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# Regulation of the GABA switch by immunomodulatory signals

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# *Abstract*

*The neuronal "GABAergic switch" represents a critical event that occurs early in life before birth, during brain development, characterized by the excitatory-to-inhibitory transition of the GABAergic transmission. Impairments in the accomplishment of this event have been associated to a remarkable excitation/inhibition network imbalance, usually linked to cognitive disabilities and behavioural deficits, typical hallmarks of neurodevelopmental disorders.*

*Even though molecular mechanisms of the GABA switch have been widely described, novel regulators of this event are being continuously characterized.*

*It is well known that mesenchymal stem cells (MSCs) represent good candidates for therapeutic interventions, given their positive roles in neuroprotection against immune-mediated and neurological diseases. However, raising evidences are considering MSC-derived extracellular vesicles (EVs) better candidates than the whole cells for clinical applications, bearing more safety and less side effects.*

*Among the immunomodulatory molecules, increasing studies consider the cytokine Interleukin 6 (IL-6) as a novel trophic factor, despite its well described role in neurodevelopmental diseases, such as autism.*

*By taking advantage of a combination of functional (calcium and chloride imaging) and molecular approaches (RT-PCRs), we found that MSC-EVs but not MSCs accelerated the timing of the GABA switch and boosted the expression of the GABA inhibitory synaptic markers. Likewise, IL-6 early exposure in neurons accelerated the timing of the GABA switch by enhancing the GABAergic transmission and upregulating the expression of KCC2, in a STAT3- dependent manner.*

*Given several evidences suggesting the presence of IL-6 within the MSC-MV cargo it is possible to speculate about their synergistic action when combined. All these data open the possibility to harness such system as a new therapeutical approach, for delivering safe and nontoxic organelles to those pathological conditions characterized by a delayed GABA switch, such as neurodevelopmental disorders.*

# 1

# BACKGROUND

## 1.1 GABA: THE MASTER INHIBITORY NEUROTRANSMITTER IN THE MATURE BRAIN

Gamma- Aminobutyric acid, known as GABA, represents the main inhibitory neurotransmitter in the mature brain. It generates an inhibitory effect in most central neurons and, together with glutamic excitatory transmission, it keeps the excitation/inhibition balance well controlled [1-3]. Intriguingly, GABA is also endowed with multiple functions according to different situational needs. In fact, GABA has been found not only in the central nervous system but also in peripheral nervous tissues and even in not-nervous tissues [4] exhibiting a wide range of functions including acting as neurotrophic factor [5, 6] modulating neuronal migration [7-9] and promoting the neurite growth [10-13].

### 1.1.1 The discovery of “Factor I”

The story of its first description in biological tissues goes back to early 1910s, but its discovery within Central Nervous System (CNS) and its acceptance as the main inhibitory neurotransmitter occurred only forty years later, when, in 1950, Awapara and collegues [14] first described a novel enzyme capable to convert glutamate to GABA. They observed that after incubating rabbit and rat brains with glutamate, the rate of glutamate decrease was directly correlated with

GABA increase. In parallel, Roberts and Frenkel (1950) were able to demonstrate that the substance collected from brain *"by homogenates, washed residues and acetone powders of brain"* could be definitely identified with GABA and synthetized from glutamate. The following year the same group even described high level of glutamic acid decarboxylase (GAD) in mouse brain [15]. Finally, just few years later Florey & McLennan (1959) isolated from mammalian brain a “Factor I” (where ‘I’ represented inhibitory action) which was able to depress neuronal activity, and was then recognized with GABA itself [16].

Chemically speaking, GABA belongs to amino acids, but, despite the presence of the amino and the acidic group, it is not likely used by cells to build up proteins, because the amino group is not directly linked to the alpha carbon group, thus preventing the use of this amino acid to integrate the aminoacidic chain. The overall metabolic pathway of GABA synthesis is usually introduced as GABA shunt [17].

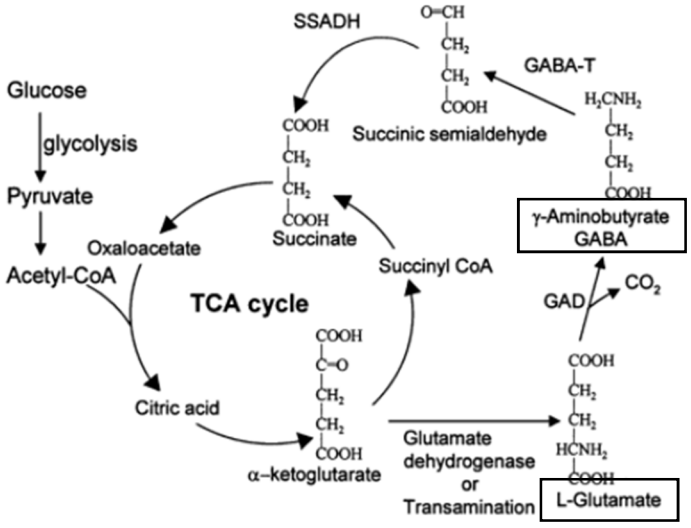


***Fig.1.1 The GABA shunt.*** *The metabolic pathway of GABA synthesis from TCA cycle. Adapted from [18].*

GABA shunt describes GABA directly synthetized from glutamate, which in turn comes from trycarbossilic acid (TCA) cycle. Glutamate from energy metabolism undergoes a decarboxylation reaction guided by the glutamate decarboxylase (GAD). This conversion is essentially irreversible and requires pyridoxal phosphate (PLP) as cofactor. After release and binding to its receptor, GABA undergoes a quick turn-over and it is converted back to succinic semialdehyde by GABA transaminase (GABA-T), rapidly oxidized and then re-up taken from neurons as succinate, so that it can enter the TCA cycle again [17].

### 1.1.2 GABA binds to ionotropic and metabotropic receptors

GABA acts basically through two main types of receptors: the **ionotropic $GABA_A$ receptor ($GABA_AR$)** and the **metabotropic $GABA_B$ receptor ($GABA_BR$)**. These two classes possess different functional properties and high heterogeneity [19].

**$GABA_A$ receptor** represents the primary target for GABA-mediated fast hyperpolarization in adult brain [19]. They are blocked by bicuculline and regulated by steroids, barbiturates and benzodiazepines [19, 20]. They represent ligand-gated chloride ion channels, assembled as hetero- or homo-pentamers, from combinations of 19 different subunits, namely six α, three ß and ten non-α /ß subunits [21-24], differentially arranged to best tune GABA fast hyperpolarization in the mature brain. Pentamers with unique subunit compositions localize at different sites to ensure distinct functions: they mediate fast phasic inhibition if shuttled at synaptic sites, like the α1/ß/2-containing $GABA_AR$s, and tonic inhibition

if placed at non-synaptic sites, as α6/ß/d-containing $GABA_A$Rs, in cerebellar granule cells [25-29].

Overall, receptors containing α1 and α 2 subunits preferentially localize in the synaptic cleft and mediate fast phasic inhibition, whereas receptors containing α4, α5, α6, and δ subunits localize extrasynaptically/perisynaptically and are responsible for tonic inhibition [27].

Intriguingly, even if tens of combinations are theoretically possible, only some of them preferentially segregate in functional channels [21, 23, 30]. Martenson and colleagues (2017) revealed that there are precise intrinsic signals guiding the fate and the composition of $GABA_A$R pentamers, as they provide the first molecular model for in vivo combinatory $GABA_A$ receptor assembly [31]. Another proven evidence helps raising the intriguing nature of $GABA_A$ receptors: not only subunits differentially segregate in distinct pentamers, but their composition and enrichment are even developmentally regulated by distinctive expression patterns [32].

After GABA release from synaptic vesicle into the synaptic cleft, it firstly activates $GABA_A$ receptors, thus triggering the opening of the channel and enabling Chloride ($Cl^-$) ions diffuse into the cell along its concentration gradient: this process will result in a fast hyperpolarization of the post synaptic mature neuron [22, 33].

Another GABA ionotropic receptor described in the nervous system is **$GABA_C$ receptor** [34], which also mediates $Cl^-$ flux in the cells and is similar to heteromeric $GABA_A$ receptor in structure, function and mechanism of action [35] However,

$GABA_CRs$ are endowed with distinctive pharmacological properties and distribution, making them of special interest. In fact, they are made up of a single type of protein subunit and they are selectively implicated in the visual system development [36, 37]. Curiously, they come to be not responsive to $GABA_AR$ antagonist bicuculline, nor to steroids, barbiturates or benzodiazepines have effect on them [20] and they represent a minor, spatially restricted, group of GABA-mediated hyperpolarization receptors.

The second major family of GABA receptors are metabotropic receptors, represented by **$GABA_B$ receptors**, which are likewise consisting of different subunits: they are hetero-oligomeric metabotropic receptors, whose subunits composition resemble that of glutamate receptors [38].Three subunits have been identified and cloned so far: GABABR1α, GABABR1β and GABABR2 [38-40] which differentially segregate into decided combinations. The active form of $GABA_B$ receptor is achieved when GABABR1α or 1β subunit binds GABABR2 subunit forming dimers or oligomers [20]. They are responsible for secondary, later, slower and prolonged component of inhibitory transmission [41], as they also serve as neurotransmitters' release modulators.

They are transmembrane receptors commonly coupled to phospholipase C and adenylate cyclase, which mediate secondary slower intracellular signal cascades [20, 33, 42].They become activated after GABA binding thus leading to release of G-protein subunits, which diffuse intracellularly and activate secondary responses, thus triggering the activation of postsynaptic K+ channels or inhibition

of presynaptic Ca2+ channels [41]. This signalling cascade leads to reduced neurotransmitter release.

In contrast to $GABA_A$ ionotropic receptors, $GABA_B$ receptors can be found both pre and post synaptically [42] and they are selectively inactivated by the phosphonic and sulfonic acid analogues of baclofen, namely, phaclofen and saclofen [43].

### 1.1.3 The GABAergic developmental switch: a developmental journey

The developing brain cannot be considered just a small adult brain in a small body. Indeed, a lot of changes occur during the first embryonic phases to shape the adult brain, not only involving the brain itself, but also endocrine [44], immunological [45] and even microbiotic systems [46, 47] undergo specific developmental changes.

Intriguingly, in the immature brain a lot of new events appear: the molecular composition is progressively modified, and neurons start synchronizing each other in order to fire and wire together so that their currents will build up functional neuronal circuits.

Indeed, in the immature brain, groups of developing neurons "sense" themselves and keep firing together, giving rise to large synchronized patterns of currents, which will be then replaced by sparse firing, time-locked and behaviourally -related currents, in adult brain [48-50]. In this scenario, the GABAergic transmission plays a very important role during development, because

its action ensures the proper brain formation and a functional network establishment [6, 51, 52].

In contrast to the inhibitory action of GABA signalling in the mature brain, during early phases of development, GABA is endowed with excitatory activity, able to elevate intracellular calcium, thus acting in a depolarizing manner [6, 50, 53, 54]. The excitatory effect of GABA at early stages of neuronal development represents an important factor early in life [49, 54-57]. Indeed, during early brain development, its excitatory activity is able to set the timing of fundamental processes such as proliferation of neuroblasts and their migration, synapse formation, and even synapse plasticity [6, 51, 52].

Notably, such an excitatory effect of GABA occurs within a limited temporal window. Indeed, GABA signalling undergoes a precise temporally-dependent biochemical modifications from the early brain-characterizing excitatory activity to the ultimate inhibitory and hyperpolarizing transmission, a critical event that is always referred to as "GABAergic switch" [3, 49, 50, 52, 54].

What basically makes GABA being excitatory rather than inhibitory are changes in ions concentration inside the neurons, and their corresponding driving force. In particular, two events happen when GABA switches from a depolarizing to hyperpolarizing neurotransmitter: 1) intracellular chloride levels decrease and 2) GABA-induced calcium transients through Voltage operated calcium channels (VOCCs) are abolished (see **Fig.1.2**).

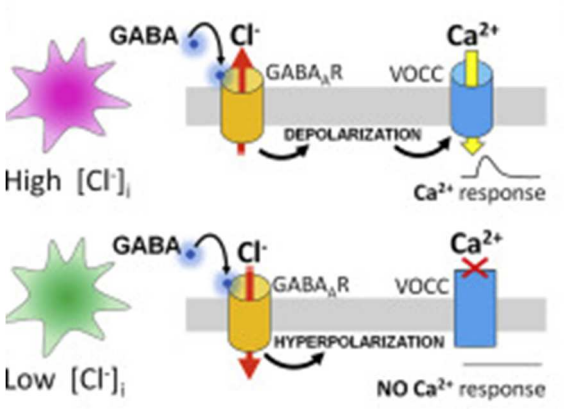


***Fig. 1.2 Schematic representation of depolarizing-to-hyperpolarizing GABAergic switch.*** *Two features mark this event: intracellular chloride levels' decrease and GABA-induced calcium transients through VOCCs opening. In immature neurons, characterized by high intracellular [Cl-] GABA depolarization opens VOCCs and calcium transients occur. In mature neurons, low [Cl-], determines GABA hyperpolarization, VOCCs remain closed, and no Ca2+ response is induced. Adapted from [58].*

### 1.1.4 $[Cl^-]_i$ shape the polarity of GABA

The most important event that define depolarizing or hyperpolarizing GABA is that chloride concentration inside the cell progressively changes during brain development [59]. In fact, the excitatory-to-inhibitory GABA shift is due to a progressive reduction in intracellular $[Cl^-]$ by sequential maturation of two chloride co-transporters, the bumetanide-sensitive **sodium-(potassium)-chloride co-transporter 1** (**NKCC1**; also known as SLC12A2), which imports chloride, and the electroneutral **potassium-chloride co-transporter 2** (**KCC2**; also known as SLC12A5), which pumps chloride outside the cells [60, 61].These chloride co-transporters are finely regulated, and their expression is opposite regulated during the brain development. KCC2 represents the main $Cl^-$ extruder in hippocampal and

neocortical principal neurons, whereas NKCC1 is almost ubiquitously expressed, and, in the nervous system, not only by central and peripheral neurons, but even by glial cells [62, 63].

Even though they are both relevant to maintain the proper chloride concentration balance inside the cell, KCC2 has been found playing a major role in the GABAergic switch, as its impaired levels and time of expression, have been related to pathological outcomes [64-67].

Additionally, KCC2 mRNA expression has been largely characterized, and it is now known its regulation by two transcriptional factors: the transcription factor early growth response 4 (Egr4 or NGFI-C) upregulates KCC2 expression [68], whereas the RE-1 silencing transcription (REST) factor inhibits KCC2 mRNA level, after binding to two repressor elements (RE-1) in the KCC2 gene [69]. As a fine intervention of these regulators, the KCC2 mRNA gradually increases during development, from spinal cord and brainstem to higher brain structures [70, 71].

Overall, what ultimately determine depolarizing or hyperpolarizing GABA is then the net flux of chloride ions through $GABA_A$ receptors, according to [Cl-] inside the cell. In immature neurons, high NKCC1 and low KCC2 expression contribute to the high chloride concentration, leading, in turn, to a depolarizing effect of GABA signalling. However, during development, NKCC1 expression dramatically declines; in contrast, KCC2 levels increase over time. As a result of this reversed expression, intracellular [$Cl^-$] becomes smaller, leading to a hyperpolarizing effect of GABA signalling [48, 54, 55].

### 1.1.5 The GABA switch is highly conserved among species

The GABAergic developmental switch is a precise sequence of events that has been found highly conserved throughout evolution, suggesting its fundamental role for the proper brain development and network setting. Indeed, it has been described in a wide range of animal species from worms, to frogs, to mammals [72, 73] and also in different brain regions [74-78].

It is important to underline that the GABAergic developmental sequence is not restricted to a limited type of neurons, but rather it applies to both GABAergic and glutamatergic neurons [53, 79, 80], indicating that this event is a common phenomenon whose objective is to firmly shape the whole functional neuronal network. In fact, GABA signalling is established not only before glutamatergic transmission, but also even before synapse formation. This explains why GABA signalling modulate cell cycle and migration, through the establishment of primitive patterns of activity, generally recognized as giant depolarizing potentials (GDPs) [81-83].

The unconventional action of excitatory GABA in immature brain, raise the question of why its depolarizing activity is being so important to become highly conserved among species. One of the most accepted theory states that GABA represents the pioneer neurotransmitter which plays trophic actions on primitive brain. By exciting immature neurons, it generates primordial oscillations, thus triggering neuronal growth and differentiation [50, 53, 74, 76]. Its depolarization generates sodium spikes, removes the voltage-dependent magnesium block from NMDA receptor channels and also activates voltage-gated calcium currents,

leading to calcium influx [48, 76, 77]. The latter process represents a fundamental hallmark for the depolarizing effect of GABA: the GABA-induced calcium transients.

### 1.1.6 Deregulation of GABA switch and brain disorders

It is very important that the brain completes the precise sequential events of GABA switch which ultimately result in lowering the intracellular chloride (due to KCC2 upregulation and NKCC1 decrease) and in an inhibitory role of GABA transmission. Such a process ensures a proper excitatory/inhibitory balance of neuronal network activity which represent a fundamental feature for a correct brain functioning.

In line with this, a deregulation of the intracellular [Cl-] and the consequence loss of the inhibitory action of GABA, has been found in several brain disorders including chronic pain [84], spinal cord lesions [85], neuronal stress [86] or seizures [87-90]. Likewise, an alteration of developmentally-dependent GABA switch was found to be associated with relevant neurodevelopmental diseases, such as epilepsy [91-94], autism-spectrum disorders [95-98], intellectual disability [99, 100], as well as schizophrenia [101].

Interestingly, it should be noted that virtually all these pathological conditions are accompanied by an inflammatory-like state which may occur at different stages of brain development. In particular it has been recently highlighted that neurodevelopmental disorders, including autism and schizophrenia, may be the result of early inflammatory conditions occurring before birth, at prenatal stages

[102-105] . Collectively, all the observations made above, raise the question whether insults occurring during either adulthood or prenatal/neonatal periods, could somehow affect this vulnerable event, which is vital for the proper brain maturation and network activity establishment.

## 1.2 MESENCHYMAL STEM CELLS: THE SLEEPING BEAUTY SYSTEM

Multipotent Mesenchymal Stromal Cells or Mesenchymal Stem Cells (MSCs) represent a powerful tool for regenerative and therapeutic purposes, as they actively survey the microenvironment, differentiate in several cellular lineages and secrete soluble and immunomodulatory factors, in order to provide trophic support for injured cells.

MSCs have been firstly isolated in early 70's by Friedenstein and his colleagues [106]. Friedenstein described a fibroblastic-like population of cells, generated by flushing them out from rat bone marrows and selected *in vitro* for their clonogenic activity and plastic adherence. After trying to transplant them subcutaneously, they observed that these cells were able to reconstitute the stromal hematopoietic microenvironment. This population of cells was only later on identified as Mesenchymal Stem Cells or Mesenchymal Stromal Cells (MSCs) and now the scientific community recognize them as multipotent stem cell population, featured with self-replicative and differentiation abilities, whose properties, potentials and perspectives are still under evaluation.

### 1.2.1 Mesenchymal Stem Cell Features

MSCs are adult multipotent stem cells, characterized by a high ability to proliferate and a low degree of plasticity, even if still able to generate few specialized lineages of cells. Furthermore, MSCs, as well as hematopoietic stem cells, own the additional ability to migrate through the blood stream from the original niche to the site of injury, according to the organism needs. MSC stemness

ability is to undertake a process that is often described as 'the mesengenic process' [107]: MSCs give rise to all cells belonging to mesodermal tissues, including bone, cartilage, fat, dermis and tendon [107]. This lifelong-lasting process ensures the continuing tissue rejuvenation and repair after damage.

Few hours after plating, after flushing them out from the whole bone marrow, MSCs adhere to plastic dishes and keep growing in distinct colonies, which are identified as Colony-Forming Unit-Fibroblasts (CFU-F), as MSCs resembles the spindle-like fibroblast morphology.

MSCs represent the heterogeneous, non-hematopoietic minor fraction of total nucleated cells of the bone marrow. [108]. In fact, they have been estimated to represent only the 0.001-0.01% of the total nucleated resident cells of the bone marrow, and their number even decreases with age: it has been estimated that they range from $1/10^4$ of nucleated cells in a new-born, to $1/5x10^5$ in a 80-year-old man.

The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cell Therapy (ISCT) set standards criteria to identify human MSCs to allow a proper in vitro characterization. In particular, defined criteria have been presented by Dominici and colleagues [109]. They propose that bona fide MSCs should meet the following three criteria: *i)* Plastic **adherence**, *ii)* **surface antigens** pattern of expression and *iii)* **differentiation** potential in specific cell lineages.

When maintained in standard culture conditions. Moreover, cultured MSCs grow *in vitro* forming distinct colony units, called CFU-F, and exhibit a fibroblastic -like morphology (**Fig. 1.3**). These conditions would allow the selection of MSCs from other cell types.

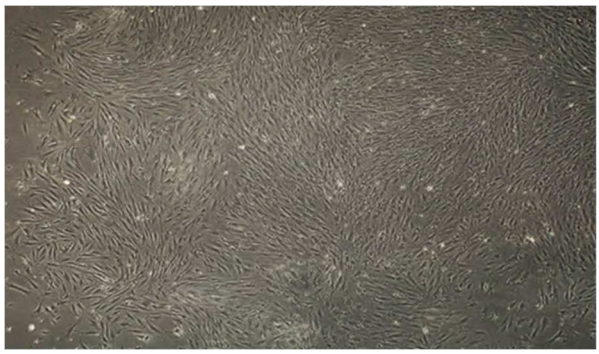

***Fig. 1.3***. *Representative microphotograph of bone marrow-MSCs in culture. Image acquired with an optical microscope. 4x magnification*

The second feature identifying MSCs is the expression of distinctive classes of antigens on their membrane surface. In fact, they are characterized by the presence of CD-73, CD-90, CD-105 markers, together with the absence of CD-31, CD-34, CD-45, CD-14 or CD-11b, CD-19 or CD-79a and HLA class II markers.

Among the positive markers, CD-73 represents the most important marker characterizing MSCs, although also highly expressed onto the lymphocyte membranes. The ecto-5'prime nucleotidase CD-73, encoded by the NT5E gene, is able to convert AMP substrates into adenosines. MSCs must priority carry CD-73 on their membranes and reveal very high CD-73 expression (>95%) [110],[111].

CD-90, also known as Thy-1, has been initially described on membranes of thymocytes [112, 113], precursors of T-cells, and represent the gold standard stemness marker for MSCs identification [109, 114-116], and although it has been originally described in T-cell precursors signalling [117, 118] and among other cells types, like neurons and glial cells [119], hematopoietic precursors [120] and also hepatocytes of foetal liver [114].

