Review Article

Advances in the use of GABAergic interneurons for the treatment of epilepsy

Federica Frisina[1,2#], Giulia Valetti[1,2#], Giulia Zuccarini[1,2#], Luciano Conti[3#] and Giorgio R Merlo[1*]

[1]Department of Molecular Biotechnologies and Health Sciences, University of Torino, Italy
[2]Maria Pia Hospital, Torino, Italy
[3]Department of Cellular, Computational and Integrative Biology - CIBIO, University of Trento, Italy

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Abstract

Forebrain GABAergic neurons, the main inhibitory type of neuron in the cortex and hippocampus, represent a highly heterogeneous cell population that has been implicated in the predisposition to epilepsy and the onset of seizure. Earlier attempts to restore inhibition and reduce seizure in animal models of epilepsy have been carried out using embryonic basal forebrain tissue as source of immature GABAergic progenitors in cell-based therapies, with promising results. For therapeutic strategies this approach appears unrealistic, while the use of pluripotent stem cells to obtain immature GABAergic neurons opens new and promising avenues. Research on neural stem cells and pluripotent stem cells has greatly advanced and protocols have been established to efficiently direct progenitor cells to differentiate towards the GABAergic lineage. However, being highly heterogeneous, these neurons are difficult to be fully represented in vitro. Better knowledge on the expressed gene profiles, at single cell level, and the differentiation trajectory of these neurons will consent a more precise monitoring of the differentiation steps. Here we review the current literature about how to obtain and characterize genuine inhibitory neurons, how these can be grafted in animal models (and one day possibly in human) and which diseases could potentially be targeted and the efficiency of therapeutic outcome. The main obstacles that need to be overcome are: a) choice of an appropriate animal model, b) availability of human cells prone to GABA differentiation, c) the full representation of all IN subtypes, their proportions and their physiological activities, d) how to monitor them on the long-term after transplant.

Introduction

Epilepsy is a severe neurological disease affecting more than 70 million people worldwide. It is characterized by unpredictable and abnormal electrical discharges resulting in recurrent seizures [1]. Epilepsy has been causally linked to altered excitatory/inhibitory neuronal balance. Based on this theory, GABAergic interneurons (INs) are regarded as the primary inhibitory neurons whose activity failure results in hyperactivity of the epileptic circuitry [2].

Anti-epileptic drugs (AEDs) are the forefront treatment for seizure control [3]. About one third of patients with epilepsy, however, suffer from intractable seizures that are unresponsive to AEDs [4]. Furthermore, patients that respond to AEDs typically face serious adverse side effects [5]. Surgical removal of affected brain tissues or implanting neurostimulator devices are effective options only for a fraction of drug-refractory patients [6]. Furthermore, current treatments of epilepsy often enhance some of the deficits in cognitive functions typically associated with epilepsy, leading to poor compliance [7]. Thus there is urgent need to find alternative strategies, especially for AED-refractory patients.

Basic research on stem cells, their cellular and molecular properties, and their potential use in cell-based therapeutic approaches has greatly advanced, in recent years. Intense efforts have been placed on the establishment of protocols to efficiently direct differentiation of stem and progenitor cells towards several lineages. Among these GABAergic INs, the

main inhibitory neuronal type in the mammalian forebrain, have been obtained with good enrichment both from mouse and human stem cells, and hold promises for their potential use in clinic [8]. However, several problems and drawbacks arise, among which the highly heterogeneous neurochemical and networking properties of INs, the difficulty to fully recapitulate their developmental trajectories, and their fate upon transplant in the long term.

In spite of difficulties, attempts to use inhibitory GABAergic INs in cell-based therapies are showing some efficacy to attenuate the epileptic phenotype in selected animal models. Here we review the most relevant literature focussing on how to obtain and characterize genuine inhibitory INs and how these can be grafted in animal models (and one day possibly in human) of epileptic conditions, showing promising results that should foster future research in this area.

Types, origin and functions of forebrain INs

The mammalian cerebral cortex is typically a six-layered organization which includes two major classes of neurons: the excitatory pyramidal cells that project to cortical and subcortical targets, and the inhibitory non-pyramidal cells, the cortical INs. The hippocampus, that has a similar embryonic derivation, is equally composed of pyramidal projection and inhibitory neurons, although with a different layering organization and composition [9].

While pyramidal cells project their axons to distant region within or outside the cortex, and predominantly transmit signal using glutamate as neurotransmitter, INs have short, locally extending axons which connect to nearby neurons and use primarily γ-aminobutyric acid (GABA) as neurotransmitter. Within the mammalian cortex GABAergic INs represent 10-20% of the total cortical neuronal population, depending on the region examined, and are the only source of GABA [9].

