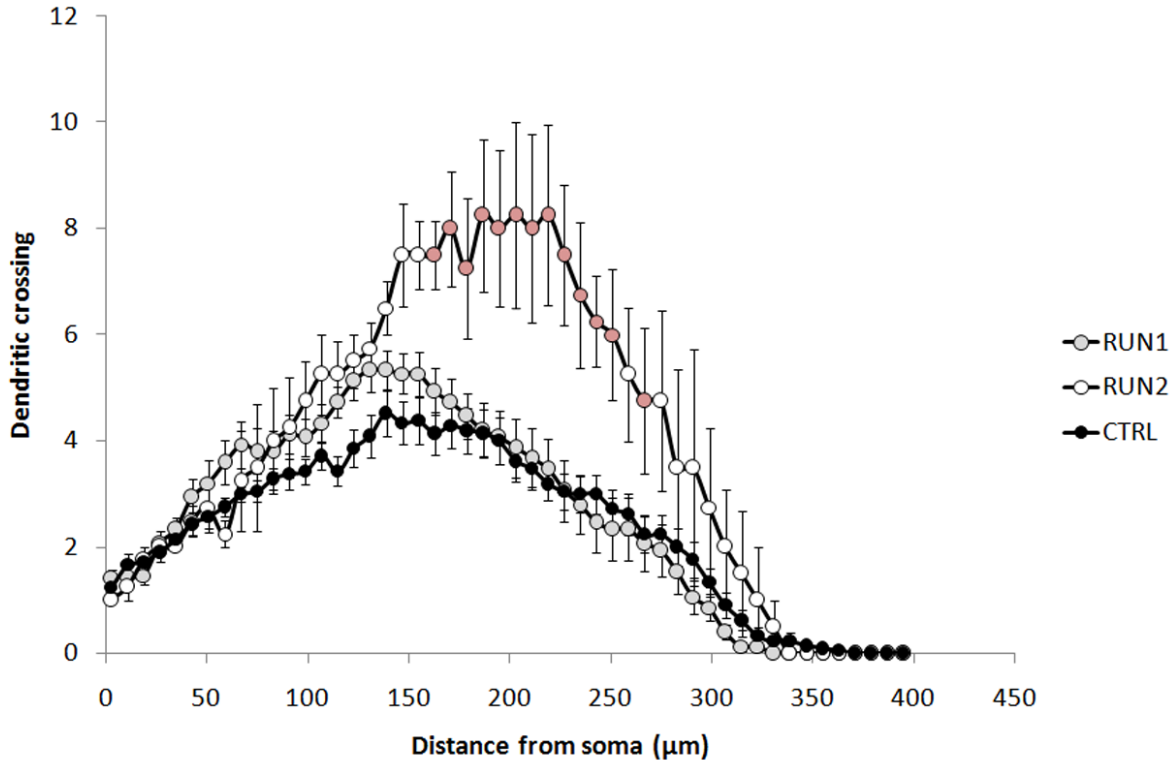

Figure 9. Sholl Analysis results showing the differences between CRTL, RUN1 and RUN2 cells. Sholl analysis provides an estimate of dendrite arborization by evaluating the dendritic crossing along the Sholl rings (starting radius = 3μm; interval between succeeding concentric circles = 8 μm). One-way ANOVA and Tukey post-hoc analysis highlight significant differences between RUN2 (n = 4 cells) vs CTRL (n = 21 cells) and RUN1 (n = 15 cells) at different distances from the soma (●). 163 μm $p < 0.01$, Tukey HSD: $p < 0.01$ RUN 2 vs CTRL; $p < 0.05$ RUN 2 vs RUN 1. 171 μm $p < 0.01$, Tukey HSD $p < 0.01$ RUN 2 vs CTRL, RUN 1; 179 μm $p < 0.05$, Tukey HSD $p < 0.05$ RUN 2 vs CTRL, RUN 1. 187 μm $p < 0.01$, Tukey HSD $p < 0.01$ RUN 2 vs CTRL, RUN 1. 195 μm $p < 0.01$, Tukey HSD $p < 0.01$ RUN 2 vs CTRL, RUN 1. 203 μm $p < 0.01$, Tukey HSD $p < 0.01$ RUN 2 vs CTRL, RUN 1. 211 μm $p < 0.01$, Tukey HSD $p < 0.01$ RUN 2 vs CTRL, RUN 1. 219 μm $p < 0.01$, Tukey HSD $p < 0.01$ RUN 2 vs CTRL, RUN 1. 227 μm $p < 0.01$, Tukey HSD $p < 0.01$ RUN 2 vs CTRL, RUN 1. 235 μm $p < 0.01$, Tukey HSD $p < 0.01$ RUN 2 vs CTRL, RUN 1. 243 μm $p < 0.01$, Tukey HSD $p < 0.01$ RUN 2 vs CTRL, RUN 1. 251 μm $p < 0.01$, Tukey HSD: $p < 0.01$ RUN 2 vs RUN 1; $p < 0.05$ RUN 2 vs CTRL. 267 μm $p < 0.05$, Tukey HSD $p < 0.05$ RUN 2 vs CTRL, RUN 1.

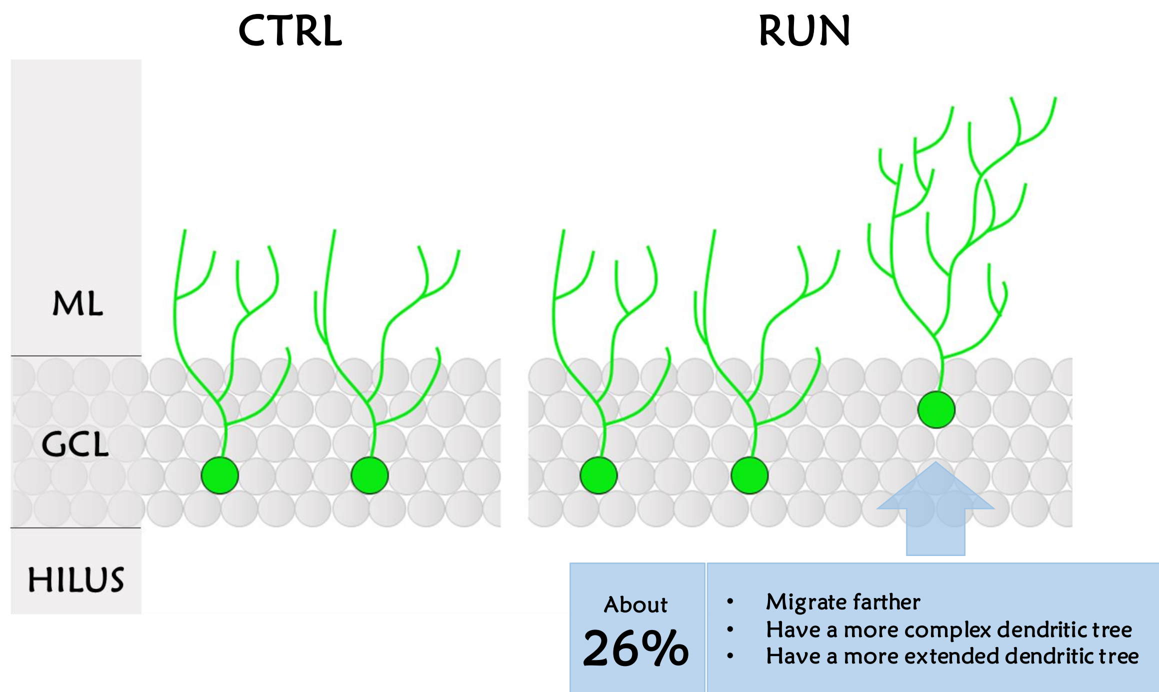

Figure 10. Differences between CTRL and RUN 30-day old GFP-positive granule cells. In the figure are outlined the features of newly generated granule cells at 30 days after GFP injection in CTRL vs RUN groups. It is possible to notice that a subpopulation of RUN cells is characterised by a greater maturation. ML: molecular layer; GCL: granule cell layer.

Discussion

Three days of physical exercise can influence the development of newly-generated granule cells with possible implications on hippocampal functions. Analysing granules of 30 days, a higher maturation degree, highlighted by the increased complexity and dendritic length, in rats of RUN group has been found. However, these modifications could be attributed to a percentage of GFP-positive cells of about 26%, which is interestingly close to the percentage found on 7-day-old cells that received a precocious GABAergic contact after the same voluntary running protocol here applied [1]. It is therefore feasible that anticipation of synaptic GABAergic contact, which antedates the exposition of cells to depolarizing GABA action, might affect the survival and morpho-functional development of granules that receive the contact, and speed up neuronal growth. Therefore, the morphological characteristics of the more developed subpopulation of 30-day-old cells, and in particular the increased extension and branching of the dendritic trees, highlight the possibility of a higher number of synaptic contacts and a deeper integration into the hippocampal network with a possible contribution to the hippocampal functions. In particular, this more developed population, which is still in the “critical period” of high excitability, might influence the synaptic plasticity related to the immature pool of cells. According to this hypothesis, the electrophysiological analysis of field potentials revealed a difference in the maintenance phase of LTP induced through the high frequency stimulation of the medial

perforant pathway: in RUN group, this phase of the synaptic potentiation is characterised by fEPSPs of higher intensity, suggesting a link between voluntary running, enhanced morphological development and hippocampal functions.

Hippocampus is a structure of pivotal importance for episodic memory, for the establishment of memory traces about “where”, “how” and “when” a precise event is happened. The DG of hippocampus, which represents its principal mnestic traces processing unit, has function of pattern separator, allowing the brain discrimination of similar experiences or objects. In contrast, adult-born granule cells pass through a “critical period” of their development characterised by morpho-functional properties suited for pattern integration function and temporal separation of memories [143, 199]. As suggested by Piatti et al. [200], since the higher number of immature granules in the development period suited to perform pattern integration function, the increased development speed of immature granules might ameliorate temporal resolution and the reliability of multiple events memorization. In addition, variation in the dendritic trees complexity and extension, might be related with increased number of synaptic contacts on the immature population; all these contact originate from not only the entorhinal cortex but also from CA3 back-projection [201] and might play an important role in the pattern integration function, supporting the correlation between similar events or comparable stimulus.

It is therefore suggested that the subpopulation of more mature newly-generated neurons found in RUN group might be subject to a deeper integration into the hippocampal network. The effects related to physical activity might be therefore able to influence aspects related to memory such as the capability to associate different mnestic traces or recognize and discern similar memory traces established with different timings. Thus, the difference found in the maintenance phase of LTP is probably related with the morphological characteristics of this more mature subpopulation of 30-days old cells, and might influence DG functions leading to the physical activity-associated improvements of cognitive performance widely reported in literature [122, 155, 191, 202]. The maintenance phase of LTP represent the trend of the synaptic potentiation in time: the more this phase is prolonged and sustained, the more the synaptic potentiation at the pathway previously stimulated will last. The LTP lead to the appearance of higher intensity EPSCs, increasing the neuronal excitability [14, 21] and, consequently, increasing the probability that a neuron might fire when stimulated. The difference in the maintenance phase of LTP here reported extend the temporal window within which two similar events gain a higher probability of activating the same neuronal population. This difference reported in RUN group might lead to the improvement in the pattern integration function of the DG since it could improve the association of events through the activation of a similar population of immature granules, which, after the complement of their development and reduction in excitability, will only respond to the events experimented during their development [199]. However, the difference in the maintenance phase of LTP in rats that undergone three days of voluntary running, starts 10 minutes after the HFS and become evident and significant in the last minutes

of the recording. The slightly of the difference reported may be related to the relatively small population of the more mature cell found in RUN group – the 26% of the total immature population – whose contribution to the LTP could be arduous to clearly highlight with a field LTP. Nevertheless, considering the significance of the last four minutes of the recording, new experiments, which will extend the recording time from 40 minutes to 80 minutes after the tetanic stimulation of the perforant pathway, might be rightful and hence will be performed soon in the forthcoming.

PART II: PLASTICITY IN DISEASE

Even though brain plasticity represents a fundamental intrinsic property, able to allow the modification of the structures and functions of the central nervous system in response to environmental demands, it could also be subject to corruption of its normal dynamics and turned into the main rival in the battle against several neurological disorders. Indeed, neuronal plasticity is a double face medal, which could also represent the central pathogenic foundation of neurodevelopmental and neurodegenerative disorders such as depression, epilepsy, Alzheimer's disease, schizophrenia and autism spectrum disorders (ASD). In the same way as plasticity in healthy brain is based on mechanisms that involve modifications at synaptic level, in the morphology of the neurons or in the number of neurons within the neural circuits, the aberrant plasticity in many neurological disease is based on the same mechanisms which, in this case, act in an uncontrolled and misleading way. Thus, if plasticity in health can be referred to as an adaptive process, acquired brain insults could trigger aberrant plastic dynamics giving rise to maladaptive plastic changes that play a role in the pathophysiology of several neuropsychiatric conditions. However, important factors to consider that likely contribute to individual differences in the efficacy of plasticity dynamics are genetic or epigenetic mechanisms, or lifetime experiences [78], which produce dissimilar set point or slope of change in plasticity and different disease manifestation and progression.

To give back a better idea about what plasticity diseases are, some examples of these disorders follow. One well-known example of maladaptive plasticity is the focal hand dystonia, often referred to as musician's cramp or writer's cramp, which causes involuntary movement, cramps or tremor of the affected hand. This disorder is a disabling condition that has been difficult to treat with physiotherapy or other methods. It has been demonstrated that recurrent and rapid time-synchronous movements during vigorous practicing, could lead to the degeneration of the sensory feedback controlling fine motor movements, which after all result in the fusion of the different cortical representation of the specific fingers [203]. The abnormal sensory representation affects the motor control and, in turn, anomalous motor control reinforces the sensory aberration: at the end, this positive feedback loop reinforces the dystonic condition. Compared to control individuals, patients with dystonia have significantly reduced levels of the inhibitory neurotransmitter GABA in the sensorymotor cortex and in the lentiform nuclei contralateral to the affected hand, as demonstrated with *in vivo* magnetic resonance spectroscopy [204]. Another disorder with a strong base on the aberrant plasticity that is worth to mention is the ASD. These neurodevelopmental disorders are associated with synaptic deficits, including imbalanced excitation/inhibition ratios and impaired synaptic plasticity [205]. In ADS a reduction in GABAergic receptors and in the enzymes that synthetize GABA, together with increased levels of BDNF has been reported. Moreover, proteins implicated in synaptic development and plasticity such as neuroligins 3 and 4, SH3 and multiple ankyrin repeat

domains 3 (SHANK3) or protocadherin-10, has been identified as candidate genes that confer increased risk of ASD [206]. Since animal models of these human single gene syndromic causes of autism demonstrate aberrant synaptic plasticity, these findings have suggested the view that autism should be thought as a synaptopathy by which proteins that are involved in synaptic development and plasticity are affected. Repetitive Transcranial Magnetic Stimulation (rTMS), which as the potential to induce long-lasting modulation of the cortical excitability and plasticity, has showed to improve some specific behavioural symptoms in individuals with ASD [206]. Further diseases that appear as manifestation of aberrant plasticity are for schizophrenia, a neurodevelopmental disorder about which several lines of evidence suggest that the neurotransmitter mechanisms mediating plasticity in the cortex are altered, and Alzheimer's disease, where the amyloid-β (Aβ) protein initiate a cascade of events ending in synaptic dysfunction and cell death. In addition, important diseases of great impact for human health that share a base of aberrant plasticity dynamics are depression and epilepsy: a detailed description of these diseases and their relation with brain plasticity could be find in the sections below.

All the disorders reported above are sadly well-known and draw attention on a severe concern since brain diseases represent a serious problem for human health and often denote a heavy burden for human society. Besides, many plasticity diseases are complex pathologies with limited known treatment options. Plasticity diseases, consequently, often cause suffering to the patients and frustration to the professionals who are asked to treat these disorders. For these reasons, the research of promising approach for future pharmacological therapies, which should be aimed at returning the neural circuitries that are in an abnormal state, back to their normal state, is an important and still unmet clinical need.

UNDERSTANDING AND TREATING MAJOR DEPRESSION

Depression is one of the most devastating illnesses, and is among the leading contributors to the global burden of disease. The proportion of the global population with depression in 2015 is estimated to be 4.4%. The World Health Organization (WHO) reported that prevalence varies throughout WHO regions, from a low of 2.6% among males in the Western Pacific Region to 5.9% among females in the African Region. Prevalence rates vary by age, peaking in older adulthood (above 7.5% among females aged 55-74 years, and above 5.5% among males). Depression also occurs in children and adolescents below the age of 15 years, but at a lower level than older age groups. This disorder is more common among females (5.1%) than males (3.6%). The WHO reported that the total number of people living with depression in the world is 322 million and the total estimated number of people living with depression increased by 18.4% between 2005 and 2015.

Major Depression Disorder (MDD) is commonly diagnosed by criteria in the Diagnostic and Statistical Manual (DSM) [207], which specifies that 5 of 9 symptoms should be present for a 2-week period: the criteria are summarized in Tab. 1.

Melancholic features include a pronounced loss of pleasure in most or all activities, early morning awakening, and a worsening of symptoms in the morning hours; psychotic symptoms often include feeling of personal inadequacy, guilt, punishment or death [207]. Lastly, depression is strongly associated with suicide ideations and attempts [208]. The course of MDD reflects its complexity: initial onset can include various physical symptoms among which pain (i.e. headache, musculoskeletal, abdominal/pelvic), neurovegetative mood symptoms, and cognitive changes. The progression of MDD is variable with some patients rarely experiencing a remission (consisting in more than 2 months with no or only a few mild symptoms) and others that go through many years with few or no symptoms between isolated depressive episodes. Many patients who have experienced only few months of depression, might be expected to recover spontaneously. On the other hand, chronicity of symptoms decreases the possibility that a full remission will follow the treatment. In addition, lower recovery rates are associated with psychotic features, symptom severity, prominent anxiety, and personality disorders. Anyhow, most patients with symptoms of MDD do eventually improve. An observational study reported that 17% of participants with MDD remained in a chronic state of depression after 39 months, and another 40% had a fluctuating course of depression, while 43% were in remission from baseline [209]. Unfortunately, depression is highly recurrent. In keeping with this statement, a prospective study found that 64% of patients recovered from an episode of MDD experienced at least 1 additional episode, with the greatest risk of recurrence in the first months after recovery. As the period of remission increase, the probability of recurrence decrease; on the other hand, each recurrence rises the risk of experiencing further episodes of MDD [210].

Table 1. DSM criteria for MDD

DSM 5th Edition diagnostic criteria for major depression
A. Five (or more) of the following symptoms present during the same two-week period and represent a change from previous functioning; at least one of the symptoms is either depressed mood or anhedonia
B. Symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning
C. Episode not attributable to the physiologic effects of a substance or another medical condition 1. Depressed mood most of the day (e.g. feels sad, empty, hopeless) 2. Markedly diminished interest or pleasure in almost all activities nearly every day 3. Significant appetite changes or significant weight loss or gain 4. Insomnia or hypersomnia nearly every day 5. Psychomotor agitation or retardation 6. Fatigue or loss of energy 7. Feelings of worthlessness or excessive guilt 8. Diminished ability to think or concentrate or indecisiveness
Adapted from Diagnostic and statistical manual of mental disorders. 5th edition. Washington, DC: American Psychiatric Association; 2013.

Etiopathology of depression

MDD is a multifaceted disease with a etiopathogenesis based upon multiple factors that might act at multiple levels such as genetic, biological, psychological and social: the mechanisms that contribute to the emergence of the disease are still not completely understood. The focus of the etiopathology of MDD is principally on its neurobiology and how it associated with genetic and environmental contributors. In particular, much attention is condensed on three major monoamine systems namely serotonin, norepinephrine (NA) and dopamine (DA).

The classical monoamine hypothesis of depression postulates a deficiency of monoaminergic transmission, and in particular serotonin. Indeed, numerous studies support a dominant role of 5-HT system on depression since the presence of evidence about alterations of the serotoninergic transmission. In particular, it has been reported a reduced activity of 5-HT neurons in post-mortem studies on depressed patients and,

a reduction in the number of serotonin transporters (SERT) in midbrain and amygdala together with an imbalance in the number of some 5-HT receptor subtypes in pre- and post-synaptic terminals [211]. Further support derives from the observation that individuals with the s allele of the promoter region of SERT gene (SLC 6A4) have a higher risk of MDD in response to early life stress such as child abuse or carelessness [212]. This allele gives rise to an attenuated promoter of the SERT transporter gene, which regulates the availability of 5-HT in the central nervous system.

Norepinephrine system is also considered to be involved in MDD. Drugs that block NE reuptake, thus increasing the levels of this neurotransmitter in the synaptic cleft, are effective antidepressant. Besides, numerous post-mortem and neurochemical studies on depressed patients, report a dysfunction of NE system in MDD. In particular, alterations of NE transmission in depression include: low levels of NE in urine and cerebrospinal fluid, increased levels of β-adrenergic receptors in the cortex and, increased MAO-A activity in the CNS of depressed patients.

Lastly, numerous findings support an important role in depression of DA circuits of the central nervous system. Moreover, it has been suggested that the suboptimal responses to monoaminergic-targeting antidepressants observed in some individuals might be related to the lack of effect of these drugs on DA circuitry [213]. The imbalance in DA transmission consists in the reduction of DA transporter binding sites and increased postsynaptic dopamine D2/D3 receptor density, suggesting a decrease in synaptic availability of DA [214]. The emergence of a dopaminergic hypothesis of depression is not surprising since many of the most important symptoms of MDD associated with eating, social or pleasure, are primarily mediated by dopaminergic neurons.

It is almost unanimously accepted that various neurotransmitters are pathologically involved in depression, yet none of them seems to be the sole responsible. As previously mentioned, depression is multifaceted disease and is probably caused by various aberrant plastic changes that ultimately result in the disruption of structural and functional connections of the neural circuit that underlie mood regulation. Many brain regions are proved to be altered in MDD but the most consistent findings regard the prefrontal cortex and the hippocampus, which are both reduced in volume in depressed individuals [215, 216]: the volume decrease correlates with length of the illness, its severity and the time of the treatment. Moreover, it has been reported a reduction of synaptic contacts in depressed subjects [217] and, rodent models revealed that, like depression, exposure to stress causes atrophy and loss of neurons in the prefrontal cortex and hippocampus [218, 219]. Synaptic plasticity is governed by an intricate interaction of signalling pathways and the alteration of these pathways has been related with the susceptibility to depression. In particular, some important key modulators of synaptic plasticity altered in depression are neurotrophic factors – which levels are found reduced – and inflammatory cytokines – which levels are found elevated in MDD – [218].

Acute traumatic or chronic stress represents a significant susceptibility factors for depression and exposure to stress, in particular during early life, cause long-lasting

alterations also linked to epigenetic modifications of DNA or histones [220, 221]. A hallmark feature of the stress response is the activation of HPA axis and the subsequent increase in circulating glucocorticoids level. MDD is often associated with an increase in the activity of HPA axis and increased levels of glucocorticoids, together with the disruption of negative-feedback mechanisms [222]. In rodents, the chronic exposure to glucocorticoids reduces the number of synapses and affects their functioning; moreover, it causes atrophy of neurons in the prefrontal cortex and hippocampus, which represent atrophied regions also in depressed humans [223, 224]. It has been suggested that chronic stress, combined with genetic and environmental factors, might result in short-term adaptive changes, such as activation of immunity system, that could lead to deleterious long-term synaptic and cerebral consequences [225]. Cytokine infusion such as interferon, induces depression-like behaviour [226] and, serum levels of pro-inflammatory cytokines IL-1β, IL-6 and TNFα, have been found increased in depressed individuals: interestingly, cytokine levels result normalized after antidepressants treatment [227]. Inflammatory cytokines are produced and released in the brain from microglial cells and have influence on brain plasticity in physiological conditions [228]. Conversely, stress, aging and inflammation, inducing an abnormal increase of inflammatory cytokines, affect the normal brain plasticity leading to damage, atrophy and loss of spine synapses [225]. The atrophy of neurons caused by stress are key contributors to the symptoms of depression: in addition to HPA axis activation, the number of synapses and function are altered by other factors, and in particular neurotrophic factors, which have been implied in depression.
Many neurotrophic factors such as Vascular Endothelial Growth Factor (VEGF), Insuline-like Growth Factor (IGF-1), Fibroblast Growth Factor 2 (FGF2) and, in particular, BDNF have been implicated in depression [225]. The possible role of all these factors in MDD has been hypothesized in the context of the neurotrophic model of depression. This hypothesis postulates that, since their significant involvement in maintenance and activity-dependent formation of synaptic connections and the evidence about the increased synaptic plasticity after the chronic administration of typical antidepressant, stress and depression can lead to decreased neurotrophins levels. In keeping with this hypothesis, FGF2 signalling seems reduced in depression [229]; likewise, stress and depression decrease the expression and function of BDNF in the prefrontal cortex and hippocampus [230].

Treatments

Several approaches for the treatment of MDD have been found to be efficacious. These approaches include behavioural interventions and self-care, psychotherapy and psychopharmacological interventions [208].