CD-105, also known as Endoglin, is a type I glycoprotein identifying hematopoietic progenitor cells and belonging to the Transforming Grow Factor (TGF) beta receptor complex (TGF-βR), [121]. CD-105 membrane protein interacts with TGF-β III and TGF-β I receptors [122] and it is involved in the regulation and progression of cell cycle, cellular migration and localization [123], all TGF-β-dependent cellular pathways.

Beside they represent exhaustive markers, they are not always sufficient to define bona-fide MSCs. In fact, it must be noted that MSCs derive from heterogeneous populations of cell harboured in bone marrow, thus, a high number of analysed antigens is required, increasing the probability to culture bona-fide MSCs.

However, the remarkable feature that unequivocally identifies *bona fide* MSCs is their ability to undergo *in vitro* differentiation process. In fact, when culturing MSCs in appropriate standard differentiating media and conditions, they give rise to three principal cell lineages: osteoblasts [124], adipocytes [125], and chondroblasts [126].

*Osteogenic differentiation* could be induced with osteo-inductive medium, a complete 20% FBS-containing medium, supplemented with a-glycerophosphate, dexamethasone and ascorbic acid [115, 127, 128]. After 21 days, cellular and/or molecular assays will confirm that the differentiation has been successful. Reliable Cellular assays include Alizarin Red or Von Kossa stainings, and bone Alkaline Phosphatase assay. qRT-PCR could eventually assess the expression of osteogenic markers, as Cbfa-1, Osteopontin, Osteonectin, Osteocalcin, Collagen type I [115, 129-131].

The *adipocytic differentiation* is committed *in vitro* by feeding cells with complete medium supplemented with dexamethasone and insulin. After about 21 days in culture, lipid droplets can be revealed with Oil Red O, as triglycerides show affinity for the dye. Adipocytic markers that commit MSCs should include: C/EBPa, PPARg1, PPARg2, adipsin, lipoprotein lipase, leptin and aP2 gene [115, 127, 132, 133].

*Chondrocytic differentiation* could be verified by culturing cells in serum-free medium, supplemented with ascorbic acid and TGF-β3 [134] or TGF-β1 [135] [115]. Alcian Blue staining, collagen type II/type IX immunohistochemistry as well as molecular evaluation (Cbfa-1; type I/IX collagen; aggrecan) could be performed to assess MSCs ability to differentiate towards chondrogenic line [115, 130].

### 1.2.2 MSCs derive from different tissues and sources

MSCs in adult tissues are usually isolated from the red bone marrow (BM): this tissue is a fundamental and continuous source of multipotent precursors of blood, the hematopoietic stem cells (HSCs) whose role is to ensure blood turnover and haematopoiesis. Red BM represents a flexible and soft tissue that in the adulthood still persists in the cavity of flat bones (i.e. vertebrae, pelvis, scapulae and ribs) and in the epiphyses of long bones (humerus and femur).

BM also contains other cell types that provide the stromal support for the haematopoietic microenvironment. The heterogeneity of this compartment is made of ‘marrow stromal cells’ that include various cell types, *i.e*. fibroblasts, stromal cells, osteoblasts and osteoclasts.

Although the non-haematopoietic stem cells represent only the 0.01% of the BM-resident cells, as mentioned above, they retain the intrinsic ability to proliferate during whole life, being activated especially after injuries to serve as an extraordinary regeneration system, able to differentiate into cells of distinct lineages (cartilage, bone, fat, tendon, ligaments; [134-136]).

MSC-like cells could be found in other tissues and organs beyond BM, and even if they originate from different tissues, they all share the same pattern of surface marker expression, and they also bear the same biological effects.

MSCs derive from several tissues. A remarkable amount of MSCs originate from foetal tissues, the umbilical cord [137-141] and the Wharton's jelly above all [138], as well as the amniotic fluid [142].

Concerning adult tissues, there are other residual sources of MSCs besides BM: among all, the adipose tissue represents a very rich source of MSCs [143], more than BM [144]. Interestingly, also in peripheral blood, lung [145], liver [146], MSCs could be found, even if very limited represented.

### 1.2.3 Are Mesenchymal Stem Cells a promising tool for medicine?

MSCs are often considered powerful therapeutic candidates, especially for transplantation and regenerative purposes. Their therapeutic potential essentially relies upon their ability to secrete trophic factors to meet organism needs via paracrine communication. There are several preclinical and clinical studies ongoing, suggesting that MSCs do not become activated like antigen-presenting cells do, as they seem to express moderate levels of human leukocyte antigen

(HLA), major histocompatibility complex class molecule I (MHCI) and negligible levels of HLA class II [147]. As a result of these low expression profile, together with low levels of the co-stimulatory molecules B7-1/B7-2(CD80/86), CD40 or its ligand CD40L [134, 148, 149], MSCs prevent lymphocytes from becoming fully activated. These properties might be a great advantage also for transplantations, and especially for allogenic ones.

Several studies further demonstrated positive effects of transplanted MSCs in immune-mediated diseases and degenerative diseases: their success in therapy is related to the immunoregulatory and neuroprotective effects that lead to the functional recovery of the organism. In fact, literature provides several evidences about their role on immune system improvement [150, 151], cardiomyocyte regeneration [152], and on experimental colitis [153]. Amniotic fluid- derived MSCs, have been tested in a mouse model of colitis [154], in bone tissue engineering [155, 156], in heart ischemia-reperfusion injury [157].

Notably, clinical trials are even being evaluated in humans, in order to assess MSC efficacy in the treatment of osteogenesis imperfecta, osteoarthritis, acute kidney injury and even myocardial infarction injury or graft-versus host disease (GVHD) [158, 159].

### 1.2.4 Mesenchymal stem cells act via paracrine signals

From the very beginning of MSC studies, the best quoted hypothesis to explain MSC therapeutic qualities was their ability to engraft in the injured organ,

where they could directly guide the regeneration process. To boost this hypothesis, *in vivo* studies suggested that transplanted MSCs could undergo a cross-lineage differentiation process, known as *transdifferentiation* [160-162] which underlines the plasticity of MSCs in differentiating towards other cell types, beyond their own classical commitment, and their successful ability to regenerate damaged tissues.

Up-to-date, it has been reported that transplanted BM-MSCs could give rise to various lineages: hepatocytes, endothelial and myocardial cells, and even neurons or glial cells [163, 164]. Notably, during the last years, MSC ability to differentiate into neuronal population after treatment with specific differentiation stimuli, or by specific gene activation, has been highlighted [164, 165]: Kopen, Prockop and Phinney [166] first provided evidences that, when transplanted in newborn mice, MSCs are able to migrate towards the brain, and to adopt astrocytic and neuronal features.

Intriguingly, other studies on hepatic and pancreatic regeneration support the hypothesis that the regenerative process might occur through *cell fusion*. Bone marrow-MSC cell fusion has been demonstrated after irradiation of the host or during ischemia *in vivo* [167].

Transdifferentiation and cell fusion hypothesis became soon replaced by much more evidences suggesting that **paracrine signaling** represents the best option through which MSCs mediate regenerative processes in damaged tissues. In fact, recent studies suggest that MSCs exert complex paracrine and endocrine modulations, through the release of soluble growth factors and cytokines [168], as well as discrete cell-derived by-products, commonly identified with Microvesicles

(MVs) and Exosomes. Overall described as Extracellular vesicles (EVs), these nanoscaled (30-1000 nm) lipidic organelles are released by all cell types under both physiological and pathological conditions, and they serve the neighborough needs along with released soluble factors, with the additional advantage to carry nucleic acids, namely messenger RNAs (mRNAs), micro RNAs (miRNAs), proteins or lipids, in their lumen, that may help the recovery of the injured cells [168].

### 1.2.5 MSCs as immunomodulators

From the beginning of MSC era, strong immunoregulatory capabilities have been exploited both *in vitro* and *in vivo*. Above all the types of stem cells, in fact, they exhibit a positive role as immunomodulators, involved in the maintenance of peripheral tolerance, transplantation tolerance and autoimmunity [169-171].

In the light of these evidences, MSCs are being considered the best encouraged tool in the development of clinical applications. The relative feasibility of isolation from different tissues and expansion *in vitro*, together with any relevant ethical concerns regarding their use (being extracted from adult tissue), made them potential candidates for the treatment of a wide range of pathologies: immune diseases, such as GVHD and aplastic anemia (AA), chronic inflammatory and autoimmune diseases such as the Crohn's disease (CD) and Multiple Sclerosis (MS) or rheumatoid arthritis (RA) and others.

In particular, MSCs seems to be able to inhibit lymphocytes T-cells proliferation, B -cells proliferation, maturation and chemotactic properties, and even Natural Killer (NK)- cells proliferation and cytotoxicity [171].

They can also immunomodulate leucocytes, both the first activating cells such as neutrophilis [171], and macrophages [169, 171], by driving their activation-dependent polarity shift, from M1, pro-inflammatory, to M2, anti-inflammatory state, and finally dendritic cells. All these positive immunomodulatory properties have been firstly validated in animal models of disease, and recently clinical trials for the treatment of human immune disorders have been accepted and started [169, 171].

### 1.2.6 MSCs for the treatment of neurological diseases

Literature is rich of evidences indicating MSC positive effects in the treatment of a wide range of diseases in animal model diseases, such as osteogenesis imperfecta [172-174], osteoarthritis [175], acute kidney injury [176], myocardial infarction [177, 178], and recent evidence show a potential role of MSCs even in neurodegenerative diseases [179]. In particular, MSC efficacy is currently being evaluated in Alzheimer's (AD) [180-182] and Parkinson's diseases (PD) [183-186], Amyotrophic Lateral Sclerosis (ALS) [187-190] and also Multiple Sclerosis (MS) [191, 192], trying to figure out their ability to transdifferentiate into neural cells, whenever specific conditions are guaranteed, or to bear immunomodulatory and neuroprotective effects, as a result of their paracrine activities.

One of the best examples of MSC positive effects derives from the attempt to cure patients with AD, whose treatment, with pharmacological intervention approved by the U.S. Food and Drug Administration (FDA), can trust mainly in asymptomatic cholinesterase inhibitors (donepezil, galantamine, reivastigmine and

tacrine) and NMDAR antagonist (memantine). Stem cell-based therapy recently has aroused interest providing a new chance to intervene in the resolution of such pathologies.

Interestingly, MSC benefits in AD have been already observed in a preclinical model of AD [193], where the systemic injection of $GFP^+$-MSCs in mice, in which the genes for Amyloid-beta (Aβ) A4 protein (APP) and presenilin 1 (PS1) have been mutated to induce the disease, successfully reduced the plaque size in the hippocampus. Noteworthy, MSCs changed microglial cell distribution and morphology, as well as reduced cytokine expression, usually found high in AD [193]. Furthermore, microglial activation lead to a reduction of the neuroinflammation process and to Aβ plaques clearance.

Simultaneously, MSCs positively acted on neurogenesis as the number of BrdU-ir and HuD (a neuronal lineage marker) positive cells, in the dentate gyrus, were found increased. In parallel, in *in vitro* experiments on neuronal progenitor cells (NPCs) exposed to exogenous Aβ, the presence of MSCs, in co-culture, significantly increased the expression of Ki-67, GFAP, SOX2, nestin, and HuD. Overall these results indicate that MSCs are able to modulate both neurogenesis and NPC differentiation into mature neurons [181].

Beside these evidences focused on the treatment of neurodegenerative diseases, it is important to underline that many other studies claimed MSC support in neuronal growth and differentiation, axonal growth and synaptogenesis [194-196].

## 1.3 EXTRACELLULAR VESICLES: INTERESTING PLAYERS IN CELL-TO-CELL COMMUNICATION

The ideal way MSCs could act through is represented by the release of special by-products which perfectly fit the needs of neighbouring cells, by influencing and reprogramming the surrounding microenvironment. Indeed, MSCs are able to secrete nano-sized lipidic organelles that serve as extracellular vehicles and are commonly known as Extracellular Vesicles (EVs) (**Fig.1.4**). Virtually all cell types are able to secrete large amount of distinctive EVs, either constitutively or upon activation signals. Since their discovery, they have been extensively studied and usually classified according to their size, sedimentation rate, lipid composition, molecule cargo and biogenesis pathway [197, 198].

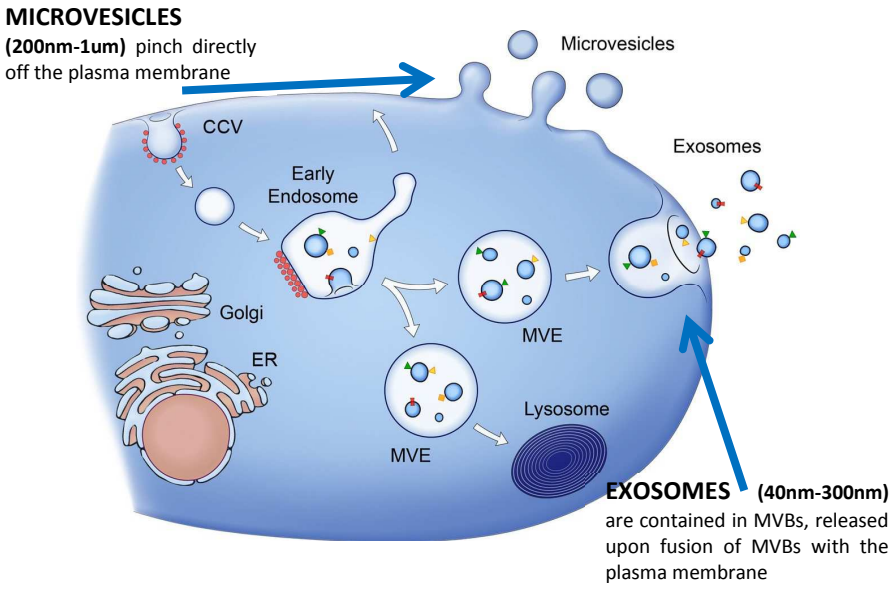


***Fig. 1.4 Microvesicles and Exosomes' biogenesis**. Adapted from [199].*

### 1.3.1 EV characterization

The most representative EVs include microvesicles, exosomes and apoptotic bodies [200].

**Microvesicles** (MVs), also referred to as ectosomes or shedding vesicles, usually represent a heterogeneous population of vesicles, whose size ranged from 50 nm up to 500-600 nm. Their biogenesis hypothesis describes them originating from outward buds in specific plasma membrane sites, usually triggered by the translocation of phosphatidylserine (PS) [201], and followed by a fission process and the subsequent vesicle release in the extracellular space [202, 203]. Phospholipid redistribution, changes in protein composition [204, 205] as well as intracellular $Ca^{2+}$ modifications [206] can influence the EV budding from plasma membrane. As a result of plasma membrane shedding, they typically express cell membrane proteins (including CD9, Mfge8 and annexin V). Some studies [207, 208] describe oncogenes and other growth-factor receptors, MHC class I molecules and integrin receptors be part of MV membranes. Their lumen is loaded with soluble proteins, such as proteases and cytokines, and nucleic acids (dsDNAs, mRNAs, miRNAs) [202]. MVs can sediment starting from 10.000xg by differential centrifugation, and can be usually detected by flow cytometry or capture-based assays.

**Exosomes** are smaller and more homogeneous population of vesicles (30-100 nm in diameter) surrounded by a lipid bilayer. They originate from the endosomal compartment; in particular, intraluminal vesicles (ILVs) which are in turn derived from a distinctive inward budding of multivesicular bodies (MVBs).

MVBs can either fuse with lysosomes for degradation, or travel back and fuse with the plasma membrane [209], thus being released. Following the release, they can target and modulate the activity of the neighbouring cells, according to their composition which is highly variable and strictly dependent on the stimulus. Exosomes are characterized by the expression, among others, of CD9, CD63, CD81, Alix and tumour susceptibility gene derived protein (Tsg101) [210]. They sediment at 100.000xg and can be detected by flow cytometry, Western Blotting, mass spectrometry and transmission electron microscopy.

### 1.3.2 EV mechanisms of action and effects

EVs may interact with target cells in three different ways: *i*) they could be up taken via endocytosis processes and then trigger secondary cell responses; *ii*) EV membrane could fuse with the recipient cells' membrane, thus allowing the release of the vesicle content into their cytoplasm, which in turn would trigger the regulation of intracellular targets; *iii*) EV membrane's proteins can directly interact with cellular receptors, allowing a secondary cell response [211].

Since their features and abilities reflect the cell of origin, they may trigger conflicting effects: either beneficial or detrimental, as, for instance, anti- or pro-inflammatory actions [208].

Even though MSC employment in regenerative medicine raised great expectations, doubts about safety and their practical use in clinics are still difficult to overcome. MSC-derived EVs (MSC-EVs) might represent better candidates to address therapeutic issues. In fact, in the perspective to use them in a cell-free

therapy, MSC-EVs would carry most of the advantages of the parental cells, but avoiding concerns and side effects which are likely related to cell-based therapies.

For their nanoscaled shape, first of all, they are less immunogenic, and they do not accumulate in organs, like kidney and lungs, as MSCs do. Moreover, they do not bear those risks deriving from immune-mediated response and malignant transformation, the major issues concerning engraftment and cell administration [212].

For these reasons, researches in this field are strongly encouraged. Chen and colleagues, reviewed the possible uses of MSC-EVs in lung disease [212], focusing on EV way of action: it is now widely accepted the idea that EVs carry nucleic acids, and in particular miRNAs, which allow a fine regulation of gene expression patterns. Cell development, survival and differentiation-related as well as immune system-related miRNAs have been described so far [212].

EV unique content is the key to guarantee a bidirectional communication within neighbouring cells, as the injured cell releases EVs as delivering specific signals to the surrounding cells which, in turn, would try to meet the requested needs and trigger reparative effects.

In this perspective, is important to underline that EVs might not only trigger beneficial effects. This is what happens for example with tumours: notably, it seemed that cancer cells are prone to release huge amount of exosomes in the microenvironment, thus contributing to metastatic widespread. Furthermore, Zhu et al. [213] in a mouse model of gastric carcinoma, observed that also MSC-derived

exosomes support tumour growth and angiogenesis [214]. Also, MSC-EVs from patients with multiple myeloma, contributed to the tumour growth, in contrast with those derived from healthy patients that, instead, made the opposing effects.

Special attention has been paid on EVs originating from different organs and released into circulating biological fluids (*i.e*. serum/blood, urine, cerebrospinal fluid) as they serve as potential diagnostic biomarkers, especially in the tentative to identify diseases at early stages, possibly before the onset of related symptoms and disabilities [215-220].

If fact, due to the limited screening tools available so far, the chance to identify early biomarkers for neurodegenerative disorders, such as AD and PD, but also for MS and stroke, as well as for other neurological pathologies, would represent a great advantage in fighting them [221].

Many efforts are being made to engineer nanovesicles, in order to make them even smarter tools for functional molecule delivery. To date, most of drugs used in clinics in the treatment, for example, of neurodegenerative diseases, try to improve symptoms, but cannot replace death cells, nor increase the survival of degenerating neurons. In this perspective, for those diseases characterized by loss of neuronal cells, engineered or specific cell-derived EVs could be modified in order to target adult neurogenic niches [222]. Luarte and colleagues observed, in this regard, that MSC-derived EVs seem to positively influence angiogenesis and neurogenesis in mice [222].

### 1.3.3 Safety of EV treatments

Since EVs, compared to the whole cells, have greater chances to cross the Blood Brain Barrier (BBB) and may have a priority entry to the brain, assessing the real safety of EVs vs MSCs is a required step before really translating them to the clinical practice, for the treatment of neurological diseases.

Concerning their safety, EVs do not undergo malignant transformation, as cells might do, even though MSCs are less prone than other cell types in this regard. Moreover, EVs are less immunogenic due to their size, and even side effects related to their administration (obstruction of small vessels or accumulation into lung or liver) should be really restricted. However, concerns about the adequate druggable amount in order induce beneficial effects, or the content characterization of EV preparations, still need to be further investigated [212].

Given these open questions, researchers are continuously looking for hints to further improve EV potential, possibly by engineering them with selected active molecules or drugs. Many efforts have been done to increase specific ligand-receptor (interaction)-mediated endocytosis, or deliver targeted miRNAs and siRNAs for gene expression regulation [222]. In conclusion, due to their advantages compared to cell-based therapies, EVs remain a striking, powerful tool to be addressed for future therapies.

## 1.4 INTERLEUKIN -6 AT THE SUMMIT OF SEVERAL BRAIN DISEASES

In the late 1960s, in the attempt to characterize the capacity of both T and B cells to produce antibodies, Miller and colleagues observed that supernatants derived from cultured T cells were able to stimulate the activation and the proliferation of B cells, thus hypothesizing the existence of specific molecules produced by T cells to stimulate B cells [223, 224]. Once these molecules were characterized, Kishimoto and colleagues kept referring to them as “B cell differentiation factors”, or “B cell growth factors”.

Twenty years later, one of them was cloned and further characterized by the same group. It was later on renamed Interleukin-6 (IL-6) [225]. Truthfully, in the first years after its discovery, there were some inconsistencies about the name used to refer to it: in fact, it was also termed B cell stimulatory factor 2 (BCS-2) [226], as novel well characterized interferon beta (IFN-B2) [227], even if it was later demonstrated that such factor does not bear the IFN activity at all [226], hybridoma plasmacytome growth factor [228], or hepatocyte stimulating factor, being able to induce severe acute phases immune responses [227, 229].

In parallel, Shabo and his group observed that the macrophage and granulocyte inducer type 2A (MGI-2A), responsible for myeloid precursors’ activation, shared the same characters as that of BCS-2/IFN-B2 and was definitely recognized as interleukin 6 as well [230]. Taken together, all these distinctive evidences strengthened the hypothesis that interleukin 6 played a very important role in the immune system activation and functioning.

### 1.4.1 IL-6 is a pleiotropic cytokine

Despite the central role of IL-6 in regulating B cell response, other biological functions have been subsequently ascribed to this cytokine: if facts, it seems that IL-6 serves as a pleiotropic molecule, as it is able to support the growth and differentiation of T cells, and induce the cytotoxic T cell phenotype, through the enhancement of IL-2 receptor/IL2 cytokine expression [231]; it may also trigger macrophage differentiation and activation, or induce endothelial cells to express chemoattractant protein 1, thus in turn promoting monocyte recruitment [232]. Overall, it comes to exert a fundamental role in immune response activation; in fact, since IL-6 knock-out mice show deficit in recruiting leucocytes to the site of inflammation 16 and an impaired IgG production over immunization [233].

Interestingly, IL-6 has also been described as a signalling recruiter of mesenchymal vascular cells, contributing to neoangiogenesis *in vivo* [234]. Other evidences demonstrate that IL-6 takes part even in damages occurring in cartilage or bone [235]: it lowers aggrecans and type II collagen expression in chondrocytes.