Adult INs are highly heterogeneous from several points of view, and consequently several subclasses have been identified whose classification relies on morphologic, histologic, molecular and neurochemical features or electrophysiological properties, or combination of these criteria [10,11]. In the rodent cerebral cortex and hippocampus, more than twenty GABAergic IN subtypes have been documented. The expression of three calcium-binding proteins calretinin (CR), calbindin (CB), parvalbumin (PV), or other markers such as somatostatin (SST), neuropeptide Y (NPY), cholecystokinin (CCK), serotonin receptor 3a (5HT3aR), vasoactive intestinal peptide (VIP), reelin, and neuronal nitric oxide synthase (NOS), coupled to their distinctive morphology, connectivity, synaptic properties, and intrinsic firing properties are features that help to differentiate the various IN subtypes [12,13]. Recent in depth molecular analyses using single-cell RNAseq confirm and extends the extraordinary variety of INs [11,14,15] further indicating that such diversity might be specified as early as the progenitor state [16].

The origin and development of forebrain INs has been thoroughly studied in the rodent brain: they derive from progenitors that reside in basal regions of the embryonic brain, namely the median ganglionic eminence (MGE) (for cortex and hyppocampus), the lateral ganglionic eminence (LGE) (for olfactory bulb INs) and the caudal ganglionic eminence (CGE) [17-21]. Cortical and hippocampal INs derive mainly from the MGE and the CGE, and the majority of them express SST or PV, and originate from Nkx2.1⁺ progenitors in the MGE and the preoptic area [22-25].

Following exit from the cell cycle, the immature INs migrate into the cortical plate following stereotyped trajectories and timing, reaching the cortex and hippocampus primordial where they mature and integrate into local circuits [12,26]. INs migration follows several tangential routes and is critical for the correct establishment and integration of INs during embryonic and early postnatal life in both humans and rodents [21,27]. In human, in addition to interneuron genesis occurring within the GEs [28], IN progenitors have been detected in cortical progenitor zones, particularly at the anterior brain regions [29,30]. Intracortical interneuron genesis may start earlier than the time when tangential migration of INs from the MGE occurs [29].

As a general feature, GABAergic INs play a fundamental role in controlling the neural circuitry and network activity of the central nervous system (CNS), forming numerous connections with local pyramidal neurons or other INs and participating in the construction of functional networks. Cortical INs exert an essential inhibitory function within local cortical circuitry, modulate and coordinate signal transmission from pyramidal cells, synchronize oscillatory activities and participate in computational functions [31,32].

Each distinct IN subtype shows peculiar morphological and electrophysiological properties [32-34] and thus it is generally believed that each subtype of IN carries out specialized tasks in the control of information flow within the cortex. However, the specific function of each IN subtype, especially in the context of the global functioning of neuronal networks and their role in epileptic seizure, is still incompletely defined.

The involvement of INs and reduced GABAergic inhibition in epilepsy

Epilepsy reflects abnormal and massive hypersynchronous discharges from large assembly of neurons in cortical networks [35,36]. Temporal lobe epilepsy (TLE) is the most common form of epilepsy, characterized by the presence of epileptic foci in the limbic system, initial precipitating injury event (which induces the initial brain damage), the presence of a latent period and hippocampal sclerosis which leads to a reorganization of the neuronal network [37]. The etiology of epilepsy is complex and heterogenous, comprising structural, genetic, infectious, metabolic, immune and/or other unknown causes [38].

Epileptic seizure might be caused by a variety of factors [39-42]. Altered excitation-inhibition balance is a proposed common pathophysiological feature of multiple seizure and neuropsychiatric disorders, including epilepsy, schizophrenia and autism [43-51]. Altered IN genesis, migration and function may logically contribute to modify the excitatory-inhibitory balance, and specifically a reduced inhibition is the expected consequence of reduced number or hypoactive INs within cortical and hippocampal circuits. Failure or interference of IN migration leads to abnormal distribution of INs and alterations of the inhibitory control of the postnatal brain but also deprivation of the neurotrophic role of GABA in early development, resulting in epilepsies or other neurological disorders [26,52-56].

One specific subtype of INs has been critically involved in epilepsy, i.e. the fast-spiking PV-expressing INs [57-59]. However, the involvement of other subtypes might be under evaluated, since they are less studied. Indeed, the role of INs and altered excitation/inhibition balance in epilepsy is far from being completely understood. Recent evidence argues for the context-dependent, possibly excitatory roles that GABAergic cells play in epileptic circuitry. The dynamic, context-dependent role of GABAergic INs in seizure requires further investigation of their functions at single cell and circuitry level [60].

The causes of INs disfuctions associated with epilepsy range from genetic mutations, altered migration, altered lamination and fine histoanatomical organization, connectivity and plasticity defects. Indirectly, also metabolic and hormonal condition may influence the function of IN, and last but not least the exposure of the developing brain to stress or substances with teratological activity [61].