Behavioural interventions and self-care

Intervention based on behavioural/self-care are particularly suited for all the patients that are opposed to treat their depressive symptoms using medicines or psychotherapy, and prefer to start with interventions that they can initiate on their own instead. The increasing in MDD diagnosis is probably associated both on improved screening and awareness by patients and health providers and, as Hidaka suggested, on the modern lifestyle, which often include isolation from a family unit, weak social interactions, high levels of stress, sleep-wake alterations, and inadequate exposure to sunlight that in conclusion lead to undernourished, lonely and stressed individuals [231]. Behavioural/self-care intervention should be aimed to contrast some of the risk factors linked to the modern lifestyle and should be therefore based on a balanced and healthy diet, on doing regular and adequate levels of physical activity, on the treatment of sleep abnormalities and, on the abstinence from alcohol and drugs consumption. Moreover, meditation, music and animal-assisted therapy have some evidence for benefit in the treatment for MDD [208].

Psychotherapy

Psychotherapy is a useful intervention in the treatment of MDD. In particular, the effectiveness of the treatment has been reported in association with cognitive behaviour therapy, behavioural activation therapy, interpersonal psychotherapy, problem-solving therapy and psychodynamic therapy [208]. The application of one of these approaches, comes after an accurate evaluation about the differences between these forms of psychotherapy that might confer certain advantage and disadvantage depending on the patient's philosophy, level of insight, willingness to participate and preferences [232].

Pharmacological approach

The currently available drugs used for pharmacological intervention are divided in first- and second-generation antidepressants. First-generation antidepressant is for instance tricyclic antidepressants (TCAs) or monoamine oxidase inhibitors (MAOIs), while second-generation antidepressants include selective serotonin reuptake inhibitors (SSRIs), bupropion, and mirtazapine; the last category is generally preferred over the first because of its less problematic side effects and reduced risk of fatalness in overdose situations [208]. Pharmacological intervention is suited for treating severe states of depression and is generally used as monotherapy or together with psychotherapy and other modalities of care. It has been reported that second-generation antidepressants as mirtazapine, escitalopram, venlafaxine and sertraline are the most efficacious treatments. Among these drugs, it has been suggested that escitalopram and sertraline might be more efficacious [233, 234], probably because they are the most potent in the

binding to 5-HT reuptake inhibitors. Anyhow, an important factor to consider is the rate of dropout, which affects the success probability of the treatment and is partially dependent on the side effects of the antidepressant. Indeed, the primary reason for discontinuation of antidepressant consumption cited in studies is nausea and vomiting: these side effects fortunately resolve in about two weeks and can be mitigated with different strategies. Unfortunately, a remarkable portion of patients affected by MDD of about 30-40% will not respond to antidepressant interventions. The strict definition of treatment-resistant depression indicates the lack of improvements in response to adequate doses and duration of therapy with two antidepressant of different classes [235]. The treatment-resistance is sometimes overcome increasing the dose of antidepressant or switching between classes, but this strategy not always gains the hoped effects. Therefore, it persists a need for the research on new effective treatments in drug design, trying to address the drug delay in efficacy, the side-effects problematics, and the treatment-resistant MDDs.

Receptor-receptor interaction: discovery and their role in disease

The existence of a direct interaction between membrane receptors, namely receptor-receptor interactions, has been assessed through numerous different methodologies, spanning from classical biochemical approaches to biophysical techniques [236]. Membrane receptors act in cell signalling recognizing specific extracellular molecules and transducing the signal inside the cells through the activation of elaborated intracellular pathways. Numerous cell surface receptors are transmembrane proteins classified and divided in families basing on their topologies. Two well-known classes of membrane receptors are G Protein-Coupled Receptors (GPCRs) and Receptors Tyrosine Kinase (RTKs). GPCRs are also called seven transmembrane domain receptors (7-TM Receptors), since they pass the cell membrane seven times: their pathway involves the activation of different heterotrimeric G proteins ($G\alpha\beta\gamma$) and lead to genomic and non-genomic effects. The major G proteins are G_s, $G_{i/o}$, G_q and $G_{12/13}$. RTKs on the other hand, are receptor mostly known for their involvement in growth factor, cytokine and hormone pathways. These receptors are most generally single subunit receptors – with some exception – which dimerize once bound to their ligand: this conformational state lead to the transphosphorylation of their cytoplasmic domains and activation of intracellular pathways with subsequent genomic and non-genomic effects.

Based on the evidence that neuropeptides often coexist with monoamines in the same synaptic releasing vesicles, Kjell Fuxe's group for the first time made the hypothesis about the possibilities of molecular integration of different transmitters signals via transmembrane receptor-receptor integration [237]. They began to test this hypothesis in the early 1980s, with the studies on the neuropeptide-monoamine receptor-receptor interactions (i.e. GPCR-GPCR) in membrane preparations of various CNS regions. Interestingly, they found that neuropeptides could modulate the binding characteristics,

and in particular the affinity, of monoamine receptors [238-240]. Thus, it was proposed that their interactions in the plasma membrane took place in postulated heteroreceptor complexes of GPCRs, bringing evidence for the existence of direct R-R interactions in the plasma membrane between different types of GPCRs. The term heteromerization was later introduced, in 1993, to describe a specific direct interaction between different types of GPCRs [241]. The concept of GPCR heteromerization was confirmed in 1999 by study reporting that two non-functional receptors, $GABA_{B1}$ and $GABA_{B2}$, assemble in a signalling heterodimer at the cell surface [242]. Nowadays, the R-R interaction field in the CNS has expanded and includes monomers, homo- and heteroreceptor complexes with receptor assemblies of unknown stoichiometry and geometry together with adapter proteins. Moreover, the initial concept about R-R interaction between GPCR receptors has been extended to other receptor categories, among which RTKs. Indeed, it becomes clear that RTKs and GPCRs possess the ability for transactivation not only via GPCR-induced release of neurotrophic factors but also through shared signal pathways or via direct allosteric R-R interaction [243, 244]. The hypothesis about a direct interaction between RTKs and GPCRs was for the first time introduced by Fuxe's group about ten years ago [243], while the first evidence about a direct RTK-GPCR interaction was reported by Flajolet and colleagues between the adenosine A2A and FGF receptors using the yeast two-hybrid system; the same group brought also evidence for the enhancement of synaptic plasticity after the combined activation of the two receptors [245]. The existence of this complex was subsequently validated with BRET techniques [244].

The operational definition proposed for R-R interaction enunciates that two experimentally measurable conditions should be fulfilled for defining an interaction as R-R interaction. In particular, the binding of a ligand to one receptor must causes a detectable change in the biochemical characteristics – ligand recognition, decoding, and trafficking process – of the other receptor; moreover, the mean distance evaluated through atomic force microscope or Resonance Energy Transfer (RET)-based approach – i.e. bioluminescence-RET (BRET) or Fluorescence-RET (FRET), must be less than 10 nm [237].

The allosteric mechanisms in receptor heteromers make possible a marked rise of the range of GPCR recognition and signalling through the modulation of the orthosteric and allosteric binding sites of the adjacent protomer, of its G protein activation and selectivity, of its signalling cascade and through the appearance of novel allosteric sites (Fig. 11). Thus, the allosteric R-R interactions in heteroreceptor complexes give diversity, specificity and bias to the receptor protomers due to conformational changes in discrete domains leading to changes in receptor protomer function and their pharmacology. Therefore, it is clear that R-R interaction has attracted much attention within the scientific research world for their promising potential as novel targets for treatment of neurological and mental diseases.

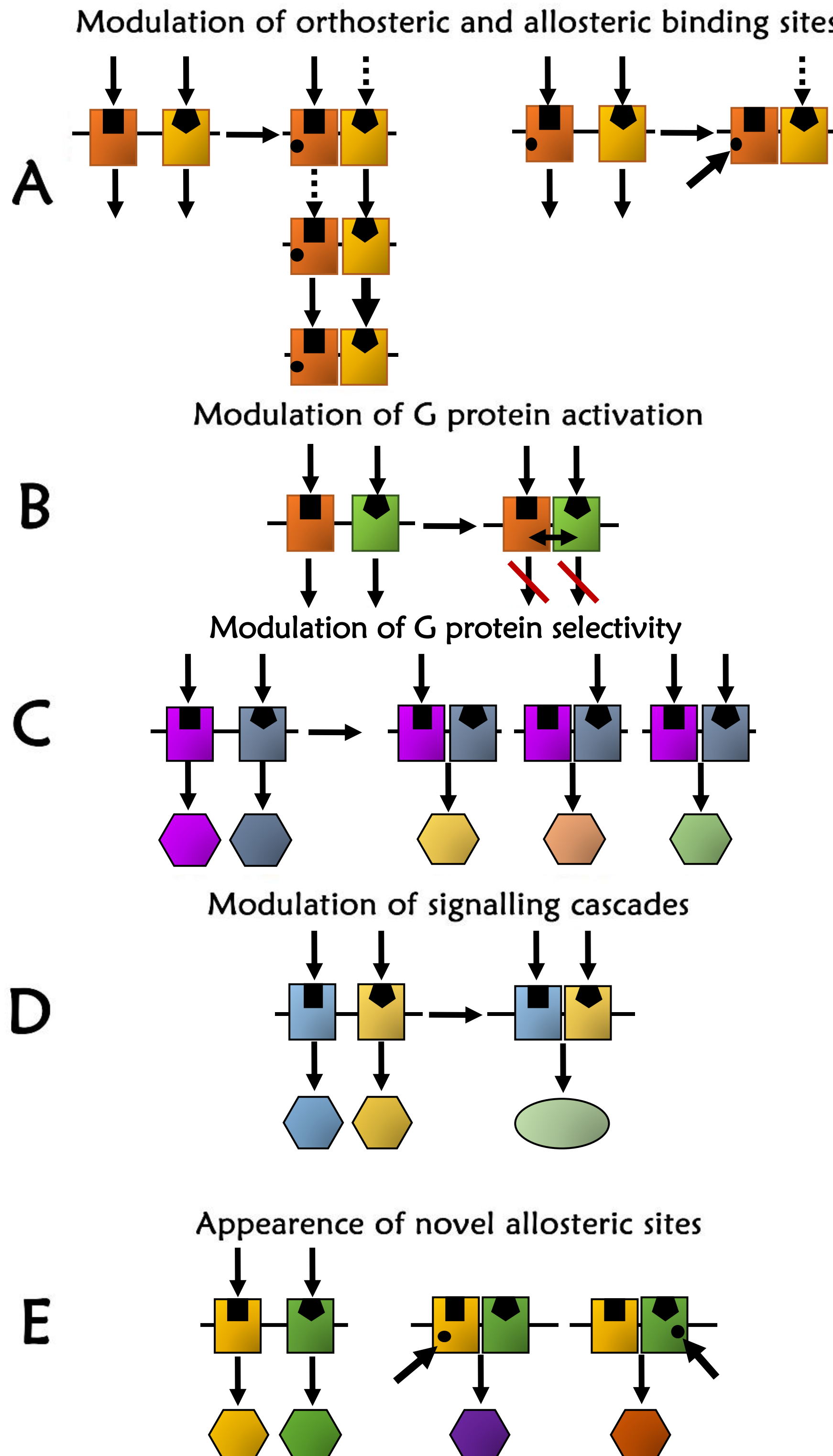

Figure 11. Receptor-receptor interactions increase the diversity of G protein-coupled receptors recognition and signalling. Upon activation of one protomer modulation of the orthosteric and allosteric binding sites of the adjacent protomer (A) can take place as well as of its G protein activation (B), its G protein selectivity (C), its signalling cascades with among others switching from G protein to β-arrestin signalling (D) and through appearance of novel allosteric sites that may alter for instance G protein coupling and selectivity (E). Adapted from “The changing world of G protein-coupled receptors: from monomers to dimers and receptor mosaics with allosteric receptor–receptor interactions” [237].

Indeed, neuropsychiatric disorders and GPCRs are strictly related. The involvement of a wide range of GPCRs in this kind of diseases has been known for a long time. However, an interesting new entrance is related with the GPCR heteromers involvement and their important role in receptor function. The connection between heteroreceptor complex has been proven for many pathologies among which schizophrenia (A2AR-mGlu5 [246] and A2AR-D2R-mGlu5R [247]), Parkinson (A2AR-D2R [248] and D2R-D3R [249]), cocaine addiction (5-HT2AR-5- HT2CR [250]), pain (μOR-δOR [251] and μOR-mGlu5R [252]) and major depression (FGFR1-5HT1A [253]).

A possible strategy to target heteroreceptor complex in disease is to develop a molecule that antagonizes the R-R interaction, that could be a protein or a oligopeptide which interferes with the complex formation [236]. Transmembrane interface interfering peptides have successfully been introduced, leading to a significant decrease of these heteroreceptor complex [2]. Nonetheless, in some cases the R-R interaction should be enhanced rather than disrupted; this is the case of FGFR1-5HT1A heteroreceptor complex, where the enhancement might lead to increased excitability and plasticity, thus counteracting depression, a disease where this complex is involved. Moreover, it is also possible that positive or negative modulators of one protomer might exert major role in modulating the function of the other protomer involved in the heteroreceptor complex through similar mechanisms [236]. However, since is currently fairly unknown how allosteric modulators at one protomer influence the function of the other in a R-R interaction, understanding these modulatory actions might probably lead to gain new opportunities for the introduction of novel therapeutic drugs.

FGFR1-5HT1A heteroreceptor complex as a novel target for the treatment of major depression

The existence of GPCR containing heteroreceptor complexes which, even in absence of neurotrophic factor bound to the RTK, can lead to transactivation of RTKs with effect on neuronal plasticity, has the potential to open a new research field for the treatment of several diseases, including Major Depression [243, 245, 254]. It is strongly suggested that the interaction between the receptor for the basic Fibroblast Growth Factor (or FGF2) FGFR1 and the serotonin receptor 5HT1A may play an important role in MDD [2, 3, 236, 253, 255]. FGFR1-5HT1A heteroreceptor complex was for the first time observed by Kjell Fuxe's group using the *in situ* Proximity Ligation Assay (*in situ* PLA) and co-immunoprecipitation (Co-IP) techniques in rat dorsal hippocampus. The presence of these complexes were found in the pyramidal cell layers of CA1 to CA3 fields and in the dorsal leaflet of DG, but not in the cerebral cortex. In addition, the existence of this interaction has been confirmed using BRET technique in cellular cultures; the specificity of this interaction has been also validated using small transmembrane peptide which interferes with the complex formation [2]. The same paper showed that allosteric R-R interactions in hippocampal cultures involved a 5HT1A agonist-induced

FGFR1 transactivation and pathways activation, as seen from its increased phosphorylation, especially after the combined agonist treatment, of FGFR1 and ERK 1/2 [2]. The agonist regulation of this heteroreceptor complex, in particular with a combined agonist treatment, leads to an increase in the affinity of the interaction of the two receptor protomers and in the number of receptor complex formed. In addition, it has been found that FGF2 and 5HT1A agonist promote neuroplasticity *in vitro*, suggesting a potential effect of a treatment based on this heteroreceptor complex in counteract and reverse the atrophy found in hippocampus in MDD. However, no *in vivo* data currently exist in support of such suggestion, thus limiting its value. Nevertheless, it is worth to note that these first evidence open up the possibility that in the hippocampus, the combined activation of 5HT1A and FGFR1 protomers in the FGFR1-5HT1A heteroreceptor complex might also potentially contribute to antidepressant-like actions [255]. The hippocampal formation is connected with key regions of the emotional and mood circuits of the brain and is therefore in a position to effectively influence the operation of these emotional networks, which is known to be related with MDD pathophysiology.

The hippocampal results were later reinforced by the finding of FGFR1-5HT1A heteroreceptor complex in large number of midbrain 5HT neurons in dorsal and median raphe nuclei of Sprague Dawley rats and rat medullary raphe cell cultures [3, 253]. In the raphe nuclei, the 5HT1A receptor is known to have a function of autoreceptor, being localised on 5HT neurons and regulating the serotonin release [256, 257], due to their regulatory effect on G protein-coupled inwardly-rectifying potassium channel (GIRK). The raphe nuclei are a heterogeneous collections of neurons, with poorly defined cytoarchitectonic limits and characterised by distinct morphologies, projections and neurochemical characteristics. These neurons surround the midline, along the rostro-caudal extension of the brainstem, both in animals [258] and human [259]. The 5HT neurons are the main neuronal constituents of the raphe nuclei, which provide parallel and overlapping ascending and descending projections that constitute the main serotoninergic inputs of the CNS. The serotonergic projections participate in the regulation of different functional – i.e. motor, somatosensory, limbic systems –, and have been associated to the control of diverse physiologic and behavioural endpoints, including emotional states and emotional behaviour [260]. In the raphe cells has been proven that combined treatment with FGFR1 and 5HT1A agonists produce a marked enhancement of the number of processes formed by each medullary raphe neuron and of the amounts of serotonin immunoreactivity per cell [253], indicating a promising effect on increasing plasticity and serotonin production in 5HT cells. Indeed, the ascending midbrain 5HT neurons might be dysregulated in depression and could have a reduced trophic support also in relation to eventual disruption or dysfunction of their allosteric R-R interactions, among which FGFR1-5HT1A heteroreceptor complex.

Taken together, the findings on hippocampus and midbrain raphe suggest that synergistic allosteric R-R interaction develops within FGFR1-5HT1A heteroreceptor complex upon agonist co-activation. It is suggested that the formation of these

complexes could contribute to antidepressant effects by recruiting 5HT1A autoreceptors into FGFR1-5HT1A complexes, potentially leading to their uncoupling from GIRK channels. As a result, both reduced 5HT1A autoreceptor function and increased trophism may develop in the midbrain 5HT neurons; moreover, improved trophism could take place also in other brain regions as the hippocampal area. Therefore, combined agonist treatment has the potential to increase neuronal activity and remove the atrophy found in numerous regions of the CNS among which hippocampus and raphe, representing a possible major event for long-term antidepressant actions. Nonetheless, the role of FGFR1-5HT1A heteroreceptor complex in a depressed brain remains to establish and for that reason, the evaluation of possible alteration of this heteroreceptor interaction represents an important requirement for the development of antidepressant strategy targeting the FGFR1-5HT1A complexes.

Thanks to the collaboration between the section of Physiology at University of Urbino and Kell Fuxe's group at Karolinska Institutet, during my second year of PhD I got the chance to spend six months in the laboratory of Stockholm to study the FGFR1-5HT1A heteroreceptor complex and its emergence as a promising target for antidepressant treatments design. Based on the previous findings from this group, a first evaluation about the therapeutic potential of combined FGFR1 and 5HT1A agonist treatment on Sprague Dawley rats has been performed. Subsequently, the hypothesis was tested if disturbances in the FGFR1-5HT1A heteroreceptor complex can take place at the behavioural and neurochemical levels in Flinders sensitive line rat model of depression.

Experimental procedures

The electrophysiological experiments were performed in the laboratory of Physiology in Urbino, while the behavioural and biochemical analyses where carried out in the Karolinska Institutet laboratory.

Animals

Concerning the experiments performed in Urbino, the animals used –Sprague Dawely rats (SD) – were housed as previously reported ("Animals", pag. 25). The study was performed in accordance with the current Italian legislation (D.lgs 26/2014) on animal experimentation, which is in strict accordance with the European Council Directives on animal use in research (n. 2010/63/EU).

The experiments carried out at Karolinska Institutet were performed using 3-4 months old male Sprague Dawley rats (SD) (Scanbur, Sweden) or male FSL rats (bred in-house), which were housed under standard laboratory conditions (20-22 °C, 50-60% humidity). Animals that underwent surgery (see "i.c.v. drug treatment", pag. 50) were single-housed after this procedure. For the behavioural testing, the rats were handled for a minimum of six days before testing to minimize stress effects. Each animal was used

for one test only. All experiments at the Karolinska Institutet were approved by the Stockholm North Committee on Ethics of Animal Experimentation.

Electrophysiological analysis of GIRK currents

The experiments were carried out on adult male SD rats (n = 15). After anesthetization with isoflurane and killing by decapitation, brains were processed and cut to obtain slices using the same procedures mentioned above ("Slices preparation" pag. 25). The patch clamp technique in whole cell configuration was used. The experiments were performed under visual guidance using a Zeiss Axioskop microscope equipped with an infrared camera connected to a monitor. Recordings were carried out using an Axopatch-200B amplifier and WinWCP software for data acquisition and analyses. The recordings pipettes were filled with an internal solution containing in mM: 126 potassium gluconate, 8 NaCl, 0.2 EGTA, 10 HEPES, 3 Mg_2ATP, 0.3 GTP (pH = 7.2; 290 mosM) and the slices where continuously perfused with oxygenated ACSF (for the composition see "Slice preparation" pag. 25). No correction was made for junction potential between internal and external solutions. Somata of neurons to be recorded were identified in CA1 pyramidal cell layer based on their typical shape. The cells were recorded in voltage-clamp mode. In each cell, resting membrane potential (RMP) and input resistance (IR) were determined. The RMP was evaluated immediately after the break-in; IR was assessed applying hyperpolarising steps of 5 mV and 300 ms. During the recordings, membrane potential was kept constant at -70 mV. The recordings were rejected if the series resistance was greater than 30 MΩ, if was changed at the end of the experiment (± 10 MΩ) or if DC offset exceed 5mV after the withdrawal from the cell. Cells displaying non-neuronal features were rejected.

Is well established that serotonin 5HT1A receptors are able to open GIRK channels, generating a hyperpolarising outward potassium current [261, 262]. Thus, 5HT1A activation and its modulation related to FGFR1-5HT1A heteroreceptor complex formation, can be monitored using whole-cell patch-clamp following the holding current (I_h) trend and IR deviations. Indeed, changes in K^+ conductance across the plasma membrane lead to input resistance decrease and I_h – required to keep membrane potential constant at the prefixed value of -70mV – increase. To evaluate if FGFR1-5HT1A interaction might result in a modulation of 5HT1A activity, GIRK current dynamics were analysed in presence of single or combined treatment with the agonists for the receptors under investigation. In particular, after break into whole-cell configuration, the cytoplasm goes through a dialysis with the pipette recording solution that induce a I_h modification and stabilization. Therefore, in each cell a baseline of stable I_h was obtained before agonists application. Once I_h reached a stable value – for at least 10 minutes – a single or combined bath application of the 5HT1A agonist 8-OH-DPAT (5 μM), FGFR1 natural ligand FGF2 (10 ng/mL) or selective FGFR1 agonist SUN11602 (5 μM), were performed.

i.c.v. drug treatment

For intracerebroventricular (i.c.v.) drug delivery animals were implanted with a guide cannula (Plastics One, Roanoke, VA). To this aim, SD and FSL rats were anesthetized with isoflurane through a breathing mask. For some FSL rats, pentobarbital was given to achieve full anaesthesia. Guide cannulas were implanted at the following coordinates relative to bregma and dura surface: mediolateral: - 1.2 mm; anteroposterior: −1.0 mm; dorsoventral: − 3.7 mm; at a 0° angle from the vertical axis in the coronal plane [263]. Animals were allowed to recover 6-7 days after surgery before experimental testing. Drugs were delivered 24 hours before the testing day of the forced swim test (i.e. right after the training session) and then again 24 hours before sacrifice and collection of the brain (i.e. right after the testing session) (Fig. 12). Drugs were dissolved in ACSF and injected via a guide cannula using a microsyringe pump (1 μL/hemisphere) at the following final concentrations: FGF2 50 ng, 8-OHDPAT 200 nmoles.

Forced Swim Test (FST)

Immobility in the forced swim test is used to measure behavioural despair and has good predictive value for testing antidepressant effects [264]. The test consisted in two sessions. For the first session, each rat (n = 7-12 rats/group) was placed for 15 min in a vertical Plexiglas cylinder (height: 50 cm; diameter: 30 cm) containing 37 cm of water (25±1 °C). For the second session (test session), the animals were placed in the cylinder for 5 minutes and filmed for the subsequent video-analysis of the behaviour. The drugs (see "i.c.v. drug treatment") were administered immediately after each of the two sessions of FST through i.c.v. injections; the animals were sacrificed 24 hours later for in situ PLA analysis (Fig. 12). Immobility time was scored manually by an observer who was blinded with respect to the experimental conditions and is defined as the cessation of activity aside from the absolute minimum movement required to remain afloat. Immobility items were compared as averages and statistical tests were made using GraphPad Prism Software.

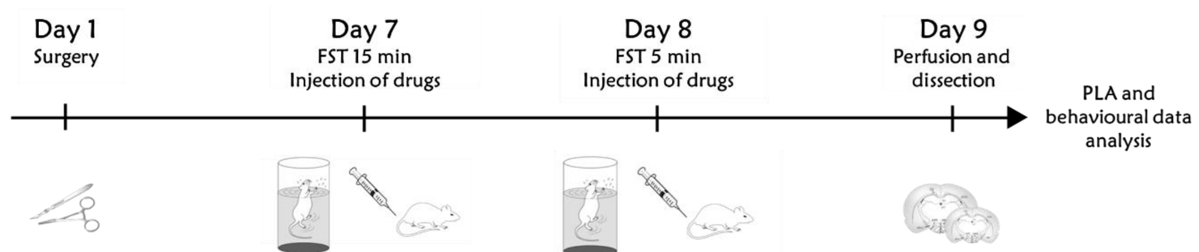

Figure 12. ***In vivo*** **treatment and behavioural experimental design.**

In situ proximity ligation assay

In situ proximity ligation assay (PLA) was performed according to manufacturer's instructions (Duolink in situ PLA detection kit (Olink, Sweden)). The principle of the technique is shown in Fig. 13. Briefly, animals were first euthanized by a lethal dose of pentobarbital (200 mg/kg) and perfused intracardially with 30–50 mL of ice-cold 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS), pH 7.4, solution. After perfusion, brains were collected and transferred into 4% PFA fixative solution for 6 h. Then, the brains were placed in sucrose 20% in PBS and incubated for 24 h until sections (10–30 μm thick) are generated and serially collected using a cryostat.