Indeed, functional pleiotropy and redundancy are characters of all cytokines, a superfamily of signalling molecules which include interleukins, interferons, colony-stimulating factors, and many growth factors. It is now well known that due to the high number of existing cytokines, they are often playing overlapping roles. As an example, IL-6, together with IL-1β and TNFα represent remarkable players in inflammatory states [232].

However, although it is mostly described as a pro-inflammatory cytokine, IL-6 seems to play beneficial roles such as promoting tissue regeneration and anti-inflammatory effects, depending on the molecular pathway IL-6 undertakes [236].

### 1.4.2 IL-6 molecular signalling pathway

IL-6 represents a small molecule that requires the corresponding IL-6 receptor to get activated after release, thus triggering secondary signalling pathways in the recipient cells.

IL-6 receptor has been long studied and characterized [237]. The active form of IL-6 receptor is composed of two polypeptidic chains, represented by the receptor *per se*, an 80 kDa polypeptidic chain (IL-6 R), and the glycosylated type I 130 kDa membrane protein, which stands for a signal transducer (gp130) [232, 238-241]. When IL-6 binds to its receptor, which can exist either in a soluble (sIL-6-R) or a transmembrane (mbIL-6-R) form, it associates to gp130, thus triggering all downstreaming events. Specifically, in order to get the functional IL-6/IL-6 receptor complex, a hexameric complex composed of two IL-6 molecules, two IL-6 of 80 kDa and two gp130 proteins is built up [242-244]. Other authors suggest that even a tetrameric complex (IL-6/IL-6-R/2 gp130) may occur [243]. It is indeed the gp130 dimerization that triggers the next sequential events.

Intriguingly, the expression of gp130 is widespread among a variety of cells. Hibi and colleagues found significant mRNA levels in different cell lines, from myeloma cells, to lymphoma, hepatoma line, glioblastoma, and even bladder carcinoma cell lines. [238]. Moreover, other members of IL-6 superfamily share gp130 as the ultimate signal transducer, such as, leukaemia inhibitory factor (LIF)

[245], IL-11 [246], oncostatin M [247], cardiotrophin [248], This commonly shared pattern of expression may justify IL-6 redundant functions.

Gp130 protein does not carry a specific catalytic domain and lacks intrinsic kinase activity, but it is non-covalently associated with Janus family tyrosine kinases (JAKs): once gp130 is activated by IL-6 binding to IL-6 R, it itself determines the activation of JAK tyrosine kinases [249]. JAK in turn triggers the tyrosine phosphorylation of STAT3 (signal transducer and activator of transcription 3), which dimerizes and becomes a fully functional transcriptional factor: in this way, it can exert both cytoplasmic effects and gene expression modulation after translocating into the nuclei.

There are two different molecular pathways IL-6 that can influence the final properties of IL-6 as pro- or anti-inflammatory molecule. When IL-6 is able to directly interact with mbIL-6 R on target cells, it undergoes the **classic signalling** pathway. Notably, this pathway has been found to evoke the activation of anti-inflammatory pathways on target cells in several mouse models [250] associated with STAT3-dependent pathways [236]. As an example, it has been demonstrated that IL-6 classical signalling supports regenerative effects on intestinal epithelial cells after damage and prevents epithelial cell death *in vivo* [251].

The second pathway through which IL-6 can be activated is named **trans-signalling** pathway and it relies on its binding with soluble IL6-R, [252]. This event occurs in chronic inflammatory disorders like Crohn's disease and rheumatoid arthritis, and is characterized by the direct recruitment of immune cells in the inflamed area [253, 254], with the activation of monocytes and the inhibition of both T-cell apoptosis and T-reg differentiation.

### 1.4.3 IL-6 in health and disease

As a result of the wide expression of its signal transducer, IL-6 is involved in a plethora of activities, and it takes part not only in the immune system activation during disease, that have been deeply investigated and widely accepted by the scientific community [255-261], but also plays a role as novel trophic factor.

The positive role of IL-6 in the system becomes clear when observing its pro-survival effects on sensory neurons [262, 263], on retinal ganglionic cells [264] and pancreatic beta cells [265]; it also supports the growing of neuronal arbors in mice [266], and tissue regeneration, as observed for liver [267, 268], intestinal epithelium [269, 270] and muscle, after injury, by directly stimulating muscle stem cells [271]. A role of IL-6 in the control of glucose- lipidic metabolism has also been described [272-274], being this cytokine capable to decrease insulin resistance and exert a protective role on the liver.

All these evidences demonstrate that IL-6 action is nearly ubiquitous, and its roles cannot only be ascribed as pro-inflammatory, inducing autoimmune activation, but somehow, also beneficial.

### 1.4.4 IL-6 in brain

The brain is another organ where IL-6 signalling plays a role [275-277]. Both IL-6 mRNA and protein expression were found in several brain regions and released by different cell types, in basal conditions [275, 278-281] suggesting its beneficial role in the normal development. Despite this evidence, IL-6 basal levels in the brain are very limited represented, under physiological conditions. However, its levels appear dramatical upregulated in a wide range of neurological diseases,

such as neurodegenerative diseases AD [281-285], PD [280, 286-288] or MS [289-292].

However, as aforementioned its pleiotropic activity, IL-6 can indeed play a role in normal, physiological processes in the brain [293] as suggested by an increasing number of evidences. In fact, IL-6 induces survival, proliferation, differentiation and regeneration of neurons [279, 294-300]; it controls neurotransmitters' release and has a positive influence on the cellular fate of neural progenitor cells (NPCs) [301]. IL-6 also promotes the oligodendrocytes differentiation [302] as well as peripheral nerves regeneration after injury [303].

Conversely, depending on the activated pathway and the environmental stimuli, IL-6 can also trigger very destructive effects in the nervous system, driving neurotoxicity which is usually followed by neuronal degeneration, and functional impairments. Conrey and colleagues observed that IL-6 treatment brings about highly cell damage and death, in cultured cerebellar granule cells [304], but another group described a neuroprotective effects of IL-6, against N-methyl-D-aspartate (NMDA)-induced excitotoxicity, in cerebellar granule neurons [305]. Significant levels of IL-6 have been described also in the serum of schizophrenic patients [306-308], in the context of maternal immune activation [309], and even in autism [276, 277, 310-315].

### 1.4.5 IL-6 and the autism

Autism represent one of the most severe neurodevelopmental disorder, which is characterized by cognitive impairments, restricted sociability, verbal and non-verbal communication deficits, as well as disrupted behaviours. There are

several evidences that suggest the presence of dysregulated immune responses, in association with aberrant cytokine patterns [276, 277, 311, 312, 316, 317].In particular, IL-6 levels in autistic patient are found increased in more than one district: in plasma [318], lymphoblasts [319], peripheral blood cells [320] as well as in the brain [321-323], suggesting a significant contribution of IL-6 to pathology of this disease.

Intriguingly, there are opposing evidences describing that patients with high functioning autism do not bear high levels of IL-6, but of other cytokines, such as IL-1beta, IL-1RA, IL-5, IL-8, IL-13, IL-1, in their plasma [324].

Indeed, an objective role of IL-6 in patient with autistic spectrum disorders (ASD) is not possible to be described, because the same molecule can behave in a good or a bad manner, according to the cellular environment and molecular signals the releasing cell is subjected to.

Considering the current state of the art, no evidences have been described about the role of IL-6 on the functional network development, nor, more specifically, on the GABAergic system development.

# 2

# AIMS OF THE WORK

The aim of the present study was to add pieces of knowledge in the comprehension of the molecular underpinnings that govern the “neuronal GABAergic switch”, a critical event that occurs early in life before birth, during brain development, and whose impairments have been associated to a remarkable excitation/inhibition network imbalance [55, 325]. These impairments would in turn lead to severe mental retardation, cognitive disabilities and behavioural deficits, typical hallmarks of neurodevelopmental disorders [95, 326].

In particular, actions of two different types of regulators have been evaluated: 1. *the modulation played by mesenchymal stem cells and their derived vesicles and* 2. *the specific role of one of the most important immune system molecules, interleukin 6*.

Hence, the attempt of this work could be to answer to few questions regarding these two distinct projects.

***1) Do MSC-released vesicles affect the GABAergic switch?***

It is well known that MSCs exert positive roles as immunomodulators [327] and they also provide the trophic support for neighbouring cells, by influencing and reprogramming the surrounding microenvironment. In addition to that, several studies described MSC immunoregulatory and neuroprotective effects in immune-

mediated diseases and neurological diseases, as they support neuronal growth, neuritogenesis [328] and axonal regeneration [329], as well as in preventing neuronal death [328, 330].

Hence, in the attempt to consider their potential therapeutic use in neurodevelopmental diseases, where the GABAergic developmental switch is known to be impaired or delayed, the questions we tried to address are presented as following:

*i) May MSCs positively contribute to the neuronal GABAergic switch, by impacting the intracellular chloride levels?*

*ii) Do MSCs exert their action on neuronal switch by directly communicate with neurons or via constitutively -released soluble factors?*

Great evidences from literature are considering extracellular vesicles better candidates than the whole cells for clinical applications. The advantages of their use are related to more safety and least side effects than cell-based therapies [199, 331-333]. Indeed, they would still bear all the advantages of the parental stem cells, MSCs, but avoid all that concerns related to the presence of whole cells (i.e. immune activation) [334-337]. Moreover, their positive role in supporting neuronal growth and survival have been widely described [222, 338-340]. For these reasons, our interest focused on the action of the EVs in the modulation of the GABA switch:

*iii) Do the MSC-derived pool of vesicles have a different impact on the GABAergic signalling, compared to whole cells?*

*iv) What is a possible mechanism of action of MSC-derived EVs on the GABAergic switch?*

***2) May IL-6 be involved in the regulation of the GABAergic switch?***

Given its fundamental role in the immune system activation [225, 232, 236] and also within the brain, increasing number of studies introduce IL-6 as a pleiotropic cytokine and a novel trophic factor, as it induces neuronal survival, NPCs proliferation and even neuronal regeneration [232, 264, 266, 293, 341]. Nevertheless, several observations also found IL-6 involvement in neurodevelopmental diseases, such as autism [276, 310, 318, 321, 322].

For all these reasons, we wanted to explore the role of IL-6 in the timing of the GABAergic switch.

In particular, we carried out this study with the attempt to achieve the following goals:

*a) How does IL-6 affect the neuronal GABAergic switch?*

*b) What is the pathway IL-6 acts through?*

*c) Is the GABAergic transmission modified by IL-6?*

Experiments performed for these two distinct lines of work have been designed to discover novel regulators of the GABA switch and to reach a better comprehension of the basis underneath this regulation: the first line has been carried out to find out potential safe candidates that can be considered for possible interventions in pathological conditions, where the switch is impaired. In parallel,

the second line of study aimed at describing the novel contribution of one of the major players in immune activation, IL-6, in modulating this critical event.

# 3

# MATERIALS & METHODS

### 3.1 Primary bone marrow MSC cultures

MCSs were isolated from bone marrow extracted from tibias and femurs of P30 male C57Bl/6 mice. Animals were euthanized according to ethical procedures driven by European Directive 2010/63/EU and the subsequent Italian Law of 6th August 2013, n.96. Tibias and femurs were removed and collected in cold PBS without Calcium/ without Magnesium (-/-) 1X.

After muscles and tendons removal, bones were rinsed in fresh PBS -/- 1X. Epiphysis were removed, and the bone marrow was collected into a petri dish filled with fresh culture medium (alpha-MEM, Lonza), by flushing out from the bone with an insulin syringe filled with the same medium. Bone marrow from all bones was flushed out in the same medium, then collected, centrifuged at 800 rpm for 10 minutes.

After medium removal, the pellet was re-suspended in ammonium chloride $NH_4Cl$ and subjected to red blood *cell lysis*, then centrifuged again and re-suspended in complete medium (alpha-MEM; 20% FBS; 1% Pen/Strep (Lonza); 1% UltraGlutamine 200 mM (Lonza).

MSCs were seeded in 25 $cm^2$ culture flasks in a humidified atmosphere at 37°C and 5% CO2. After 48 hours, non-adherent cells were removed, rinsed with

PBS -/- 1X and then fresh medium was added. Fresh medium was replaced every 3 days. Five days after the seeding, a fibroblast-like colony started to become evident. When reached sub-confluency, cells were detached with 0.25% trypsin containing 1mM EDTA for 5 min at 37°C, and subsequently expanded, first in 75 $cm^2$ flasks, and then in 175 $cm^2$ flasks. Cultures from passage (P) 6- to P14 were used to perform all the experiments.

### 3.2 Primary hippocampal cultures

24 hours before dissection, 6 well plates have been prepared. 24mm diameter coverslips were placed into each well and coated with filtered Poli-Lysine (SIGMA) in 0.1% in Borate buffer (Boric Acid 50 mM, Borax 15 mM) pH 8.5, at 37°C. The day of dissection, plates were washed with sterile water and let them dry under sterile hood.

Primary cultures of hippocampal neurons (HNs) were obtained from 17-18-day-old mouse embryos (E17-18). Brains were collected in fresh 1X HBSS (Hank's Balanced Salt Solution) (starting from HBSS 10X (Life technology), 1% Pen/Strep, Hepes 10 mM) and dissected under the stereomicroscope, in sterile conditions. After meninges were removed, hippocampi were excised out from the brain and collected in fresh HBSS 1X. Hippocampi from all embryos underwent enzymatic dissociation (Trypsin 1%) followed by a mechanical dissociation.

Finally, neurons were counted and 80.000 were seeded in single drop onto each coverslip. After 3 hours from plating, 2 ml of complete Neurobasal [(Life

Technology), with 2% B27 supplement (Life Technology), 1% Glutamax (Life Technology), 1% Pen/Step] were added to each well.

### 3.3 Immunophenotyping MSCs

When maintaining in culture, MSCs were spontaneously selected for their plastic –adherence, whereas contamination from other cell types gradually disappear. After few passages, needed to enrich the culture with MSCs and to reach an appropriate number of cells, MSCs were immunophenotyped in order to check their stemness properties.

Fluorescence Activated Cells Sorting analysis was performed to check for positive signal to SCA1, CD73, CD105 (stemness markers) and negative markers as LIN, CD117, CD31. Cells were sorted with FACS Canto II, analysed and plotted with Flowjo Software.

### 3.4 MSCs in vitro commitment towards osteogenic and adipocytic lineages

MSC ability to differentiate towards adipocytic and osteogenic lineages has also been tested.

Regarding osteogenic differentiation, MSCs were plated into 6-well plates at 25.000 cell/well and cultured for 21 days in complete medium supplemented with 5% FBS, dexamethasone $10^{-8}$ M, ascorbic acid 0.3 M and h-glycerophosphate 10 mM (SIGMA), refreshing the medium every 2 days. After 21 days, Alizarin Red staining was performed in order to reveal the presence of bone minerals.

In parallel, also adipogenic differentiation was committed. MSC were cultured in alpha-MEM 5% FBS**,** dexamethasone $10^{-8}$ M, insulin 0.5 Ag/ml (Sigma), glutamine 2 mM and 1% Pen/Strep or Alpha MEM only, as control (CTRL) group. After 21 days, the presence of lipid droplets was assessed by staining with Red Oil O in order to verify the adipocytic differentiation.

### 3.5 MSCs - HNs co-cultures

Co-cultures with HNs and MSCs were set up. MSCs were seeded in 6-wells as monolayer (until reaching sub-confluency) and neurons were plated onto coverslips endowed with paraffin dots, in order to avoid any contact between the two cell types. Co-cultures was maintained in Neurobasal complete medium, by refreshing half of the medium every two days, until the day of the experiment.

### 3.6 EV isolation and characterization

EVs were purified according to Thery's protocol [342] modification. Briefly, MSCs, grown alone, were stimulated with Neurobasal medium (i.e. in serum deprivation). for 24 hours. The following day, the medium was collected and centrifuged at 400xg at 4°C for 10 minutes to discard dead cells; the supernatant was further centrifuged at 2000xg for 20 minutes to eliminate cellular debris.

At each step, the supernatant was collected and transferred to new tubes for the next step and the pellet was discarded. The supernatant from the last step underwent an additional centrifugation at 16000xg. The pellet from this passage

selected the microvesicle population (referred to as p3 or MVs). MVs were re-suspended in PBS 1x and stored at 4°C until usage.

The remaining supernatant was then transferred to ultracentrifuge tubes (Beckman Coulter) and subjected to ultracentrifugation at 280000xg for 70'. The pellet from this passage identify Exosomal pellet (referred to as p4 or Exo). Exosomes were rinsed in PBS 1X and centrifuged again before resuspension.

Protein quantification was made with bicinchoninic acid assay kit (Micro BCA Protein Assay Kit, Thermo Fisher Pierce™) according to the manufacturer instructions. EV (both MVs and exo) preparations were checked and also characterized with Nanoparticle Tracking Analysis (Nanosight) for particle size and concentration.

### 3.7 Calcium imaging

Cultured neurons from 4 to 7 DIV (Days In Vitro) were analysed by calcium imaging recordings, to assess the temporal window of the GABA switch and 6 DIV has been chosen as the best time for all experiments.

Cells were loaded with 5 μM Oregon Green™ 488 BAPTA-1, AM, cell permeant (Life Technology) in culture medium at 37°C for 45 minutes, then rinsed with external solution [Krebs'–Ringer's–HEPES (KRH): 125 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $KH_2PO_4$, 2 mM $CaCl_2$, 6 mM glucose, and 25 mM HEPES–NaOH], pH 7.4.

The coverslip was then mounted onto a round chamber and accommodated onto an Olympus inverted microscope equipped with Metafluor software (20x magnification). After choosing the proper Regions of Interests (ROI) centered to the nuclei, the recordings have been started. A puff of 100 µM GABA was then administered to assess GABA-dependent Calcium transients.

Analysis were made by considering the fluorescence $dF= F - F_0$, where $F_0$ represented the initial signal, and F the signal after GABA stimulus. $dF/F_0$ was considered as the final value describing the fluorescence increase after GABA stimulus. Changes over baseline $dF/F_0 < 0,10$ units in response to 100 µM GABA were considered depolarizing events. Hence, for each experimental condition, the number of depolarizing cells over that threshold was then plotted.

### 3.8 Chloride imaging

Intracellular chloride evaluation was performed using the N-(Ethoxycarbonylmethyl)-6-Methoxyquinolinium Bromide (MQAE) (Biotium) chloride sensor.

Neuronal cultures on coverslips were loaded with 5 µM MQAE for 1 hour at 37°C in culture medium. Coverslips were then rinsed with KRH and transferred to the recording chamber for acquisitions. Olympus IX81 inverted microscope, under 20X dry objective (Olympus, UPLFLN NA 0.5) provided with MT20 widefield source and control system with excitation 340 nm and emission filter centered at 500 nm was used.

Experiments set up and samples' recordings were made with Xcellence RT software (Olympus). For each sample, a minimum eight fields of interests (mean of cells observed for field, 20) have been chosen to perform live imaging recordings. Offline analyses were then made by drawing ROIs on cell bodies and measuring MQAE mean intensity during the time recording window, by using the same software. Normalized data of the mean intensities for each experimental group were plotted and subjected to statistical analysis with Graphpad Prism.

### 3.9 Experimental design

Regarding the first line of the work, hippocampal neurons have always been co-cultured with MSCs or fibroblasts from the first day after the culture establishment (1 DIV) and maintained in culture together until 6 DIV. Likewise, neurons alone were fed con MSC-CM or Fibro-CM from 1 DIV until 6 DIV, and then loaded with MQAE for chloride recordings or lysed for RNA extraction. Eventually, neurons alone were treated with 3 μg/die MVs or Exo from 1 DIV and after processed for chloride imaging recordings or RNA extraction.

For the second part of the work, according to the goal of each type of experiment, neurons were treated with IL-6 20 ng/ml, STATTIC 1 μM, STATTIC 1 μM + GABA 100 μM, Bicuculline 50 μM, Bicuculline 50 μM + IL-6 20 ng/ml at 1 DIV and 4 DIV and then used at 6 DIV for processing either with MQAE chloride imaging or mIPSC recordings, lysed for RNA extraction or fixed for immunolabelling.

### 3.10 mIPSCs on hippocampal cultures in vitro at 7 div

Hippocampal neurons were recorded at 7 Days In Vitro (DIV) in whole cell configuration using patch clamp recording.

Miniature Inhibitory Post-Sinaptic Current (mIPSC) were measured at a holding potential of -70 mV with Axopatch 200B (Molecular Devices) using the following pipette internal solution: 68 mM KCl, 68 mM KGluconate, 0,5 mM EGTA, 2 mM MgSO4, 20 mM Hepes-NaOH, 4 mM MgATP, 0,2 mM GTP, pH 7.2. Electrode with a tip resistance of 3-6 MΩ was pulled from a borosilicate glass capillary through a horizontal puller.

External recording solution contained.125 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $KH_2PO_4$, 2 mM $CaCl_2$, 6 mM glucose, and 25 mM HEPES–NaOH. TTX 1µM, AP5 50 µM and CNQX 25 µM (Tocris) were added to the batch in order to pharmacologically isolate mIPSCs. All records were performed at room temperature.

Data were filtered at 5kHz through a low-pass Bassel filter and collected at a frequency of 20kHz using Digidata 1322A digitizer (Molecular Devises). Only neurons with access resistance (Ra) < 20 MΩ were included in this study. Data were collected with pClamp10 (Molecular Devices) and traces analyzed with Mini Analysis (Synatosoft).

### 3.11 qRT-PCR

After completing the experiments, neurons at 6 DIV were rinsed with fresh PBS 1X and then lysed in 500 µl TRI Reagent® (Zymo Research).

RNA was isolated with RNA Direct-Zol™ MiniPrep Isolation Kit (Zymo Research) according to the manufacturer guidelines. RNA was finally eluted in 25 µl DNAse/RNAse – free water, quantified with NANOdrop 2000c spectrophotometer (Thermo Fisher Scientific) for RNA concentration and optical density 260/280 nm ratios.

500 ng RNA for each condition was reverse transcripted in cDNA with High Capacity cDNA RT kit (Applied Biosystems). Quantitative Real-time polymerase chain reaction (qRT-PCR) was performed with Sybr Green detection kit (SensiFAST SYBR Lo-ROX, Bioline) with RT-PCR Viia7 software system (Applied Biosystems) in a final volume of 10 µl. For each sample, every single gene was subjected to at least duplicate measurements.