Dysfunction of cortical INs has been implicated in a wide range of neurological and cognitive/behavioral disease [33,62-64], leading to the term "interneuronopathy", now widely used. The variety of neurological and cognitive disturbances linked to altered IN development and functions is not surprising, considering also the infantile transitional stage in the maturation of the GABAergic system, including the migration of INs in the cortical cortex, development of GABAergic synapses and dendritic arbor, as well as the changing expression, composition, and function of GABA-A receptors, resulting from the depolarizing to hyperpolarizing switch in their responses.

A broad set of evidence implicate INs disfunctions in epilepsy [65,66]; and these include:

1. Human neurodevelopmental conditions comprising epilepsy, associated with mutations in IN-relevant genes; such is the case of the West and the Dravet syndromes. Mutations in over 25 epilepsy-associated genes in human have been proposed to promote over-excitability, some of them acting by reducing inhibition [66]. In tissue from patients with TLE,

hippocampal seizure foci were characterized by the loss of SST-expressing INs [67,68].

2. The association between a spectrum of early-onset epilepsies and neurodevelopmental or other neurological disorders that manifest as interneuronopathies in animal models. Such is the case of mice with mutations in *Arx*, in *Scn1A* and in *Apc*. Linked to this, the reduced IN population in rodent models of genetic (*Arx* mutant) and induced infantile spasms [65]. On the same line of evidence, the genetic mutation of *Snc1a* disrupts IN function and physiology and recapitulates the Dravet syndrome, characterized by infantile-onset drug-resistant epilepsy. In mice, the impairment of the activity of PV$^+$ and SST$^+$ INs leads to a disinhibition of the cortical network and a Dravet-like phenotype [69]. In a non-human primate model of neocortical focal epilepsy, a strong decrease in the number of GABAergic synapses was observed at epileptic foci [70].

3. The association between altered high-frequency oscillations (HFO) (such as γ and θ oscillations) and epilepsy [71,72]. HFO are typically recorded from brain regions capable of generating TLE, and altered HFO have been recorded in models of other epileptic disorders, such as neocortical epilepsy, genetically-caused epilepsy and infantile spasms [72]. Recent improvements in recording technologies and the introduction of optogenetics into epilepsy research has led to a better elucidation of the cellular substrates of epileptic HFO and of the role of altered neuronal networking. Although the role of INs in the generation of HFO is not fully defined, there are indications that a specific subpopulation of INs, the fast-spiking PV$^+$ INs, are central to the emergence and control of γ oscillation. Altered PV$^+$ neuron number and function have increasingly been associated to increase risk of epilepsy [73]. Likewise, INs have been implicated in the control of θ frequency oscillations [71].

4. Recent optogenetic studies have shown that enhancing the inhibitory function of GABAergic INs efficiently leads to suppression of seizures, in accordance with the concept that the excitatory-inhibitory balance shifts towards the excitatory regime in epilepsy [60,74,75].

5. Treatment with AEDs acting on the GABAergic synapse represents the most widely used clinical approach to treat epilepsy, and clinical data show that in these alleviate the manifestation during seizures, at least in the major of cases [76]. Treaments with AEDs however do not cure epilepsy, while on the other side systemic treatments with AED to prevent episodes may lead toserious side effects. More effective and local treatments are needed, that attempt to restore normal connectivity and excitation-inhibition balance.

Deriving GABAergic neurons from progenitor cells *in vitro*

As a most straight-forward approach, MGE embryonic cells can be dissociated directly from explants of the MGE

tissue and maintained *in vitro* in adherent conditions. These cells can be plated in culture and can undergo maturation into INs expressing *bona fide* mature markers [77]. This approach has been widely exploited with mouse tissues for many experimental works in which single cells can be labeled with fluorescent tracers and monitored for migration, neuritogenesis and synaptogenesis. However, this method is poorly applicable to human settings since the approach is limited by the scarceness of tissue (i.e. fetal MGE from aborted human embryos).

To overcome this barrier, in the last decade several protocols to obtain *bona-fide* MGE cells from pluripotent stem cells (PSC) have been described [8]. Mouse and human PSCs have been shown to have a broad potential to generate multiple CNS neuronal subtypes [78]. The possibility to derive mature forebrain INs directly from PSC holds great promises for research and therapeutic applications. Since both embryonic stem cells (ESCs) and iPSC (induced PSC) have been successfully derived from rodents and human, these represent the ideal source of cell-based therapies therapies, raising hopes for their applications in the field of neurodevelopmental or neurodegenerative disorders.