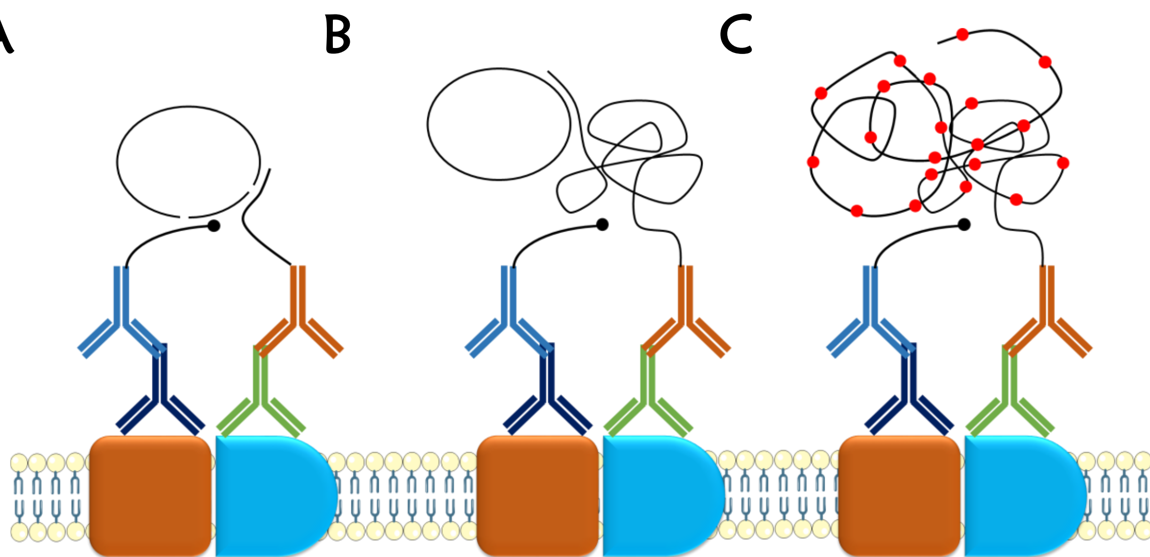

Figure 13. *In situ* Proximity Ligation Assay principle. (A) Using PLA, it is possible to display proteins interaction using primary antibodies against the proteins of our interest and a secondary antibodies linked to special oligonucleotide sequences (secondary proximity probes). Specific hybridization oligonucleotide sequences are then added. (B) If these antibodies are close enough, about 16 nm, after the addition of ligase, the nick between the hybridization sequences are closed, and a circle is formed. This circle contains the sequence for the subsequent rolling circle amplification, that extends the tail of one of the secondary proximity probe with a repeated sequence. (C) Adding a fluorescent marked probes (red dots in the image), it is possible to reveal these repeated sequence and analyse PLA blobs, using a fluorescence or confocal microscope.

The fixed free-floating sections (n = 5 rats per group) were washed four times with PBS and then incubated with the blocking solution (5% Bovine Serum Albumin –BSA – in Odyssey® Blocking Buffer – Li-cor Biotechnology –). The slices were then incubated at RT for 60 minutes with the following primary antibodies: rabbit monoclonal anti-5HT1A (VTG Biosciences) and mouse monoclonal anti-FGFR1 (abcam). The slices were subsequently incubated with the secondary proximity probes 1:10 in the blocking solution 2h at 37 °C. After this step, hybridization-ligation solution was added and the slices incubated 1 hour at 37 °C. Lastly, rolling circle amplification were performed at 37 °C for 150 minutes. Control experiments employed only one primary antibody or

cells transfected with cDNAs encoding only one type of receptor. The PLA signal was visualized and quantified by using a confocal microscope Leica TCS-SL (Leica, USA) and the Duolink Image Tool software.

Data Analysis

Data are expressed as the mean±SEM. The number of samples (n) in each experimental condition is indicated in figure legends. All data were analysed using the commercial program GraphPad PRISM 4.0 and 6.0 (GraphPad Software, USA). When two experimental conditions were compared, statistical analysis was performed using an unpaired t test. Otherwise, statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's (electrophysiology, neurochemistry) or Dunnett (behavioural analysis) Multiple Comparison post-test. The significance threshold was established at $p=0.05$.

Results

Electrophysiological analysis of 5HT1A activated hippocampal GIRK currents and their modulation by FGF2 and SUN 11602 in Sprague-Dawley rats

In line with literature, activation of 5HT1A induced an outward $G_{i/o}$-mediated current in recorded CA1 hippocampal neurons due to GIRK channels opening (Fig. 14) [265]. This outward current induces a shift from the baseline of the I_h (ΔI_h) (Fig. 14A) and IR (ΔIR) (Fig. 14B): the two parameters change following a linear correlation in all groups (Fig. 15), thus a higher shift of I_h correspond to a greater IR variation and vice versa. After application of 8-OH-DPAT (5 µM) in bath perfusion, the 5HT1A agonist produced a shift of the holding current, demonstrating a hyperpolarization that increased over 10 min and was associated with a decrease of input resistance (IR) (Fig. 14). In contrast, FGFR1 activation, by using the specific agonist SUN116052 (10 µM) or FGF2 (10 ng/mL), did not result in any effect on holding current in CA1 pyramidal neurons (Fig. 14C), indicating a failure of channel opening. Importantly, following co-application of 5HT1A and FGFR1 agonists (5µM 8-OH-DPAT + 10 µM SUN11602), the outward current was greatly reduced in the CA1 pyramidal neurons (Fig. 14).

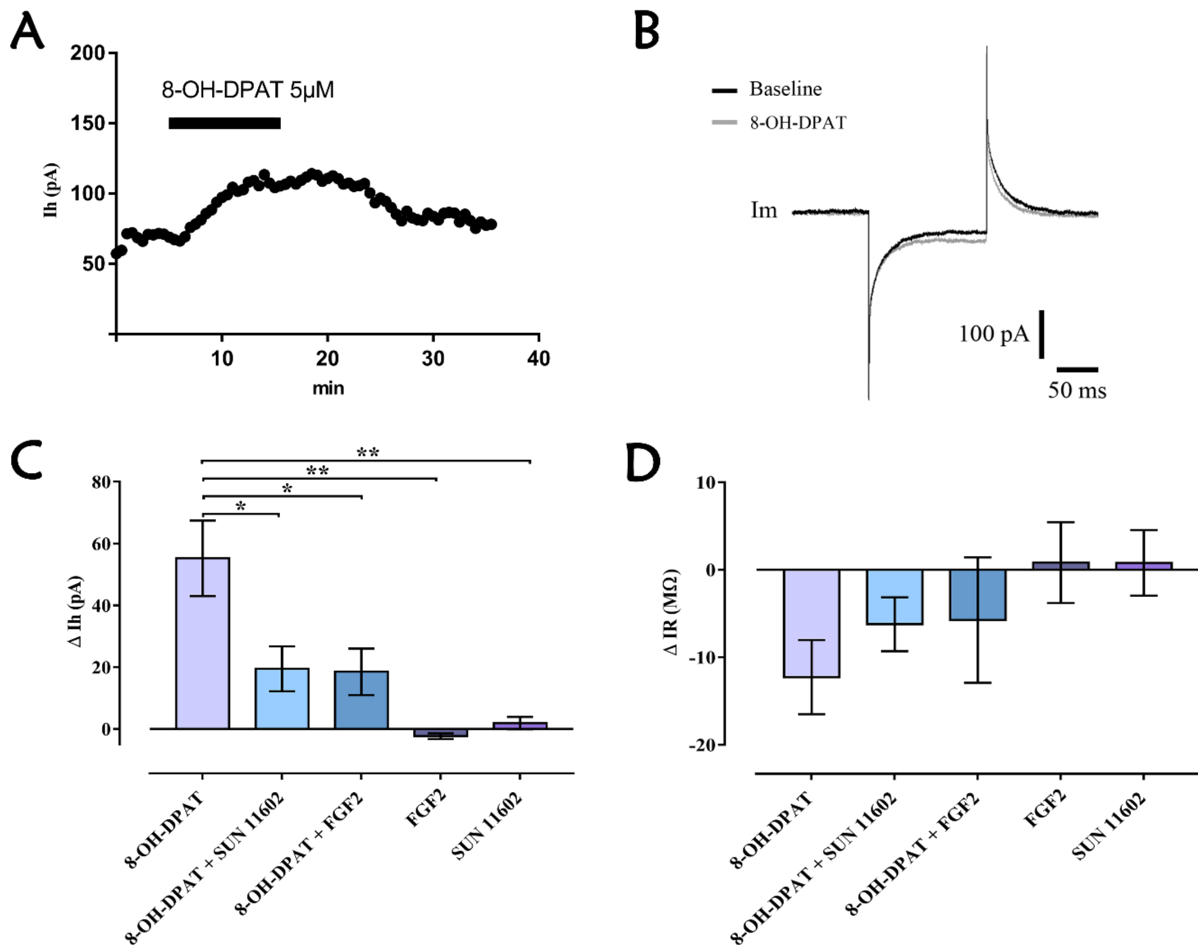

Figure 14. Reduction of G Protein-coupled inwardly rectifying K⁺ channel (GIRK) currents induced by combined bath application of 8-OH-DPAT and FGFR1 agonists on CA1 hippocampal neurons of Sprague-Dawley rats. A. Representative graph showing the holding current (I_h) shift following 5 µM 8-OH-DPAT application, indicating the occurrence of hyperpolarization. B. 8-OH-DPAT application induces an IR decrease, suggesting a membrane channels opening C. Summary graph including all agonists tested. Combined application of 8-OH-DPAT together with FGFR1 agonists (10ng/mL FGF2; 10 µM SUN 11602) reduces the amplitude of the GIRK current induced by 5HT1A activation. One-way ANOVA, Tukey's post-hoc: *p < 0.05; **p < 0.01. D. GIRK channel opening decrease input resistance (IR) of CA1 neurons. In line with the effect exerted on holding current, combined agonist treatment tends to reduce the IR drop elicited by 5HT1A-induced GIRK activation. All data are expressed as Mean±SEM. Number of recorded cells (n): 8-OH-DPAT (8), 8-OH-DPAT + SUN 11602 (10), 8-OH-DPAT + FGF2 (8), FGF2 (6), SUN11602 (6) [266].

The same effect was also achieved by applying a mixture of 5 µM 8-OH-DPAT and FGF2 at a concentration of 10 ng/mL (Fig. 14). These data suggest that FGFR1 activation was able to produce a significant reduction of the GIRK current induced by 8-OH-DPAT. All observed effects were reversed by washing out with ACSF. There was a non-significant trend for the FGFR1 agonist and FGF2 to counteract the reduction of input resistance produced by the 5HT1A agonist (Fig. 14D).

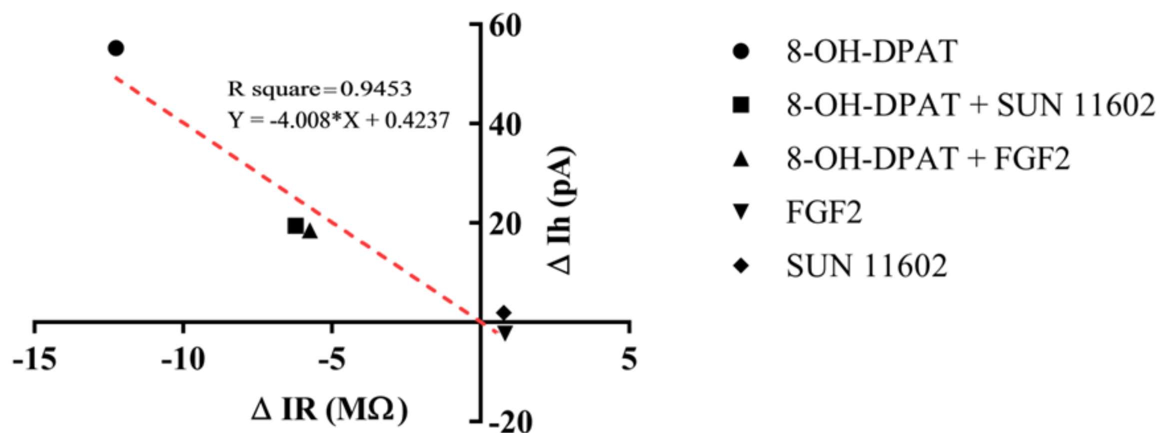

Figure 15. Mean value of delta input resistance (ΔIR) plotted against mean value of delta holding current (ΔI_h) recorded in the experimental groups. Linear relationship between ΔIR and ΔI_h parameters (red dashed line) displays how to an increase of ΔI_h, due to GIRK channel opening, corresponds a decrease of ΔIR.

Effects of acute i.c.v. treatment with FGF2 and/or 8-OH-DPAT in the forced swim test

Sprague-Dawley rats

Each drug treatment by itself did not significantly affect the behaviour in the forced swim test (n = 9-11) compared to ACSF treated littermates (n = 16). The combined treatment of FGF2 (50 ng) and 8-OH-DPAT (200 nmoles) caused a decreased immobility time compared to ACSF treated littermates (Fig. 16A).

Flinders sensitive line rats

In FSL rats, a single i.c.v. treatment with 8-OH-DPAT alone (200 nmol/L) caused a significant reduction in the immobility time compared to ACSF treated littermates (Fig. 16B). In contrast, neither the combined treatment, nor FGF2 by itself had any significant effect on immobility time in the forced swim test.

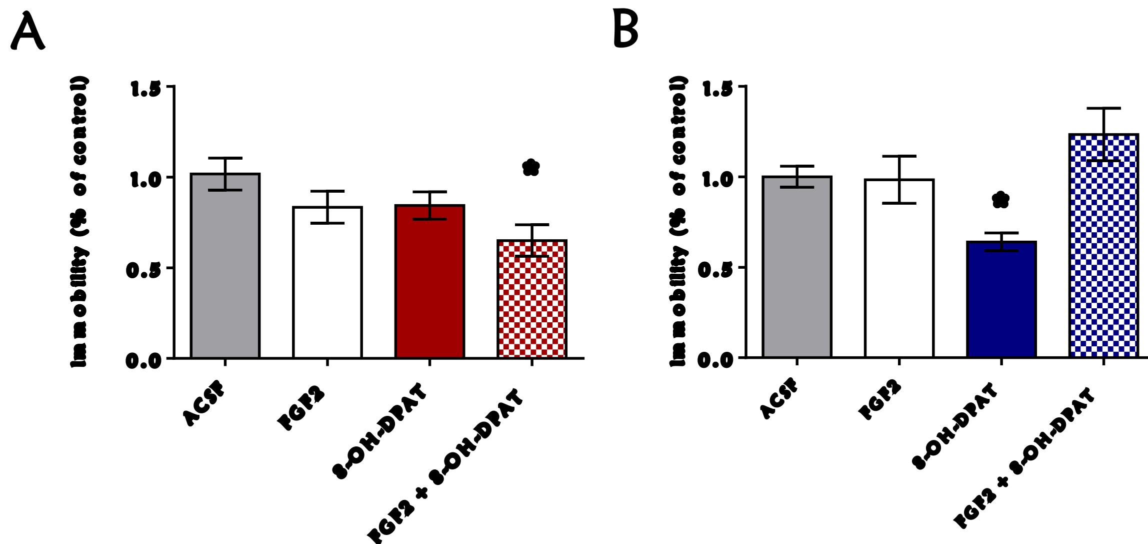

Figure 16. Forced Swim Test in SD and FSL rats. (A) SD rats showed a significant reduction of immobility time upon combined 8-OH-DPAT and FGF2 i.c.v treatment in the forced swim test compared to vehicle controls. All drugs were administered i.c.v. 24 hours before testing. Immobility time was scored during a 5 min test session by an experimenter blind to treatment conditions and expressed as percent of immobility time in vehicle treated controls. Neither FGF2 (50 ng) nor 8-OH-DPAT (200 nmoles) alone did significantly decrease immobility time in the Sprague Dawley rats. However, co-administration of the drugs showed a significant decrease in immobility time as compared to ACSF controls. ACSF n = 16; FGFR2 n = 9; 8-OH-DPAT n = 11; FGF2 + 8-OH-DPAT n = 15. (B) FSL "Depressed" rats showed a significant reduction of immobility time in 8-OH-DPAT alone (200 nmoles i.c.v.) treated rats in the forced swim test compared to vehicle treated rats. All drugs were administered i.c.v. 24 hours before testing. Immobility time was scored during a 5 min test session by an experimenter blind to treatment conditions and expressed as percent of immobility time in vehicle treated FSL rats. Flinder's sensitive line rats demonstrated a significant decrease in immobility time with 8-OH-DPAT alone, but not with FGF2 alone nor with combined treatment (FGF2 + 8-OH-DPAT). ACSF n = 10; FGF2 n = 8; 8-OH-DPAT n = 10; FGF2 + 8-OH-DPAT n = 11. One-way ANOVA followed by Dunnett multiple comparison test, *$p < 0.05$ [266].

Effects of acute i.c.v. treatment with FGF2 and/or 8-OH-DPAT on hippocampal FGFR1-5HT1A heteroreceptor complexes using the in situ Proximity Ligation Assay

<u>Sprague-Dawley rat</u>

The CA1, CA2 and CA3 areas of the dorsal hippocampus were analysed by confocal laser microscopy (Fig. 17). The specific PLA clusters had a similar density in all these areas as seen from the number of PLA clusters per nucleus per sampled field. In Fig. 17 it is seen that the only change found in the different treatment groups, in which the forced swim test had been performed, was in the CA2 area. A significant increase in the density of FGFR1-5HT1A PLA clusters was observed in this region in the combined treatment (FGF2 + 8-OH-DPAT) group. A possible trend for such an increase was found in the CA3 but not in the CA1 area in the combined treatment group (Fig. 17). The panels below the quantification graphs illustrate the increases obtained with the combined treatment in the CA2 area vs 8-OH-DPAT alone and ACSF alone. The number

of PLA positive cells in percent of total number of nuclei per sampled field did not change in any region (data not shown). Thus, the synergistic increase in the number of PLA blobs per nucleus per sampled field in CA2 reflects an increase in the density of clusters in already PLA positive cells.

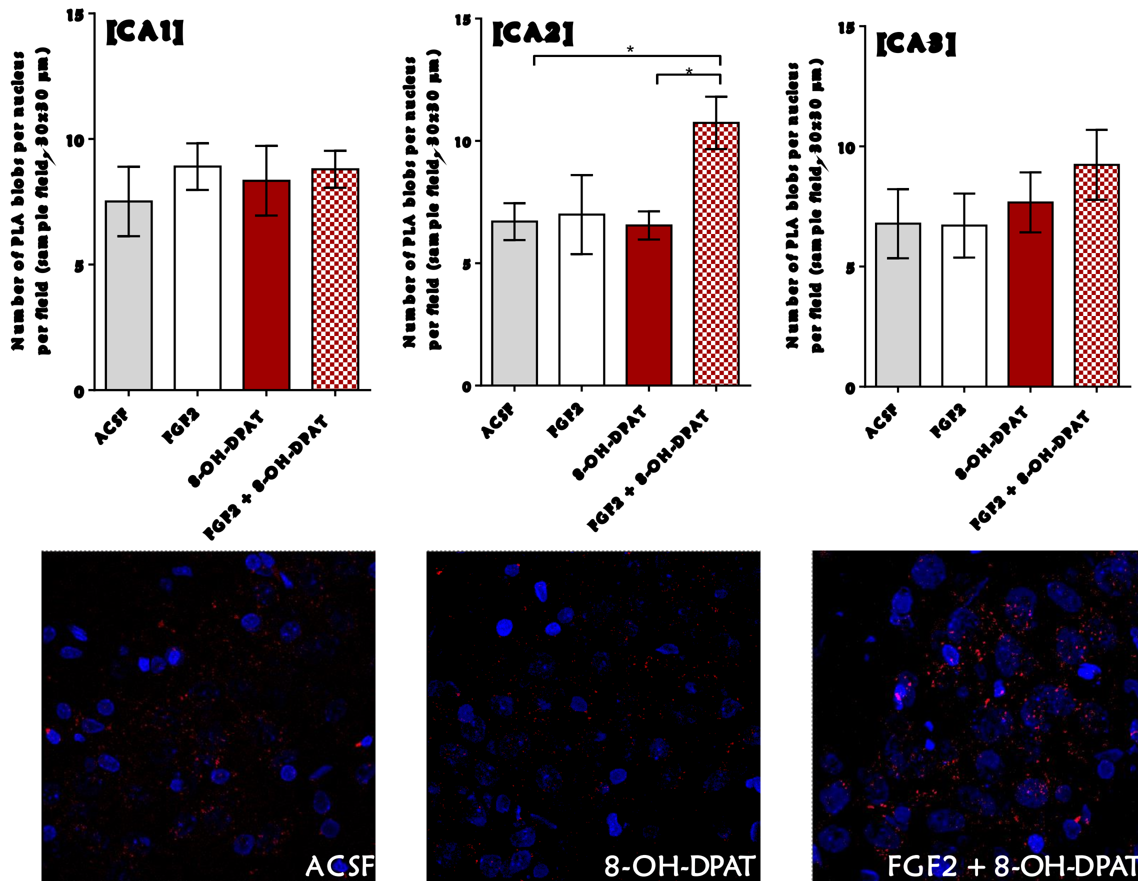

Figure 17. FGFR1-5HT1A heteroreceptor complexes in SD rat hippocampus detected by *in situ* PLA. SD rats showed a significant increase in FGFR1-5HT1A heteroreceptor complexes (Proximity ligation assay (PLA) positive clusters) in the CA2 area of the dorsal hippocampus following a combined, but not single, i.c.v. treatment of the two agonists, 24 h after drug administration. 4 rats per group, duplicates, One-Way ANOVA with the Bonferroni post-hoc test * $p < 0.05$ [266].

Flinders sensitive line rats

A significant increase in the density of specific PLA clusters was found after i.c.v. treatment with 8-OH-DPAT treatment alone in the CA2 and CA3 areas but not in the CA1 area of the dorsal hippocampus vs both the ACSF controls and combined treatment groups (Fig. 18). The increase induced by i.c.v. FGF2 treatment alone did not reach significance. No effects were found in the CA1-3 areas by combined i.c.v. treatment with FGF2 and 8-OH-DPAT. In the panels below the graphs of quantification, the increases of PLA positive FGFR1-5HT1A heteroreceptor complexes found are illustrated in the CA2 area (Fig. 18). The number of PLA positive cells in per cent of total number of nuclei per sampled field did not change in any region. Thus, the increase induced by 8-

OH-DPAT in the number of PLA clusters per nucleus per sampled field in CA2 and CA3 areas reflects an increase in the density of blobs in already PLA positive cells.

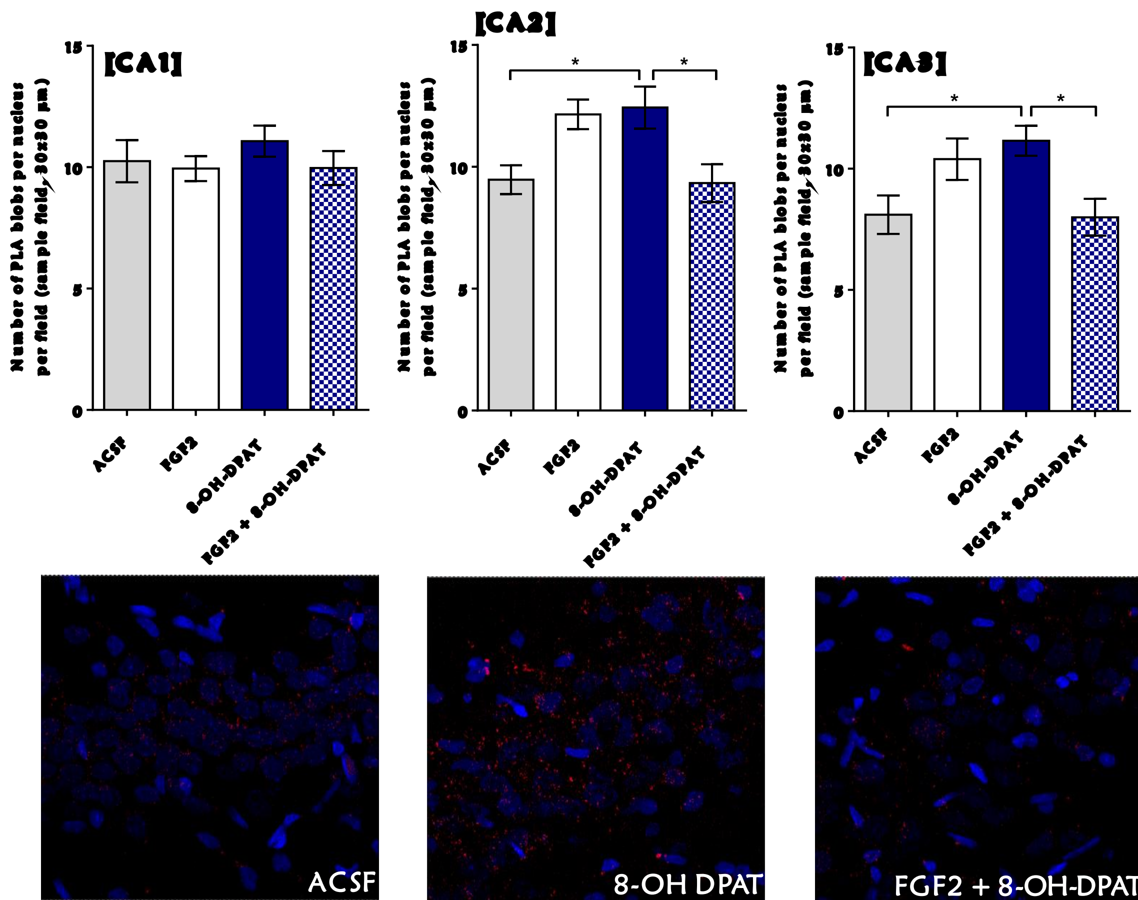

Figure 18. FGFR1-5HT1A heteroreceptor complexes in FSL rat hippocampus detected by *in situ* PLA Hippocampal FGFR1-5HT1A heteroreceptor complexes (PLA clusters) increase in the CA2 and CA3 areas of the dorsal hippocampus in FSL rats following i.c.v. injections of 8-OHDPAT alone but not with combined treatment. FGFR1-5HT1A heteroreceptor complexes remain unchanged in FSL rats in the CA1 area after acute treatment. 5 rats per group, duplicates, One-Way ANOVA with Bonferroni post-hoc test * $p< 0.05$ [266].