Data analysis were performed with the comparative ΔΔCt method. RNA levels were normalized to housekeeping genes *gapdh* or *β3-Tubulin* as indicated. Sybr used oligos: mouse *kcc2*, Fw GGACCACTAGCTGACCTC, Rv CACCTGAGCCGTTTGATG; mouse *nkcc1*, Fw CCACCAGGAAACCATACCA, Rv AAGGCAGGCAAGTCTACC; mouse *gabrb1*, Fw TCCCGTGATGGTTGCTATGG, Rv CCGCAAGCGAATGTCATATCC; mouse *gabrg2*, Fw GCAACCGGAAACCAAGCAAGGATA, Rv GGTGGGTGGCATTGTTCATTTGGA; mouse *gabra3* Fw

ATGTGGCACTTTTATGTGACCA, Rv CCCCAGGTTCTTGTCGTCTTG; mouse *Tgfβ2* Fw ATCGATGGCACCTCCACATATG), Rv (GCGAAGGCAGCAATTATGCTG).

### 3.12 Immunofluorescence staining for GABAergic synaptic markers

Immunostaining for synaptic markers has been carried out on neurons at 6 DIV fixed in 4 % (w/v) PFA, 4 % (w/v) sucrose, 20 mM NaOH and 5 mM MgCl2 in PBS, pH 7.4, for 8 minutes at room temperature (RT).

Briefly, cultures were permeabilized and non-specific binding sites of proteins blocked with Goat Serum Dilution Buffer (GSDB;15 % (w/v) goat serum, 0.3 % (v/v) Triton X-100, 450 mM NaCl, 20 mM phosphate buffer, pH 7.4 for 30 minutes. Protein of interests were then stained with the following primary antibodies (Invitrogen): anti-guinea pig vGAT 1:1000; anti-mouse pGephrin 1:400, anti-rabbit β3-tubulin 1:100 in GSDB for 3 hours, at RT. After rinsing with PBS, coverslips were then incubated with the corresponding fluorophore-conjugated secondary antibodies (Invitrogen): 1:200 dilution was used for Alexa Fluor -488-conjugated anti-guinea pig IgG, Alexa Fluor 555-conjugated anti-mouse IgG and Alexa Fluor-647 anti-rabbit IgG, in GSDB for 1 hour at room temperature. Coverslips were then rinsed and mounted on using PBS with 70% glycerol mounting reagent containing 1 µl DAPI. Finally, they were sealed with nail polish and let dry before acquisition.

Images were acquired with Fluoview FV1000 Olympus IX81 (Center Valley, PA, USA) confocal microscope system provided with an 60X 1.4 NA oil immersion

objective (Plan-Apochromat; Olympus) using laser excitation at 405, 488, 594 and 647 nm, and processed using Fiji. Laser settings were unchanged for all images obtained from the different conditions.

### 3.13 Statistical analysis

Raw data from each experiment were plotted with GraphPad Prism 5 software and results were presented as means ± s.e.m. The normal distribution of experimental data was firstly assessed using D'Agostino-Pearson Kolmogorov Smirnov normality test. When comparing two normally distributed sample groups, Student's two-tailed unpaired test was considered. In case of sample groups not normally distributed, Mann–Whitney's non-parametric test was used to perform the statistical analysis. When comparing more than two sample groups, one- or two-way ANOVA, coupled to Bonferroni's multiple comparisons test was considered. Values of $p < 0.05$ units were considered statistically significant and showed on the graphs.

# 4

# RESULTS

## 4.1 SETTING UP THE SYSTEM

Before exploring the role of Mesenchymal Stem Cells and the released by-products (extracellular vesicles, EVs), as well as the role of the pleiotropic cytokine IL-6, in affecting the timing of the GABAergic switch in hippocampal neurons, a required premise was to set up all the procedures of our experimental systems. This premise included the validation and proof of reliability of cells and tools we took advantage of and the characterization of the temporal window where the GABA switch is accomplished, in naïve neurons.

### 4.1.1 Characterization of bona fide MSCs

Mesenchymal stem cells (MSCs) were isolated from tibias and femurs of adult mice bone marrow and cultured for several passages in order to promote MSC selection and enrichment. They appeared for their typical fibroblastic spindle-like morphology (**Fig. 4.1**) and they were used from passage from 6 to 14.

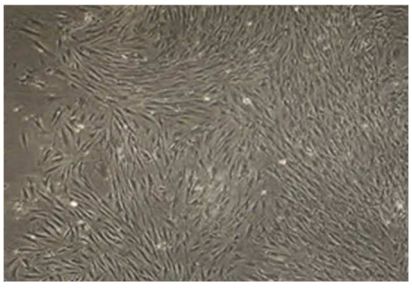

**Fig. 4.1 Representative image *of in vitro cultured MSCs.*** *MSCs showed fibroblastic spindle-like morphology and grown in colony forming units (CFUs).*

Moreover, stemness markers were monitored to ensure that cells maintain their properties in culture. By the Fluorescence Activated Cell Sorting (FACS) analysis (**Fig. 4.2**), cells showed very high expression of the ultimate stemness marker, ScaI, in the vast majority of cells (99.5%). The high ScaI positivity was accompanied by high CD-105 (92,8%).

Even if lower expression of CD-73 (8.5%) and CD-117 (7.5%) was determined, the presence of ScaI and CD-105 together with the virtually lack of expression of the "negative markers" such as CD-31 and LIN (Lineage) markers (0.1% and 3.5% respectively) encouraged us to verify differentiation ability *in vitro*, which is the most important feature for defining *bona fide* MSCs (**Fig. 4.3**).

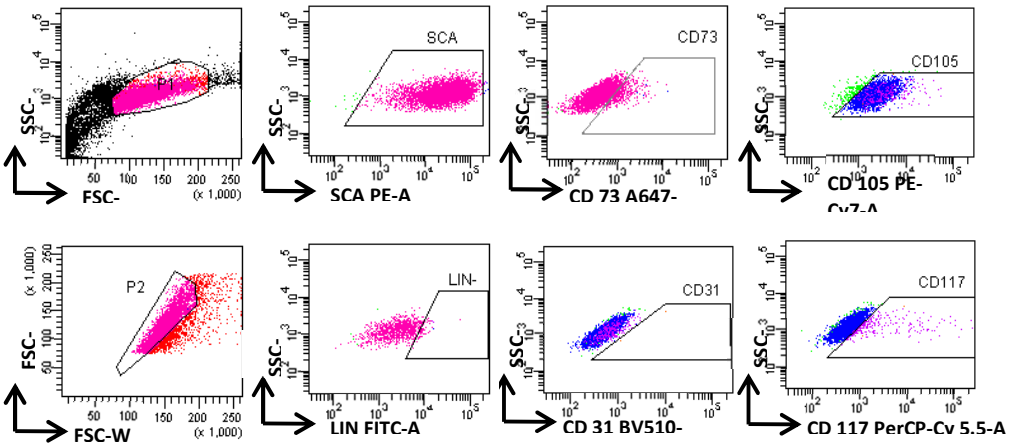


**Fig. 4.2** ***Representative traces of FACS analysis of cultured MSCs****. A considerable population of cells showed remarkable ScaI and CD105 markers' expression. Very low expression levels found for CD31, CD117 and Lin. Virtually lack of cells of Lin-.*

The differentiation protocol was successfully carried out, as after 21 days of culturing MSCs in defined differentiation media resulted in adipocytes and

osteocytes' differentiated cells, that have been confirmed with Oil Red O and Alizarin Red staining, respectively (**Fig. 4.3**).

MSCs from passage 6 (P6) showed strong differentiation ability, that was preserved until passage 14 (P14). These evidences made us confident to use MCS cultures within P6- P14 temporal window after culture establishment.

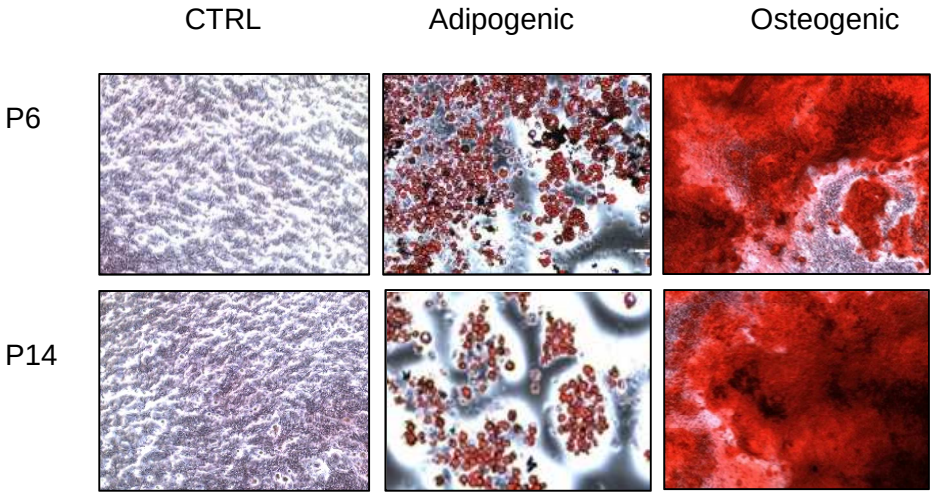


**Fig. 4.3** ***MSC differentiation ability at different passages (p6 and p14).*** *From left to right: un-differentiated control MSCs; Oil Red O-stained MSCs to identify lipidic droplets in adipogenic differentiation; Alizarin Red staining to visualize calcium deposits in osteogenic differentiation.*

### 4.1.2 Extracellular vesicle isolation and characterization

Extracellular vesicles (EVs) were isolated through differential centrifugation steps (see method section, chapter 3). The MV-enriched and Exo-enriched pellets were lysed for protein quantification. We ascertained that the yield was usually reproducible.

Moreover, with Nanoparticle Tracking Analysis (**Fig. 4.4**) we observed the presence of whole particles suitable for characterization and use, even though the centrifugation did not separate exclusively larger or exclusively smaller particles, but rather a MV- or Exo-enriched population. MV fraction included particles that covered a wide range of sizes, abundantly represented by particles 135 nm-, 200 nm-, 400 nm-sized. We found even 600 nm-sized (**Fig. 4.5, A**) vesicles. In contrast, the Exo-enriched pool showed sharp and more restricted size limits, with most of the particles representing around 105 nm and 165 nm size (**Fig. 4.6, B**).

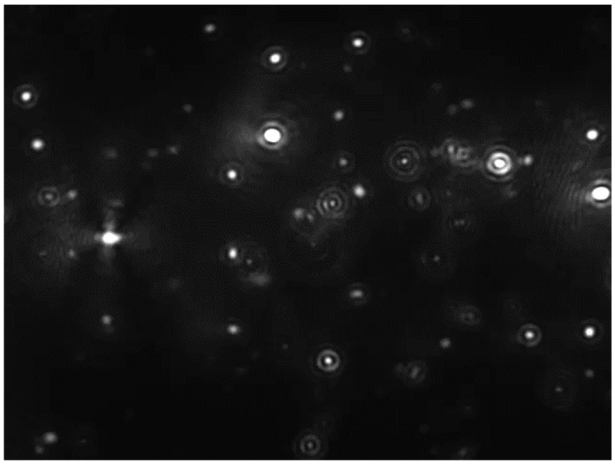

**Fig. 4.4** ***Representative image showing vesicle tracking for Nanoparticle Tracking Analysis (NTA) with NanoSight.*** *Note population of different sizes*

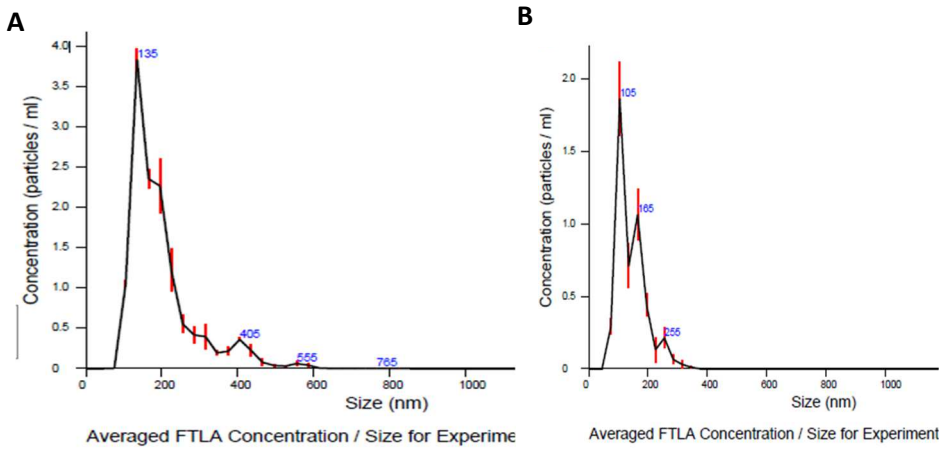


**Fig. 4.5** ***Representative NTA output analysis*** *of MV-enriched pool (**A**) and Exo-enriched pool (**B**)*

Regarding the concentration of the pools, by NTA software analysis we found that under appropriate 24-hour stimulus, MSCs could release a considerable amount of vesicles, being in the order of 10^8. and even if more precise and reliable, the measurement of whole particles with NTA was at least in line with protein quantification, that we used as a standard method for defining the amount of vesicles necessary for the treatment.

### 4.1.3 Monitoring the GABA switch in cultured hippocampal neurons

To precisely evaluate the timing of the GABA switch during the development of hippocampal neuron in vitro, we took advantage of both calcium and chloride imaging techniques. As already pointed out, the excitatory-to-inhibitory transition of GABAergic signalling implies changes in the intracellular chloride concentration leading to a different calcium responsiveness upon extracellular GABA stimulation.

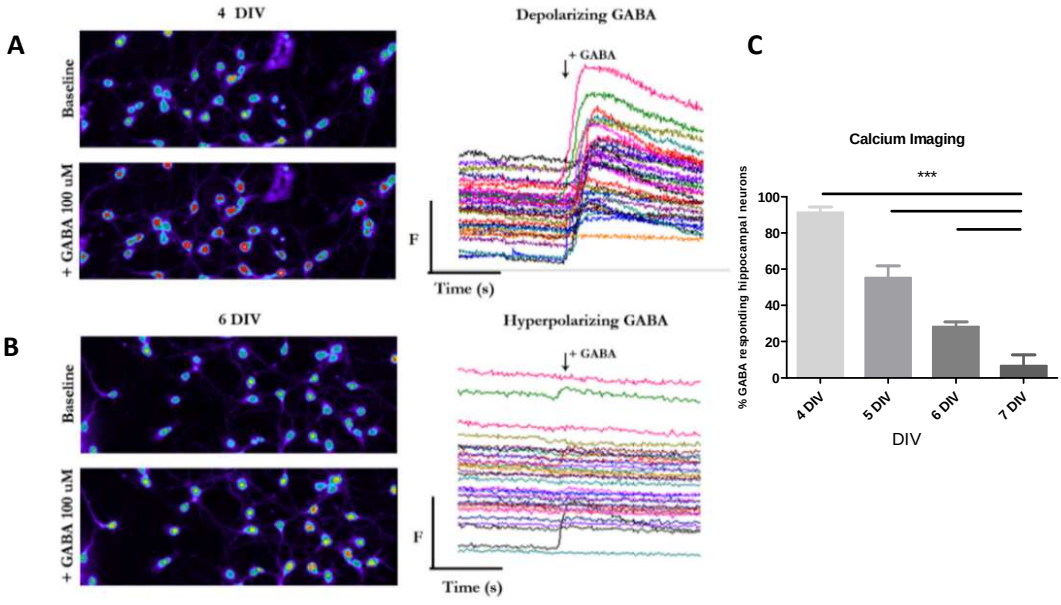


**Fig. 4.6** ***Representative traces of calcium imaging showing a progressive reduction of the depolarizing effect of GABA on neuronal development****. **A**: GABA-induced calcium transients occurred in virtually all neurons at 4 DIV; representative image (left) and traces (right), of depolarizing GABA. **B**: GABA-induced calcium transients at 6 DIV are still occurring although in a lower number of cells; representative image (left) and traces (right), of depolarizing GABA. **C**: Quantification of GABA responding-neurons during the 4-7 DIV time window. p<0.001*

Based on these considerations, we first measured GABA-induced calcium transients of cultured hippocampal neurons at different developmental stages (**Fig. 4.6)**. In parallel, through a specific chloride-sensitive fluorescence dye, MQAE, we directly measured intracellular chloride levels at the same stages assayed by calcium imaging, in order to associate the calcium response to GABA with intracellular chloride levels **(Fig. 4.7)**.

To this aim, neurons cultures were loaded with the calcium sensitive dye Oregon-Green and imaged by single-cell calcium imaging upon the exposure to 100 μM GABA. As shown in **Fig. 4.6, C,** at early stage of neuronal development (4 DIV), GABA elicited a calcium transient in most of the neurons in the recording field (>90%), indicating an excitatory action of GABA.

As neurons developed, such a percentage was progressively reduced; indeed only 60% of neurons showed a significant GABA-induced calcium transient at 5 DIV, 30% at 6 DIV, 10% at 7 DIV, becoming totally absent at later stages of development (10-12 DIV, not shown). This evidence is in line with a gradual loss of GABA excitatory effect reflecting a developmentally-regulated switch of GABA signalling.

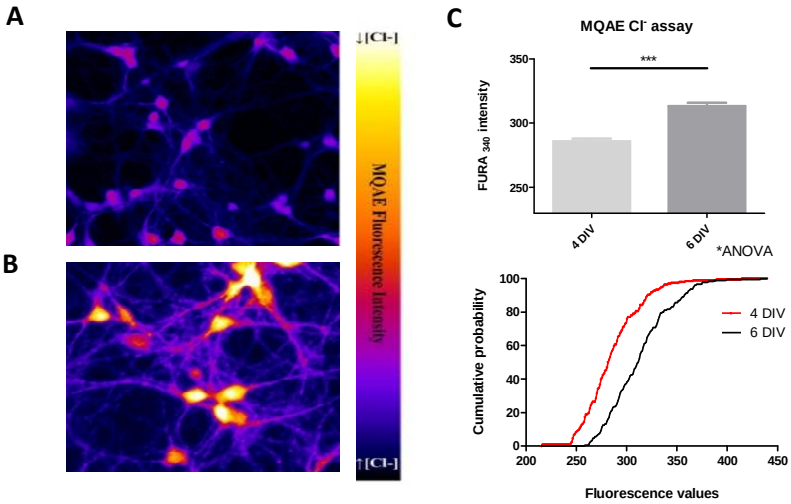


**Fig. 4.7** ***Chloride imaging recordings showing a progressive chloride decrease in neurons during development****. Representative images of MQAE-loaded neurons in* ***A:*** *high chloride concentrated neurons (low MQAE signal)* ***B****: low chloride concentrated neurons (high MQAE signal) and. Pseudo-colour ImageJ LUT applied to appreciate the contrast.* ***C****: MQAE mean intensity quantification (top) and cumulative probability (bottom) graphs for neurons 4 and 6 DIV showing the significant difference in intracellular chloride concentration where the GABA shift occurs.*

In parallel we directly evaluated intracellular chloride by means of the chloride sensitive dye concentrations MQAE (**Fig. 4.7**), whose fluorescence becomes quenched upon complexing with chloride ions [343-345]. Neurons were loaded with MQAE and fluorescence was evaluated at 4 and 6 DIV, the developmental window in which the percentage of neurons showing GABA-dependent calcium transients was strongly reduced (**Fig. 4.6**).

Interestingly, we detected a significant increase in MQAE fluorescence between 4 DIV and 6 DIV, as shown by a leftward shift of the cumulative probability of MQAE fluorescence intensities at these two stages (**Fig. 4.7, C**). It should be noted that although the transition of neurons from 4 DIV to 6 DIV causes a

reduction of about 70% of GABA-responding neurons, the associated change of MQAE fluorescence intensity was only of 10%. In line with previous results [55, 346], this suggests that slight alterations in intracellular chloride levels may be linked with a significant reduction of GABA excitatory action.

This data clearly indicates a marked reduction of intracellular chloride concentrations during this developmental stage thus explaining the reduction of neurons exhibiting a GABA-induced calcium response. Overall these results let us to state that cultured neurons established from E18 embryos are characterized by a well-defined temporal window (lasting almost 7 days) in which neurons undergoes a progressive switch of GABA signalling from excitatory to inhibitory.

In order to assess whether changes in intracellular chloride levels were associated with modifications of chloride co-transporters expression, NKCC1 ($Cl^-$ importer) and KCC2 ($Cl^-$ exporter) mRNA levels were monitored during hippocampal neurons development. The analysis of qRT-PCR (**Fig.4.8**) indeed revealed that NKCC1 mRNA levels was halved from 4 DIV to 8-12 DIV, whilst, in contrast, KCC2 mRNA levels showed a strong increase over the same temporal window, reaching the maximal peak at 12 DIV.

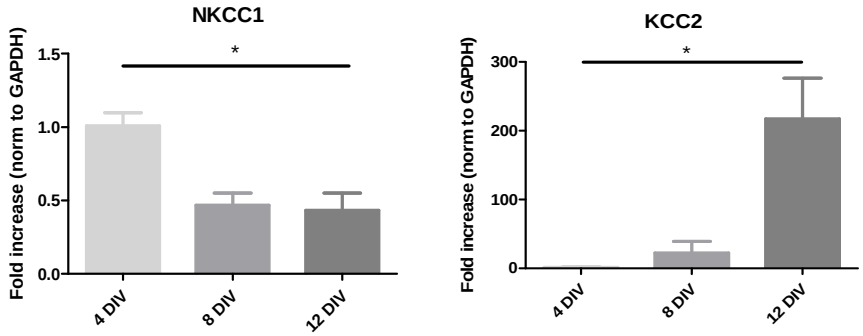


**Fig.4.8** *qRT-PCRs on hippocampal neurons showing the relative mRNA expression levels of NKCC1 and KCC2 chloride co-transporters over 4-to-12 DIV time-course. Note the different scale bar. * refers to p< 0.05.*

## 4.2 HOW DO MSCs AFFECT THE GABA SWITCH?

### 4.2.1 MSC co-cultured with HNs seem to delay the timing of $[Cl^-]_i$ decrease

In order to understand whether and possibly how MSCs could affect the neuronal GABAergic switch, as a first step we set up a co-culture system, where MSCs and HNs shared the same culture medium without being in direct contact (**Fig. 4.9, A**).