Initial studies have been performed with ESCs or iPSC of mouse origin and then extended to the human counterpart. In general, these protocols are based on the exposure of PSC-derived neuroectoderm tissue to specific morphogens/signals known to instruct embryonic MGE cells specification during development [79,80]. Watanabe and colleagues described that stage-specific Wnt pathway inhibition in mouse ESCs can induce the generation of rostral Foxg1$^+$ (i.e. telencephalic) progenitors and that their subsequent exposure to sonic hedgehog (SHH) drives the regionalization to a ventral Nkx2.1$^+$ forebrain MGE progenitor identity [81]. Although this study represented a milestone in the field, the efficiency was quite low and the cultures contained also Pax6$^+$ telencephalic dorsal progenitors (i.e. progenitors of cortical excitatory neurons).

Attempts to generate more pure cultures have been performed by exploiting reporter-based selection approaches. This strategy allows to better define factors for effective and more selective MGE regionalization and demonstrates that while FGF8 exposure can promote MGE identity, FGF15/19 had a negative effect, being effective in promoting CGE cell fates [82]. The most significative example of reporter-based selection approaches has been the use of Lhx6::GFP-expressing mouse ESCs, which allowed to select enriched populations of MGE progenitors *in vitro* [83]. This strategy showed to be successful (although with low efficiency) in generating SST$^+$ and PV$^+$ GABAergic INs *in vivo* and also showed their *in vivo* relevance after intracerebral transplantation [83].

It was then shown that forced expression of Nkx2.1 resulted in higher and prolonged expression of Lhx6 and improved efficiency of cortical IN generation, in the absence of SHH [45]. Likewise inducing the expression of Nkx2.1, Dlx2 and Lmo3 resulted in an ever-higher efficiency of MGE-type IN generation, with a preference of PV$^+$ over SST$^+$ young neurons [84]. Transplantation studies using these neurons substantially confirmed these findings [85]. Thus recapitulating the physiology of development *in vitro* results in the generation of apparently genuine INs.

Still, the differentiation of progenitors towards INs entails formidable problems, due to extreme diversification of IN subtypes and their early cell fate determination. One of the key issues is the lack of adequate molecular and functional markers to fully define them, and the poor knowledge on the temporal dynamic of their expression. Until few years ago INs were characterized at a population level by using few neurochemical markers, with the caveat that these analyses represents averages of cell behavior and cell states. While individual differences cannot be appreciated. Today we begin to appreciate the full complexity and variability of the molecular states of these neurons at the single-cell level [15,16]; in this respect Close and colleagues examined stepwise changes in gene expression profiles of *in vitro* differentiating progenitors, using a single cell approach, and then compare these with fetal cortical INsat various ages [86]. Their analysis yielded results that substantially confirm all key finding *in vivo*, while at the same time pointing to novel disease-relevant differentiation determinants, to be included in future monitoring efforts.

Differently form mouse PSCs, the generation of cortical IN-like cells from human ESCs and iPSCs lagged behind mainly because of the much longer time required for differentiation of human cells, their tendency to die after replating, and the lack of methods to purify IN progenitors or post-mitotic precursors from the mixed population that occur with every protocols. While some study demonstrated to capacity of human iPSCs to generate telencephalic-like progenitors [87], two studies were critical in moving the field forward. First, the optimization of aprotocol based on the strong dual inhibition of the TGFβ pathway with the peptide noggin and the small molecule SB431542 during initial phases of differentiation, showed that neural ectoderm can be efficiently obtained from pluripotent stem cells [88]. Second, also in human, differential WNT signal agonism or antagonism can be used to drive telencephalic (Foxg1$^+$) human ES-derived cultures into cortical-like (Pax6$^+$) or MGE-like (Nkx2.1$^+$) progenitors [89].

Now, a relatively rapid and uniform generation of telencephalic progenitors has been achieved. These progenitors can be "dorsalized" or "ventralized" into a variety of subfields and differentiated into a variety of neuron types, including cortical INs [90-92]. While these studies are promising, caution needs to be applied as they show two major challenges towards safe and reliable generation of human INs. First is the protracted maturation stage of the human MGE progenitors to give rise to functional INs. Second,

only few cells actually acquire a PV+ identity, which in turn could be the consequence of the longer differentiation time of these particular neurons. As much attention is drawn towards this disease-relevant neuron type, our knowledge is possibly biased towards these neurons.

Despite the challenge in generating reliable and stable human INs, two studies showed some degree of efficacy in the treatment of seizure in mouse models, *in vivo* [93,94]. Based on this, research in the field is rapidly proceeding trying to answer these crucial questions: a) can we obtain the desired IN type and subtype? b) can we retain their ability to establish inhibitory synapses on the correct neuronal targets? c) can we avoid long and complex protocols, inevitably leading to considerable cell loss and increased variability? Considering the high molecular variability, a valid monitoring method will be single cell RNAseq analyses [86].