Discussion

The therapeutic value of antidepressant drugs is widely proved but the mechanism behind their effectiveness is not actually understood. Ascendant serotoninergic projections and 5HT receptor subtypes expression are disturbed and contribute to the pathogenesis of depression, becoming target for the treatment of this mental disorder [267]. Besides, hippocampal and prefrontal atrophy and, FGF2 signalling system downregulation, have reported to occur in depression suggesting that alteration of this growth factor pathway may represent another contributing factor in MD etiology [229, 268, 269]. The existence of FGFR1-5HT1A heteroreceptor complex was demonstrated in raphe nuclei and hippocampus [2, 3, 253], indicating this R-R

interaction as a potential molecular meeting point between the serotoninergic and the neurotrophic factor hypotheses of depression. Furthermore, the activation of FGFR1-5HT1A complex, in particular after a combined agonists administration, leads to an enhancement of neuroplasticity *in vitro* [2, 253] and is linked to possible anti-depressive effects *in vivo* [2]. Therefore, it has been suggested that activation of FGFR1-5HT1A heteroreceptor complex may be related with antidepressant effect of serotonin in the brain, and combined activation of both receptors could results in more rapid and stronger antidepressant action than found with SSRIs.

Following this point of view, the antidepressant effect of combined treatment on SD rats has been assessed using the forced swim test. For this test, the animals were placed in a cylinder filled with water and received the treatment after the two constituting sessions. In FST, immobility – characterised by absence of movements except for those necessary to remain above the water level – is considered a depressed-like behaviour: if the treatment act on serotoninergic system and has antidepressant effect, a reduction in immobility time is observed [270]. Interestingly, combined, but not single i.c.v. treatment with 8-OH-DPAT and FGF2 produced antidepressant effects in SD rat as seen from the significant reduction of the immobility time.

The brains of rats used in the FST were lately taken for *in situ* PLA 24 hours after the forced swim test. In line with the behavioural results mentioned above, the combined treatment, but not the single treatments in the SD rat, resulted in a significant and differential increase in the number of FGFR1-5HT1A heteroreceptor complexes in the pyramidal cell layer of the CA2 field. Such a recruitment of FGFR1-5HT1A complexes in the CA2 area may contribute to the antidepressant-like effects observed in the FST by the combined i.c.v. treatment. The enhanced integrated response in the CA2 area through the increase of these heteroreceptor complexes, may be beneficial for the emotional brain circuits modulated by CA2 and participate to the antidepressant-like effects observed in the current study. Indeed, it was demonstrated that the CA2 area is crucial for social memory [271] highlighting a link of the CA2 area to emotional circuits and a putative role of CA2 field in depression, as indicated from the current findings.

5HT1A receptors are widely expressed both at post-synaptic level – where the work as heteroreceptor, mediating the serotoninergic effect on target neurons – and at somato-dendritic level of raphe 5HT neurons – where are known to function as autoreceptor [272]. Depression is associated with an increase in 5HT1A autoreceptors, which, in accordance with their regulatory role on the firing of 5HT neurons, reduce the serotonin release and thus, the 5HT transmission [272]. The neuronal inhibition and firing decrease due to 5HT1A activation is dependent on GIRK channels activation and, consequently, to cellular hyperpolarization [273]. The effectiveness of SSRI anti-depressive treatments seems to be linked with the desensitization of 5HT1A receptor and decrease of 5HT1A-mediated auto-inhibition, which, in turn, lead to increased serotonin release [272].

In line with these assertions, we evaluated by means of electrophysiological approach, if FGFR1-5HT1A combined agonist treatment, might exert its beneficial effect

through the modulation of 5HT1A-mediated GIRK currents. The electrophysiological analysis of 5HT1A-activated GIRK currents gave evidence that FGF2 and the FGFR1 agonist SUN 11602, can substantially and significantly reduce the GIRK current elicited in hippocampal CA1 pyramidal neurons by 8-OH-DPAT. Thus, an uncoupling of the 5HT1A $G_{i/o}$ -mediated opening of the GIRK channels may take place upon agonist co-activation of the FGFR1-5HT1A protomers, probably recruiting 5HT1A receptors in the heteroreceptor complexes. The 5HT1A receptor have a modulatory effect on the internal circuitries of the hippocampus upon activation by serotonin released from widespread 5HT nerve terminal networks [274, 275] originating from the midbrain raphe region [276, 277]. The decrease of neuronal hyperpolarization found with the combined agonist treatment might lead to increased excitability and hence relevant implications on depression treatment. Indeed, the increased neuronal excitability in the dorsal hippocampus is related with anxiolytic and anti-depressive effects [278]. The combined agonist reduction of 5HT1A-mediated GIRK current might be also exerted on 5HT neurons of midbrain raphe and, together with the action on hippocampal area, might lead to a faster antidepressant effect and promote a beneficial trophic outcome. However, to confirm this hypothesis, electrophysiological experiments on midbrain raphe are required. In addition, since this short-term effect was recorded on CA1 field while lasting increase in FGFR1-5HT1A blobs were found only in CA2 area, the electrophysiological experiments should be extended to this neighbouring area, which might exert a more important role in the antidepressant effect observed with the combined treatment.

In keeping with the results collected on SD rats, it became necessary to study the FGF2 and 5HT1A agonist regulation of the FGFR1-5HT1A heteroreceptor complexes in a depressed brain, and in particular in a genetic rat model of depression (FSL).

In the forced swim test there was no reduction in the immobility time of the FSL rats after combined treatment but only after i.c.v. injection of 8-OH-DPAT alone. These results might suggest that the FGF2 and 8-OH-DPAT interactions are disturbed in this genetic rat model of depression, indicating the presence of alterations in the FGFR1-5HT1A allosteric R-R interactions. The reasons behind these changes could be numerous. Alterations in the receptor-receptor interactions may be related to changes in the composition and stoichiometry of the FGFR1-5HT1A heteroreceptor complexes in the FSL vs SD strain. It could also involve changes in the allosteric receptor-receptor interactions due to differences in the transmitter panorama between the two strains [279]. Therefore, the molecular mechanism may involve differential changes in the agonist regulation of the FGFR1-5HT1A heteroreceptor complexes through differential R-R interactions in the FSL vs SD rat. Nevertheless, 5HT1A supersensitivity has been demonstrated in the FSL rats versus control [280, 281] and might in part be responsible for the results obtained.

In situ PLA results point out that in the FSL rats the combined treatment failed to change the density of the PLA clusters in the CA2 and CA3 fields in line with the failure of the combined treatment to produce antidepressant-like actions in the FST. On the

opposite, it is of interest to highlight that 8-OH-DPAT alone produced a significant increase in the FGFR1-5HT1A heteroreceptor complexes in CA2 and CA3 fields of the dorsal hippocampus, which was linked to the development of significant antidepressant-like effects in the FST. These results give support to the view that 5HT1A agonists can produce antidepressant-like effects involving the CA2 and CA3 areas of the hippocampus [282]. Thus, it seems likely that disturbances develop in the agonist regulation of the FGFR1-5HT1A heteroreceptor complexes in the CA2 and CA3 regions of FSL rats and that these alterations block the recruitment of these complexes upon combined i.c.v. FGF2 and 8-OH-DPAT treatment. The malfunctioning of FGFR1-5HT1A interaction described in FSL rats might be associated with lack of antidepressant-like effect observed in the forced swim test upon combined agonists treatment. As discussed, one reason for these differential actions of the treatments in the FSL and control rats may be attributable to a possible different composition and stoichiometry of these heteroreceptor complexes in the two strains that will likely lead to differences in their allosteric receptor-receptor interactions.

Taken together, the combined results obtained are compatible with the view that in a genetic model of depression using the FSL rats, malfunctions may develop in the allosteric receptor-receptor interactions of FGFR1-5HT1A heteroreceptor complexes located in the CA2 area of the dorsal hippocampus. The combined agonist regulation of their densities in this region also becomes disturbed. Future work will require the use of interfering peptides [2] to finally determine the role of these heteroreceptor complexes in this genetic model of depression. In addition, it is worth to highlight that raphe nuclei have a pivotal role in MDD and should therefore be considered in the study of the FGFR1-5HT1A heteroreceptor complexes role in depression. In line with this statement, some important PLA results have already been obtained at Karolinska laboratory and support the suggestion about a malfunction in the allosteric R-R interaction of FGFR1-5HT1A complexes also in this crucial brain area [266]. Nonetheless, further experiments, and in particular electrophysiological analyses of these heteroreceptor complexes in the dorsal raphe and in the hippocampus of the FSL model of depression, are required.

In conclusion, the FGFR1-5HT1A heteroreceptor complex has emerged as a promising target for depression treatment development, since the important actions on neuroplasticity [2, 253], the potential modulatory effect on 5HT1A-mediated GIRK current and the evidence of its malfunctioning in depression. Nevertheless, remain to further establish how this complex might be related with depression investigating FGFR1-5HT1A heteroreceptor complex in other rat model of depression, such as chronic mild stress model or the olfactory bulbectomized model. Therefore, a deep knowledge of this complex and its role in disease might contribute to drug development, hopefully leading to the emergence of better and faster antidepressant drugs, which in this field still remain a major concern.

MALADAPTIVE PLASTICITY IN TEMPORAL LOBE EPILEPSY AND ITS PREVENTION

Epilepsy is a heterogeneous and debilitating disease which affect approximately 1-2% of the population and is a brain disorder characterised predominantly by recurrent and unpredictable interruptions of normal brain function, during the so-called seizures. This neurological condition is known since antiquity but it was only in the late nineteenth and early twentieth centuries that, thanks to the advent of electrophysiological recordings [283], quickly became apparent that during seizures there is a dramatic change in the electroencephalogram (EEG) attributed to excessive and synchronous neuronal activity [284]. As the neurophysiology field improves through the years, investigators demonstrate that during seizures and interictal EEG spikes (i.e. altered EEG recording in the "normal" state of a period between seizures; interictal events are too short to give rise to a manifest clinical behaviour) some neurons in the cortex fire abnormally. Between the early 1950s and 1970s, many studies on animal models of acutely provoked seizures in neocortex and hippocampus, gave back many information about the origin and spread of seizures [285]. In the early 1970s, the development of the brain slice and dissociated cell cultures allows to investigate functional mechanisms at the cellular and subcellular levels giving important contributions to our understanding of the mechanisms underlying epileptic seizures [285]. However, despite the progressive emergence of new details about epilepsy through the years, the mechanisms that convert a normal brain into an epileptic one it is not currently fully understood.

Seizures, which are paroxysmal events due to unusual, excessive and hypersynchronous electrical activity in the brain, give rise to several clinical/behavioural manifestations ranging from intense convulsions and loss of consciousness to not readily discernible manifestations. Seizures, even if are not necessarily more important than other brain alterations intrinsic to seizures such as metabolic, blood flow, receptor, gene activation and network connectivity changes, are the most well-known neurological symptom of epilepsy. The formulation of an exhaustive definition of seizure could be tricky. Thus, during the years many different definitions of seizure has been proposed. Recently, a task force of the International League Against Epilepsy described a seizure as "a transient occurrence of signs and symptoms due to abnormal or synchronous neuronal activity in the brain" [286]. However, it is worth to specify that not all seizures imply epilepsy, particularly for single seizures with low likelihood of recurrence or for provoked seizures in an *in vitro* preparation or in a normal animal brain. Therefore, a seizure is any clinical event related to an anomalous electrical discharge in the brain, whereas epilepsy is the tendency to have recurrent seizures. Accordingly, the term "Epilepsy" refers to recurrent and unprovoked seizures. Many studies performed through the last decades have suggested that "seizures beget seizures". Hence a first seizure, elicited by a series of degenerative, reactive or regenerative events triggered for

instance after a traumatic events, infections and febrile status, is capable to trigger a cascade of molecular and biochemical events that lead to reactive maladaptive plasticity and enhanced network excitability which, in turn, transform the naïve network in an epileptic one [287]; this process is referred to as "epileptogenesis". In accordance with the notion that "seizures beget seizures" some patients have a higher risk of new seizures in dependence of the number of seizures they have undergone [288]. Many types of epilepsy, however, exist and not all of them are characterised by the progressive course of the disease. Benign occipital epilepsy, childhood and juvenile absence epilepsies [289], juvenile myoclonic epilepsy, benign familial neonatal, infant and neonatal-infant epilepsies [290] and benign childhood epilepsy with centrotemporal spikes [291], are all examples of non-progressive epileptic syndromes where seizure activity decreases or even disappears with age progression [291, 292].

On the other hand, mesial temporal lobe epilepsy (MTLE), the most common form of human epilepsy [293], despite the existence of milder forms [294], is a serious progressive and chronic disease characterised by seizures originating in hippocampus, entorhinal cortex or amygdala [295]. Additional hallmarks of MTLE are mesial temporal lobe sclerosis, also referred to as hippocampal sclerosis [296], and its high rate of drug resistance (30-40 % of patients with drug resistant epilepsy suffer of MTLE) [297] which often make surgical resection of epileptic tissue the only therapeutic alternative. Thus, although many advancements in the research on MTLE has been made during years, the need for new and, in particular, efficient drugs remains an important and still not attained medical achievement.

Epileptogenesis and aberrant circuit modifications in MTLE

Epileptogenesis, as mention above, can be triggered by genetic or acquired factors and is the process by which the normal brain network is transformed into a tissue capable of generating spontaneous and recurrent seizures [298]. This term is referred to both the initial conversion in epileptic tissue and, according to the new terminology, the following expansion of that tissue during the disease development, after the diagnosis. Epileptogenesis is a process that can take as little as minutes to hours or as long as months to years [299]. Thus, between the first insult and the appearance of new seizure, there is a period of transition referred to as "latent" or "silent" period. During the latent period, several molecular, cellular and circuital alterations follow one another and evolve over time. At the end of the latent period, non-convulsive EEG-detectable seizures always precede the appearance of the first convulsive seizure [300, 301]. Most of the information about epileptogenesis process comes from animal models of epilepsy but recently evidence that validate the information obtained in animals, has been gathered also on human tissue from biopsy or autopsy. Based on these data, it seems that the incidence of epilepsy is higher in the first years after the injury (for instance traumatic brain injury, stroke and others) and, after a decade, it become progressively, though still present, lower [302]. In addition, other relevant information obtained

concern the importance of the first insult onto the epileptogenesis. Indeed, there is a variation in the latency and frequency of seizure depending on the model of epilepsy studied: in post-stroke and traumatic epilepsy, the hippocampal damage is milder than what observed in Status Epilepticus (SE)-induced epilepsy [303] (Status epilepticus "is a special circumstance with prolonged or recurrent seizures" [286]). Most of the information on epileptogenesis has been obtained in studies on induced SE and focus in hippocampal area. Epileptogenesis is a process characterised by alterations that come in succession with overlapping timing of development. From few minutes to several hours after the seizure insult, the massive release of neurotransmitters, and in particular glutamate, is followed by ion channels activation and calcium influx which induce short-lasting biochemical modifications of pre-existing target molecules such as receptors, Ca^{2+} translocation systems and neurotransmitter trafficking. All these phenomena could induce excitotoxicity and cell death. In addition, a complex series of delayed but long-lasting cellular events, such as proteins phosphorylation and immediate early gene activation, occur [299]. After several hours to days, it persists an altered and upregulated gene transcription and cellular death, growth factor expression and release, a strong activation of inflammatory cascades and, glial and vascular responses. Lastly, after weeks to months, many remodelling events take place including axonal sprouting, synaptogenesis, gliosis, angiogenesis, network reorganisation and increased synchronous activity with an altered excitation/inhibition balance [299].

It is suggested by many histopathological and electrophysiological data that two alterations in local synaptic circuits are essential for acquired epileptogenesis: (1) the decreased GABA-mediated inhibition and (2) the amplified recurrent excitation relative to the increases in axonal sprouting.

Reduced GABAergic inhibition

GABA is the most important inhibitory neurotransmitter in the CNS. GABA could bind to its ionotropic receptor $GABA_A$, which is a hetero-pentameric channel formed by various combination of α, β, γ, δ, ε, π, θ and, ρ subunits. In general, GABA receptor containing $\gamma 2$ subunit mediates fast inhibitory currents while receptors containing $\alpha 4$, $\alpha 5$, or $\alpha 6$ and/or a δ subunit instead of γ, mediate a tonic inhibition. GABA inhibition in the brain is linked to a variety of cognitive processes in health and its alteration is involved in several neurological diseases among which epilepsy [304]. The observation about the link between $GABA_AR$ polymorphisms/mutations and numerous epilepsies in human, emphasizes the importance of this neurotransmitter and its mediated inhibition in this neurological disease [305, 306]. In addition, the loss of GABAergic inhibition is observed prior to a seizure [307]. Depending on the type of epilepsy, the alteration of GABAergic drive is generally associated to inhibitory interneuron loss and/or reduction in the number of inhibitory synapses on principal neurons [308, 309]. Accordingly, there is evidence from morphological and physiological techniques that a modest loss of inhibitory interneurons is associated with epileptogenesis [310]. Thus,

immunohistochemical and *in situ* hybridization techniques reveal a loss of specific type of GABAergic neurons in epilepsy, in cortical areas and in the hippocampus [311, 312]. Although GABAergic neurons death has been identified in several brain regions, the loss of somatostatin-positive GABA neurons in the DG hilus represents one of the most consistent findings [313]; loss of somatostatin-positive GABAergic neurons in CA1 *stratum oriens* have also been proved. Inhibitory interneuron loss, if not compensated by axonal sprouting, could cause GABAergic tone reduction. Whole-cell electrophysiological recordings revealed a reduced frequency of miniature Inhibitory Post-Synaptic Currents (mIPSC) in hippocampal granule cells and CA1 pyramidal neurons, supporting the hypothesis of inhibitory drive reduction and neuronal loss [314, 315]. This reduction of frequency, in keeping with the preferential degeneration of somatostatin-positive interneurons, is mostly localised on principal neuron dendrites [308, 316]. Despite the changes in dendritic inhibition, the mIPSC frequency in the somata of principal neurons is preserved [308, 316], thus contrasting an ongoing status epilepticus. This pattern of GABAergic interneuron loss gave rise to the suggestion that neurons innervating dendrites are more susceptible of damage in epilepsy. Nonetheless, despite this suggestion, in CA1 of a mouse model of temporal lobe epilepsy, cholecystokinin-positive interneurons, which provide perisomatic inhibition, appeared to be decreased while other perisomatic inhibitory interneurons remain unaltered [317]; in DG of several animal models, loss of parvoalbumin-positive basket cells and axo-axonic cells has been proven [318]. Therefore, it is inferable that neuronal loss affects diverse typologies of GABAergic neurons and it seems that the most affected type of interneuron might vary in relation to the brain region analysed [313, 319]. Despite the findings reported above, the relationship between seizures, neuronal death and epilepsy continues to be object of several controversies and debates. Indeed, if on one hand it seems clear that some types of seizures, especially if isolated and brief, do not lead to neuronal death, on the other hand, more prolonged seizures, as those typical of MTLE, which often progress to tonic-clonic convulsions, are much more likely to lead to neuronal loss [320]. Additionally, the prolonged and repetitive seizure that characterise the SE are proved to cause brain damage and extensive neuronal death [309, 321]. The demonstration of a clear relationship between GABA neuron loss and epilepsy could be difficult to realize in part because GABAergic interneuron loss does not occur in isolation but also involves principal neurons death [313].

In spite of the evidence for loss of GABAergic neurons in epilepsy, some inhibitory GABAergic cells remain and alteration and compensation in this survived population might lead to inadequate control of principal neurons and possibly contribute to epileptogenesis. In keeping with this idea, deficit in DG basket cell function, which lead to an increased failure rate on their synapses onto granule cells, has been identified [322]. In addition, receptor alterations on survived GABAergic interneurons may also reduce the activity of these cells. δ subunit-containing $GABA_A$ receptors, which are responsible for tonic inhibition, have an increased expression in GABAergic neurons of pilocarpine animal model of depression [323, 324] and this

alteration might reduce their excitability and their inhibitory drive onto principal excitatory neurons [323]. Lastly, worth of mention is another important finding regarding GABAergic alterations in epilepsy, which concern $GABA_A$R-mediated current - I_{GABA} -. Thus, in MTLE patients it is proved that I_{GABA} is subject to a rundown of its intensity [325, 326], suggesting a contribution of this abnormality to the reduced GABA inhibition on epileptic neuronal circuits. Taken together it seems possible that, despite the presence of compensatory network and cellular adjustment, all these alterations of GABAergic neuron population could lead to occasional failure in their inhibitory drive, which might result in the appearance of sporadic seizure activity.

Axonal sprouting and amplified recurrent excitation

In the latent period, network reorganisation is observed. Studies performed in 1970s showed that lesions of the perforant pathway lead to an input reorganisation characterised by the formation of aberrant connections [327]. A decade later, it emerged that after seizures, new mossy fibers are formed [328, 329], and this phenomenon has been successively confirmed in numerous animal model of epilepsy and in human MTLE [330]. Some research data highlight that this synaptic reorganisation also consists in mossy fiber sprouting, that is the emergence of new DG granule cell axons. The hypothesis associated with the mossy fiber sprouting here described is that, considering many electrophysiological and ultrastructural data which suggest that almost all mossy fiber are excitatory projections, the increased sprouting observed in the latent period might increase the recurrent excitation during acquired epileptogenesis, since sprouted mossy fiber make contact on dendrites of DGCs and interneurons [331]. In particular, recent quantitative ultrastructural research data point out that the sprouted excitatory synapses preferentially contact DGCs versus interneurons [332, 333], thus creating an excitatory loop.

Research data shows that in epilepsy, after an initial loss of excitatory synapses onto DGCs, the number of those synapses partially recover in the inner ML (about 84%) and outer two-thirds of ML (101%) in 3-6 months, probably in relation to mossy fiber sprouting [285]. This phenomenon is not exclusively related to the latent period but it continues after the epileptogenic brain injury [285], probably in relation to its nature of homeostatic mechanism aimed to maintain a set-point level of excitatory drive on DGCs. It is possible that this mechanism could be pushed over the limits by epileptogenic injuries and contribute to the creation of a network able to generate spontaneous seizure. However, the role of mossy fiber sprouting in epileptogenesis remain to clarify in order to establish if this phenomenon could be either necessary or sufficient for hyperexcitability.

During epileptogenesis, axonal sprouting and synaptic reorganisation take place not only in DG but are observed also in other regions, such as CA1 field [334, 335]. Indeed, CA1 is a region highly vulnerable to excitotoxic damage and neuronal death in this area represents a well-known marker of hippocampal sclerosis in MTLE. In this

scenario, the mechanism of axonal sprouting in the *alveus* and in *stratum oriens* [334] may give rise to the formation of new recurrent excitatory circuits that could contribute to the acquired epileptogenesis.

Despite the sprouting considered in human and animal models of epilepsies is mainly principal neurons sprouting, even GABAergic neurons undergo axonal sprouting in MTLE, and may contribute to, or compensate for, the excitation/inhibition imbalance found in this neuropathology. Studies on GABAergic sprouting used several markers, such as GABA, its synthesising enzymes such as GAD (Glutamate Decarboxylase – an enzyme that catalyse the decarboxylation of glutamate to GABA) or GABA transporters, such as GAT-1 (GABA transporter 1). A wide portion of reports on GABAergic axonal sprouting have focused on the hippocampal portion relative to the dendritic trees of DGCs. In pilocarpine model of epilepsy, GAD-positive fibers in the supra-granular region and the external two-thirds of the DG molecular layer have been found reduced [336]; in addition, GAT-1 and GAD positive fibers in the hilus have been found decreased as well [318, 336]. However, this condition distinguishes the first days after the induction of epilepsy, while GAT-1 and GABA positive fibers are found increased in the molecular layer after 6-9 days and remain higher until the period of recurrent seizures. GAD positive fibers, on the other hand, increase following a slower timing and can be found increased in the outer ML around and after two months following the SE induction. The increased GAD and GAT-1 immunoreactivity has been confirmed in several other animal models [337, 338] and in human hippocampus [339, 340]. Taken together, these findings suggest an early loss of GABAergic interneurons followed by an increased axonal sprouting onto the dendrites of hippocampal DGCs in epilepsy. GABAergic interneurons sprouting has also been observed in hippocampal CA1 field but instead of contacting pyramidal neurons, it is suggested that sprouted calbindin (CB)- and GAD-immunoreactive fibers make synapses onto dendrites of other interneurons [341].