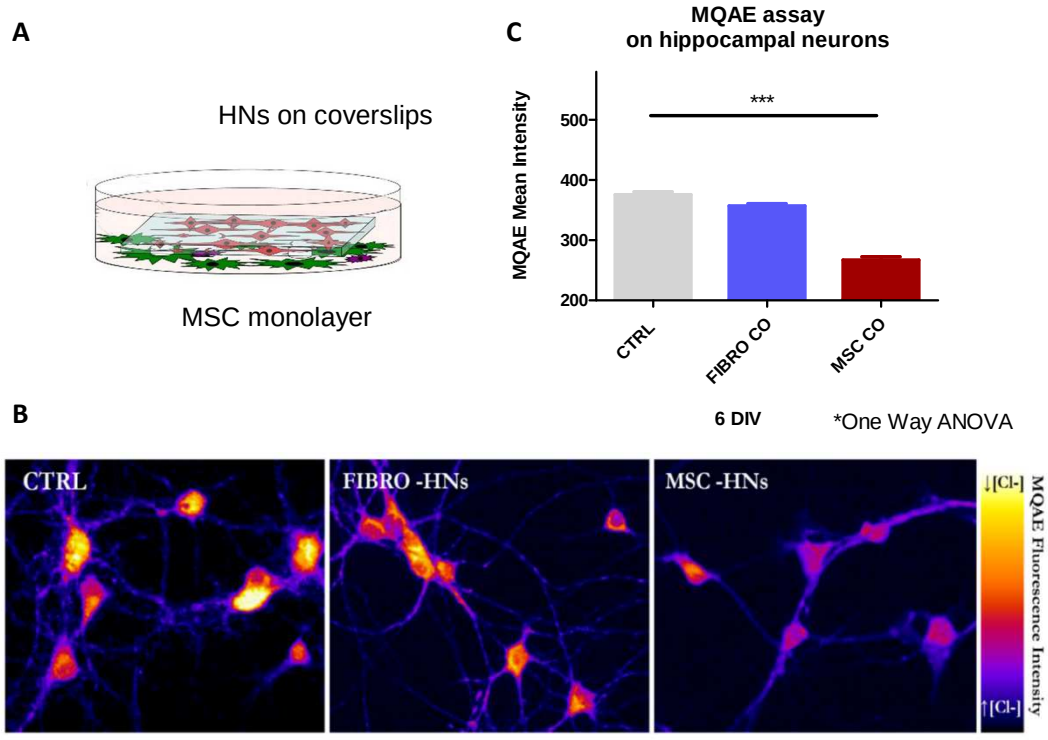


**Fig. 4.9** *HNs co-cultured with MSCs showed a delay in the timing of the chloride decrease.* ***A****: representative image of the MSC-HN co-culture;* ***B****: representative images of MQAE loaded neurons for chloride recordings;* ***C:*** *quantification of MQAE mean intensities in HNs in different experimental conditions. CTRL: control neurons, Fibro-HNs: neurons co-cultured with Fibroblasts as a negative control and MSC-HNs: neurons co-cultured with MSCs. HN-MSC cocultures were compared with HN grown alone, or HN grown in the presence of a monolayer of fibroblasts as a control cell type.*

MSCs were put in co-cultured with HNs from 1 DIV and maintained until 6 DIV. At 6 DIV, MQAE recordings were performed (**Fig.4.9, B**): the analyses revealed that neurons in co-culture with MSCs showed lower fluorescence intensity, meaning higher levels in intracellular chloride, when compared to fibroblast-HNs or HNs grown alone, both considered as control groups.

As shown in **Fig. 4.9, C**, neurons co-cultured with MSCs showed a strong statistically significant MQAE intensity reduction (up to 50%) in comparison with the control groups.

### 4.2.2 MSC-conditioned medium delay the timing of $[Cl^-]_i$ decrease

In the attempt to figure out whether soluble factors and/or EVs could be responsible of the observed increase of chloride concentration, MSC were conditioned in neuronal medium that after 24h has been collected (referred to as conditioned medium, CM) and administered to feed neurons from 1 DIV, until 6 DIV (**Fig. 4.10, A**).

At 6 DIV, neurons were monitored by MQAE assay (**Fig. 4.10, B**). Interestingly, MQAE average intensity indicated a delay in the timing of chloride decrease, as shown by the high intracellular chloride concentration, in line with what observed in MSC-HN co culture and even upon CM administration, the entity of MQAE decrease between treated and control neurons was found statistically significant ($p<0.001$; ***) (**Fig. 4.10, C)**.

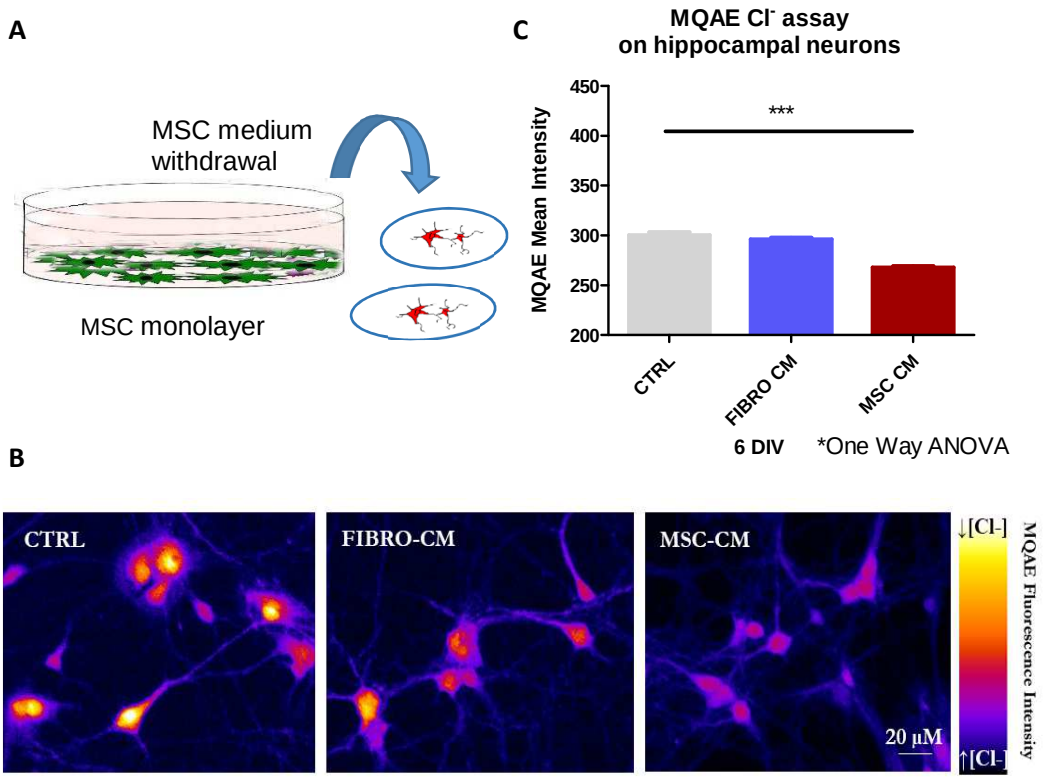


**Fig. 4.10** ***HNs maintained in the presence of MSC-conditioned medium (MSC-CM) showed a delayed GABA switch***. ***A****: representative cartoon of the experimental plan;* ***B****: representative images of MQAE loaded neurons for chloride recordings: comparison between control neurons (CTRL), neurons fed with Fibroblasts-conditioned medium (FIBRO-CM) as a second control, and neurons fed with MSC-conditioned medium (MSC-CM).* ***C****: quantification of MQAE mean intensities showing differences in HNs fed with media derived from control neurons, from Fibroblasts (FIBRO-CM) and MSCs (MSC-CM), (p<0.001 One Way ANOVA).*

### 4.2.3 MSC-derived EVs selectively accelerate the timing of $[Cl^-]_i$ decrease

The following step was treating neurons with the different extracellular pools of vesicles (MVs vs. exosomes) isolated from MSC-derived medium, by distinguishing neurons treated with microvesicles (MVs) and neurons treated with exosomes (Exo) (**Fig. 4.11, A**), with the aim of understanding if there could be a differential effect on the previously described chloride level changes.

Interestingly, by selecting extracellular vesicles, we appreciated chloride changes with MQAE recordings and in particular, we observed a different behaviour of neurons treated with MVs and neurons treated with Exo.

As showed in the figure (**Fig. 4.11, B**), neurons treated with EVs induced higher MQAE intensity levels in neurons when compared with controls, or treated with Exo.

In particular, the quantitative analysis of chloride dye mean intensities of recorded neurons (**Fig. 4.11, C**) reflects a statistically significant difference between untreated neurons and MVs-treated neurons, as clearly revealed by the cumulative probability graph (**Fig. 4.11, D**).

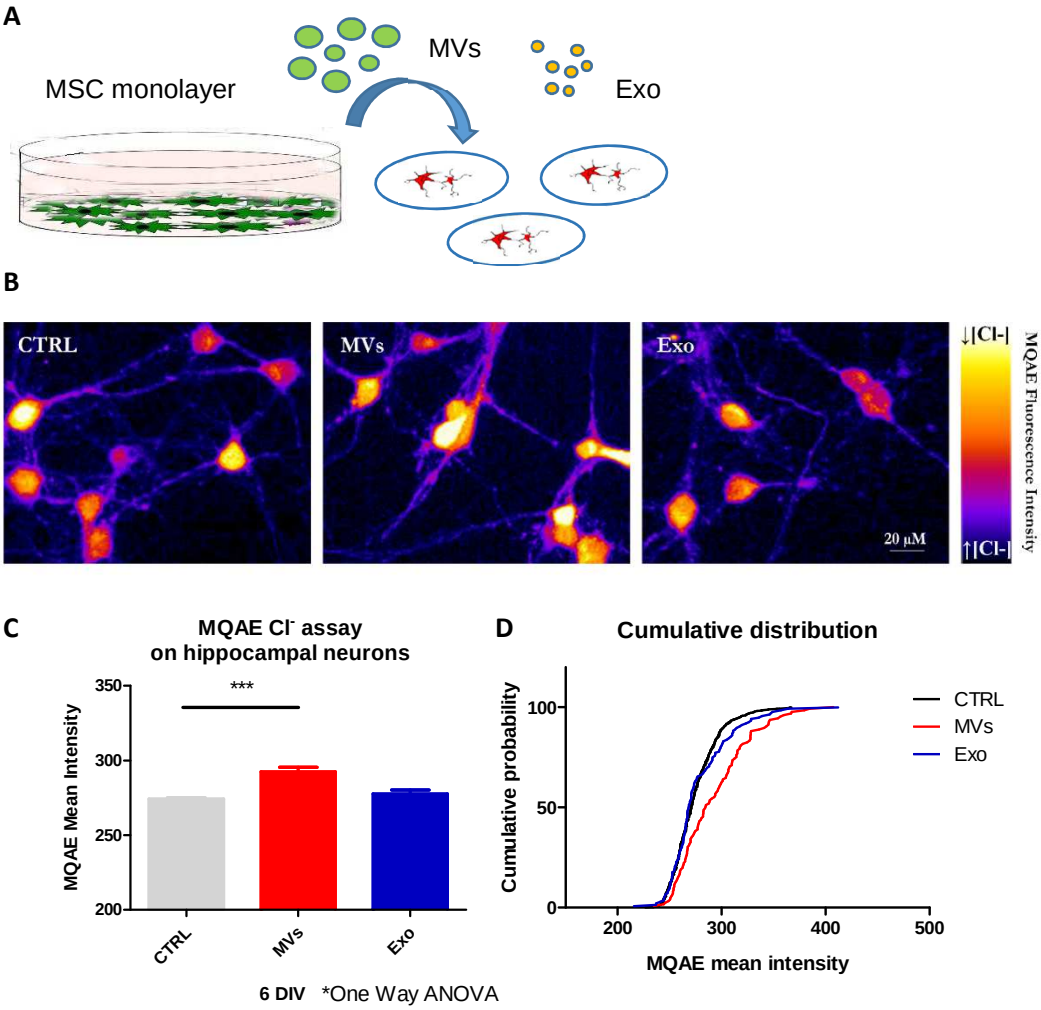


**Fig. 4.11** ***MSC-derived MVs or Exo differentially impacted the timing the GABA switch in treated HNs***. ***A****: representative cartoon of the experimental plan;* ***B****: representative images of MQAE loaded neurons for chloride recordings: comparison between control neurons (CTRL), neurons treated with MSC-derived MVs (MVs), and with MSC-derived exosomes (Exo).* ***C****: quantification of MQAE mean intensities and* ***D****: cumulative probability distribution showing differences in the three experimental conditions (p<0.0001, One Way ANOVA).*

### 4.2.4 MSC- MVs increase neuronal vGAT/gephyrin puncta

Given the interesting results obtained with MVs treatment, we explored how this pool of vesicles could impact the neuronal fate.

In particular, since the GABAergic switch is strictly related to the arrangement of GABAergic machinery, we assessed whether MSC-MVs could modify the expression of GABAergic inhibitory synaptic markers: the pre-synaptic vesicular GABA transporter, vGAT (**Fig. 4.12**) and a post-synaptic marker gephyrin (**Fig. 4.13**).

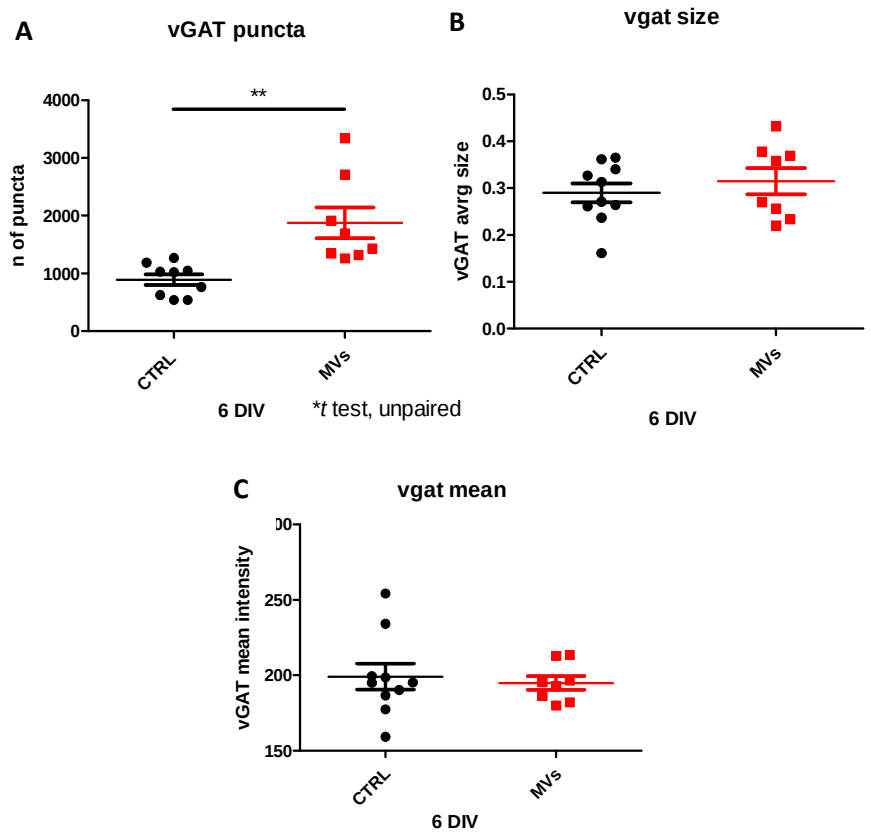


**Fig. 4.12** ***Quantitative analysis of vGAT immunolabelling***. *MV-treated and control neurons showed a significant increase in the number of vGAT puncta (p=0.0025; **, t test), but not in vGAT average size (p=0.4754) nor in the intensity of its expression (p=6965).*

After treating neurons with MVs from 1 DIV to 6 DIV with 3µg/MVs/die, we observed a significant increase in the number of vGAT positive puncta (p=0.0025; **), but not in the intensity of expression (p=6965), nor in their size (p=0.4754 (**Fig. 4.12, A, B, C**) on neuronal branches.

On the other side, we did not find any differences in the number of gephyrin positive puncta (p=0.3887) (**Fig. 4.13, A**), although a significant increase in the mean intensity of expression (p<0.001; ***) (**Fig. 4.13, B**), accompanied by a decrease in the size of the postsynaptic marker (p=0.0368; *) (**Fig. 4.13, C**) were detected.

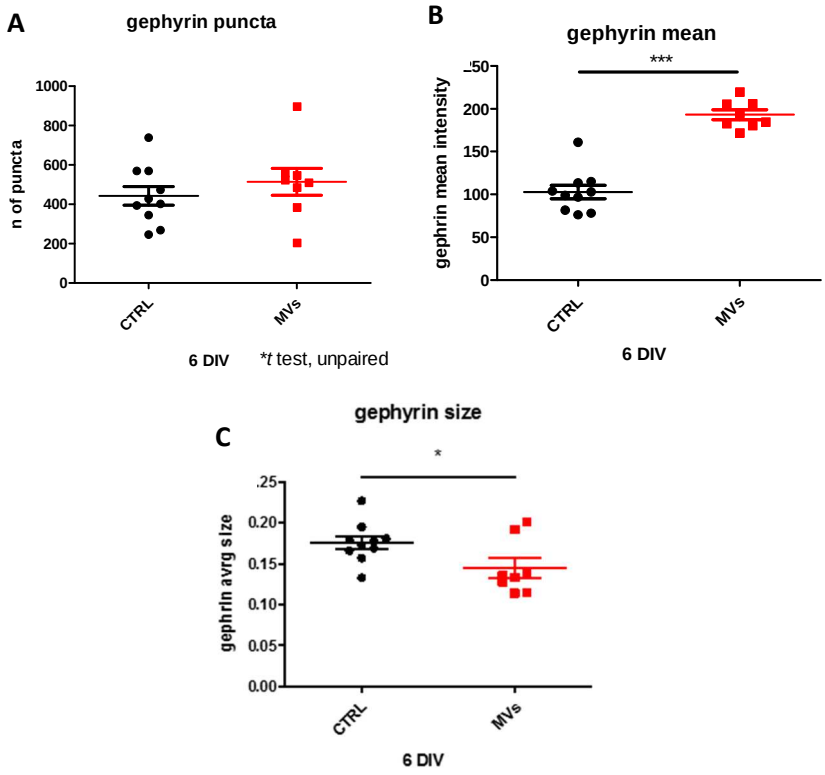


**Fig. 4.13** ***Quantitative analysis of gephyrin immunolabelling****. MVs-treated and control neurons showed a significant increase in the mean intensity of gephrin expression (p<0.001; ***, t test) accompanied by a decrease in the number of the average size (p=0.0368; *, t test), without any changes in the number of puncta (p=0.3887).*

When we checked the co-localization of the pre- synaptic and post-synaptic markers, we found a significant increase of co-localizing vGAT/gephyrin puncta in MV-treated neurons compared to control neurons (p=0.0326) (**Fig 4.14**), suggesting that MVs treatment boosted the development of GABAergic synaptic markers.

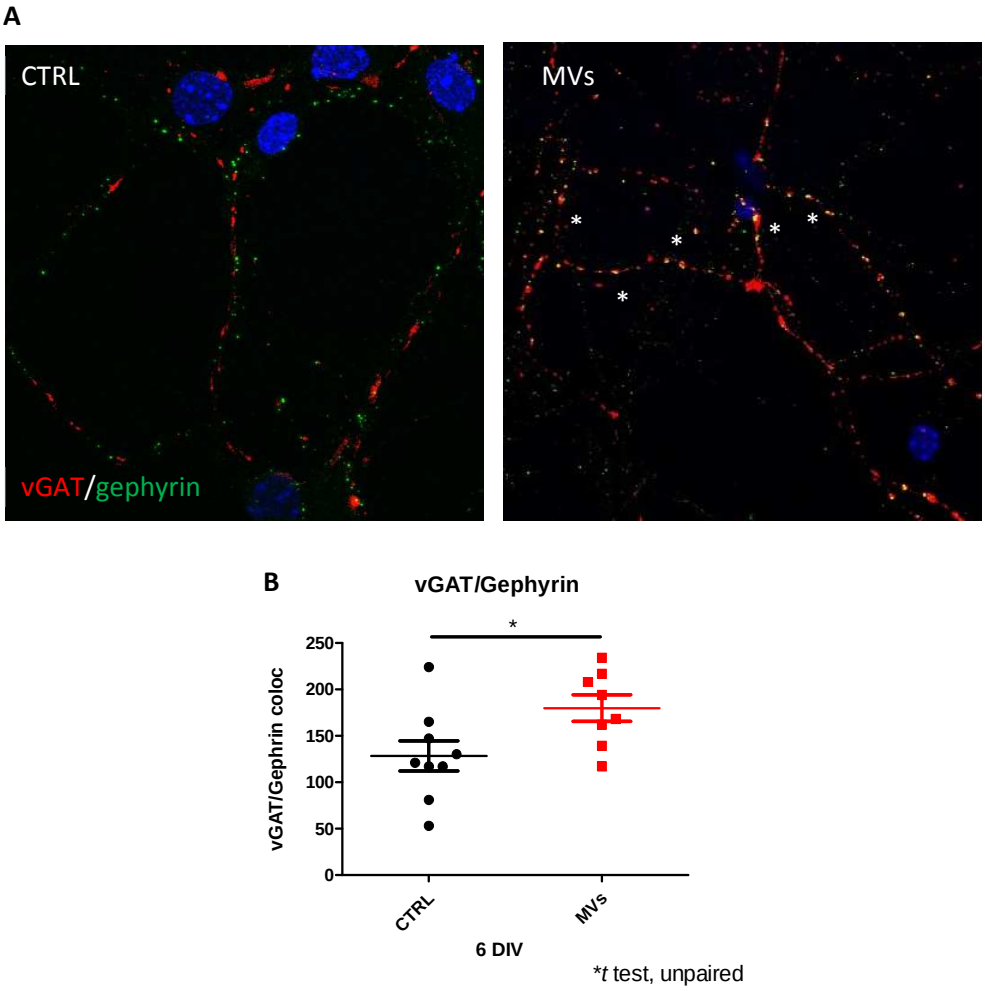


**Fig 4.14 MVs treatment increases vGAT and vGAT/gephyrin colocalizing puncta. A:** *Representative images of 6 DIV control and MVs-treated neurons immunolabelled for vGAT (red) and gephyrin (green); DAPI counterstain (blue), 60x magnification*. **B:** *Quantitative analysis of v GATt/gephyrin showing a significant increase in the number of co-colocalizing puncta in MV-treated neurons (p=0.0326; *, t test).*

### 4.2.5 MSC- MVs do not alter KCC2 and NKCC1 expression

In order to understand whether MSC-MVs could affect the intracellular chloride by targeting the transcriptional machinery, we then investigated the expression profile of the two chloride co-transporters NKCC1 and KCC2 in neurons treated with MVs.

Interestingly we did not find alterations at transcriptional levels in neurons that underwent MV treatment in comparison with control. In fact, as shown in the graphs, both NKCC1 (p=0.7813) (**Fig. 4.15, A**) and KCC2 (p=0.2208) (**Fig. 4.15, B**). mRNA relative expression levels of transcript seemed to be not significantly different.