Alternative strategies to the direct generation of INs from PSCs have been also developed.

Among them there is the possibility to derive stable and homogeneous lines of adherent neural stem cells from iPSCs and maintain them in monolayer culture while preserving a full and homogeneous commitment towards the GABAergic lineage [95,96]. These adherent monolayer cultures show limited capacity to maintain a defined dorso-ventral identity [97,98] and to generate multiple brain cell types, other than GABAergic neurons [99,100]. This approach has been shown to work also for human cells [101-103].

Finally, direct conversion strategies that allow to directly convert somatic cells (i.e. fibroblasts) into forebrain INs by forced expression of specific transcription factors have been recently described [104,105]. This technique can produce functional forebrain INs without the requirement to pass through a pluripotent state but the overall efficiency, in particular for the human system, is quite limited.

The choice of cells used in models of epilepsy

Cells from various sources and maintained in different conditions have been tested in preclinical models of epilepsy following grafting into distinct regions of the brain (Figure 1). The donor cells examined include hippocampal precursor cells, neural stem cells (NSCs), primary GABAergic neurons or GABAergic precursor cells derived from either embryonic LGE or MGE, or from mouse and human ESCs and iPSCs [106]. All these cells have been shown to modulate hippocampal plasticity, modify epileptogenesis, reduce the frequency of spontaneous recurrent seizures and alleviate related co-morbidities, although with varying efficiency. The outcomes varied depending on the animal model employed, the timing of grafting intervention after the first seizures and the time-point of measurement of SRS [106].

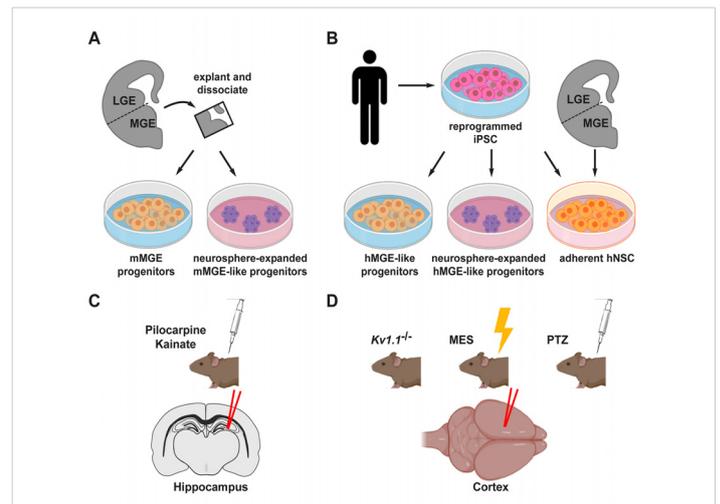1. NSCs short-term expanded from the embryonic MGE or from the postnatal subventricular zone (SVZ) are good

Figure 1: Schematic representation of the main cell-based experimental strategies to epilepsy.

A,B. The source of IN progenitor cells of rodent (A) or human (B) origin, used in experimental therapies. In rodents, most studies use progenitors obtained from dissociated MGE, or neurosphere derived from the same progenitors. In human, research is focusing on the use of iPSC derived from somatic cells as a source of IN progenitors to be used in cell-based therapies.

C,D. The most widely used models of epilepsy (induced or spontaneous) and the corresponding experimental strategies adopted for cell-based treatments in the hippocampus (C) or in the cortex (D). Spontaneous epilepsy is the consequence of genetic manipulation disrupting disease-relevant genes. On the other side, seizure can be induced physically by MES, and chemically with PTZ, pilocarpine or kainate. Abbreviations are indicated in the text..

candidates as donors, being multipotent and self-renewing [107]. To be noted, NSC grafting implies the possibility to replenish both new GABAergic INs and new astrocytes into a brain area, the later secreting a multitude of neurotrophic factors and anticonvulsant proteins such as GDNF [108,109]. Due to their ability to engraft into the dentate gyrus and influence hippocampal neurogenesis [107], NSCs might also be able to ameliorate those cognitive functions which typically decline in chronic TLE.

2. Cells dissociated from embryonic MGE can be maintained in culture and generate INs able to migrate in the host brain when transplanted in early postnatal mice, expressing INs markers PV, SST, NPY and CR [22,110]. These cells are characterized by the expression of markers such as NeuN, doublecortin (DCX) and Hu24, but they are negative for non-neuronal markers such as glial fibrillar acidic protein (GFAP), vimentin and Olig2 [111]. Most importantly, MGE-derived neurons establish synapses and direct evidences suggest that these cells can functionally integrate and influence GABA-mediated inhibition in the host brain [110]. Moreover, *in vitro* electrophysiology studies confirm that these cells have firing properties typical of mature INs [111].