Axonal sprouting has been reported not only on interneurons that target the dendritic region, but also on perisomatic GABAergic neurons in DG and CA1 fields of hippocampus. In line with the evidence on dendritic sprouting, perisomatic neurons generate new fibers which make contact on DGCs and in CA1 on somata of other inhibitory GABAergic neurons [341]. Nevertheless, the functional consequence of GABAergic circuit reorganisation in epilepsy remains to establish also considering all the GABAergic changes in epilepsy such as $GABA_A$ subunits composition and pharmacology changes or G protein modulation of GABAergic drive [342-344]. In addition, despite inhibitory sprouting is seen as compensatory mechanism, it is strongly suggested that this phenomenon might play a contribution to the emergence of epileptic seizures [341].

MTLE and adult neurogenesis

The functional role of DG in the hippocampal network it not completely understood yet but another and intriguing role proposed is a “gate” function for the excitatory input entering the hippocampus [345]. Accordingly, it has been observed,

using voltage sensitive dyes, that following the activation of entorhinal cortex and subsequent spread of neuronal activity to DG, the further propagation of the impulses was blocked at this anatomical level [346]. Thus, failure of DG gating property has been highlighted in many different model of epilepsy, suggesting that in this neuropathology, the alteration of this feature might exert effects on the excitation/inhibition balance in the hippocampus. The failure of this DG activity could be related with many alterations among which those described above, such as interneuron loss, but an important component might be also represented by the features regarding dentate granule cells and neurogenesis in epilepsy.

Adult neurogenesis is a DG natural feature of mammalian brain and has received large attention for the role of hippocampus in learning, memory and cognition. Several research data highlight the existence of neurogenesis dysregulation in several brain diseases among which epilepsy [347]. The first evidence on a possible connection between neurogenesis and epilepsy was gathered in 1990s, on animal models, when was discovered that in the latent period it is possible to assist to a substantial increase of granule generation in temporal lobe epilepsy [348, 349]. Indeed, among all the pro-neurogenic stimuli that affect DG granule generation, epilepsy is considered one of the strongest. Increased granule neurogenesis is a feature of most epilepsy models [350, 351], but it is obviously linked to all those epilepsies that involve the hippocampus, among which the prominent is MTLE. Here, it was established that these newly-generated cells survive for at least one year [352]. Despite the increased neurogenesis that follow the induction of epilepsy, animal models clearly show a dramatically decreased rate of neurogenesis with the passage of time [353], a condition that is probably connected with the hippocampal sclerosis often reported in MTLE in both animals and humans [347].

In epilepsy, abnormal granule cells could be found in the DG and the majority of this population has been proven to be newly-generated [352, 354-356]. These abnormalities consist in: mossy fiber sprouting onto granule cell dendrites in the inner molecular layer (see above); ectopic granule cell migration, which consist in newly-born cells that localises in the hilus - instead of integrating into the granule cell layer - thus giving rise to recurring excitatory circuits once reached by DGCs axons [357-359]; basal dendrite persistence in mature granule cells [360, 361] that project into the hilus and receive recurrent innervation from neighbouring DGCs [362, 363].

A legit question that arises from these finding is: do the altered features of adult-generated DGCs impairs DG gating and contribute to epileptogenesis? It has been proven that ectopic granule cells display an higher ratio of excitation/inhibition inputs [364] and burst, which is a property not common in normal DGCs that could be seen as a pro-epileptogenic feature [357]. On the other hand, normal DG granule cells correctly located display a decreased level of excitability suggesting the possibility of a homeostatic compensation [365]. In addition, many newly-generated cells in this region have a decreased number of dendritic spines; by contrast, other newborn DGCs, characterised by long basal dendrites and robust innervation by sprouted mossy fibers,

display an increased number of spines and are probably reached by a higher excitatory drive [356]. Despite all the data collected, the contribution of aberrant DGCs to epileptogenesis is still not clear and needs a deeper investigation. Nonetheless, considering the features here reported, it is obvious to believe that DG in epilepsy might not function in the same manner as a normal dentate gyrus does.

Role of inflammation in epilepsy

Inflammation is a physiological response to infections, injuries (i.e. ischemic, traumatic and excitotoxic) or stress and is characterised by the production and/or release of many different mediators from the cellular component of the innate and adaptative immunity. Inflammatory mediators are normally present at very low levels in healthy brain, but they can be upregulated by several different stimuli and therefore increase their brain levels.

In the brain, innate immunity is mediated by microglial cells, which can be considered as the resident macrophages of the CNS; in addition, also astrocytes and neurons take part to the immune response [366, 367]. Brain damage or infections induce the release of cytokine-related mediators that activate and recruit, in the affected area, microglia and blood-derived macrophages. Microglia in the presence of cellular debris or damaged cells, become rounded, circular migratory macrophages and start releasing cytokines and growth factors which can exert both damage and beneficial effects on the surrounding cells [368]. Some key inflammatory mediators are: IL-1β, IL-6, TNF-α, cell adhesion molecules, toll-like receptors prostaglandins and complement factors.

The relationship between inflammation and epilepsy have gathered increasing attention in the recent period. Thus, many research data suggest a direct association between seizures and acute and chronic activation of inflammatory pathways [369-371]. Indeed, inflammation has been assessed not to be a rare phenomenon in the epilepsy. Moreover, the finding that anti-inflammatory treatments exert anticonvulsant effect in some cases of drug-resistant epilepsy [372, 373] supports the view of neuroinflammation as a mechanism implicated in seizures and epilepsy. The expression level of IL-1β, one of the two molecular form constituting the IL-1 cytokine family, is found to be rapidly upregulated after the induction of SE in animal models of MTLE (1.5 hour post-injection of pro-epileptogenic compound) [374] and the rapid increase of this cytokine in hippocampus it is proved to be probably dependent on microglial activation [375]. In the kindling model of epilepsy, IL-1β, TGF-β and TNF-α expression levels are increased in many limbic areas [376]. The effect of TNF-α on epilepsy seems to be related to the amount of exposition. Indeed, it has been found that low concentration of this factor could exert anticonvulsant effects [377], while a chronic over-exposition of TNF-α leads to neurological dysfunction among which seizures [378]. Another important part of the immune response activated in MTLE is the complement cascade [379]. Remarkably, injection into rat hippocampus of complement components induces

electroencephalographic and behavioural seizures together with cytotoxicity, confirming a potential role of complement in epileptogenesis [380].
Together with the production of pro-inflammatory molecules, inflammatory response is also characterised by the release of anti-inflammatory mediators and binding molecules which modulate this response limiting the development of deleterious effects [381]. Accordingly, in epilepsy, an up-regulation of IL-Ra - an antagonist of IL-1β - has been reported [382], but contrary to normal inflammatory response, in this neuropathology this factor is released with a delayed time course with respect to the release of IL-1β [383], thus making the brain less responsive and effective in terminating the action of a sustained increase of IL-1β.

A key component which contributes to epileptogenesis and is related with immune activation is the loss in the blood brain barrier (BBB) functions, which has been suggested to be caused by seizure activity [384, 385] and/or systemic factors, such as the release of immune molecular mediators which increases the endothelial permeability [384, 386]. In addition, BBB integrity in epilepsy is also affected by VEGF, a potent modulator of endothelial permeability, which is released by neurons in response to seizures and triggers vascular remodelling and angiogenesis [387, 388], leading to the formation of new leaky blood vessels.
One of the consequences of BBB disruption is the presence of serum proteins in the brain. Albumin, one of the most abundant protein in the blood, it has been demonstrated to be taken up or bound to neurons, astrocytes and microglial cells after SE [385]. Albumin has influence on neuron excitability and inflammation. In particular, the extravasation of albumin due to leaky BBB and the consequent uptake of this protein by astrocyte through a TGF-β dependent mechanism [389], is followed by a reduction in Kir 4.1 potassium channels and the subsequent increase in extracellular potassium concentration, which depolarizes neurons and enhances neuronal firing [390]. Moreover, it has been proven that albumin uptake in neurons can increase the synthesis and release of glutamate [391], which in turn enhances neuronal excitability. BBB damage has consequences also for brain inflammation. Indeed, brain inflammation is often observed in areas where the BBB is damaged [392, 393]. Extravasated albumin can induce brain inflammation through transcriptional changes that lead to upregulation of pro-inflammatory cytokines and IL-1β and TGF-β pathway activation [394-396]. In addition, peripheral leukocytes can infiltrate from the blood to the brain through ICAM-1 (Adhesion Molecule 1) and VCAM-1 (Vascular Adhesion Molecule 1) adhesion molecules, contributing to the epilepsy-related brain inflammation [397]. Interestingly, interfering with this process, through the block of the interaction between leukocytes and endothelial cells after acute seizures, avoids the development of chronic epilepsy in the pilocarpine model of epilepsy [397].

Oxidative stress and epilepsy

Epilepsy, characterised by recurrent seizures, can lead to increased levels of reactive oxygen species (ROS) and reactive nitrogen oxygen (RNS) in the brain. The generation of free radicals is mainly imputable to mitochondrial phosphorylation chain [398]. The term ROS is referred to species such as hydroxyl radical ($HO^{\cdot}$), superoxide anion radical ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2) or peroxyl radicals ($HOO^{\cdot}$); RNS term is referred to nitric oxide (NO) and molecules derived from NO, such as peroxynitrite ($ONOO^{\cdot}$), nitrosyl (ON^{-}), and nitrogen dioxide (NO_2). The increase in oxidative and nitrosative stress is generally linked with a condition in which cellular antioxidant defences fail to deactivate all the ROS and RNS generated, and could be associated to the intense metabolic activity.

A wealth of studies has shown the existence of a relation between oxidative stress and epilepsy [399] and it seems that an increase in free radical production is related with prolonged seizures that might result in mitochondrial dysfunction in hippocampus, which is in turn linked with neuronal death and epileptogenesis [399, 400]. Research data on hippocampus in KA model of epilepsy reported evidence of a reduced GSH/GSSH (reduced form /oxidized form of glutathione) ratio together with increased glutathione peroxidase and glutathione reductase activity [401]. This increase in redox status might contribute to seizure-related neuronal death [399, 402]. Oxidative stress levels, as those induced by seizures, can be measured from blood samples. Several studies reported a decreased levels of antioxidant such as glutathione peroxidase or Cu-Zn Superoxide dismutase in patients affected by progressive myoclonic epilepsies [403]. Besides, erythrocytic GSH, total antioxidant status in plasma and vitamin E levels have been found lower in patients affected by refractory epilepsy [404]. Moreover, lipid peroxidation levels have been found to improve after treatment in epilepsy confirming a potential implication of free radicals in this neuropathology [405]. Lipid peroxidation (LPO) is a key feature of oxidative stress and is mediated by a radical activity on the polyunsaturated ω- 3 and ω-6 fatty acids which leads to the formation of lipid peroxyl radicals ($LOO^{\cdot}$). This compound in turn gives rise to several lipid hyperoxide products such as malondialdehyde (MDA), 4-hydroperoxy-2-nonenal (HPNE), 4-oxo-2-nonenal (ONE), and 4-hydroxy-2-nonenal (HNE) [406, 407].

Considering the strong implication of oxidative stress in epilepsy, many research teams have started to evaluate the therapeutic potential of several endogenous or exogenous antioxidants. One of the mostly studied antioxidant assessed in epilepsy is melatonin. Its anticonvulsive effects have been confirmed in several animal models of epilepsy leading to the demonstration that this hormone reduces seizure activity and neurodegeneration in KA and pilocarpine model of epilepsy [408, 409]. Melatonin reduce ROS, RNS and lipidic peroxidation [408, 410] and the maintenance of its antioxidant contribution has been proven to mediate important anti-convulsive and neuroprotective effects [411].

Another antioxidant compound studied in epilepsy is ascorbate. Ascorbate seems to improve convulsive behaviour and neurodegeneration in many animal models [412]. It has been suggested that this compound may act inhibiting oxidative stress and maintaining GSH homeostasis [413].
α-tocopherol (α-T – the vitamin E isoform with the highest *in vivo* biological activity and bioavailability) is a lipophilic antioxidant which is able to cross the BBB and accumulate in the brain [6, 7]. Many studies highlighted that α-T levels are reduced in the plasma of epileptic patients, suggesting its potential beneficial role in the treatment of epilepsy [405]. Besides, it has been proven that α-T showed beneficial effects in animal model of epilepsy reducing seizure activity and neuronal death [412]; this antioxidant compound reduces RNS and lipidic peroxidation in the pilocarpine model of depression [414], and it has been proven to decrease BBB disruption [415]. The potentiality of α-T in epilepsy and its potential ability to contrast epileptogenesis will be further discussed in this thesis (see "Post-seizure α-tocopherol treatment for preventing epileptogenesis").

microRNA dysregulation in MTLE

The analysis of brain tissue from patients with MTLE highlights the presence of extensive gene expression dysregulation that is connected to recurrent seizures and affects several genes related with inflammation, gliosis, synaptic reorganisation and neuronal function [416]. New levels in gene expression regulation have recently emerged after the discovery of microRNAs (miRNAs or miRs). miRs are endogenous and short (about 22 nucleotides) RNA oligonucleotides that recognize partially complemented mRNA targets and generally inhibit their transcription [417]. To function, a microRNA should form the RISC (RNA-induced silencing complex) after the bound with the protein Argonaute (Fig. 18); once formed, this complex binds its mRNA targets and induce their degradation or blocks their translation [417].

Interestingly, about the 60% of human proteins seems to be directly regulated by miR [418]. A single miR has numerous targets, thus controlling several different genes together [419]. Since their discovery, the understanding about miR potential role in many different diseases and their importance as new target for innovative therapies has been progressively grown. Concerning epilepsy, it has recently been established the first curated and dedicated database of miR related with this neuropathology called EpimiRBase [420]. The first study on miR in human epilepsy was performed in 2010: the data obtained showed a clear increase of astroglial expression of miR-146a in patients with MTLE and hippocampal sclerosis [421]. In epilepsy, the up-regulation of miR-146a is the most reliable result obtained among animal and human studies. miR-146a is strictly connected to the control of inflammatory response: indeed, its expression level is increased by IL-1β [422]. miR-146a, however, is a suppressor of pro-inflammatory mediators as IL-1 receptor-associated kinase-1 (IRAK-1), IRAK-2 and TNF receptor associated factor 6 (TRAF-6). In addition, mir-146a is also a suppressor of IL-

1β, indicating a functioning of this miR as negative feedback aimed to control and regulate the astroglial-mediated inflammatory response [422]. Thus, since the mir-146a up-regulation acts reducing the expression of pro-inflammatory cytokines, it has been suggested that this could represent a compensatory mechanism in epilepsy. However, in both rats and human this miR remains up-regulated during latent and chronic period of epilepsy [422, 423].

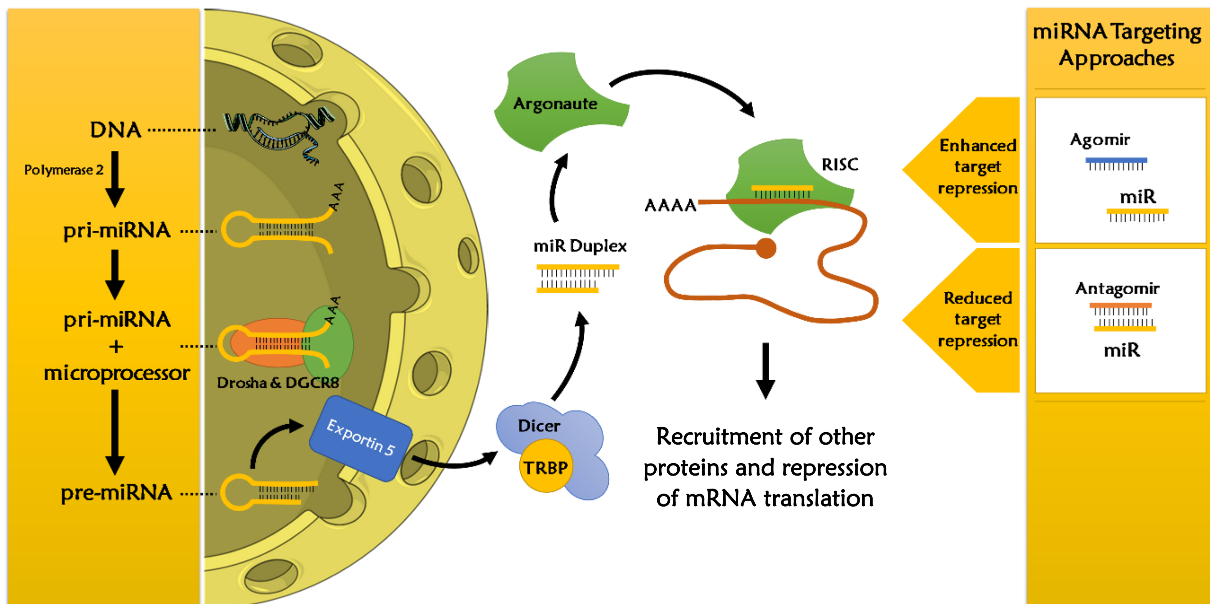

Figure 18. miRNA biogenesis and some miRNA manipulation approaches. microRNA biogenesis starts with the transcription of a pri-miRNA (primary microRNA) from introns of protein-coding genes and specific loci. Then, the microprocessor complex (containing the RNase Drosha and Di George Syndrome Critical Region 8 - DGCR8 -), cleave the pri-miRNA to produce the pre-miRNA, an approximately 60-70 nucleotide-long hairpin structure. The pre-miRNA is successively exported to the cytoplasm by exportin 5 and processed by the RNase Dicer to produce the mature duplex miRNA (approximately 22-nucleotide): the process is enhanced by transactivation-responsive RNA binding protein (TRBP). One strand of the miR is then bound by an argonaute protein, forming the miRNA-induced silencing complex (RISC), which traffics along target mRNAs until it binds its 7–8 nucleotide complementarity sequence. This binding produces stable miRNA–mRNA complexes that facilitate mRNA decay or translational repression following recruitment of other factors, such as GW182 proteins. (On the right) The introduction of a miRNA agomir (mimic) will facilitate miRNA-dependent silencing of targets. On the other hand, the use of antisense oligonucleotide sequence complementary to the miRNA of interest can block miRNA function and thereby de-repress a target mRNA.

Many other microRNAs that might play a role in epilepsy have been identified through years [424]. For instance, miR-124 is a brain-specific microRNA considered as a key regulator in neuronal differentiation and the development of the nervous system. This miR, as suggested by a recent research, could also function at the growth cone or at synapses levels where it exert modulatory effects on synaptic activity and neuronal connectivity [425]. In addition, mir-124 has also influence on inflammation where it plays a dual and contrasting role. Indeed, this microRNA is a master regulator of neuron-restrictive silencer factor (NRSF) which is a transcription factors repressor whose activity

contrasts epileptogenesis; however, mir-124 also exerts pro-inflammatory function in epilepsy promoting microglia activation [426].
Research on miR-155 showed that it represses microglia functions, suppressing several genes required for microglial activation, phagocytosis and inflammatory response; targeting miR-155 with an antagomir (which physically blocks a miR functioning) (Fig. 18) protects against neurodegeneration [427] and improves postictal behaviour after SE in mice [428].
It has been demonstrated that miR-181a is up-regulated in pilocarpine model of epilepsy and children affected by MTLE. According to research data on rat model an i.c.v. injection of this microRNA antagonist reduces neuronal death after SE [429]. In addition, this miR can also target AMPA receptor containing the GluA2 subunit reducing dendritic spine and mEPSCs [430].
An intriguing feature related with disease diagnosis and follow-up is the presence of circulating miRs in biofluid as blood or CSF (Cerebrospinal Fluid), which highlights the usefulness of this regulatory oligonucleotides as biomarkers of brain injury [424]. The circulating microRNA might originate from controlled tissue release by exosomes or directly from brain extracellular fluids as a consequence of a leaky BBB. Studies suggested a precise pattern of circulating miRs which delineates a disease profile for different brain injuries, including prolonged seizures [431]. A set of circulating miR in epilepsy has been identified and increased serum levels of miR-146a is part of it [432]. In addition, differences in miR blood levels between patients with refractory and controlled seizures have been found, suggesting a potential use of circulation miR as biomarkers for identify drug-resistant epilepsy [433].

Animal models of epilepsy

The understanding of the mechanisms underlying epileptogenesis and seizure development in MTLE and other typologies of epilepsy its hardly achievable from clinical research on human patients. Therefore, several different animal models of epilepsy have been developed. In this paragraph, some of the most commonly used animal model of epilepsy are described.

The kainic acid model of epilepsy

Chemoconvulsant drugs are one widely used method to induce epilepsy in animals. Among chemoconvulsant compounds employed, kainic acid (KA) represents one of the first compounds used to obtain a MTLE model of epilepsy [434]. Kainic acid is an L-glutamate cyclic analogue, antagonist of ionotropic KA receptors. This compound was firstly isolated in 1950s from *Digenea simplex* [435], a red alga, with the meant to be used against infestation of *Ascaris lunbricoides*, a parasitic nematode. Nevertheless, it was successively found that KA induces a powerful excitatory response in cortical neurons of treated rats [436], thus becoming used as glutamate analogue. This finding

leads to the discovery of new glutamate receptors named KA receptors and to the development of KA model of temporal lobe epilepsy, characterised to a progress that resembles human MTLE, with a latent period followed by refractory seizures [437, 438]. Soon after an intra-hippocampal injection of KA, it is possible to record EEG changes and hippocampal epileptiform discharges before seizures occurrence [439, 440]. These first events are followed by the manifestation of seizure that starts in the hippocampus and then might spread to ipsilateral and contralateral amygdala, contralateral hippocampus and frontal cortex; intra-amygdaloid injection of KA gives rise to a similar pattern of propagation [441].
The hippocampus is a structure highly vulnerable to the neuropathological changes that take place after KA injection and, interestingly, this high susceptibility is maintained even if hippocampus it is not the injection site of KA. In particular, kainic acid preferentially damages CA3 area but leaves almost untouched CA1 and DG regions. However, in CA1, and particularly in the *stratum oriens*, takes place a relevant loss of GABAergic interneurons and specially parvalbumin- and somatostatin-positive neurons [441]. Animals injected with KA develop a chronic epilepsy and start suffering for spontaneous seizures between 5 days to one month after the induction of SE [440, 441]. Nevertheless, alterations in the EEG and interictal spikes can be recorded before and after the onset of the first spontaneous seizure [441, 442]. Noticeably, mice and guinea-pig don't manifest convulsive seizures if used to obtain the KA model, while rats undergo non-convulsive seizures only during the latent period, while manifest convulsion in chronic phase [441, 442].

Kainic acid can be also administered systemically and the main advantage is that many animals can be injected at the same time without surgical procedures. However, this way of delivery has a principal drawback that concerns the bioavailability of KA in the brain often leading to the necessity of multiple injections [441]. Many research data suggest that using this way of KA administration, SE occurs around 1 hour post-injection [443, 444]. About 30 minutes after KA injection, EEG shows an epileptiform pattern that has been described to first appear in the entorhinal cortex and then in CA3 field and amygdala, followed by thalamus, CA1 field and frontal cortex [445]. The data collected through years suggest that systemic injection of KA induces a seizures onset principally reliant on hippocampal formation [445, 446]. The typology of damage induced by systemic administration of kainic acid is similar to what can be observed after the intracelebral injection, but the degree of the induced damages is grater [441]. Systemic administration induces a loss of pyramidal cells in CA1 and CA3 hippocampal fields [441, 447] and a degeneration of parvalbumin-positive inhibitory interneurons in CA1, entorhinal cortex and subiculum [441, 448]. In most animals, non-convulsive seizures start between 10 and 30 days after the injection; the implementation of EEG monitoring has revealed that the latent period after this kind of administration last approximately 14 days [441, 449].

The pilocarpine model of epilepsy

Pilocarpine is a chemoconvulsant drug that targets muscarinic acetylcholine receptors. To induce SE, this compound could be administered, as seen for KA, systemically or through an intracerebral injection. The pilocarpine model of epilepsy has many similarities in network and pathways involved with human MTLE. In addition, EEG features and neuropathological alteration are similar to the KA model of epilepsy. This model has been reported to show mossy fiber sprouting in DG and morphological changes in several brain areas such as thalamus, DG hilus, amygdala, and cerebral cortex [450]. In addition, similarly to KA model, pilocarpine induces damages of the BBB and it has been suggested that this event strongly contributes to SE in the pilocarpine model [451]. One advantage of this chemoconvulsant usage is its efficacy since almost all animals that survive to SE develop recurrent spontaneous seizures [441].