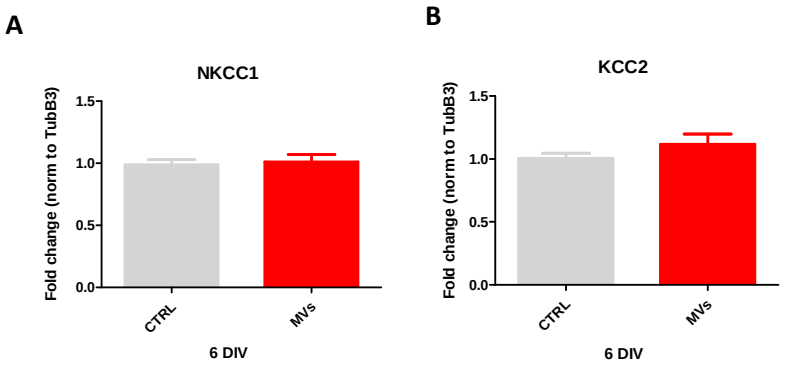


**Fig 4.15** ***MV treatment did not affect NKCC1 or KCC2 expression in hippocampal neurons****. qRT-PCRs on control, MV-treated neurons did not show any changes between in the relative mRNA expression levels of (**A**) NKCC1 (p=0.7813) nor of (**B**) KCC2 (p=0.2208).*

### 4.2.6 MSC-derived MVs increase TGF-β2 mRNA levels

Having observed unaltered transcriptional KCC2 profile, we wondered whether enhanced KCC2 membrane shuttling could be related to the lower chloride levels in neurons treated with MVs. To address this issue, possible involvement and changes in TGF-β2 expression, known in literature to promote KCC2 shuttling into the membrane, have been investigated.

As a result of MV treatment, 6 DIV neurons showed an increased TGF-β2 mRNA levels, in comparison with control neurons (**Fig. 4.16**). Even though a significant effect could not be detected, possibly due to the low number of observations performed, a clear trend of TGF-β2 increase could be indeed observable.

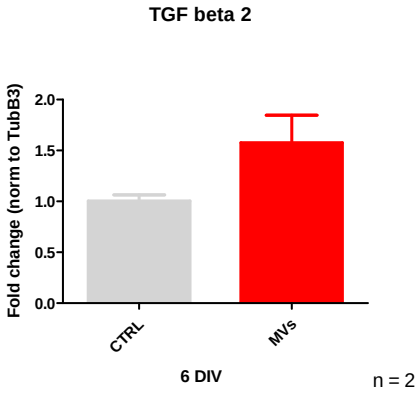


**Fig. 4.16 MVs seem to increase *TGF-β2 mRNA levels***. *A trend in TGF-β2 upregulation upon MV treatment evaluated by qRT-PCR is detectable.*

## 4.3 EXPLOITING THE ROLE OF IL-6 IN DEVELOPING NEURONS

In a parallel project, we wanted to exacerbate the role of the pleiotropic cytokine interleukin 6 in the modulation of the GABAergic switch in developing neurons. Although evidences reported in literature indicate a role of IL-6 in modulating neuronal function, whether IL-6 can affect the GABA switch is still unknown.

### 4.3.1 IL-6 promotes the GABAergic switch in cultured neurons at 6 DIV

To address this issue cultured neurons were treated with IL-6 at 10 and 20 ng/ml from 1 DIV to 6 DIV, and subsequently assayed through calcium imaging and MQAE chloride imaging recordings.

Calcium imaging experiments performed at 6 DIV revealed that IL-6 treatment reduced the percentage of GABA-responding neurons (**Fig 4.17, A-B**) in a dose-dependent manner, becoming statistically significant at 20 ng/ml ($p<0.05$; One Way ANOVA).

In parallel, we also observed that IL-6 treated neurons exhibited a higher MQAE mean intensity value if compared to control condition ($p<0.001$; One Way ANOVA) (**Fig 4.17, C**)., thus indicating that the lower percentage of GABA-responding neurons may be the result of a reduced intracellular chloride levels.

Being the chloride imaging a more precise and quantitative method to study the GABA polarity, we then relied to this tool for our next approaches, and we used IL-6 20ng/ml concentrated.

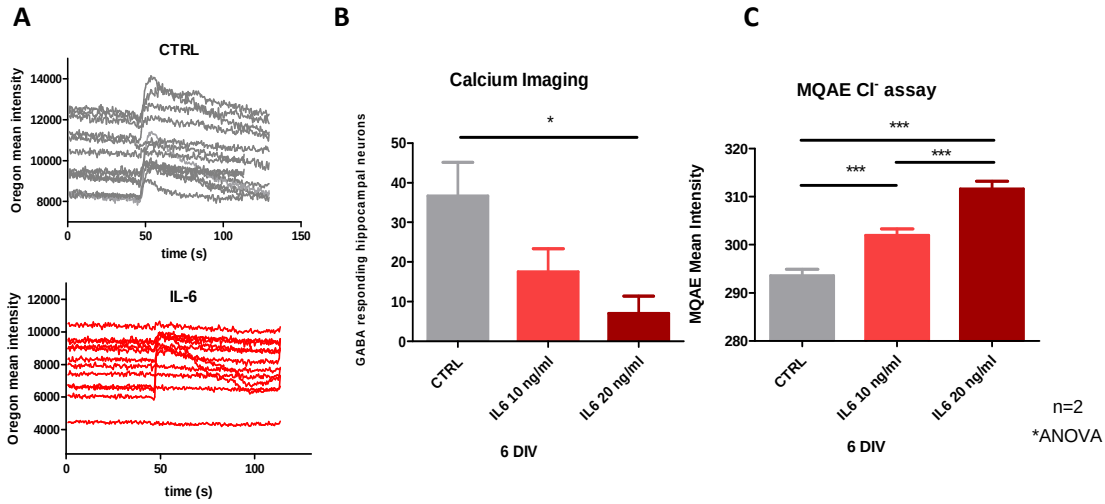


**Fig. 4.17** ***IL6 accelerates the timing of the GABAergic switch***. ***A****: Representatives traces of calcium imaging recordings, CTRL and IL-6-treated neurons, and* ***B*** *the relative graph showing IL-6 dose-dependent way of action, becoming significant at 20 ng/ml.* ***C****: MQAE chloride imaging showed that IL-6 promoted high MQAE intensity, meaning low intracellular chloride levels.*

Moreover, even a single transient exposure of IL-6 at 1 DIV for 2 hours was sufficient to reduce intracellular chloride level, as indicated by a higher MQAE fluorescence intensity (**Fig 4.18, A**). Eventually, we also observed that the effect of IL-6 on chloride levels is a long-lasting phenomenon, indeed a single exposure of IL-6 increased MQAE intensity even at later stage of development, 12 DIV, as observed in the graph (**Fig 4.18, B**).

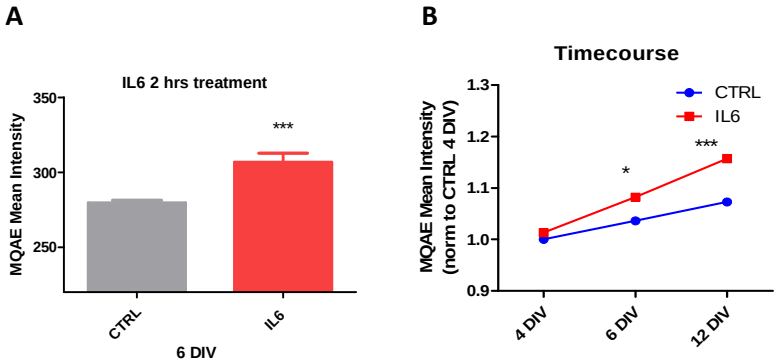


**Fig 4.18** ***Fast and long-lasting effects of IL-6 on neuronal chloride levels****. **A**: a single early exposure of IL-6 to neurons, resulted in lower chloride levels (increased MQAE intensity) at 6 DIV neurons (p<0.0001, t test). **B**: IL-6 effects on chloride decrease are observed even at later timepoint: 6 DIV p<0.05; 12 DIV p<0.001; Two Way ANOVA.*

Taken together, all these findings indicate that IL-6 exposure at early stages of neuronal development can promote the timing of the excitatory to inhibitory shift of GABA signalling.

### 4.3.2 IL-6 exposure impacts KCC2 expression in developing neurons

In order to explore the molecular underpinnings of the IL-6-dependent effect on the GABA switch, by means of qPCR we investigated the transcriptional profile of the two main chloride co-transporters (NKCC1 and KCC2) upon IL-6 treatment, which are responsible for this process.

Interestingly we found that neurons treated with IL-6 showed higher KCC2 mRNA levels respect to control (**Fig. 4.19, A**), whereas NKCC1 mRNA levels were found to be unaltered (**Fig. 4.19, B**). Since KCC2 acts as a chloride importer, its

higher expression is in line with the lower intracellular chloride level previously showed.

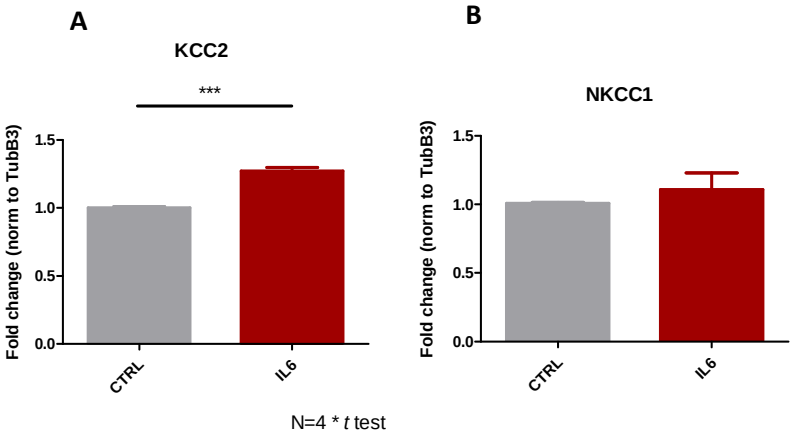


**Fig. 4.19** ***IL-6 treatment increased KCC2 expression in hippocampal neurons****. qRT-PCRs on control and IL-6 treated neurons at 6 DIV showed significant increase in the relative mRNA expression levels of KCC2 (*p<0.0001) (***A***), but NKCC1 was found unaltered (p=0.4252) (****B****).*

### 4.3.3 IL-6 impact on [$Cl^-$] levels involves STAT3 signalling activation

Since IL-6 signalling pathway requires the activation of the Signal Transducer and Activator of Transcription 3 (STAT3), we explored whether the effects produced by IL-6 on the GABA switch might be due to STAT3 signalling.

STAT3 activation depends on the phosphorylation at tyrosine 505 [347] which leads to the translocation of STAT3 into the nucleus thus acting gene expression. In order to prevent STAT3 activation, we took advantage of a selective STAT3 inhibitor, STATTIC, which is known to specifically block STAT3 phosphorylation.

At first, we set out the working concentration of STATTIC effective in preventing STAT3 phosphorylation without inducing neuronal death. We found that Stattic 1 uM was able to prevent STAT-3 phosphorylation without damaging neuron. Hence, cultured neurons at 1 DIV were treated with either IL-6, IL-6 + STATTIC 1 uM or STATTIC 1 uM alone and subsequently assayed for chloride imaging at 6 DIV.

As expected IL-6 treated neurons exhibited a higher MQAE fluorescence intensity, interestingly such effect was totally prevented by Stattic, thus demonstrating a causal link between STAT3 phosphorylation and IL-6-mediated effect on the GABA switch (**Fig. 4.20**).

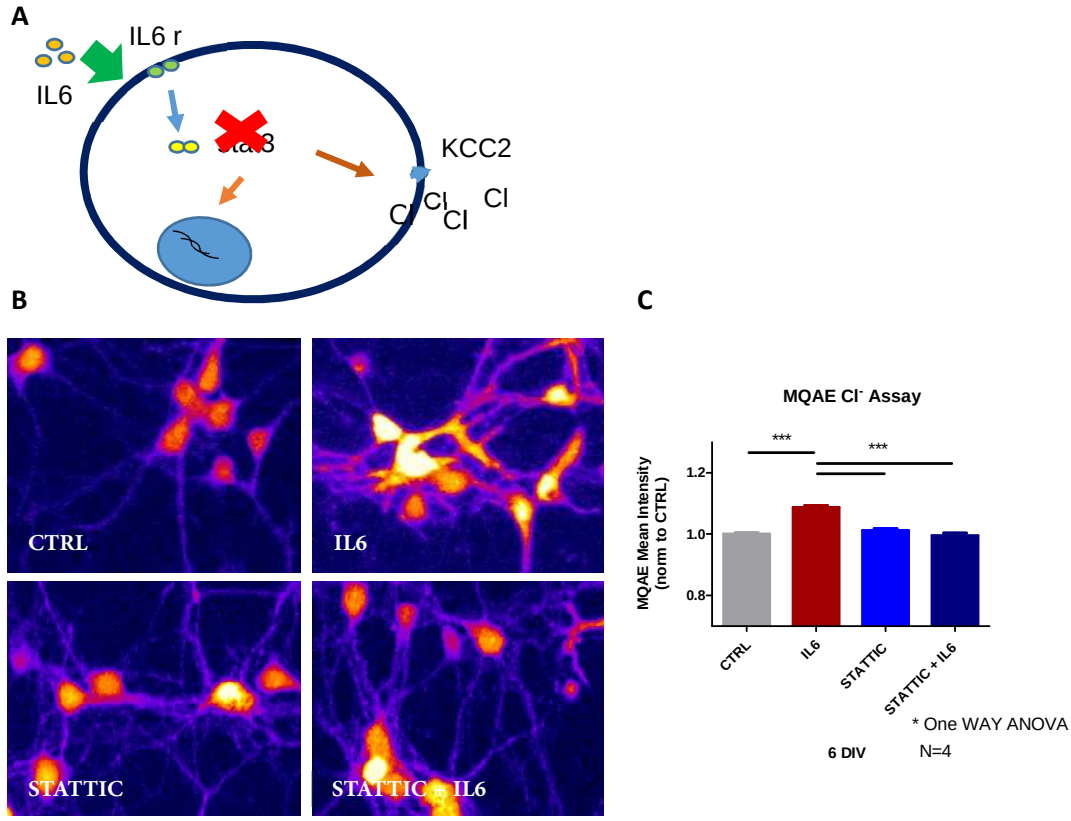


**Fig. 4.20** ***IL-6 decreased the neuronal chloride concentration via a STAT3 -dependent pathway.*** ***A****: representative cartoon showing the experimental plan.* ***B****: representative images and* ***C:*** *quantitative analysis of neurons treated with IL-6 and STATTIC. The blockage of STAT3 completely prevented the effect of IL-6 in decreasing the chloride levels (MQAE mean intensity decrease). P>0.001, One Way ANOVA.*

We next wondering whether STAT3 activation was also responsible for the increase of KCC2 expression upon IL-6 treatment. For this issue, we measured KCC2 mRNA levels in 6 DIV neurons treated with IL6 and with IL6 + STATTIC and we observed that the exposure to STAT3 inhibitor, led to a considerable decrease in KCC2 expression (p=0.0067, **) (**Fig. 4.21**).

Overall these findings demonstrate that IL-6 could decrease the intracellular chloride, through KCC2 upregulation, in a STAT3-dependent manner.

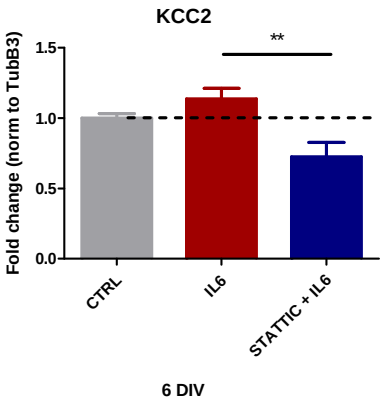


**Fig. 4.21** ***Inhibition of IL-6-dependent STAT3 activation with STATTIC resulted in KCC2 mRNA downregulation****. Significant decrease in expression has been found between IL-6 and IL-6+STATTIC-treated neurons (p=0.0067; One Way ANOVA).*

### 4.3.4 GABA signalling itself modulates the timing of the GABA switch

In the attempt to find the molecular mechanisms responsible for such effect, we focused our attention on possible signalling which are known to regulate the GABA switch. Based on the literature [348], it has been reported that by potentiating or inhibiting the GABAergic transmission, the GABA switch could be accelerated or reduced respectively. We first confirmed that in our model GABA signalling can modulate the GABA switch.

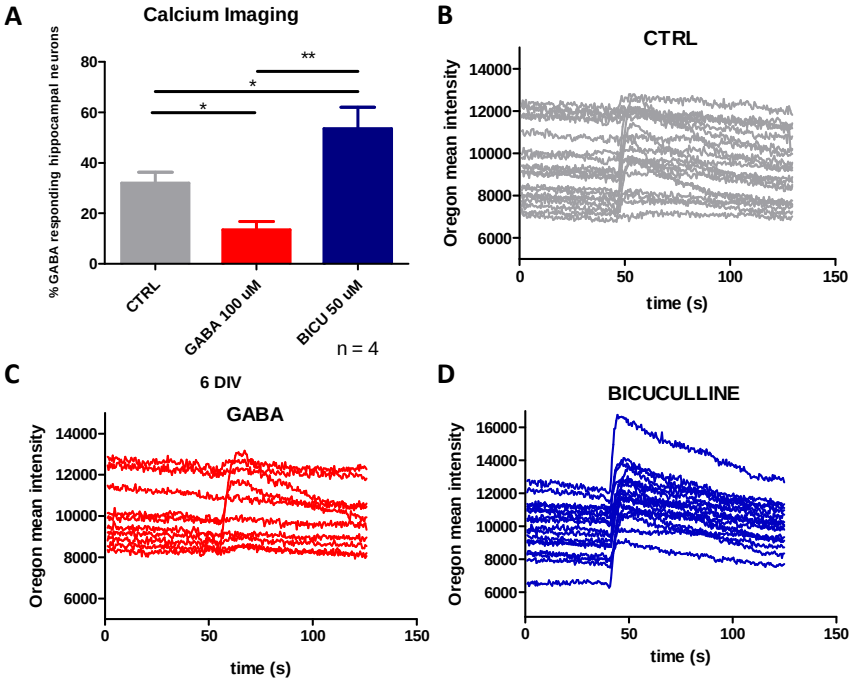


**Fig. 4.22** ***The GABA signalling itself modulated the depolarizing -towards-hyperpolarizing GABA shift. A:*** *quantitative analysis graph showing the percentage of GABA responding neurons in different experimental conditions. Significant decrease between CTRL and GABA-treated neurons $p<0.05$ e increase between CTRL and Bicuculline-treated neurons $p<0.05$.* ***B, C, D:*** *representative traces of calcium imaging recordings in control, GABA-treated and Bicuculline-treated neurons.*

In line with the literature, cultured neurons treated with GABA 100 µM from 1 DIV resulted in a lower percentage of GABA responding neurons (**Fig 4.22**) ($p<0.05$; *) and a higher MQAE signal, thus reflecting a lower intracellular chloride levels (**Fig 4.23**) ($p<0.0001$; ***). On the other hand, by treating neurons with $GABA_A$ receptor antagonistic, Bicuculline 50 µM, a significant delay in the GABA switch was observed, being calcium transients higher ($p<0.05$, *) (**Fig. 4.22**) and MQAE levels lower ($p<0.001$, **) (**Fig 4.23**) than control neurons.

Overall these results confirmed that even in our model, GABA itself exerted a trophic role in boosting the timing of the GABA switch. This prompted us to investigate whether IL-6 can promote the GABA switch by enhancing GABAergic transmission.

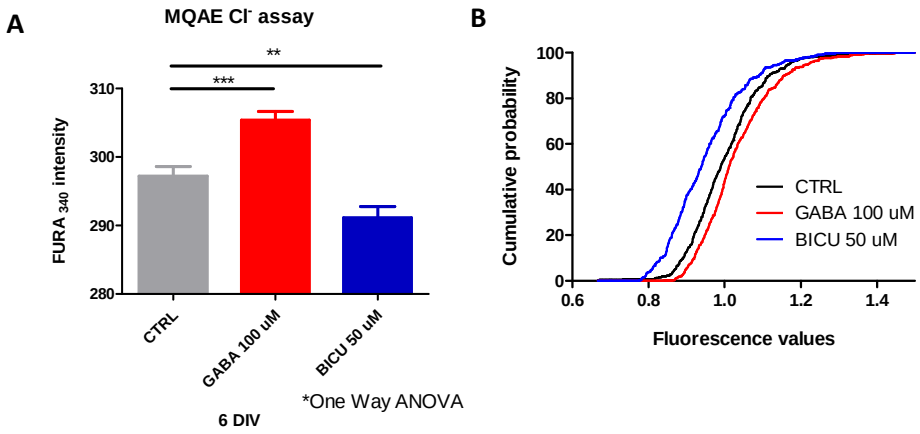


**Fig. 4.23** *The GABA signalling modulated the levels of intracellular chloride concentrations.* **A:** *quantitative analysis graph showing MQAE mean intensity in different experimental conditions. Significant MQAE increase between CTRL and GABA-treated neurons ($p<0.0001$) and decrease between CTRL and Bicuculline-treated neurons ($p<0.001$), mean lower and higher chloride concentration, respectively.* ***B****: cumulative probability graph showing significant differences between the three experimental conditions.*

### 4.3.5 IL-6 enhances the GABAergic transmission in developing neurons

Cultured neurons were treated with IL-6 and subsequently subjected to electrophysiological evaluations, with the aim to investigate whether changes in chloride levels may have a functional impact on the GABAergic transmission.