3. Obtaining genuine INs from hiPSC to be used in cell-based therapy circumvents two key issues: the limited availability of fetal brain material and the use of autologous, patient-specific cell [112]. hiPSC-derived MGE-like progenitors potentially yield both cortical-like and striatal-like GABAergic INs that express SST, CR, and CB. Electrophysiological analyses have

shown a slow maturation of these INs. Following injection into the mouse brain, hiPSC-derived IN precursors dispersed from the injection site, matured into subtypes, and functionally integrated into the host cortex. They did not migrate as extensively as dissociated embyonic MGE cells and some cells remained at the injection site. This suggests that optimization of the iPS differentiation and/or development of methods to purify migratory MGE-like cells may be needed [92].

Advances in the use of GABA-committed progenitors in models of epilepsy

There is urgent need to develop new therapies that directly target epileptic foci restoring proper inhibition and synchronicity, rather than more systemic interventions [93,113]. One strategy that has been proposed in recent years is to restore normal circuit function by transplanting cells capable to differentiate into GABAergic neuronsat the seizure focus. This approach has been recently tested experimentally and validated, owing to improved tools and methods for cell propagation and differentiation *in vitro* [106,114] (Figure 1).

In this section we will illustrate the most recent developments and discuss critical issues in moving these experimental therapies into clinic. We will also highlight aspects such as cell-survival, migration, differentiation into INs subclasses and functional integration in the host network, and especially emphasizing the long-term effects of different grafting approaches. For clarity, we have subdivided the results of grafting into the hippocampus from those obtained in the cortex, justified by the fact that TLE, the most common type of epilepsy in adults, initiates at the hippocampus. Key observations are summarized in table 1 (hippocampus) and table 2 (cortex).

Hippocampus: TLE in rodents can be pharmacologically induced by systemic injection of pilocarpine or kainic acid (KA). These treatments recapitulate the *status epilepticus* (SE), followed by a latent period and the subsequent appearance of spontaneous recurrent seizures (SRSs) representing the chronic phase. These treatment also induce functional and histoanatomical changes in the hippocampus, such as degeneration of dentate hilar neurons and some pyramidal neurons in CA1 and CA3 regions, differentiation of dentate granule cells, reduction in the number of GABAergic INs, aberrant sprouting of dentate mossy fibers, hippocampal hyperexcitability and learning and memory deficits [37,93,107,115,116].

A first attempt exploited an established cell line of uncommitted human pan-neuronal stem cells (hNSCs) obtained from dissociated fetal brain tissue. These cells were grafted in the hippocampus of rats 24 hrs after the cessation of pilocarpine-induced SE [31], and the anti-epileptic effects were evaluated 28-35 days after. The grafts resulted in a significant reduction of seizure frequency, duration and severity, associated with reduced aggressiveness. Six weeks after grafting, hNSCs had migrated in various hippocampal

areas, in the amygdala and piriform cortex; 42 days later the number of grafted cells in CA1, CA3 and hilus was diminished, while it remained unchanged in CA2 and granule cell layers. GABAergic INs, representing 26% of grafted cells, were found in the hippocampus and piriform cortex, and 30% of these expressed PV. A small percentage of glutamatergic (GluR2$^+$) and astrocytes (GFAP$^+$) was also found in the CA1 area. The grafted cells did not acquire granular or pyramidal phenotypes, suggesting that hNSCs mostly differentiated into GABAergic INs. This was further confirmed by recording field excitatory postsynaptic potential (fESPS) in CA1 and stimulation of Schaffer collateral fibers. These experiments showed that the fEPSP amplitudes of hNSCs-transplanted group was significantly smaller compared to controls. Thus, the attenuation of seizure was associated with an enhancement of inhibition due to increased GABAergic activity in the damaged hippocampus [31]. In this study, the long-term fate of the transplanted cells, in terms of survival, senescence and stability of the differentiated phenotype, was not assessed.

In a subsequent study, GABAergic progenitors obtained from dissociated mouse embryonic LGE were used [117]. The cells were pre-treated with FGF2 and caspase inhibitor bilaterally grafted into the hippocampi on KA-treated rats. Grafing was done four days after the appearance of SE. The majority of surviving grafted cells (33% of the total) differented into NeuN+ cells, a large percentage of which was GABAergic, further subdivided in CB$^+$, PV$^+$, CR$^+$ and NPY$^+$ neurons (Table 1). This study performed a long-term follow-up, i.e. 9-12 months after grafting, and showed a much reduced frequency (between -67 and -89% compared to controls) of SRSs but observed no rescue of the aberrant mossy fiber sprouting [117]. Interestingly, although the LGE is not the physiological source of cortical and hippocampal INs, these results suggest that LGE and MGE progenitors are interchangeable when used as a source of cells for grafting, reasonably because their behavior might be strongly context-dependent.