Electroshock-induced seizures

Electrical stimulation approach to induce epilepsy in animals has the great benefit to generate epilepsy features with high reproducibility and low mortality [452]. Electroshock is a widely used method of electrical stimulation to induce seizure and is mainly employed to study the cellular and molecular mechanisms that relate epileptogenesis to synaptic plasticity abnormalities or cognitive deficits [453, 454]. It can be divided into minimal or maximal electroshock-induced seizures (ES) depending on the intensity of stimulation, which do not require a stereotaxic implantation of electrodes but could be achieved with a stimulation through corneal electrodes. Minimal ES is assumed to represent a model of myoclonic seizures while maximal ES represent a model of generalized tonic-clonic seizures [452].

Kindling model

Kindling model of epilepsy represents the most studied model based on the electrical stimulation approach. The kindling model is obtained inducing repeated afterdischarges by electrical stimulation in precise brain regions (using electrode implantation), which induce a seizure-related plasticity phenomenon and the enhancement of seizure susceptibility. Repeated afterdischarges lastly result in the development of spontaneous seizures and chronic epileptic state [455]. Kindling has been considered a useful model for studies on epileptogenesis mechanisms considering the reproducible sequences of molecular and cellular alterations that this model induces [456, 457]. A clear drawback of kindling, however, is the time and costs required for the procedures together with the risks associated with the long periods required, such as the possibility of damaging or losing the electrodes implantation [452]. Despite all the risk associated with the kindling model procedure, it represents an excellent approach to study epileptogenesis. Indeed, contrary to MTLE induced in other models,

as pilocarpine or KA, which induce an SE associated with relevant temporal and extra-temporal damages, kindling provides a progressive development that leads to neuronal loss and cellular alterations and lastly results in spontaneous seizures [452].

Post-seizure α-tocopherol treatment for preventing epileptogenesis

Status epilepticus is one of the most frequent neurological emergency, associated with significant morbidity and mortality if not treated promptly. Thereby, it requires rapid assistance to avoid brain damage, systemic complications, or death [458]. Antiepileptic drugs (AEDs) are a wide and heterogeneous group of pharmacological agents characterised by a varied range of pharmacokinetic and pharmacodynamic effects. The effect of these treatments is generally aimed to enhance inhibitory processes or to contrast excitatory processes. In the last 20 years, many other new AEDs has been developed, thus leading to a general distinction between traditional (commercialized before 1990) and newer antiepileptic drugs [459]. Newer AEDs generally differ from the traditional for their improved tolerability but a comparable efficacy. However, it has also been suggested that increased prescription of new AEDs vs traditional has increased the risk of refractoriness [459]. Therefore, it is clear that several clinical needs still persist such as resistance to AEDs or adverse side effects which further affect the quality of life in patients with epilepsy. Besides, as previously discussed, the development of chronic epilepsy passes through a series of maladaptive molecular, cellular and circuital detrimental rearrangements, which ultimately lead to the birth of a seizure-generating circuit. Here comes the pivotal importance of setting up new therapies able to block the process before the onset of new seizures and development of the epileptic disease.

The classical view of epilepsy is centred on neurons and leads to the development of treatments that target ion channels, GABA and glutamate receptors. Recently, oxidative stress and inflammation have emerged as processes able to precipitate seizures or sustain seizure activity. Considering that several research data highlight the contribute of free radical to epilepsy [399, 400, 403, 404], natural compounds with antioxidant properties were considered in preventing seizure-induced pathology [412, 413]. Among these, as previously mentioned, vitamin E was proved to have beneficial effects in epilepsy, reducing convulsions and brain oxidative stress [414, 460]. Thus, a treatment with vitamin E reduce ROS and RNS generation following a delay of minutes to hours [414, 461-463], thereby decreasing the severity of seizures and their detrimental effects. In addition, patients with drug-resistant epilepsy got benefit from vitamin E treatment, which has been showed to decrease their blood levels of LPO products and induce positive changes on their EEG [464, 465]. The effects of vitamin E on epilepsy it has been assumed to be connected to the antioxidant properties of this compound.

Nonetheless, vitamin E – mainly as α-T – has several non-antioxidant properties [466], suggesting a conceivable antioxidant-independent mechanism involved in mediating its effects on epilepsy. Accordingly, vitamin E can regulate cell signaling through the modulation of miRs [467], which regulate gene expression and have influence on cellular pathways including inflammatory cascades.

Recent findings from our group, demonstrated that four-day application of α-T after the induction of SE, reduces neuroinflammation and neurodegenerative processes in the KA model of epilepsy as highlighted by the reduction of neuron degeneration, dendritic spine loss, astrocytosis and microglia activation [6]. These findings might be coherent with mechanisms of α-T that are beyond the antioxidant one.

Considering the data previously collected, we decided to extend the α-T treatment throughout the latent period to further investigate the antiepileptogenic potential of this compound and the mechanisms involved. Thus, we used the KA model of epilepsy that we obtain through an acute administration of kainic acid on adult rats. We investigate the excitability of hippocampus circuitry with and without α-T postictal treatment 15 days after SE performing electrophysiological field recordings in CA1 of brain slices. Besides, GABA and AMPA currents were investigated in *Xenopus* oocytes microinjected with hippocampal membranes from rats treated and not with α-T after SE induction. The influence of α-T treatment on neuroinflammation markers, oxidative stress, and neurodegeneration, were assessed in hippocampi from the different experimental groups. Lastly, we tested the potential relevance of selected circulating miRNAs as epileptogenesis biomarkers.

Experimental procedures

Animals

Adult male Sprague-Dawley albino rats (n = 72) (Charles River, Italy) were used in accordance with the Italian law on animal experimentation (D.lgs 26/2014; research project permitted with authorization N. 465/2015-PR by Italian Ministry of Health), and housed as described at page 25. The experimental design is summarized in figure 19.

To induce SE, rats were administered an acute intraperitoneal (i.p.) injection of kainic acid (KA; 10 mg/Kg b.w. in physiological saline) and seizure behaviour was monitored and scored according to the Racine scale, up to the manifestation of a full SE [468]. In particular, typical wet dog shakes appear about 30 min after injection while full limbic motor seizures (including rearing and loss of postural control) follow, being manifested about 1.5 hours after KA injection.

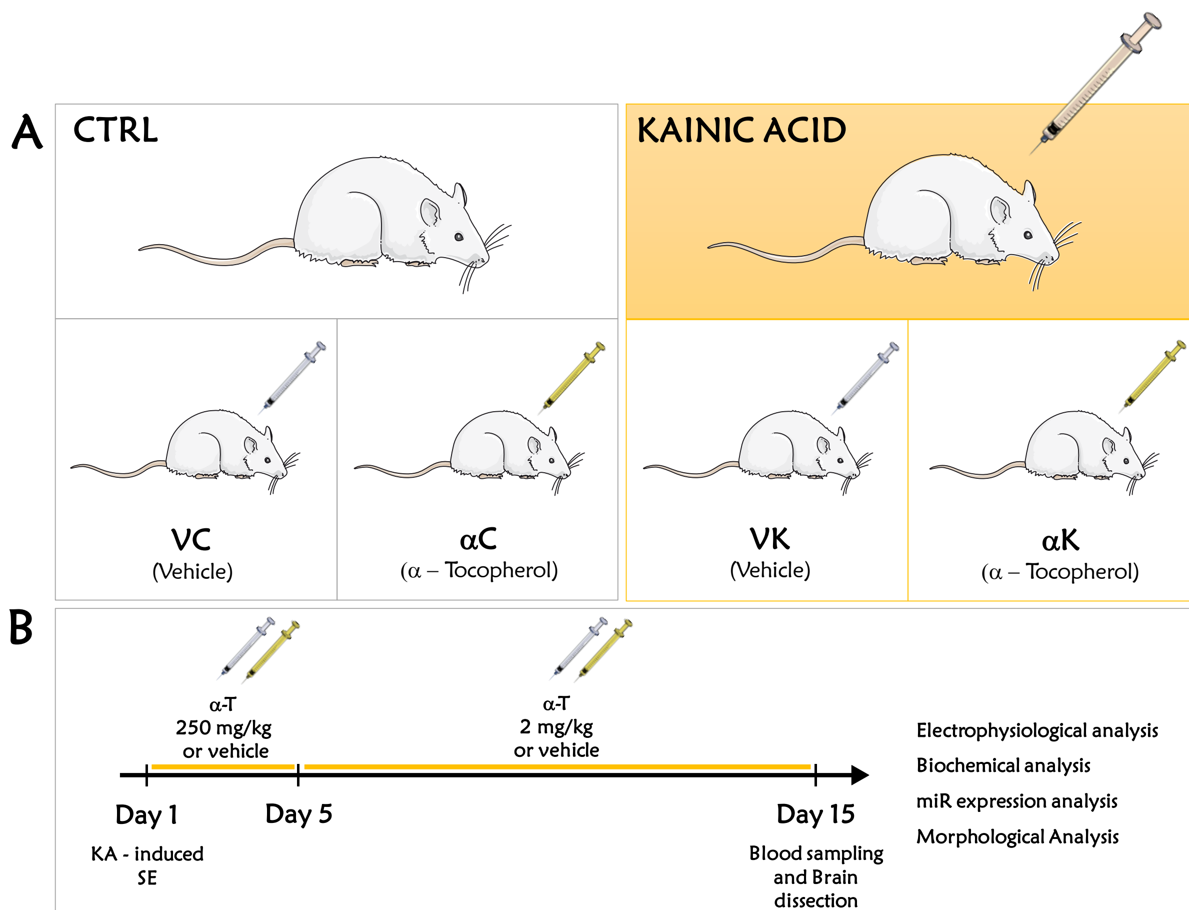

Figure 19. Experimental design. (A) Schematic representation of animal groups and relative treatment: αK, treated kainate-exposed; VK, untreated kainate-exposed; αC, treated non-epileptic; VC, untreated non-epileptic. (B) Time-course representation of the experimental design.

After approximately 3 hours, animals showed profuse salivation, circling and jumping, and SE. Three hours after the appearance of SE, seizures were suppressed by diazepam injection (2 mg/Kg b.w.). Animals showing all the Racine scale steps were considered (n = 48); about 20% of the kainate-injected rats died during or early after the SE, while approximately 14% did not clearly show the full progression of Racine stages. Epileptic rats were randomly divided in two groups: i) animals treated with an i.p. bolus of α-T once a day up to the fifteenth day (n = 24; αK) (250 mg/kg b.w. for the first 4 days followed by 2 mg/kg for the remaining days); ii) rats (n = 24; VK) injected with the vehicle solution (Fig. 19). In addition, other animals (n = 42) were injected with physiological saline instead of kainic acid (non-epileptic rats): half of them (n = 21; αC) were treated with α-T (once a day, for 15 days following the same schedule described above) and the other half (n = 21; VC) with the vehicle solution (Fig. 19). Epileptic rats were monitored, using the Racine scale steps, for spontaneous seizure arising up to the conclusion of treatment protocols. At the end of the 15-day treatment, rats were anaesthetized and sacrificed as previously described (pag. 25).

Electrophysiological field recordings on CA1

Brain from kainate injected (αK, VK) and saline-injected (αC, VC) rats (n = 8 each group) were processed for electrophysiological field recording on slices as described at page 25.
Recording and bipolar stimulating electrodes were both filled with ACSF (for the composition see "Slice preparation" pag. 25): the former electrode was placed in CA1 pyramidal cell layer and the latter in the *stratum radiatum*, maintaining approximately 500 µm of distance between them. Slices giving fEPSPs of at least 1 mV in amplitude were considered for recordings. To test basal synaptic transmission, input/output curves were performed eliciting fEPSPs through the application on slice of current square pulses of increasing magnitude (from 0 to 160 pA, increments of 20 pA; 300 µs in duration) with A385 stimulus isolator (World Precision Instruments, USA). Afterward, baseline responses (60% of maximal fEPSP amplitude) were evoked using low-frequency test pulses (at 30-s intervals) and recorded over 30 min, a period sufficient to ensure stability. Population spikes arising from fEPSP were analysed.
Once obtained a stable baseline, $GABA_A$-receptor antagonist bicuculline (BMI, 50 µM) and potassium channel blocker 4-aminopyridine (4-AP, 50 µM) in ACSF solution were added to bath perfusion for 30 min, during which spontaneous drug-induced extracellular field potential events (interictal) and fEPSPs were recorded. The latency to the onset of epileptiform-like activity was estimated.
Electrophysiological data analyses were performed offline using the WinWCP software. Experiments and data analyses were performed in blind by the operators.

$GABA_A$R and AMPA current evaluation in transfected Xenopus oocytes

<u>Tissue collection and membrane preparation</u>

Brain tissue from αK and VK rats (n = 3 for each group) was snap frozen immediately after collection in liquid nitrogen and stored at -80 °C until further use.
For membrane preparation, tissues were homogenized using a Teflon glass homogenizer in 2 mL of glycine buffer composed of (in mM): 200 glycine, 150 NaCl, 50 EGTA, 50 EDTA, 300 sucrose; plus 20 µL protease inhibitors (Sigma); pH 9 adjusted with NaOH. The homogenate was centrifuged for 15 min at 9.500 x g. The supernatant was collected and centrifuged for 2 h at 105 x g at 4 °C. The pellet was washed, re-suspended in assay buffer (glycine 5 mM) and used for microtransplantation by injection in *Xenopus laevis* oocytes. The use of female *Xenopus laevis* were performed in accordance to institutional policies and guidelines of the Italian Ministry of Health (n°. authorization 78/2015-PR).

Electrophysiology

Twelve to 48 hours after injection, membrane currents were recorded from voltage clamped oocytes by using two microelectrodes filled with 3 M KCl. Oocytes were placed in a recording chamber (volume 0.1 mL) and perfused continuously, 9-10 mL/min, with oocyte Ringer's solution (OR) at room temperature (20-22 °C) composed of (in mM): NaCl, 82.5; KCl, 2.5; $CaCl_2$, 2.5; $MgCl_2$, 1; HEPES, 5, adjusted to pH 7.4 with NaOH.
GABA current rundown was defined as the decrease in percentage of the current peak amplitude after six 10 seconds-applications of GABA 500 μM at 40 s intervals [469, 470]. The I_{GABA} desensitization was defined as the time taken for the current to decay from its peak to half-peak value (T 0.5). GABA was delivered by pressure applications (10-20 psi for 1 s with a General Valve [Fairfield, NYUSA] Picospritzer II) from glass micro pipettes positioned near to the oocyte.
Current–voltage (I–V) relationships were constructed holding the oocytes at -60 mV and stepping the membrane potential for 2-4 min at the desired value before applying the neurotransmitter. For these experiments, electrodes were filled with K-Acetate (3 M) [471] to reduce the excessive leakage of Cl^- from electrodes into the oocytes. However, the experiments gave the same results when KCl filling solution was used (not shown). To determine the GABA reversal potential (E_{GABA}), I–V relationships were fitted with a linear regression using the Sigmaplot 12 software (Systat Software Inc.). Unless otherwise indicated, for all the experiments, GABA 500 μM was applied for 4 s.
In experiments involving AMPA currents, the oocytes were pretreated for 20 s with cyclothiazide (CTZ, 20 µM - a positive allosteric modulator of AMPAR), before application of 10 s of AMPA, 20 µM [472], to block the receptors desensitization. Experiments involving IEM 1460 (a voltage-dependent open-channel blocker which preferentially blocks GluA2-lacking AMPARs [473]) were performed holding the oocytes at -80 mV; the current inhibition was calculated as the ratio of the current blocked by IEM 1460 (I GluA2-lacking) over the total I_{AMPA} , expressed as a percent.
Chemicals were dissolved in sterile water (GABA, AMPA, IEM 1460), or DMSO (CTZ) and stocked at -20 °C until use. For all the experiments, solutions were freshly prepared and drugs and neurotransmitters were diluted to the desired concentration in OR. The final concentration of DMSO was always lower than 1:2000 after dilution. All drugs were purchased from Tocris Bio-science (Minneapolis, MN, U.S.A.) and OR salts from SIGMA (Saint Louis, MO, U.S.A.).

Biochemical Analyses

Rats (n = 16, 4 for each group) were killed by an overdose of sodium tiopenthal via i.p. and hippocampi, after transcardial perfusion with ice-cold physiological saline, were quickly excised, and stored at -80 °C up to use. Hippocampi were then homogenized and lysed with 0.5 mL of ice-cold lysis buffer (50 mM Tris–HCl, pH 7.8,

0.25 M sucrose, 1% (w/v) SDS, 1 µg/mL pepstatin, 10 µg/mL leupeptin, 2 mM sodium orthovanadate, 10 mM NaF, 5 mM EDTA, 5 mM nethylmaleimide, 40 µg/mL phenylmethylsulfonyl fluoride, and 0.1% Nonidet-P40) and sonicated for 45 s at 50 W. Oxidative stress evaluation and protein expression quantification were performed.

Oxidative stress evaluation by spectrophotometric detection of protein carbonyls (PCO)

The amount of oxidative stress was determined measuring PCO levels in hippocampus samples using the molecule 2,4-dinitrophenylhydrazine (DNPH), which is a specific probe able to react with PCO leading to the formation of protein-conjugated dinitrophenylhydrazones (DNP). Protein-DNP adducts are characterised by a peak absorbance at 366 nm, allowing a quantitative determination of PCO content by spectrophotometer.

A step by step protocol to assay PCO was carried out in accordance with Colombo and colleagues [474]. In particular, 200 μL of 10 mM DNPH solution was added to 1 mL of protein samples; at the same time, blank samples were prepared adding 200 μL of 2N HCl (without DNPH) to 1 mL of protein sample. The samples are than vortexed and left in the dark at RT for 60 min where are mixed every 15 min. 1.2 mL of 20% TCA solution are then added to protein samples which are subsequently incubated on ice for 15 min. After this step, the samples were centrifuged at 10,000 × g for 5 min, at 4 °C. The supernatants were discarded and the proteins washed with 1 mL of 20% TCA. The samples were centrifuged at 10,000 × g for 5 min, at 4 °C and the supernatants discarded. Pellets were than washed twice with 1 mL of 1:1 (v/v) ethanol:ethyl acetate and vortexed to remove free DNPH, until supernatants are completely transparent. The pellets were collected centrifuging at 10,000 × g for 5 min, at 4 °C and discard supernatants. The pellets were dried for about 5 min to allow complete solvent evaporation. After this step, the protein pellets were re-suspended in 1 mL of 6 M guanidine hydrochloride (dissolved in 50 mM phosphate buffer, pH 2.3) and incubated at 37 °C for 15-30 minutes. Once protein pellets were completely dissolved, spectrophotometric measurements of PCO content were performed at 366 nm by using a molar absorption coefficient of 22,000 M^{-1} cm^{-1}, and results were expressed as nmol of PCO per mg of protein (nmol/mg protein).

Electrophoresis and western blotting analysis

Hippocampus samples were boiled for 4 min and then centrifuged for 10 min at 14,000 x g to remove insoluble debris. Supernatants were mixed 1:1 (vol/vol) with sample buffer (0.5 M Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 4% of 2-mercaptoethanol, and 0.05% bromophenol blue) and 30 µg of sample proteins were loaded onto 10%, 12% and 15% SDS-polyacrylamide slab gels and separated by electrophoresis. Pre-stained molecular mass markers (Bio-Rad, Milan, Italy) were run in adjacent lanes. The gels were electroblotted and stained with Coomassie brilliant blue

R250. For immunoblotting, the following antibodies were used: mouse monoclonal antibodies against Claudin-5 (Thermo Fisher Scientific, Waltham, Massachusetts, USA), GFAP (Sigma: Saint Louis, MO, USA), interleukin-1 β (IL-1 β; Santa Cruz Biotechnology: Santa Cruz, CA, USA), and interleukin-6 (IL-6; Santa Cruz Biotechnology: Santa Cruz, CA, USA); rabbit polyclonal anti-TNF-α (Sigma), anti-ionized calcium-binding adapter molecule 1 (IBA1; Wako Life Sciences Inc., USA) and anti-actin (Sigma: Saint Louis, MO, USA). Blots were incubated with specific primary antibodies (1:1.000) and subsequently with the appropriate secondary antibodies conjugated with horseradish peroxidase (1:3.000; Bio-Rad, Milan, Italy). Immune complexes were visualized using an enhanced chemioluminescence Western blot analysis system (Amersham - Pharmacia, Milan, Italy) following manufacturer's specifications. Blot images were then digitized (Chemidoc, Bio-Rad) and areas of all labelled bands were quantified using the computerized imaging system software (QuantityOne; Bio-Rad). After antibody probing, nitrocellulose membranes were stripped for 30 min at 50 °C with stripping buffer (62.5 mM Tris–HCl, pH 6.7, containing 10 mM β- mercaptoethanol and 2% SDS) and re-probed with anti-actin (1:200). Immune complexes were visualized using an enhanced chemioluminescence. In each series, relative optical densities (arbitrary units, AU) were normalized for densitometric values obtained from actin-labelled bands.

microRNA Expression Analyses

Total RNA extraction from serum and hippocampal homogenate

Peripheral blood samples were collected from anesthetized rats (sodium tiopenthal, 45 mg/kg body weight via i.p.; n = 3 for each group) in appropriate tubes and, following 30 min resting at RT, serum was obtained by centrifugation at 2500 rpm for 5 min at 4 °C.

Hippocampi were then excised from the anesthetized rats after suppression and microRNAs were isolated from supernatant of homogenates prepared as described above (see Biochemical Analyses paragraph, page 80). After two subsequent spins, total RNA was extracted from 100 µL of rat serum and homogenates using an RNA purification kit (Norgen Biotek Corporation, Thorold, ON, Canada). RNA was stored at -80 °C until use.

Quantitative Real Time PCR (qRT-PCR) for mature microRNAs analysis

miR-124, mir-126 and miR-146a expression were analysed. microRNA expression was quantified using a real-time approach with the TaqMan miRNA reverse transcription kit and a miRNA assay (Applied Biosystems, Foster City, CA). The TaqMan MicroRNA reverse transcription kit was used to reverse transcribe the total RNA following manufacturer's instructions. Briefly, 5 μL of RT mix contained 1 μL of each miR-specific stemloop primer (miR-126, miR-146a and miR-124), 1.7 μL of input RNA,

0.4 μL of 10 mM dNTPs, 0.3 μL of reverse transcriptase, 0.5 μL of 10× buffer, 0.6 μL of RNAse inhibitor diluted 1:10 and 0.5 μL of H_2O. The mixture was incubated at 16 °C for 30 minutes, at 42 °C for 30 minutes, and at 85 °C for 5 minutes. Afterwards, qRT-PCR was performed in 20 μL of PCR mix containing 1 μL of 20× TaqMan MicroRNA assay (containing PCR primers and probes; 5′-FAM) 10 μL of 2× TaqMan Universal PCR Master Mix No UNG (Applied Biosystems), 1.33 μL of reverse-transcribed product and 7.67 μL of nuclease-free water. The reaction was first incubated at 95 °C for 10 minutes, followed by 40 cycles at 95 °C for 15 seconds and at 60 °C for 1 minutes. To obtain accurate and reproducible results, the relative expression of circulating miRNAs was quantified using synthetic C*aenorhabditis elegans* miRNA (cel-miR-39) as the reference miRNA, which was spiked into rat serum before RNA extraction. MicroRNAs expression in homogenate was evaluated using the spliceosomal RNA U6 as the reference. Each reaction was performed in duplicate.
The qRT-PCR was performed on an ABIPRISM 7500 RealTime PCR System (Applied Biosystems). Data were analysed using a 7500 system software (1 1.4.0) with the automatic comparative threshold (Ct) setting to adapt baseline. Detection thresholds were set at 35 Ct. The relative amount of miR-146a, miR-126 and miR-124 was calculated using the Ct method:

- Serum: ΔCt = Ct (miR-146a/miR-126/miR-124) − Ct (cel-miR-39); $2^{-\Delta Ct}$

- Homogenate: ΔΔCt = [Ct (miR-146a/miR-126/miR-124 treated sample) − Ct (U6 treated sample)] - [Ct (miR-146a/miR-126/miR-124 control sample) − Ct (U6 control sample)]; $2^{-\Delta\Delta Ct}$

Results are expressed in the figures as fold change related to control sample (VC) and values less than 1 indicated down-regulation, whereas values higher than 1 indicated up-regulation.

MicroRNA Fluorescence *in situ* hybridization (FISH)

To perform fluorescence *in situ* hybridization rats (n = 12; three for each group) were deeply anesthetized with sodium tiopenthal (via i.p.) and transcardially perfused with physiological saline followed by 4% paraformaldehyde (PFA) in phosphate buffer saline (PBS, 0.1 M; pH 7.4). Brains were removed, post-fixed in 4% PFA for 48 h, and then transferred in PBS. Brains were embedded in paraffin and cut by a rotative microtome in 6 μm-thick coronal sections, which were processed for *in situ* hybridization, using TSA™ Plus Fluorescence systems through the miRCURY LNA™ ISH optimization Kit (Exiqon, Euroclone, Italy); procedure for FISH detection of microRNA

was performed according to manufacturer's instructions. Every step of the procedure, including tissue sectioning, was took place in a clean and nuclease free environment. Briefly, PFA-fixed, paraffin-embedded sections were mounted on slides, dewaxed, rehydrated with PBS and treated with Proteinase K solution at 37 °C for 20 min at the concentration of 2 μg/mL, in a Dako Hybridizer machine. Slides were then dehydrated in ethanol and air-dried. The FISH probes (LNA™ microRNA probe, LNA™ scrambled microRNA probe and LNA™ U6 snRNA to set optimal hybridization conditions) were denatured at 90 °C for 4 min, before proceeding with the hybridization of the probe with the specimen on the slides; a coverslip was applied, sealed with rubber cement, and sections were incubated at 55 °C for one hour in a Dako Hybridizer machine. Then, after coverslip removal, the slides were placed in a glass jar containing Saline-Sodium Citrate solution (SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7), put into a water bath set to the hybridization temperature to ensure sufficient stringency. Following PBS washes, the slides were placed in a humidifying chamber and incubated with blocking solution (containing 2% sheep serum, 0.1 % Tween-20, 1% BSA in PBS) for 15 min at RT. Slides were then incubated with anti-DIG-POD (1:400 in dilutant solution containing: 1% sheep serum, 0.05% Tween-20, 1% BSA in PBS) for 60 min at RT. Finally, TSA™ Plus Fluorescein substrate (Perkin Elmer) substrate was applied to the sections and incubated 2x5 min at RT. PBS buffer washes were used to stop the reaction. The slides were covered directly with SlowFade Gold antifade reagent (Life technologies - Thermo Fisher Scientific) and examined under a confocal microscope (Leica TCS-SL), with suitable filter set.
To perform the densitometric analysis of FISH staining, six slices for each experimental group were considered. Signal intensity of miRNAs and U6 expressions were measured using ImageJ (https://imagej.nih.gov/ij/) to calculate the product of area and mean gray value (Integrated density) as percentage of modulation (miRNAs percentage value versus U6).