Interestingly, we found that (**Fig. 4.24**) IL-6 significantly increased the frequency of miniature inhibitory post synaptic currents (mIPSCs) ($p<0.0001$) and, again, that such effect was STAT3-dependent, since the pharmacological blockage of STAT3 prevented the effect on mIPSCs

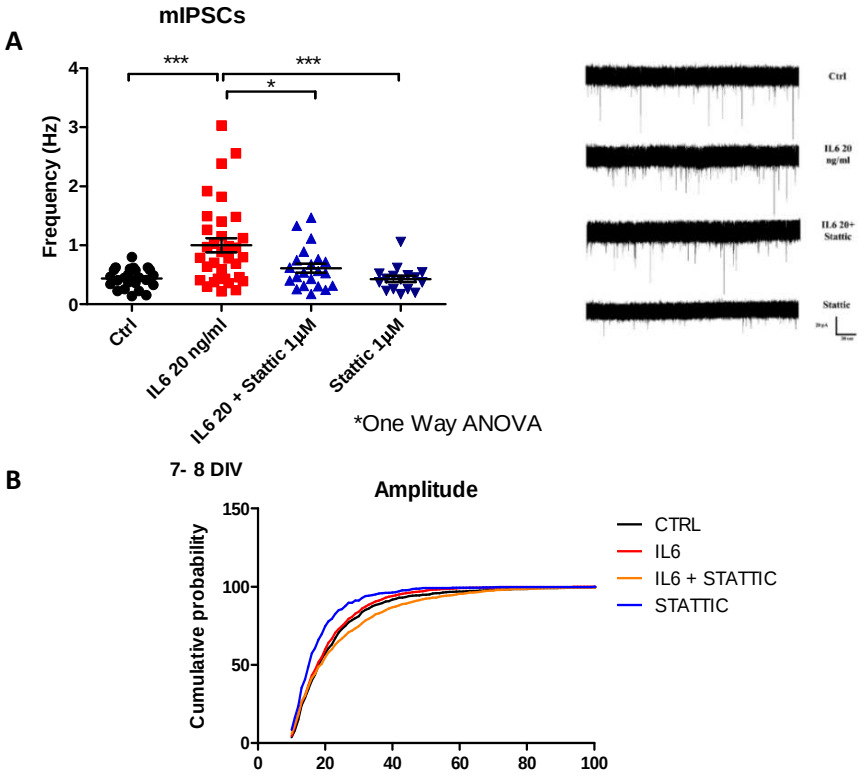


**Fig. 4.24** ***IL-6 enhances the GABAergic inhibitory transmission in developing neurons****. Patch clump recordings and representative traces of mIPSCs on neurons at 7-8 DIV showing an increased frequency (**A**) in IL-6-treated versus control neurons ($p<0.0001$), and no effect in the presence of STAT3 inhibitor. **B**: cumulative distribution probability of mIPSCs amplitude in the different experimental conditions.*

There was no relevant change in the amplitude of mIPSCs currents between control and IL-6-treated neurons (**Fig. 4.24, B**), even though a slight decrease in neurons treated with STATTIC alone was detected.

### 4.3.6 IL-6 might act through the GABA signalling potentiation

To further evaluate the impact of IL-6 on GABAergic machinery, the transcriptional profile of different GABA receptor subunits was examined.

To this aim, mRNA levels of the following subunits receptors - GABRB1, GABRG2, GABRA3, were quantified by means of qRT-PCR.

We found that IL-6 treatment induced an increase in all the analysed subunits (**Fig. 4.25**), although only the gene coding for *gabrg2* was found to be statistically relevant, (p=0.0399) whereas a positive trend was detected for the other genes (*gabrb1*, p=0.0694; *gabra3*, p=0.1716).

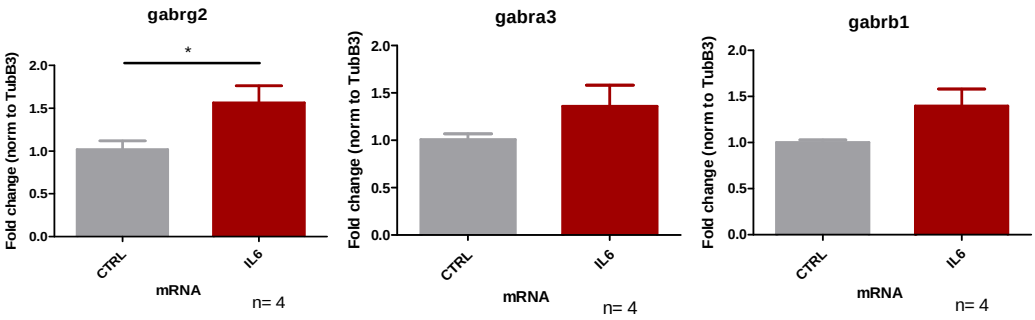


**Fig. 4.25** ***IL-6 treatment increased GABA receptor subunits expression in hippocampal neurons.*** *qRT-PCRs on control and IL-6 treated neurons showed significant increase in the relative mRNA expression levels of gabrg2 gene (p=0.0399). Increased trends have been observed for gabra3 (p=0.1716) and gabrb1 (p=0.0694).*

### 4.3.7 IL-6 promotes vGAT/gephyrin puncta increase

In order to strengthen our hypothesis that IL-6 promotes the GABAergic switch through the enrolment of the GABAergic machinery, we performed *immunolabeling* of both pre- and post- synaptic inhibitory markers on cultured neurons treated with IL-6.

As expected, we found that IL-6 significantly increased the density of the presynaptic vesicular GABA Transporter (vGAT) positive puncta (p=0.0289) (**Fig. 4.26.A**) in neurons at 6 DIV, as well as their total area (p=0.0086) (**Fig. 4.26, B**).

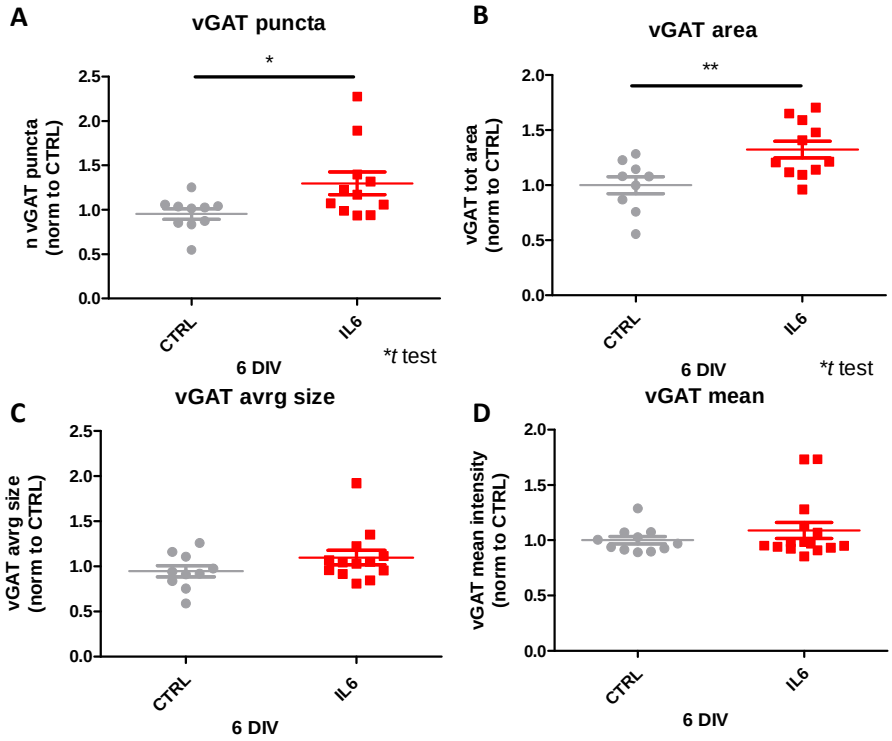


**Fig. 4.26** ***Quantitative analysis of vGAT immunolabelling on IL-6-treated and control neurons****. A significant increase in the number of vGAT puncta (p=0.0289; *, t test), and in their area of expression (p=0.0086), but not in the vGAT average size (p=0.1461) nor in the intensity of its expression (p=0.3334) have been observed.*

Conversely, any significant changes in neither the size (p=0.1461) (**Fig. 4.26, C**) nor in mean intensity (p=0.3334) of vGAT positive puncta (**Fig. 4.26, D**) were identified.

On the other side, the postsynaptic inhibitory marker gephyrin was also evaluated through immunolabelling. Notably, no differences have been reported between IL-6 -treated and control neurons in the number of gephyrin positive puncta (p=0.7327) (**Fig. 4.27, A**), area of expression (p=0.9549) (**Fig. 4.27, B**) size (p=0.6377) (**Fig. 4.27, C**) and mean intensity (p=0.1894) (**Fig. 4.27, D**).

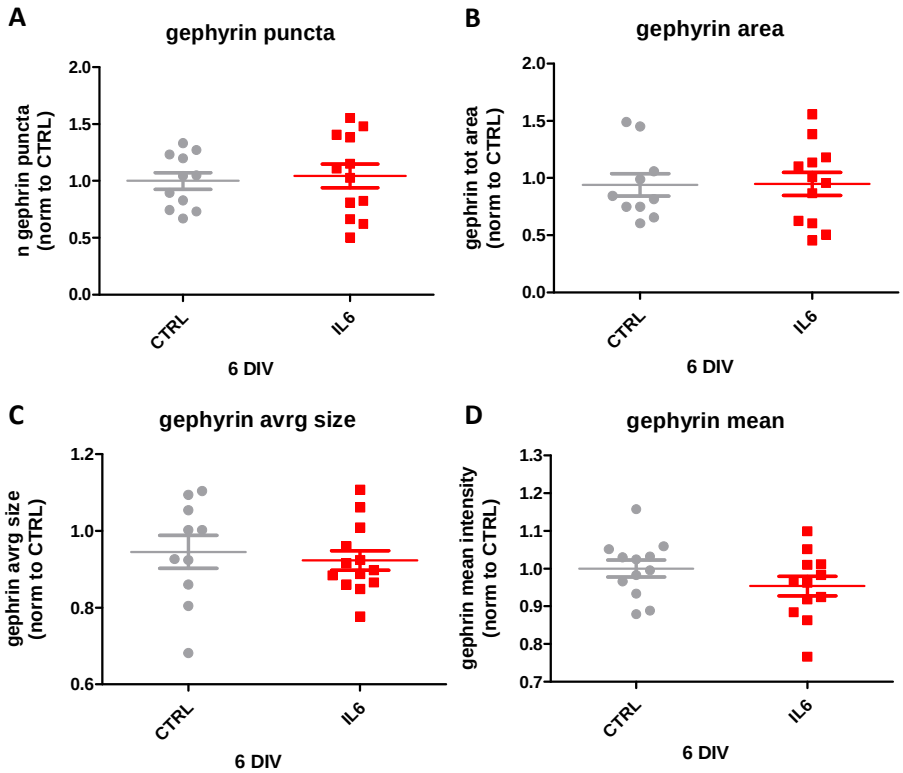


**Fig. 4.27** ***Quantitative analysis of gephyrin immunolabelling on IL-6-treated and control neurons.*** *Any significant changes in the number of gephyrin puncta (p=0.7327), in their area of expression (p=0.9549), nor in the average size of the puncta (p=0.6377) or in the intensity of its expression (p=0.1894) have been identified.*

Despite gephyrin positive puncta remained unchanged, the density of gephyrin clusters co-localizing with vGAT were increased in IL-6 -treated neurons compared to control neurons (p=0.0339) (**Fig. 4.28**).

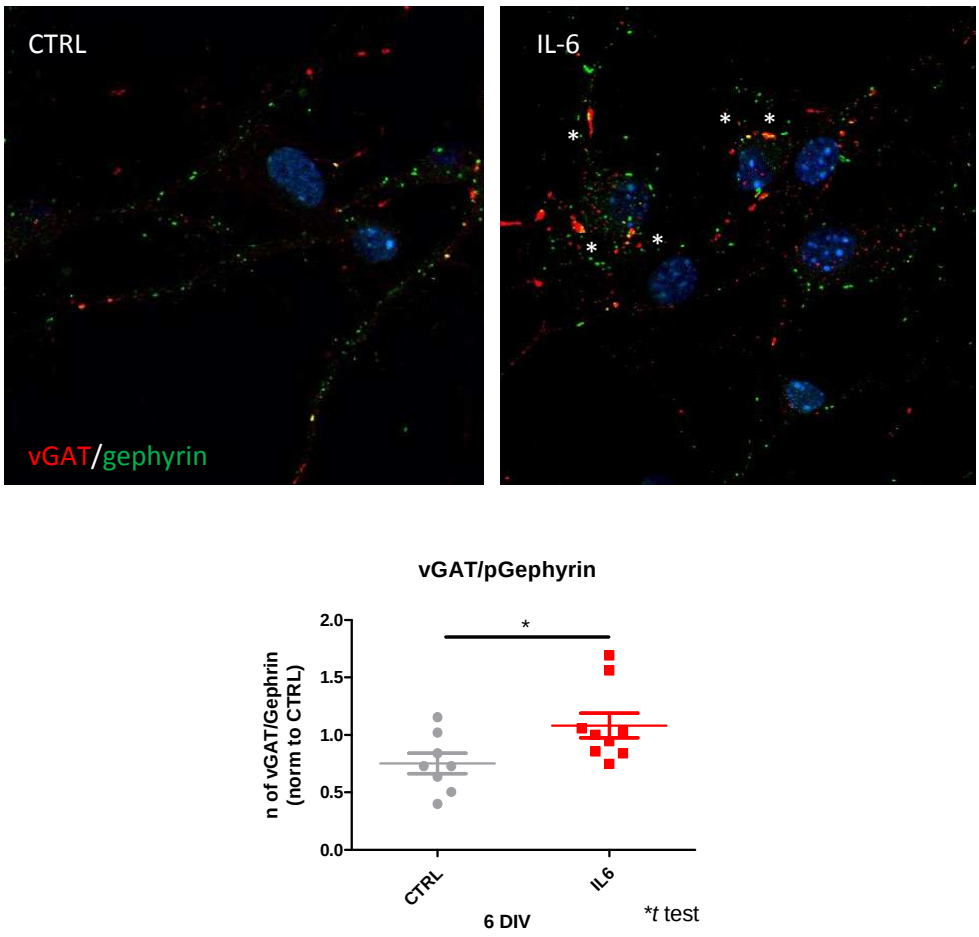


**Fig. 4.28 IL-6 treatment increases vGAT and vGAT/gephyrin colocalizing puncta.** ***A:*** *Representative images of 6 DIV control and IL-6-treated neurons immunolabelled for vGAT (red) and gephyrin (green); DAPI counterstain (blue), 60x magnification.* ***B:*** *Quantitative analysis of vGAT and gephyrin immunolabelling showing a significant increase in the number of co-colocalizing puncta in IL-6-treated neurons (p=0.0339; *, t test).*

### 4.3.8 IL-6 promotes the GABAergic switch through the enhancement of the GABAergic transmission

Based on these data indicating that IL-6 is able to enhance the GABAergic transmission, we wanted to provide the causal link between the IL6 dependent positive effect on the GABA switch and the enhancement of the GABAergic transmission.

Indeed, we reasoned that if IL-6 accelerates the GABA switch by potentiating GABA signalling itself, one would have expected that the pharmacological blockade of $GABA_A$ ionic receptors would prevent the IL-6-mediated reduction of intracellular chloride.

To address this issue, we treated neurons with IL6 alone and with Bicuculline in order to block the GABA transmission. The MQAE experiment showed that bicuculline was able to occlude the effect induced by IL-6 (**Fig. 4.29**) indicating that IL-6 can accelerate the GABA switch through a potentiation of GABA signalling. These pleiotropic effects would finally determine an acceleration of the GABAergic switch, in terms of intracellular chloride levels' decrease.

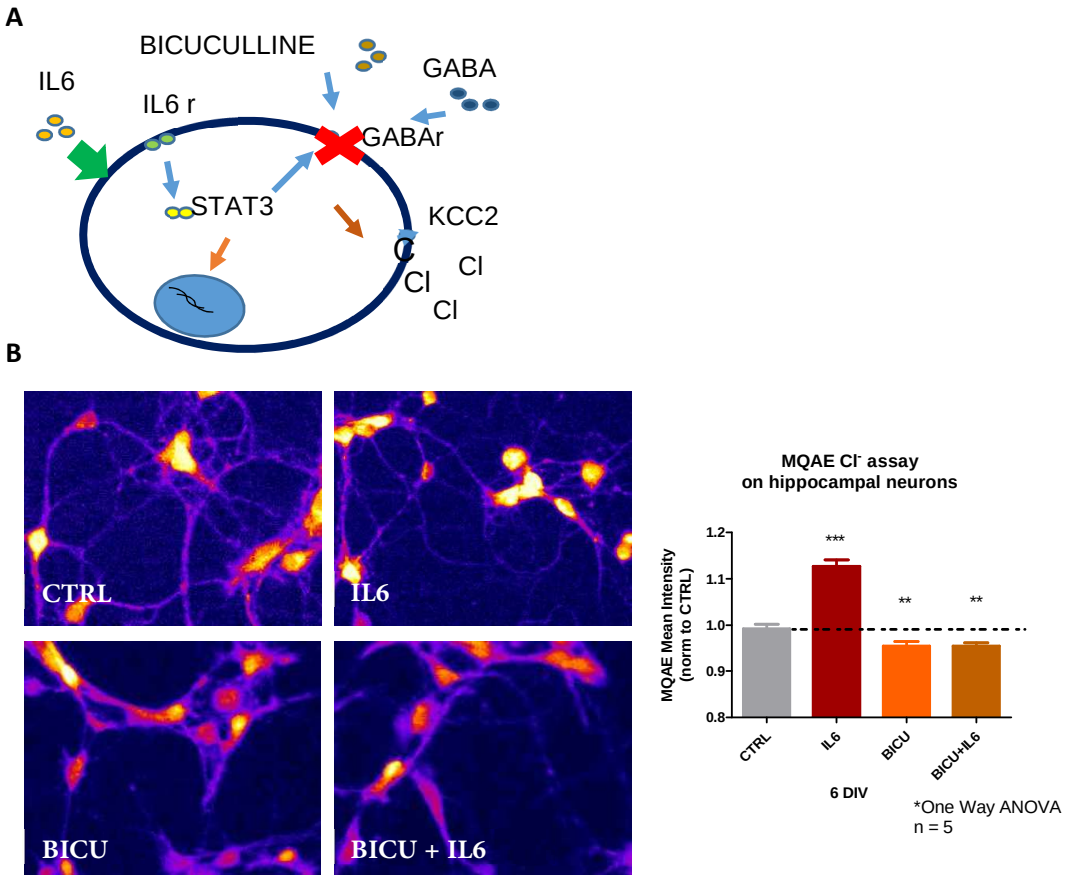


**Fig. 4.29** *IL-6 decreased the neuronal chloride concentration via the GABAergic machinery recruitment.* ***A****: representative cartoon showing the experimental plan.* ***B****: representative images and* ***C*** *quantitative analysis of neurons treated with IL-6 and Bicuculline. The blockage of $GABA_A$ receptor completely prevented the effect of IL-6 in decreasing the chloride levels (showed as MQAE mean intensity increase). P>0.001, One Way ANOVA.*

# 5

# DISCUSSION & CONCLUSIONS

The present study provides new evidences in the understanding of the neuronal GABAergic switch, a critical event that occurs early in life before birth, during brain development, and whose impairments have been associated to mental retardation, cognitive disabilities and behavioural deficits, typical hallmarks of neurodevelopmental disorders [95, 326], all conditions which are characterized by a remarkable excitation/inhibition network imbalance [55, 325].

The depolarizing-to-hyperpolarizing GABA switch has been largely described and already accepted in literature, but, truthfully, for long times the “real” existence of the developmental switch has been highly debated. Despite the abundance of literature supporting the depolarizing action of GABA in immature neurons, few works [349, 350] tried to undermine this principle, suggesting this phenomenon as an experimental artefact due to insufficient metabolic substrate availability *in vitro*, or, in a more recently published study, to intracellular $Cl^-$ accumulation in injured neurons after the slicing procedure [351]. Indeed, all these observations have been strongly refused [352, 353] and the GABA switch is currently seen as a real critical event occurring during brain development.

According to this, our study further strengthens the existing literature and describes the presence of a limited temporal window characterized by excitatory, depolarizing GABA transmission in cultured hippocampal neurons.

Even though molecular mechanisms underneath the GABA switch have been widely described, regulators of this event are being continuously characterized. In this view, our study has been carried out with the attempt to investigate novel modulators of this event. We focused our attention on immune factors, namely IL-6 and MSCs, that could be overall described like "t*he brawn and the brain*", since although they both play a role as immune modulators, the former represents one of the main effectors of the immune system [232, 236, 275, 354], while the latter actively surveys the microenvironment to meet the neighbourhood needs [355-358].

However, these two lines of investigation will be discussed separately because, although our results show a similar effect of these two candidates on the GABA switch and GABA transmission maturation, a possible link between them is still missing. However, it is not trivial that these two pathways might, at a certain point, meet each other and act via a shared final process.

Before starting in investigating the role played by these two regulators, we first needed to characterize the GABAergic switch in *in vitro* hippocampal neurons, with the purpose to setup the proper experimental conditions for the subsequent experiments. Among the techniques available to investigate the GABA switch, calcium imaging represents a fundamental tool to study the neuronal network responsiveness and the effect of GABA in cultured neurons. Indeed, the depolarizing (excitatory) action of GABA can be demonstrated in terms of calcium transients evoked by GABA [359]. Most of the knowledge we currently know comes

from pioneering works in which the developmental switch of GABA signalling has been described by means of this technique [360] [348].

Hence, based on the literature, we monitored calcium transients upon GABA acute application at different time points of the in vitro development of hippocampal neurons in order to identify the precise temporal window at which the GABA switch takes place. We found that, in line with what reported in literature [58], neurons progressively decreased their ability to exhibit GABA-mediated calcium transients stimuli from 4 to 7 DIV, becoming completely silent to GABA at 7-8 DIV. In particular, at 4 DIV nearly all neurons were responsive to GABA, as a consequence of their immature status, however, such percentage decreased to 70% at 5 DIV, to 30% at 6 DIV, and to less than 10% of responding neurons at 7 DIV, suggesting that the developmental switch occurs within this time window.

Based on this evidence, we decided to set our experimental day at 6 DIV, which represent a developmental time point in which 30% of neurons are still responsive to GABA.

Interestingly, consistent evidences about the timing of the GABA switch have been described even in other brain areas beside hippocampus. One example was provided by Barkis and colleagues [361] in retinal ganglionic neurons, where the GABA switch was completed around 8-11 DIV, in cultured retina explants.

Given the shift of GABA polarity tightly dependent on intracellular chloride levels, we aimed at assessing the chloride changes in our experimental model through the membrane-permeable $Cl^-$-sensitive MQAE dye whose fluorescence is

inversely proportional to intracellular chloride levels. [345]. In line with the calcium imaging data, we detected a significant increase in MQAE intensity between 4 and 6 DIV, thus reflecting a significant intracellular $Cl^-$ reduction.

It is worth noting that even small changes in the MQAE intensity, coupled to the high sensitivity of the dye, indeed corresponded to high functional differences of GABA signalling, as observed with calcium imaging. In fact, considering the same temporal window, from 4 to 6 DIV, MQAE exhibits only 10% significant differences, whereas the percentage of GABA-responding neurons dramatically dropped from 90% to 30%. Moreover, being intracellular chloride level the main regulator of GABA polarity, we decided to use MQAE imaging as principle method to directly assess the GABA switch in this study.