MGE-progenitor cells dissociated from mouse embryonic brains were also used for transplantation studies into the hippocampi of adult mice, 9-20 days after pilocarpine-induced SE [115]. Starting from 7days after transplantation the grafted cells exhibited a bipolar migratory morphology and showed a survival rate of >30%. Sixty days after grafting, cells were found to participate in host inhibitory local circuits, with a survival rate of about 15% and showing features of mature INs. Within 2 months, the grafted cells differentiated into GAD67$^+$ INs, expressing a wide set of neurochemical markers including SST, nNOS and PV (Table 1). Very few cells expressed glial lineage markers. The authors characterized the grafted cells for their electrophysiological properties and RNA expression profiles. The profiling results (26% fast spiking, 41% regular nonpyramidal spiking, 9% late spiking and 24% burst spiking; all expressing gene markers typical of the MGE lineage of INs such as Lhx6) were consistent with an MGE-derived mature IN

Table 1: Grafting approaches in the hippocampus.

Tissue Of Origin	Transplanted Cells	Transplantation Strategy	Engraftment And Migration[1]	Neuromarkers	Phenotypic Effects[2]	Reference
hNSC	hNSC	*mouse model*: pilocarpine *how many*: 5×10^6 cells in 500µl	42 DAT [3]: cells found in CA1, hilus, dentate gyrus, CA3, amygdala and piriform cortex	42 DAT: 26% GABA+; 2% GluR$_2$+; 2% GFAP+	1 MAT [4]: increase of GABA inhibition; reduction of seizure frequency, duration and severity	31
LGE	GABAergic progenitors	*mouse model*: kainic acid *how many*: 8×10^4 cell/µl/site	12 MAT: cells found partly inside the hippocampus and partly in the lateral ventricle	12MAT: 69% GABA+ (30% CB+, 29% PV+, 31% CR+ and 8% NPY+)	9-12 MAT: 67-89% reduced seizures	117
MGE	MGE progenitors	*mouse model*: pilocarpine *how many*: 3×10^4 cells/injection	7 DAT: MGE-cells migration; 60 DAT: cells localized in host inhibitory local circuits; functional integration into hippocampal network (spontaneous excitatory post-synaptic currents)	2 MAT: 92% NeuN+; 63% GAD67+; 41% SST+; 21% NOS+; 7.7% PV+; 1.8% olig2+; 1% GFAP+	60 DAT: 92% reduced seizure frequency and behavioral comorbidities	115
MGE	MGE progenitors	*mouse model*: pilocarpine *how many*: 3×10^4 cells/injection	210 DAT: MGE-cells migration; 270 DAT: functional integration into hippocampal network (spontaneous excitatory post-synaptic currents)	6-7-12 MAT: Tuj1+; GA67+; SST+, PV+, NOS+	2-6-12 MAT: increase of GABA inhibition; reduced seizure frequency, rescue of behavioral deficits	118
MGE	MGE-derived neurospheres (GF): 2,7% bTub +; 20-30% GFAP-Nestin	*mouse model*: pilocarpine *how many*: 400 cells/4µl per animal	120 DAT: migration in DG and CA1	120 DAT: 1,9% NPY+; 2,4% PV+; 0,7% CR+; 5,6% GFAP+	90 DAT: reduced seizure frequency (glia-mediated)	119
MGE	MGE-derived neurospheres (GF-RA): 20% βTub; 10% GFAP 30% nestin	*mouse model*: pilocarpine *how many*: 400 cells/4µl per animal	120 DAT: migration in DG and CA1	120 DAT: 1,5% NPY+; 2% PV+; 3,4% CR+; 3% GFAP+	90 DAT: no anticonvulsant effects and lower cell-survival rate	119
MGE	MGE progenitors	*mouse model*: pilocarpine *how many*: 400 cells/4µl per animal	120 DAT: migration in DG and CA1	120 DAT: 12% NPY+; 7% PV+; 6% CR+; 0,7% GFAP+	90 DAT: reduced seizure frequency (IN-mediated)	119
hESC	MGE-like progenitors	*mouse model*: pilocarpine *how many*: 5×10^4 cells in a 0.5 µl/site	14 DAT and 4MAT: migration; 2-5 MAT: integration into hippocampal network	14DAT: 80% GABA+ (Nkx2.1; NeuN+; Lhx6); 4 MAT: 80% GABA+ (Nkx2.1; Sox6; Lhx6; SST; CB; PV; CR; NPY; VIP);	3 MAT: reduced seizure frequency and rescue of behavioral deficits, of GABA release and induced inhibitory postsynaptic responses in host neurons; excitatory synaptic inputs from host glutamatergic neurons	93
hiPSC	MGE-like progenitors	*mouse model*: kainic acid *how many*: 8-10 $\times 10^4$ cells/µl/ site	5 MAT: proliferation and migration in DG, CA1 and CA3 Integration into hippocampal network (formation of synapses between transplanted and host excitatory neurons)	5 MAT: 86% NeuN+: 76% GABA+ (27% PV+, 11% NPY+)	5 MAT: reduced seizure frequency, duration and severity; reduced anhedonia and cognitive deficits	112
MGE	NSC	*mouse model*: kainic acid *how many*: 8-10 $\times 10^4$ cells/µl/ site	migration in DG	NeuN+; GABA+; GFAP+; NG2+	reduced seizure frequency, duration and severity	107