Morphological Analysis

The animals (n = 12 rats; three for each group) were deeply anesthetized with sodium tiopenthal (via i.p.) and transcardially perfused with physiological saline followed by 4% PFA in PBS. Brains were removed, post-fixed in 4% PFA for 48 h and then transferred in PBS. Vibratome was used to cut brains in serial sections (50 μm thick), which were collected in PBS and mounted on slides. To perform FluoroJade staining, slides were sequentially immersed in 100% ethanol for 3 minutes and then in 70% ethanol for 1 minute. After rinsing in distilled water, slides were incubated in 0.06% $KMnO_4$ solution for 15 minutes, rinsed in distilled water and transferred to a 0.001% solution of FluoroJade dissolved in 0.1% acetic acid for 20 minutes. Slides were then rinsed thrice in distilled water for 1 minute, dried, immersed in xilene and coverslipped with Entellan. FluoJade-positive cells were observed by Axioskop fluorescence microscope (Carl Zeiss, Germany) equipped with a filter suitable for visualizing

fluorescein isothiocyanate; cells with evident neuronal morphology were counted in at least six sections for each animal taken along rostro-caudal extension of CA1 hippocampal field. Eighty-five fields/animal (270 x 250 μm each) were considered.

Statistical Analysis

Data were expressed as mean±SEM. For multiple variable comparison, results were analysed using appropriate ANOVA test (one-way or two-way) followed by Tukey's post-hoc test; for single comparison, Student's t test and Chi-square test, unpaired t-test or Wilcockson Signed rank test, after normal distribution testing (Shapiro-Wilk test) were appropriately used. Significance threshold was established for $p = 0.05$.

Results

Electrophysiological recordings

Population spike analysis

Differences in the hippocampal network excitability related to α-T postictal treatment in epileptic and non-epileptic rats were evaluated by stimulations of Schaffer collaterals in *stratum radiatum* of brain slices and recordings in CA1 pyramidal cell layer. To this aim, some excitability indexes as the number of population spikes in fEPSP responses, input/output curves, and the latency of the first interictal event after drug-induced network dysregulation, have been assessed.
Non-epileptic rats (αC and VC) during baseline recording, typically showed a fEPSP characterised by a single population spike (Fig. 20A); in these groups, a second population spike in the fEPSP response was rarely found (Fig. 20B).
On the contrary, slices from KA-injected rats (αK and VK) showed a fEPSP which gave rise to one to three/four population spikes (Fig. 20A; VK mean value: 2.71 ± 0.24); notably, only slices from vehicle-treated epileptic rats exhibited up to four population spikes, while most of αK slices evoked only one population spike, thus similarly to non-epileptic rats (αK mean value: 1.68 ± 0.17). In addition, input/output curve at increasing stimulus magnitude showed that αK group displayed a significantly lower number of population spikes compared to VK, starting from 40 pA stimulus intensity (Fig. 20B), indicating a reduced network excitability following α-T postictal treatment in epileptic rats.

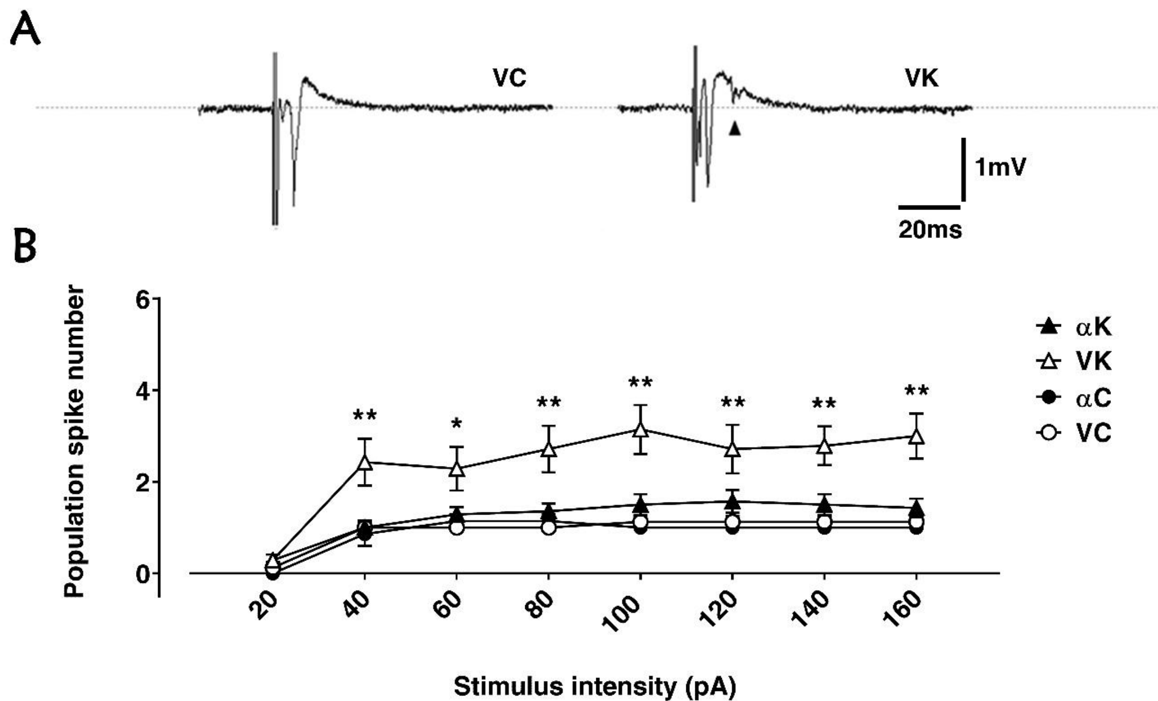

Figure 20. Population spike number. (A) Field excitatory post-synaptic potential recorded in CA1 pyramidal layer in treated kainate-exposed (αK) and untreated kainate-exposed (VK) rats. The arrowhead indicates multiple population spike recorded in VK rat slice. (B) fEPSP population spike number recorded in all the experimental groups in response to Schaffer's collaterals stimulation. Two-way ANOVA repeated measure followed by Tukey's post-hoc, $*p < 0.05$; $**p < 0.01$. Data are expressed as mean±SEM. αK, treated kainate-exposed; VK, untreated kainate-exposed; αC, treated non-epileptic; VC, untreated non-epileptic. Data are currently under submission.

Induced epileptiform-like network activity

The appearance of spontaneous epileptiform bursting events was exclusively found in VK group (about 40% of recorded slices; n = 15; Chi-square test: $p < 0.01$), supporting the higher hippocampal excitability in this group.
BMI and 4-AP co-application in perfusion bath gave rise to epileptic-like activity in slices from all groups. The adopted blocker concentrations were able to mainly evoke interictal events, characterised by a duration of less than 400 ms, with a positive and negative peaks clearly distinguishable from baseline activity (Fig. 21A); ictal event, defined a spontaneous activity constituted by at least three interictal events in rapid succession, were rarely observed. As index of hyperexcitability, we evaluated the latency for the onset of the first interictal event. Epileptic rats treated with α-T showed a latency of the first interictal event comparable to that of non-epileptic rats with and without treatment, and significantly different from that detected in VK rats (Fig. 21B), indicating a less marked hippocampal excitability under α-T treatment following SE.

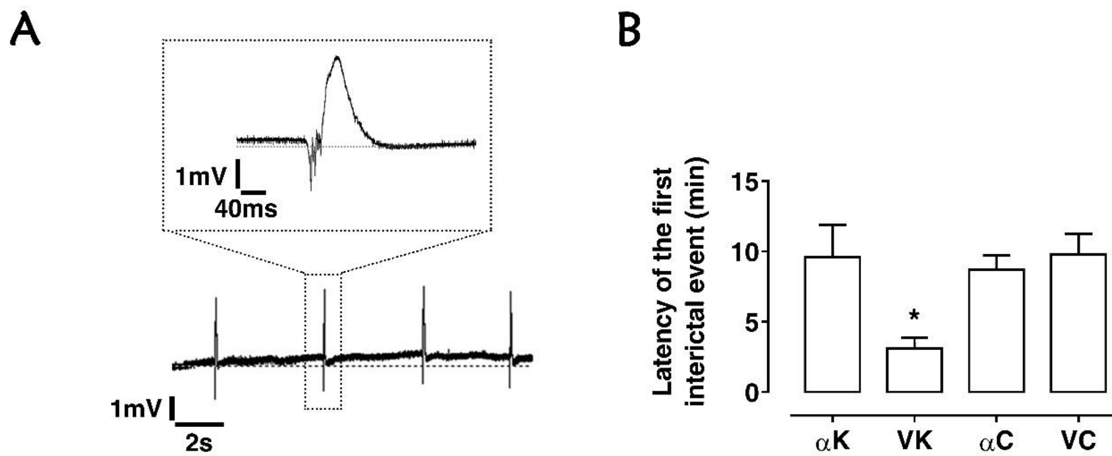

Figure 21. Bath application of 50 μM BMI together with 50μM 4-AP leads to the appearance of spontaneous interictal events. (A) Enlarged view of a single interictal event. (B) Latency of the first interictal event recorded in the experimental groups. One-way ANOVA followed by Tukey's post-hoc, *p < 0.05. Data are expressed as mean±SEM. αK, treated kainate-exposed; VK, untreated kainate-exposed; αC, treated non-epileptic; VC, untreated non-epileptic. Data are currently under submission.

$GABA_A$R and AMPA current evaluation in *Xenopus* oocytes

The effect of α-T treatment on electrophysiological properties of $GABA_A$R and AMPAR was here evaluated taking advantage of membrane microtransplantation in *Xenopus* oocytes. First, a good expression of $GABA_A$ and AMPA receptors using tissues from treated and untreated rats (VK vs αK: mean I_{GABA} = 81.2 nA ± 26.5 vs 101.8 ± 31.4 nA, n = 14 vs 13, p = 0.369; mean I_{AMPA} = 75 nA ± 33.5 vs 118.5 ± 43 nA, n = 10 vs 14) was obtained. The small variability in the current amplitude between the groups is due to the difference of expression between cells and donors (frogs) as reported in [475, 476]. Successively, the evaluation of the electrophysiological properties of the two receptors was performed. As for $GABA_A$R, a significant current rundown was present in both αK and VK hippocampal tissues (VK vs αK: 43 ± 7.5 % vs 54.8 ± 6.9 %, n = 10 vs 12, p = 0.259), suggesting that α-T does not act on the degree of current desensitization as previously reported for BDNF or fracktaline [470, 477].

Additionally, no differences of E_{GABA} were observed between the two groups (VK vs αK: mean E_{GABA} = -4.7 ± 1.2 mV vs -22.6 ± 1.4 mV; p > 0.05 (n = 10)), thus indicating that a modification of chloride homeostasis is not likely to be involved in the effect evoked by the administration of α-T.

Furthermore, I_{AMPA} normalized to I_{GABA} amplitude [478] were analysed: the result point out that this parameter was similar in the two groups (AMPA/GABA: VK= 304 ± 82%; αK = 239 ± 47%, p > 0.05; n = 10).

To further characterize AMPA responses, IEM1460, a selective GluR2-lacking AMPA receptor blocker, was tested since GluR2 function is crucial in development and various pathologies [473]. The results showed that the percentage of IEM1460 block was similar

in the two groups (VK= 60.3 ± 16%; αK = 53 ± 14; $p > 0.05$, $n = 8$) indicating no difference in the expression of GluR2.

Biochemical Results

Oxidative stress evaluation

The formation of PCO has been considered a marker of oxidative stress in hippocampi derived from the different experimental groups. In detail, the hippocampi of vehicle-treated epileptic rats (VK) showed a significantly larger amount of PCO as compared to α-T treated epileptic group (Fig. 22), which in turn resulted similar to non-epileptic rats. Furthermore, no difference was found in PCO content when α-T was administered under control conditions. These findings indicate α-T treatment lead to a reduction in oxidative stress levels 15 days post-SE induction.

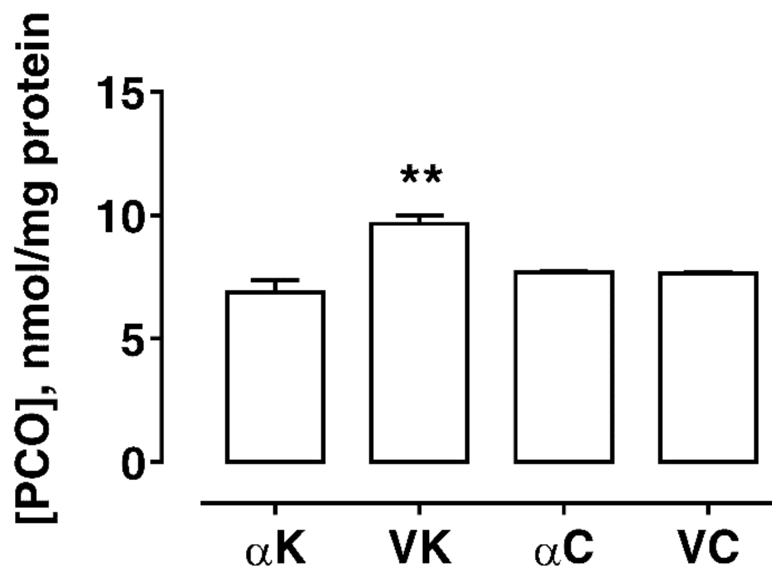

Figure 22. Effect of α-tocopherol treatment on protein carbonyls formation used as marker of oxidative stress. Histograms represent PCO content of three independent measurements in each experimental group (means±SEM). One-way ANOVA followed by Tukey's post hoc test, *$p < 0.05$; **$p < 0.01$. αK, treated kainate-exposed; VK, untreated kainate-exposed; αC, treated non-epileptic; VC, untreated non-epileptic. Data are currently under submission.

Neuroinflammation assessment

Fifteen days after SE, the expression levels of GFAP and IBA-1 (used as markers of reactive astrogliosis and microglial activation, respectively), were both significantly increased in vehicle-treated epileptic animals (VK) when compared to non-epileptic vehicle-treated rats (VC) (Fig. 23). Consistently, the expression of pro-inflammatory cytokines IL-1 β and TNF-α was also up-regulated (Fig. 23), whereas, IL-6 protein levels appeared to not change. In αK rats, the levels of all the neuroinflammatory markers

here considered were significantly lower than in VK (Fig. 23), except IL-6 which levels resulted increased.
As consequence of neuroinflammation, disruption of the BBB can occur, losing its capability to protect brain environment. Thus, we evaluated the expression levels of claudin-5, a molecule forming tight junctions, which limit the paracellular permeability, in order to gain insight regarding the integrity of the BBB 15 days after SE. Densitometric analysis of immunoblots revealed a marked decrease in claudin-5 protein levels in KA-injected groups, which was recovered by α-T treatment (Fig. 23).

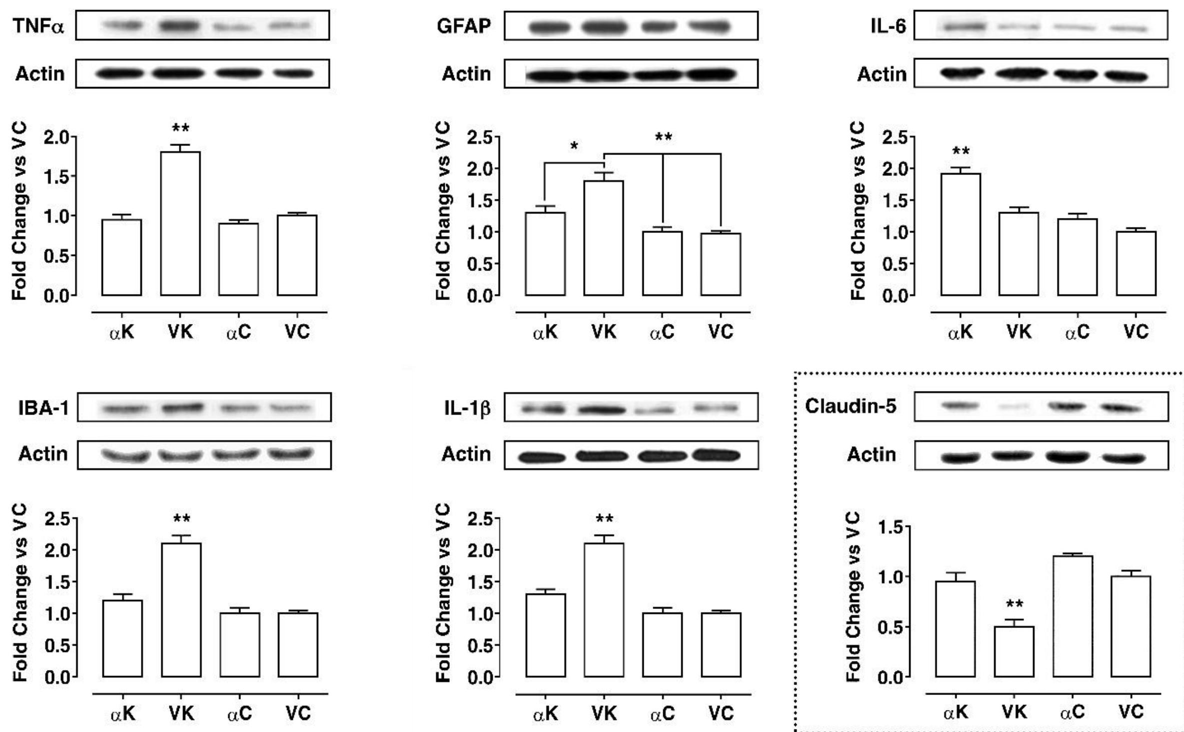

Figure 23. Effect of α-tocopherol treatment on neuroinflammatory and markers blood–brain barrier in control and kainate-induced epileptic rats. Western blot analysis of the expression levels of neuroinflammatory markers and claudin-5 (used as marker of blood-brain barrier integrity - dashed line), in hippocampal homogenates obtained from rats of all the experimental groups. Per each experimental group: representative immunoblots are displayed and anti-actin blots are shown as loading control. Note that immunoblots are shown in the same sequence as bars in the corresponding histograms. Histograms represent densitometric analyses of blots from three independent experiments (means±SEM). One-way ANOVA followed by Tukey's post hoc test, *$p < 0.05$; **$p < 0.01$. αK, treated kainate-exposed; VK, untreated kainate-exposed; αC, treated non-epileptic; VC, untreated non-epileptic. Data are currently under submission.

microRNA Expression Modulation

Aberrantly expressed microRNAs detected in the hippocampus of MTLE patients and TLE mice models [479], include miR-124, miR-126 and miR-146a. In particular, much attention have received the well-characterised miR-146a which play an important

role in the regulation of astroglia mediated inflammatory response [422], revealing its overexpression in glioneuronal lesions from patients with drug-resistant epilepsy. In addition, miR-146a has been found to modulate IL-6 expression by targeting IRAK-1 and TRAF-6, key regulator proteins of TLR-4 signalling activation [480].
Our results on three selected miRNAs – miR-124, miR-126 and miR-146a – among the experimental groups, revealed a significant up-regulation of miR-146a in hippocampi of vehicle-treated epileptic rats (VK) (Fig. 24A) as compared to non-epileptic ones (VC); strikingly, this SE-induced effect, was completely prevented by α-T treatment (αK) (Fig. 5A). On the contrary, a trend of decrease in miR-126 and miR-124 expression levels was observed in epileptic hippocampi in comparison to vehicle-treated non-epileptic rats (VC); after α-T treatment the differences became statistically significant. Interestingly, this effect was induced by α-T also in absence of epileptic insult (αC).

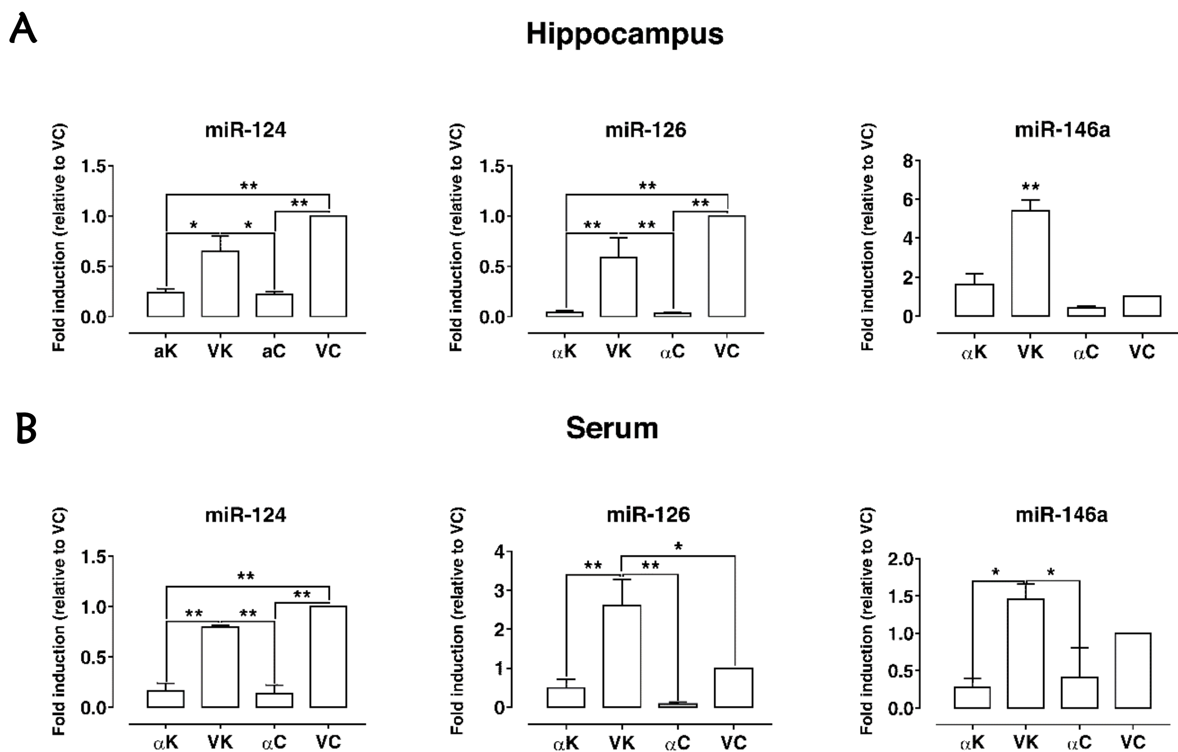

Figure 24. Effect of α-tocopherol treatment on microRNA expression. (A) Quantification of miR-124, miR-126 and miR-146a expression in hippocampal homogenates and, (B) quantification of serum miR-124, miR-126 and miR-146a expression obtained from rats of the different experimental groups. One-way ANOVA followed by Tukey's post hoc test, *$p < 0.05$; **$p < 0.01$. All data have been normalized on VC miR expression level and expressed as mean±SEM. αK, treated kainate-exposed; VK, untreated kainate-exposed; αC, treated non-epileptic; VC, untreated non-epileptic. Data are currently under submission.

In addition, in the serum of same rats we measured circulating microRNAs. We uncovered changes for miR-146a and miR-124 that fit with those observed in the related hippocampi (Fig. 24B); on the contrary, miR-126 serum levels were strongly up-regulated following SE (VK), a phenomenon promptly prevented by α-T treatment.

miR-124 expression was found significantly down-regulated both in hippocampus and in serum by α-T.
To further investigate its low hippocampus expression, FISH for miR-124 was performed in pyramidal neurons of CA1 hippocampal fields: this technique confirmed its down-regulation. miR-124 signal was bright in pyramidal cells of CA1 hippocampal field and restricted to cytoplasm (Fig. 25). The brightness of the FISH signal was quenched in neuron cytoplasm of α-T treated epileptic rats and was very similar to that of the corresponding α-T non-epileptic ones (αC) (Fig. 25).
Taken together, these results suggest an influence of α-T on microRNA expression. In addition, circulating microRNAs indicate that miR-126 and miR-146a may be biomarkers for the epileptic disease and useful in the evaluation of the effectiveness of treatment.