**5.1 MSC-derived MVs promote the GABAergic switch**

The first line of work aimed at exploring the role of MSCs in the possible regulation of the GABA switch. The final goal of this investigation is to identify potential druggable candidates to be addressed for further applications, especially in the study of neurodevelopmental disorders, where often, but not always, the GABA switch is delayed. In particular, an important finding emerges from this study: extracellular vesicles derived from MSCs (MSC-EVs) seemed to accelerate the timing of the GABA switch, whereas the whole cells failed to achieve the same result.

Indeed, a recent work from our lab demonstrated that MSCs could enhance the GABAergic transmission in adult hippocampal neurons, when co-cultured

together [355]. However, this study did not investigate the developmental switch of KCC2, since it focused on adult neurons, where the GABA polarity has already shifted. As a matter of fact, even though several studies showed that MSCs promoted the neuronal post mitotic differentiation and survival [328, 362-364] and neurite elongation [195], overall suggesting their positive role on neuronal growth, they referred to engineered MSCs, and, remarkably, to our knowledge, no one paid attention to the GABA switch nor to how these cells could affect this event.

In our system, it seems that MSCs delay the timing of the switch, suggesting that, in the attempt to translate this study into clinical applications, they cannot be the proper candidate for diseases where the GABA switch is already delayed and characterized by network hyperexcitability.

Hence, it is true that MSCs could be considered good candidates for the treatment of several pathological conditions, including neurological diseases [179, 183, 194, 365], as bearing the beneficial therapeutic potentials of stem cells. However, a stemness-based therapy could not be always the right option for all diseases and cannot have the claim to target whatsoever pathological condition or perfectly suit whoever needs. In agreement with this statement, despite convincing evidences of MSC- beneficial roles even for the neuronal development, there are some concerns about their practical use that still need to be ameliorated, like the standardization of their features, their safety for implantation as well as the graft-versus-host rejection [364, 366, 367].

Better candidates could be then represented by vesicles isolated from the culture medium of these cells, bearing many advantages of the parental cells, by minimizing the aforementioned drawbacks.

For these reasons we proceeded by evaluating neuronal changes in MQAE levels avoiding the presence of the whole MSCs. In the attempt to figure out whether soluble factors, that are constitutively secreted in the medium by MSCs, could similarly impair the timing of the GABA switch, we first fed neurons with the MSC pre-conditioned medium (CM), and we still observed lower MQAE intensities in treated versus control neurons, suggesting the presence of soluble factors that can heavily affect the chloride homeostasis.

This result appeared contrasting what the literature suggest: MSCs can release beneficial molecules, such as, among the others, brain-derived neurotrophic factor (BDNF) [368] [369] , claimed as responsible for the MSC-pro-survival effect on neurons. In our support, it is worth noting that 1) these authors used the MSC-CM to treat neurons starting from 6 DIV and not from the beginning of the establishment of the culture, or 2) that the MSC-CM derived not from naïve MSC, but from MSCs previously co-cultured with HNs. Hence, it is possible to speculate that the pro-survival and beneficial effect of MSC-CM, might be a consequence of the benefits given by trophic factors that neurons themselves physiologically secreted in the very early stages of culture. It might be reasonable that the treatment from 1 DIV, when neurons are juvenile, with media deriving from naïve stem cells, could differently *prime* the neuronal growth or at least affect intracellular chloride levels.

Moreover, this effect is specifically attributable to stem cells, as both co-culture and CM derived from fibroblasts, always conceived as ”bystander” cells, not endowed with stemness properties – and for this reason used as control cells - did not alter the chloride levels at all.

Eventually, since at 6 DIV the temporal window for appreciate the GABA switch has been already overcome, it might be possible that MSCs could not be proposed for interventions on very early juvenile neurons, at least in the contest of the GABA switch regulation.

Remarkably, after isolating the pool of MSC-derived vesicles, the treatment of neurons from 1 DIV resulted in an acceleration of the timing of the switch, and, in particular, in a MV-type specific manner. In fact, usually EVs can be enriched with, primarily, but not exclusively, MV or exosome fraction (see chapter 1), even though literature not always aims at distinguishing these two populations, but simply refers to them as extracellular vesicles [199, 335, 370-373].

Interestingly, we found a significant acceleration in the timing of chloride decrease in neurons treated with the MV-enriched fraction, but not in those treated with the exosome-enriched fraction, suggesting a specific role of MVs in the control of chloride concentrations. We ascertained that these treatments did not affect the growing of the cultures, as neurons appeared morphologically and functionally well developed.

Indeed, literature provides several evidences about the safety and beneficial role of EVs that convinced us about the positive effects of vesicles on

the neuronal maturation. Moreover, more than one study focuses on the MV effects on neurons [371, 374, 375], even if research on exosomes is currently growing up, and a lot of publications have already described them as good candidates for personalized therapy [331, 333, 336, 337].

However, given the size range covered by these two populations, that partially overlaps, current techniques do not make possible to separate them perfectly, and for these reason exosomes could be considered a sub-population of the MV-pool [199]. Even though the ultracentrifugation process does indeed cut an Exosome pool out of the EV complete pool, as verified with nanoscale instrument (Nanosight), it is not possible to claim that the positive effect of MVs would be due to exclusively larger-sized vesicles, but, rather, to the higher concentration of the larger sized-vesicles than the smaller ones.

As a proof of this evidence, it is worth noting that neither the CM nor the co-culture system - where all types of vesicles were resealed, and diluted - triggered the same effect, suggesting that: 1- the whole medium may contains not only too much diluted vesicles or soluble factors, but also apoptotic bodies and cellular debris that could have a negative impact on the neuronal chloride homeostasis, and 2 -in the whole medium the pool of MVs did not reach the proper concentration to be efficient and trigger positive effects, as observed after concentrating the MV pool alone.

Due to their effect on the intracellular chloride levels, we indeed focused on MV population, and we wondered what the possible mechanisms beyond this regulation could be. Eventually, we hypothesized that MVs could modulate the

GABAergic machinery and trigger cellular rearrangements that in turn would accelerate the maturation of the GABAergic transmission itself. To test this hypothesis, we indeed treated neurons with MVs and observed a significant increase in the number of the inhibitory pre-synaptic marker vGAT and even a significant co-colocalization with its post-synaptic counterpart gephyrin, suggesting a possible role of MSC-MVs in boosting the maturation of inhibitory synaptic connections.

This result could be interesting when we consider those pathological conditions where the GABA transmission is impaired, or even disorders known as “developmental synaptopathies” [376], that share an impaired protein homeostasis at the synapse [377]. Due to the critical importance of the synaptic homeostasis, both a loss and gain of synaptic function would be responsible for the mental retardation and cognitive impairments, typical of neurodevelopmental disorders.

In this perspective, a possible practical use of MVs could be targeted to those conditions where the synaptic imbalance is due to a loss of synapses, or, ideally, where the GABA switch is delayed and the network hyperexcitable. One of these critical condition, may include, for example, the delay of the excitatory-to-inhibitory GABA switch in offspring coming from mothers subjected to immune system activation during pregnancy. In fact, Corradini and colleagues [378] recently demonstrated a significant GABA transmission impairment and GABA switch delay in a mouse model of maternal immune system activation (MIA).

Interestingly, other authors paved EVs as good candidates for the treatment of neurological disorders characterized by excitation/inhibition imbalance and/ or

synaptic deficits, not only during development. Long and colleagues [379] provide a recent evidence of the beneficial neuroprotective effects of intranasal injection of MSC-derived vesicles in mice subjected to pilocarpine-induced status epilepticus, accompanied with a great protection against the glutamatergic and GABAergic neuronal loss. Ophelders and colleagues [371] demonstrated that the *in utero* intravenous injection of MSC-EVs in ovine foetuses, subjected to global hypoxia-ischemia by transient umbilical cord occlusion, improved brain function in the offspring, by reducing the number and duration of seizures and also preventing hypomyelination. Deng and colleagues (2017) [374] even demonstrated the therapeutic effects of EVs on synaptic deficits and spatial learning and memory impairments, as a consequence of transient global ischemia.

Taken together, these evidences support our hypothesis that the administration of extracellular vesicles, rather than intact MSCs, could be sufficient to exert therapeutic effects on neuronal deficiencies, with the advantage to eliminate potential concerns related to the administration of living cells.

Surely, several studies demonstrated the efficacy of MSCs in ameliorating GABAergic system impairments, but differently from the present work, MSCs derive from different sources or, most importantly, their functions have been ameliorated through genetical engineering, pointing out, again, that stemness therapy with naïve MSCs could sometimes bear hindrances. As a matter of fact, Long and collaborators [366] demonstrated that Hes1-silencing could promote MSC differentiation into GABAergic neuron-like cells, and that their administration improved the functional recovery of the epileptic rats. Eventually, they proved that

the MSC engineering was indeed responsible for the functional recovery from epilepsy.

However, it is also true that other sources of MSCs, coming from other species, maybe more suitable for clinical interventions, as Zanier and colleagues indeed demonstrated the beneficial effects of human MSCs in the neuronal survival and growth, in brains after trauma [380] and ischemic stroke [381]. Nevertheless, literature has not proved yet the efficacy of MSCs in the modulation of chloride homeostasis, linked to the GABA functional switch.

In order to look deeper into the mechanism of MV-related chloride regulation, we evaluated the mRNA levels of the two chloride co-transporters NKCC1 and KCC2 in treated and naïve neurons, and, even though any differences have not been observed for NKCC1 $Cl^-$ importer, a slight, even though not yet significant, increased trend, has been observed for KCC2 $Cl^-$ exporter. This unclear result obliges us to provide further clarification of the molecular mechanism underneath the chloride decrease, possibly, as first step, by increasing the statistical events. Indeed, the levels of KCC2 protein expression will be evaluated.

Moreover, since the active form of KCC2 in strictly related to its dimerization and localization to the plasma membrane, we wondered whether MV treatment affected the membrane shuttering of KCC2 from the cytoplasm. Roussa and colleagues indeed identified the novel contribution of TGF-β2 in promoting this event [382]. The same authors did not observe any significant changes in the total expression levels but an enhanced localization of KCC2 in the plasma membrane, due to higher TGF-β2 expression. Hence, we hypothesized MVs targeting the

expression of TGF-β2, and we then investigated the levels of its mRNA in MV-treated neurons, finding out increased levels of expression in comparison with untreated neurons.

Even though this finding requires the direct verification of KCC2 -enhanced expression to the plasma membrane, which is indeed in our perspective evaluation, it is at least a first evidence that let us speculate about the MV-mediated mechanism of action.

In fact, overall all these results could suggest that MVs, but not exosomes, nor MSCs, are surely able to promote intracellular [$Cl^-$] decrease, related to an acceleration of the timing of the GABA switch, and that MVs also target the GABAergic machinery by increasing the expression of synaptic inhibitory markers. In parallel, it might be possible that they also trigger molecular rearrangements and influence the mRNA expression levels of KCC2 and TGF-β2, which, in turn, might be responsible for the modulation of the chloride homeostasis.

This hypothesis could be advantageous in the perspective way to translate the MV use in practical applications. Indeed, MVs are considered as valid candidates for therapeutic interventions, as they act as "*Trojan horses*", by shuttling modulatory molecules [199, 332, 333, 335, 337, 372, 375, 383], among others, miRNAs [333, 334, 339, 384, 385], cytokines and a lot of other proteins [383]. In support to these statement, Eirin and colleagues recently provided a fine evaluation of transcriptomic and proteomic vesicles' cargo, in comparison with MSCs [383].

Eventually, this EV-special feature might represent the real power of these organelles: the possibility to functionalize the vesicles with any desirable molecule, in order to make them smart candidates for direct and specific targeting, thus bypassing all concerns related to living cell-based therapies [373].

### 5.2 IL-6 accelerates the GABAergic switch through the enhancement of the GABAergic transmission

The results obtained with EV-MSCs seem be in line with what obtained by another study that is being carried out in our laboratory aimed at exploring the possible role of the pleiotropic cytokine interleukin 6 (IL-6) in the regulation of the GABAergic switch.

We overall found that the exposure of IL-6 to neuronal development was sufficient to accelerate the excitatory-to-inhibitory GABA functional transition.

In particular, a single IL-6 exposure at first day of the in vitro development of hippocampal neurons, was enough to trigger a significant reduction of the intracellular [$Cl^-$] at 6 DIV, suggesting that early exposure to this cytokine may induce long lasting effects. Furthermore, the effect of IL-6 on the GABA switch appeared to be a STAT-3-mediated event, as the pharmacological blockade of STAT3 phosphorylation, by means of Stattic, was sufficient to prevent the chloride decrease in neurons at 6 DIV.

In support to our evidences, literature provides several examples of STAT3-dependent IL-6 activities. In fact, Kamimura et al, demonstrated that IL-6 is indeed one of the molecules triggering the phosphorylation of STAT3 and its subsequent

dimerization, which, in turn, would activate downstreaming processes leading to both non-genetic and genetic rearrangements, according to the environment and the cell type [239]. IL-6 -dependent STAT3 activation has been implied in several pathological conditions, like in cancer [386], cardiovascular [387] and neuronal diseases [388], but the effects on the GABA transmission development have never been demonstrated yet.

The novelty of our study concerns the possible role of IL-6 in the modulation of the GABA switch. We here demonstrated that IL-6 was able to target neurons and determine neuronal rearrangements through STAT3, having observed not only modifications in the chloride homeostasis, but also in the GABAergic transmission itself.

As a matter of fact, our study also showed that IL-6 treatment in juvenile neurons significantly enhanced the maturation of the inhibitory synaptic connections at 6 DIV, as well as the inhibitory transmission establishment. In fact, the number of pre-synaptic vGAT and vGAT/ gephyrin puncta co-colocalization have been found significantly increased in IL-6-treated neurons. Most importantly, those connections were actually functional, as verified through electrophysiological recordings, and the occurrence of miniature Inhibitory Post Synaptic Currents (mIPSCs) was even found higher in treated neurons, compared to control ones.

These results paved the way for further insights about the role of IL-6 in immature neurons. In fact, several studies reported in literature have always considered this cytokine as a pro-inflammatory signalling molecule, being involved in the immune system activation, together with other important players, like TNF-a

and IL-1β. However, during the last years, novel roles have been attributed to this cytokine, suggesting its action in a pleiotropic manner [232, 236].

Few examples in support to this statement are provided. A beneficial role of IL-6 has been described in more than one pathological conditions, as described by Sulistio and colleagues [389]. However, more recently, Yamada [298] and Peng [305] attributed neuroprotective role to IL-6 after neuronal toxicity whereas Kushima and Oh further demonstrated the ability of IL-6 to act as trophic factor in promoting neuronal differentiation [294, 301].

Supported by the literature, we first hypothesized that in our conditions IL-6 may act as a trophic factor during early phase of neuronal development, thus participating in the growth and differentiation of neurons, very similarly to GABAergic system. In fact, it is true that the tonic GABA currents, long before the establishment of synapses, are indeed responsible to boost the development of the neuronal progenitor cells (NPCs), the migration of juvenile neurons from subvetricular zone (SVZ), (the stemness source of the brain), and their correct placement in the hippocampus and cortex [390].

Eventually, a study established in Mu-Ming Poo's laboratory [348] demonstrated that GABA itself, acting like a trophic factor for immature neurons, could trigger the GABA switch and that the pharmacological blockade of the $GABA_A$ ionotropic and ligand-gated ion channel receptors, by means of the $GABA_A$ receptor antagonist Bicuculline, could delay the timing of such process.

According to these evidences, we hypothesized that IL-6, through STAT3 activation, could enhance the GABAergic signalling pathway thus promoting the expression of GABA signalling molecules responsible for the GABA switch.

This reasoning prompted us to investigate whether the expression of GABA receptors subunits could somehow be positively modulated after IL-6 exposure. The GABA receptor subunit γ2 represents one of the master regulators of the GABA transmission development, as highlighted by the evidence that mutations in the *gabrg2* gene, linked to a reduction of the inhibitory transmission, have been associated to epilepsy [391-395]. We interestingly found a significant upregulation in the *gabrg2* mRNA expression and a slight trend in the *gabrb1* and *gabra3* subunits upon IL-6 application, suggesting that this cytokine could affect not only the protein expression of the inhibitory vGAT/gephyrin synaptic markers, but also the GABA receptor subunits.

To unequivocally demonstrate that IL-6 triggered intracellular [$Cl^-$] decrease by targeting the GABAergic signalling, we treated neurons with IL-6 together with the pharmacological inhibitor of the $GABA_A$ receptors, Bicuculline, in order to block GABAergic system. The evaluation of the intracellular [$Cl^-$] changes at 6 DIV showed that Bicuculline was sufficient to prevent the effect induced by IL-6 on the GABA switch suggesting a causal link between IL-6, GABA switch and inhibitory transmission.

Moreover, since the GABA switch is related to the net flux of ion chloride through $GABA_A$ receptors, we also wondered whether IL-6 could modulate the expression level of KCC2 and NKCC2. The mRNAs levels of the two chloride co-

transporters have been assessed and we interestingly found a significant KCC2 upregulation in treated versus control neurons. Overall this finding strengthened our hypothesis that IL-6 promotes the GABA switch.

Many studies reported in literature provided important evidences about KCC2 dysregulation and many pathological diseases. Fiumelli and colleagues demonstrated that the premature expression of KCC2 in immature neurons resulted in an alteration of spine morphology [396] and underlined that the fine regulation of KCC2 represents the *conditio sine qua non* for the well establishment of the inhibitory GABAergic transmission.

Kelley and colleagues recently showed that aberrant KCC2 activity enhanced the development, duration and severity of seizure events, in status epilepticus (SE) [65]. On the other hand, Silayeva and collaborators demonstrated that the development of SE, due to lower KCC2 expression, could be limited through a potentiation of KCC2 activity via its S940-phosphorylation [66]. Recently, a novel evidence described KCC2 as a critical downstream target gene of methyl CpG binding protein 2 (MeCP2), and its contribution to Rett Syndrome disease: in fact, MeCP2-deficient neurons showed significant deficits in KCC2 expression and a delayed GABA functional switch, with subsequent functional deficits, that were finally rescued after KCC2 overexpression [67].

Despite several evidences supporting detrimental effects of KCC2 downregulation on neuronal functioning, the possible molecular underpinnings of KCC2 regulation still remain controversial. Here we proposed that IL-6 is able to

accelerate the GABA switch in a STAT3-dependent manner, through the potentiation of GABAergic transmission

Moreover, IL-6 upregulates KCC2 expression and promotes the decrease of intracellular [$Cl^-$] in juvenile neurons.

Despite this clear evidence in vitro, whether this cytokine can also affect such process *in vivo* needs to be carefully considered. To address this issue, we took a step forward moving to a *in vivo* model. An explorative and very preliminary experiment (not shown) indicated that cultured neurons derived from mothers injected with IL-6 showed significantly increased MQAE levels, indicating lower intracellular [$Cl^-$], compared to neurons derived from saline- injected mothers.

This result would suggest that IL-6 prenatal exposure alters the GABAergic switch in offspring *in vivo*. However, further experiments are needed to corroborate this evidence. Hence, analyses of the inhibitory synaptic markers as well as KCC2 expression in the offspring brains, as well as behavioural and cognitive tests, will be evaluated.

As a general and intuitive view, there is the tendency to consider the enhancement of neuronal growth and network establishment as a positive event.

In line with this assumption, the role played by IL-6 might be considered as beneficial for the GABAergic switch. However, raising evidences strongly suggested that an early maturation of neurons, including a premature expression of KCC2, occurring when the brain is still developing, during pregnancy, may be

detrimental for the whole embryonic brain development, leading to functional impairments in the offspring.

Support of this statement comes from two considerations. Firstly, few works are suggesting that KCC2 early upregulation may sometimes negatively impact the neuronal fate and the network establishment, as well as lead to pathological conditions. Cancedda and colleagues, in fact, demonstrated that the premature KCC2 overexpression led to remarkable deficits in the morphological maturation of the neuronal progenitor cells, underlining the critical importance of GABA excitatory action in immature neurons, for the proper network establishment [397]. More recently, Award and colleagues found out that a premature and sustained KCC2 overexpression increased febrile seizure susceptibility and dendritic spine alterations, in a rat model of atypical febrile seizures [64].

Secondly, recent works demonstrated that IL-6 prenatal exposure during inflammatory states, may account for severe behavioural and cognitive impairments in the offspring. Smith et al, demonstrated that IL-6 was responsible for brain alterations and behaviour deficits in a model of maternal immune activation (MIA), induced by PolyI:C injection [309]. Furthermore, Wischhof showed that the prenatal LPS-exposure, a neurodevelopmental model of schizophrenia, caused deficits in cognitive functions, myelination and parvalbumin expression, in a sex related manner [103]. Most recently, Graham and colleagues provided a novel evidence in humans linking maternal inflammation during pregnancy, with newborn brain and behavioural phenotypes, that can be relevant for psychiatric disorders [102].

For all these reasons, it is a fair assumption that IL-6 prenatal exposure might also impair the GABAergic transmission leading to a pathological maturation of the GABA switch in the offspring, via the KCC2 premature overexpression.

To summarize, multiple effects have been observed in neurons treated with IL-6 *in vitro*, and this scenario necessarily begs important questions: how are these events sequentially activated after IL-6 exposure, in order to promote the GABA switch? In other words, does the chloride decrease represent the origin or the destination, in the IL-6-mediated action?

Altogether these data suggest that IL-6, through STAT3 activation, can enhance GABA signalling machinery via the expression of several GABA-related genes thus promoting the transition of GABA signalling from excitatory to inhibitory. It has been proposed that STAT3 can affect synaptic plasticity via non-genomic effect [398]. Based on this evidence an alternative scenario could be that IL-6-dependent STAT3 could activate several *cytoplasmic effectors* which in turn might converge to multiple layers of regulation thus controlling the switch of the GABAergic transmission.

In conclusion, the present work has been carried out with the attempt to achieve a better comprehension of the GABAergic developmental switch, suggesting two novel regulators of this event. On one hand, the efficacy of microvesicles in promoting the GABAergic switch through the intracellular [$Cl^-$] decrease, in comparison to whole cells, may represent an interesting option to be addressed for further insights, especially in the study of new therapeutic drug-carriers, targeted to cell-free based treatments.

On the other hand, we demonstrated that interleukin-6 can accelerates the timing of the GABA switch in neurons by means of a potentiation of GABAergic transmission. In parallel, IL-6 significantly promotes KCC2 upregulation, and all these events appeared to be STAT3-phosphorylation dependent.

Even though MSC-MVs and IL-6 are included in distinct research frames, and for this reason should be considered separately, it is possible to speculate about a possible connection between them. Indeed, several evidences suggest that IL-6 was found to be present within the MSC-MV contents, as weel as other cytokines [373, 375], thus suggesting a possible link between these two factors. All these data open the possibility to harness such system as new therapeutical approach, for delivering safe and nontoxic organelles to those pathological conditions characterized by a delayed GABA switch, such as neurodevelopmental disorders.

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