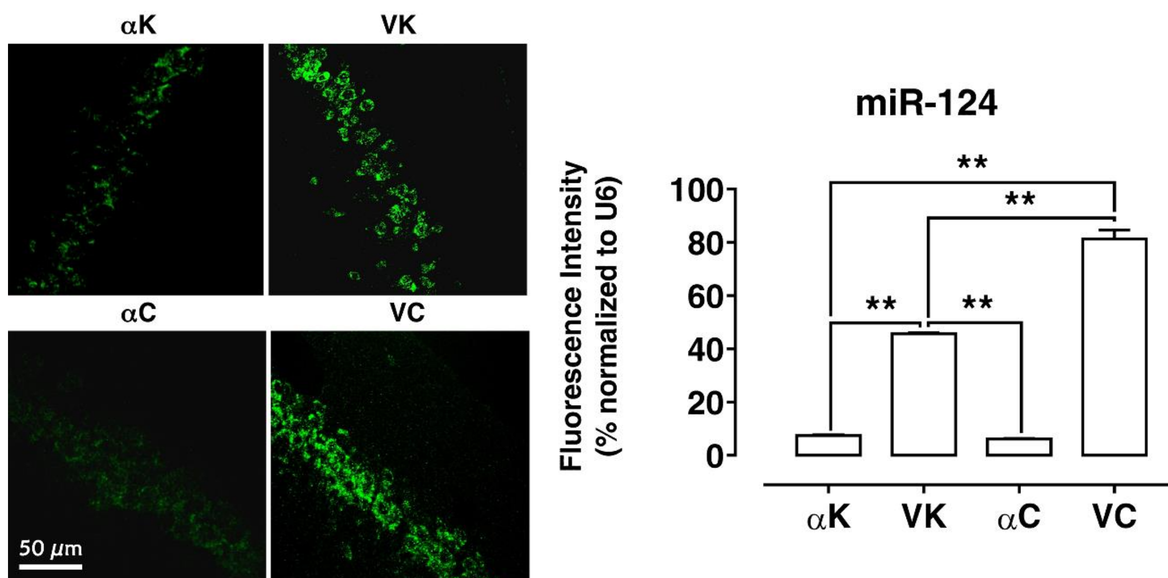

Figure 25. Effect of α-tocopherol treatment on miR-124 expression. (On the left) Representative confocal FISH images (on the left) of fluorescent in situ hybridization of miR-124 in CA1 pyramidal cells (scale bar: 50 μm). (On the right) Quantification of FISH intensity of miR-124 normalized to U6 snRNA in CA1 pyramidal cells. All data are expressed as mean±SEM. One-way ANOVA followed by Tukey's post hoc test, $^{**}p < 0.01$. αK, treated kainate-exposed; VK, untreated kainate-exposed; αC, treated non-epileptic; VC, untreated non-epileptic. Data are currently under submission.

Morphological Analysis Results

Fifteen days following SE, epileptic hippocampi showed a number of FluoroJade-positive cells, while, no degenerating neuron was found in control (saline-injected) non-epileptic rats (αC; VC). VK rats exhibited an overall greater number of degenerating neurons across rostro-caudal extension of CA1 field (Fig. 26) than αK hippocampi, which was highly significant at more caudal brain levels (Fig. 26). This finding indicates a reduction in neuronal degeneration due to α-T post-seizure treatment, thus confirming and extending previous finding from our lab [6].

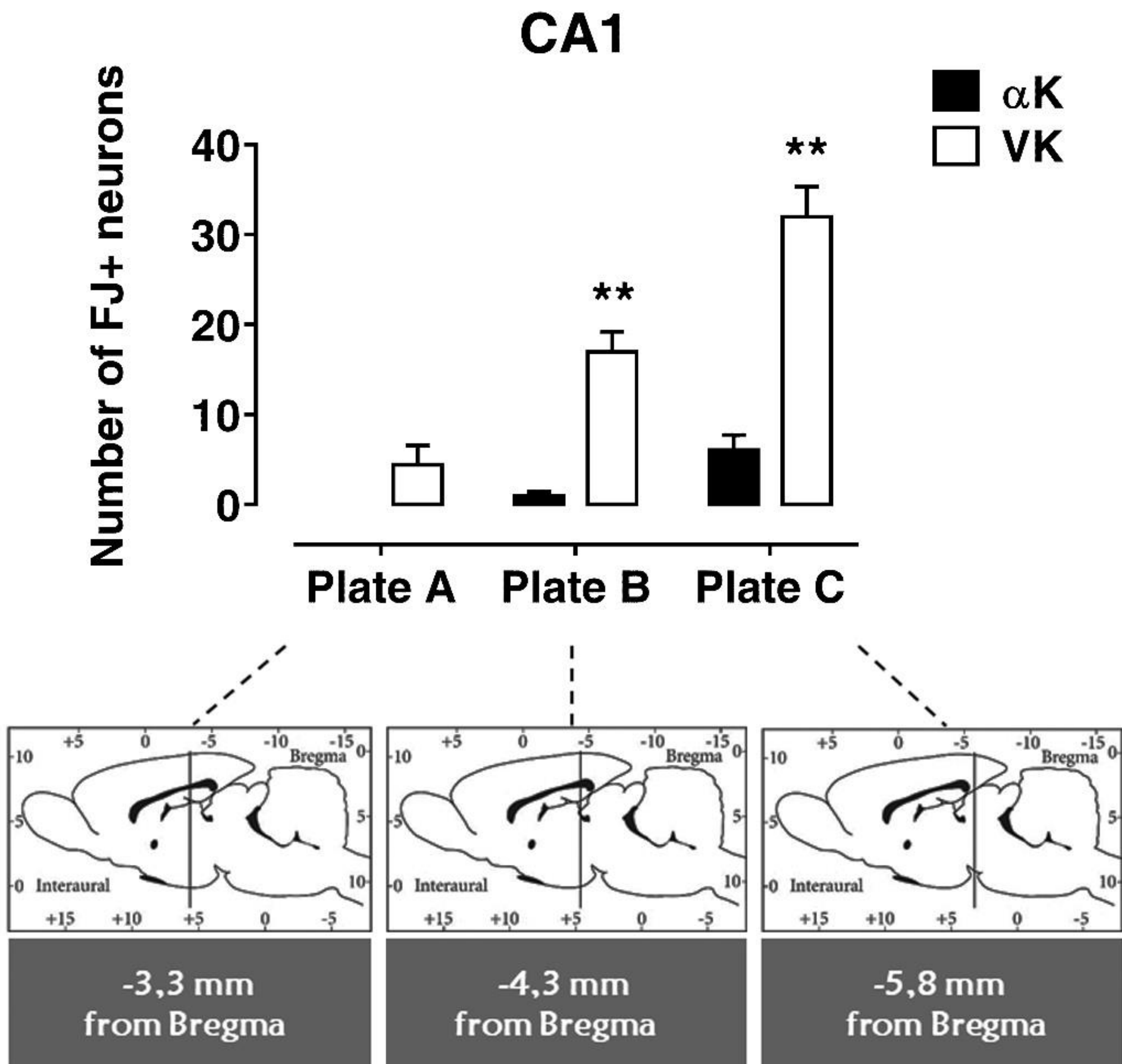

Figure 26. Effect of α-tocopherol treatment on neuronal degeneration. The histogram reports the quantification of FluorJade-positive cells in CA1 hippocampal field in αK and VK experimental groups. Plate A: -3,3 mm, Plate B: -4,3 mm and Plate C -5,8 mm from Bregma. Student's t test, **$p < 0.01$. αK, treated kainate-exposed; VK, untreated kainate-exposed; αC, treated non-epileptic; VC, untreated non-epileptic. Data are currently under submission.

Discussion

TLE represent the most common form of partial epilepsy in humans and is often preceded by a seizure-free period defined as latent period, during which maladaptive network reorganisations take place, leading to the appearance of spontaneous recurrent seizures that characterise the chronic stage [481]. The KA model of epilepsy resembles the human MTLE disease [442], thus representing a validated animal model to study epileptogenesis. The CA1 region of hippocampus following kainate-induced SE is hyperexcitable, establishing a permissive factor for the genesis and propagation of

epileptic seizures. Neuronal cell loss, synaptic reorganisation, imbalance in excitatory and inhibitory drive, gliosis and inflammatory pathway activation may contribute to alter network excitability and synchronization during the latent period.
The present study demonstrates that α-T postictal administration is able to strongly reduce CA1 hippocampal network excitability as highlighted by the electrophysiological analysis of some parameters considered good markers for the evaluation of neuronal network excitability during epileptogenesis [482, 483]. In particular, α-T has been found to decrease the number of population spikes in evoked fEPSPs and reduce the susceptibility to generate spontaneous and drug-triggered epileptiform bursting events on slices. These findings would support the use of vitamin E to improve seizure control in patients with epilepsy [465] when added to the antiepileptic drugs (AEDs). However, the mechanisms underlying the antiepileptogenic effect of this natural compound have not yet been fully investigated.
Aberrant excitatory/inhibitory drive in epilepsy is considered one of the main alteration contributing to epileptogenesis and GABA, the most important inhibitory neurotransmitter in the CNS, and its receptors are considered the main target of current and future antiepileptic drugs. The reduced efficacy of GABAergic inhibition has been strongly associated with the occurrence of recurrent seizures [484]. Indeed, it has been demonstrated that $GABA_A$ receptors in the epileptic hippocampus of both humans and in animals, become less responsive to repeated activation than those from normal tissue [325, 326, 485-487]. This change in $GABA_AR$ desensitization, called $GABA_AR$ rundown, might represent a crucial mechanism contributing to inhibitory failure and excitation/inhibition imbalance that lead to spontaneous seizures [488]. In this context, we found that α-T did not mitigate the SE-induced $GABA_AR$ rundown since hippocampal tissues from both α-T treated and vehicle-treated epileptic animals displayed a very similar marked rundown during repetitive applications of GABA. The molecular mechanisms behind the increased $GABA_A$-receptor rundown in the epileptic tissue are still unknown. However, it has been hypothesized that it could be dependent on alterations in $GABA_A$ receptor subunit composition [485] or changes in $GABA_AR$ phosphorylation/dephosphorylation [488]. Hence, our data suggest that α-T has no effect on $GABA_A$ receptor population structure nor their function regulation. In addition, chloride homeostasis is not different between treated and untreated epileptic animals, suggesting that chloride transporter expression and/or activity are not altered by the α-T postictal administration. Lastly, AMPA/GABA current ratio was not different in the two groups, as well as AMPA response and subunit composition, indicating no effects directly exerted by α-T on AMPA expression and/or functioning. Taken together, it is suggested that the observed beneficial effects of α-T treatment are due to different mechanisms.

A number of studies have often ascribed vitamin E effectiveness in mitigating the severity of human and animal epilepsy, to its well-known antioxidant property [464, 465]. In line with this view, our findings regarding PCO determinations indicate the significant reduction of oxidative stress level in hippocampus of α-T-treated epileptic

rats. Nevertheless, a series of studies revealed that the forms of vitamin E, mainly α-T, are able to function as signaling and gene regulation molecules independently from their antioxidant action [489] and in the last few years, several laboratories have described additional cellular and molecular properties for this vitamin, such as, reduction of proliferation rate [490, 491], the enhancement of immune functions, inflammatory pathway regulation and neuroprotection [6, 492-495].

Previous findings from our lab have shown that four days of α-T treatment after kainate-induced SE are able to reduce hippocampal neuroinflammation and neurodegeneration, quenching neuroinflammatory processes triggered by SE [6]. Additionally, α-T treatment counteracts glutamine synthase decline, recovering the glutamate-glutamine-GABA cycle pathway, which has been found to be disrupted in the hippocampus of MTLE affected patients [496]. Lastly, post-SE α-T treatment induces an increase in dendritic spine number and in synaptophysin immunoreactivity, suggesting a role of α-T in synaptogenesis promotion and/or synapse protection [6].

Here, the post-SE α-T administration effects of reducing hippocampal neuroinflammation and neurodegeneration are confirmed after a longer period of treatment. Thus, 15 days of α-T treatment significantly reduce astrogliosis and microglial activation together with pro-inflammatory cytokines IL-1 β and TNF-α. IL-6 protein levels, however, are up-regulated by α-T in epileptic rats with respect to all the other animal groups. IL-6 is a multifunctional cytokine with pro-, but also anti-inflammatory activities [497] and it has been reported to contribute to neuroprotection after SE [498]. Besides, IL-6 knockout mice appears to be more susceptible to some chemoconvulsant agents [499]. The mechanisms underlying IL-6 anti-inflammatory effect have not been fully elucidated, but it has been demonstrated that this cytokine can down-regulate the synthesis of other pro-inflammatory cytokines such as IL-1β and TNF-α [500, 501], thus limiting neuroinflammation contribution to epileptogenesis. According to these findings, here we highlight a low protein expression level of IL-1β and TNF-α in epileptic rats treated with α-T as compared to the corresponding non-treated ones. This down-regulation could in part be related with the IL-6 effect above mentioned. Interestingly, the small regulatory RNA molecule miR-146a, an inflammation-associated microRNA up-regulated during epileptogenesis in rat hippocampus [421], has been recognized as an endogenous regulator of cytokine signalling [502]. In this view, He et al. [503] demonstrated that miR-146a has influence on IL-6 expression, since miR-146 up-regulation increase, and miR-146a mimics decrease, the expression of IL-6 in Lipopolysaccharides (LPS)-stimulated macrophages. In our experiments, we found that miR-146a expression was significantly enhanced in VK rats in comparison to control rats fifteen days after SE, while miR-146a up-regulation in epileptic rats has been found reduced by α-T treatment. α-tocopherol was previously disclosed to down-regulate miRNA levels [467]. Thus, a plausible hypothesis is that during the latent period post-SE induction, α-T can reduce miR-146a expression and increase, as a consequence, IL-6 release in rat hippocampus. However, it is worth mentioning that mechanisms other than the anti-inflammatory one could be involved in the neuroprotective effects of IL-

6. Indeed, this cytokine facilitates the concentration- and time-dependent up-regulation of adenosine A1 receptor expression and signalling [504]: adenosine has been shown to exert a powerful anticonvulsant effect [505]. In addition, *in vitro* studies showed that IL-6 is able to protect against glutamate- and NMDA-induced excitotoxicity [506, 507]. The present findings confirm and extend at 15 days post KA injection the previous findings about the protection against SE-induced neurodegeneration exerted by α-T. FluoroJade analysis revealed that degenerating neurons were scattered throughout the rostro-caudal CA1 extension, with a particular higher concentration at more caudal hippocampal levels. Importantly, at this level we detected a significantly lower number of degenerating neurons in α-T-treated epileptic rats in comparison to the corresponding non-treated ones. The rostro-caudal gradient in vulnerability of CA1 to epileptic injury could be consistent with the increasing degree of neuronal excitability from dorsal to ventral hippocampus described by several authors [508-512] and, it is noteworthy that postictal α-T administration is able to rescue CA1 neurons, showing a corresponding region-specific pattern along the rostro-caudal axis of hippocampus.

The evaluation of the existence of a possible beneficial effect exerted by α-T treatment on preventing BBB damages has been evaluated in the present study. A compromised functioning of brain endothelial cells results in serious consequences for BBB integrity [513] and therefore for brain homeostasis. BBB breakdown can be a direct consequence of seizure activity or immune mediators, which induce shifts in vascular physiology, damaging its function of separating the circulating blood from CNS extracellular fluid, thus increasing permeability [384-386]. Tight junctions contain a complex of transmembrane proteins, including claudin, the alteration of which in the expression levels is indicative of a BBB damage. Our results clearly highlight the ability of α-T to promote BBB integrity, probably by reducing neuroinflammation levels after SE. This is a remarkable effect considering the crucial role played by BBB in maintaining cerebral homeostasis and providing neuroprotection.

Since numerous research data indicates that a number of microRNAs are dysregulated in epileptic brain of human and animal models [420, 424], we monitored changes in the expression levels of three selected miRs in all the experimental groups. Accordingly, we found that miR-146a was significantly up-regulated in hippocampus of non-treated epileptic rats fifteen days after SE induction, but α-T treatment was able to prevent its over-expression. On the other hand, miR-126 and miR-124 were significantly down-regulated in α-T treated epileptic hippocampi. Besides, it is worth mentioning that under α-T treatment, in absence of the epileptic insult, all the considered miRs showed an alteration of their expression indicating a possible direct effect of α-tocopherol on miRs expression. Consistently, it has been previously reported that α-T can induce a redox-independent down-regulation of microRNA expression by [467]. The miR-146a appears to be a crucial mediator in the neuroinflammatory response and increased level of miR-146a has been found in the latent period of epileptic disease both in rat model and in human MTLE [421], thus suggesting a potential role of this miRNAs in epileptogenesis. It is therefore conceivable that α-T, decreasing the expression levels

of IL-1β and TNF-α pro-inflammatory cytokines, is able to prevent miR-146a up-regulation induced by SE, which in turn, as discussed above, might lead to an increased IL-6 release, hence contributing to reduce IL-1β and TNF-α protein levels.
miR-126 is an highly expressed microRNA in endothelial cells and plays a critical role in angiogenesis and blood vessel integrity [514, 515]. In addition, miR-126 regulates the expression levels of endothelial adhesion molecules in inflammation [516]. According to recent findings, the BBB function is dependent on several miRNAs, among which miR-126 [517]. Our data point-out that miR-126 expression level tends to decrease in hippocampus fifteen days following SE. This reduction could be related to impaired BBB integrity as shown by the dramatic loss of claudin-5 detected in epileptic non-treated rats. It is remarkable to highlight that α-T treatment seems able to reduce miR-126 levels even in the absence of epileptic insult. This effect suggests that α-T can independently and directly regulate miR-126 expression in the brain, affecting the regulation of its targets including those related with cellular growth and survival [518]. Accordingly, miR-126 has been proven to regulate SOX2 [519] and EGFL7 – a Notch signaling modulator - [520], thus suggesting a role in the regulation and maintenance of self-renewal and pluripotency features in stem cells and neural progenitors. However, it is not possible to exclude the influence of DG neurogenesis and its modulation by α-T [521-523] and epilepsy [347] on the results obtained since miR-126 evaluation was performed in whole hippocampus.
miR-124 is widely expressed in the brain and several evidence suggests a link between its dysregulation and numerous CNS disorders [524]. miR-124 is also involved in epilepsy where it plays a dual and opposite role of pro- and anti-inflammatory modulator [426]. In our experimental conditions, hippocampal miR-124 expression was not significantly modified fifteen days after KA-induced SE, even though a trend toward down-regulation is appreciable. FISH analysis, revealed a miR-124 expression in cytoplasm of hippocampal neurons, including pyramidal neurons of CA1 field. Both RT-PCR quantitative analysis and FISH staining showed that under α-T treatment conditions, miR-124 expression was significantly down-regulated in KA-injected and non-epileptic rats, thus suggesting a direct insult-independent action of α-T on miR-124 expression. However, considering the effect of α-T on neuron network excitability, these findings could support a pro-epileptogenic role played by miR-124 in epilepsy.

Circulating miRs have been proven to be clinically relevant diagnostic/prognostic biomarkers for several human diseases [525]. Accordingly, our results on miRs expression levels in serum revealed that the modulation of miR-124 and miR-146a in the different experimental rat groups reflects the data obtained in hippocampi from the same animals, faithfully reproducing alterations induced by SE and α-T treatment. In particular, the expression level of circulating miR-124 is of special interest since it is specifically expressed in brain and has been proposed as diagnostic and prognostic tool in some brain disorders. At the opposite, miR-126 evaluation in serum from VK group resulted increased (instead of decreased as emerged in hippocampus) with respect to miR-126 serum levels detected in non-epileptic untreated rats. Notably, α-T treatment

reduce miR-126 levels in epileptic and non-epileptic rats, thus corroborating the α-T effect on miR-126 expression.
Taken together, our findings allow us to suggest miR-124, miR-126 and miR-146a expression levels in the serum as biomarkers to follow up the effectiveness of antiepileptic treatments.

In conclusion, α-T treatment during the seizure-free latent period after SE-induction allows the decrease of maladaptive plasticity processes, including neuroinflammation and neurodegeneration, which promote the occurrence of spontaneous recurrent seizures leading to chronic epilepsy. The main findings of this study disclose an α-T-mediated decrease of hippocampal network excitability following SE-induction and the possible involvement of an α-T-mediated anti-inflammatory mechanism in reducing epileptogenesis. The reduced excitability is not a consequence of α-T influence on $GABA_AR$ and AMPA currents but it is probably due to other mechanism as, for instance, reduced GABAergic neurons degeneration, decreased BBB damage or inflammatory pathways modulation. Importantly, α-T treatment on epileptic rats did not induce any adverse reaction such as significant weight loss, bleeding, diarrhea, liver structure damage, or others. This is a very significant aspect since side effects of current AEDs are a major impediment to optimal dosing for seizure control, mainly under drug-resistant epilepsy. Finally, a major discovery regard circulating miR-126 and miR-146a during epileptogenesis which might represent not only valuable diagnostic/prognostic biomarkers but also useful biomarkers to evaluate the clinical efficacy of specific treatments.

CONCLUSIONS

Learning, memory and many neuropathologies have a common underlying feature called brain plasticity, which plays a double-face role in these different conditions. It is therefore clear that the current concept of brain plasticity has a great relevance for neuroscience, but also for areas outside this field, being of potential interest for all human life.

A healthy brain is able to respond with physiological plastic changes that involve spines, synapses or dendrites which continuously rewire the network throughout our lifetime, overcoming the limits of genetic slowness, and making adaptation to a fast-changing environment possible. Among all the mechanisms that support brain plasticity, hippocampal neurogenesis represents a highly fascinating phenomenon not jet completely understood. Indeed, it remains to shed light upon the specific contribution of newly-generated granule cells to hippocampal function, a question that after several years of research on this field has not been answered yet. A possible way to understanding the functional role of hippocampal adult neurogenesis lies in its dynamicity and dependency on the network activity which could promote or prevent this process. Accordingly, rewarding experiences as physical activity, positively affect adult born DG neurogenesis, promoting the generation and integration of new granule cells. Here, we brought evidence that a brief physical activity generates short-term and long-lasting modifications in hippocampus, which could influence future network activity potentially lead to cognitive enhancement widely attributed to physical activity [122, 155, 191, 202].

Despite brain plasticity can be certainly considered an essential property of the neural network, its highly controlled dynamics might face their failure and become corrupted giving rise or contributing to several neurological disorders among which major depression and epilepsy. A common social need concerning these maladaptive plasticity-related pathologies, is the availability of new and more efficient treatments, together with the identification of fast and reliable diagnostic/prognostic tests, also considering the high incidence of treatment-resistant patients. The present thesis has analysed and emphasised the existence of promising new targets of treatment in the depressive disorder and the potential benefits of α-tocopherol-based treatment in counteracting epileptogenesis, together with the emergence of some circulating miRs for the valuable monitoring of mesial temporal lobe epilepsy.

To conclude, managing to manipulate brain plasticity is gaining great attraction for its potential in health and disease. On one hand it might allow to improve essential features of human brain as learning, memory and cognition; on the other hand, the correction of the program that govern maladaptive neural plasticity in disease might lead to the development of effective form of treatment aimed to correct the mechanisms behind the pathology and not the symptoms themselves.

ABBREVIATION LIST

5-HT – 5- Hydroxytryptamine

5HT1A – 5- Hydroxytryptamine Receptor 1A

α-T – α-Tocopherol

ACSF – Artificial Cerebrospinal Fluid

AEDs – Antiepileptic drugs

AMPAR – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

AP – Action Potential

BBB – Blood Brain Barrier

BDNF – Brain Derived Neurotrophic Factor

CA1, CA2, CA3 – *Cornu Ammonis* 1, 2, 3

CNS – Central Nervous System

DA - Dopamine

DG – Dentate Gyrus

DGCs – Dentate Granule Cells

EEG – Electroencephalogram

fEPSP – Field Excitatory Postsynaptic Potential

FGFR1 – Fibroblast Growth Factor Receptor 1

FSL rats – Flinder's Sensitive Line rats

FST – Forced Swim Test

GABA – Gamma-Aminobutyric Acid

GCL – Granule Cell Layer

GFP – Green Fluorescent Protein

GluA1, GluA2 – Glutamate receptor subunits

GPCR – G Protein Coupled Receptor

i.c.v. – Intracerebroventricular

IL-1, IL-6 – Interleukin -1 and -6 Families

***in situ* PLA** – *in situ* Proximity Ligation Assay

i.p. – Intraperitoneal

KA – Kainic Acid

LTD – Long Term Depression

LTP – Long Term Potentiation

LPO – Lipid peroxidation

MDD – Major Depressive Disorder

mEPSC – Miniature Excitatory Postsynaptic Current

mIPSC – Miniature Inhibitory Postsynaptic Current

miR – microRNA

ML – Molecular Layer

MTLE – Mesial Temporal Lobe Epilepsy

NA - Norepinephrine

NMDAR – N-methyl-D-aspartate receptor

NR2A, NR2B – NMDAR subunits

PLA – Proximity Ligation Assay

PSC – Postsynaptic Current

RNS – Reactive Nitrogen Oxygen

ROS – Reactive Oxygen Species

R-R interaction – Receptor-Receptor interaction

RTK – Receptor Tyrosine Kinase

SD rats – Sprague Dawley rats

SE – Status Epilepticus

SGZ – Subgranular Zone

SSRI – Selective Serotonin Reuptake Inhibitors

TNFα – Tumour Necrosis Factor α

VGCCs – Voltage Gated Calcium Channels

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