(1): Effects of cells integration in host brain after transplantation. (2): Effects related to seizure and/or behavioral comorbidities. (3): DAT: days after transplantation. (4): MAT: months after transplantation.

Table 2: Grafting approaches in the neocortex.

Starting cells	Transplanted cells	Transplantation strategy	Engraftment and migration[1]	Neuromarkers	Phenotypic Effects[2]	Reference
MGE	MGE progenitors	*mouse model*: WT *how many*: 4×10^5 cells/mouse	30-40 DAT [3]: cells dispersed in cortex and integrated in the network	30 DAT: GABA+; GAD67+; CR+; PV+; NPY+; SST+	1 MAT [4]: MGE-derived neurons receive inputs from host brain neurons, establish synaptic contact with dendrites of host neurons	110
hPSCs	MGE-like progenitors	*mouse model*: WT *how many*: 1-10 $\times 10^4$ cells/ injection site	6-7 MAT: cells migrated and integrated in the cortex; received synaptic inputs, generated GABAergic synaptic output	6 MAT: 53% LHX6+; 51% GABA+; 61% CB+; 72% CR+; 50% SST+ and rare PV+	N/A	92
MGE	MGE progenitors	*mouse model*: Kv1.1$^{-/-}$ mice *how many*: 4×10^5 cells/mouse	30-40 DAT: cells dispersed in cortex and integrated in the network	30 DAT: 65% GABA+; 29% PV+; 44% SOM+; 10% NPY+; 5% CR+	1 MAT: reduced seizure activity	110

MGE	MGE progenitors	*mouse model*: MES *how many*: 5 x 10^4 cells/mouse	60 DAT: cells migrated to cortex, hippocampus, striatum, SVZ, corpus callosum	60 DAT: NPY+; CR+; PV+	60 DAT: protection against tonic seizures and reduced mortality rate	120
MGE	MGE-derived neurospheres	*mouse model*: PTZ and MES *how many*: 5−15 × 10^4 cells in 0.4 µl	60 DAT: cell migrated to piriform cortex, fimbria and ventricular wall	60 DAT: PV+	2 MAT: protection against PTZ-induced seizures, but not against MES-induced seizures.	121

(1): Effects of cells integration in host brain after transplantation. (2): Effects related to seizure and/or behavioral comorbidities. (3): DAT: days after transplantation. (4): MAT: months after transplantation.

phenotype. The authors reported a 92% reduction in seizure frequency but no rescue of aberrant mossy fiber sprouting, in accordance with previous results [115]. The therapeutic effects were analyzed only in a two-month time scale.

Adopting a similar experimental paradigm, another study used freshly dissociated MGE-derived progenitors grafted in a model of pilocarpin-induced seizure, and monitored the effect at 7 and 12 months after transplantation [118]. At 7 months, the grafted cells were differentiated into GAD67$^+$ neurons expressing SST, PV and nNOS (Table 1). Two months after grafting, the animals showed seizures suppression and rescue of behavioral comorbidities; at 6 months seizures frequency was reduced by 84% and at 12 months the seizures total number was reduced by 88%. The authors also documented enhancement of GABAergic IN-mediated currents suggesting a functional integration in the host network [118].

In a recent study, MGE-derived dissociated progenitor cells were used in grafting experiments and compared to MGE-derived neurospheres for their anticonvulsant effects in a model of pilocarpine-induced TLE [119]. Specifically, the authors compared three *in vitro* states of the grafted cells: fresh MGE-cells; MGE-derived neurospheres cultured with growth factors and retinoic acid (GF-RA group); or the same cultured without retinoic acid (GF group). While the GF-RA group resulted in increased neuronal population *in vitro*, only the GF-neurospheres and freshly dissociated MGE cells showed anticonvulsant activity. Both groups showed reduced seizure frequency 3 months after the grafting, but apparently by different mechanisms: GF-neurospheres efficiently differentiated into INs and glial cells with equal efficiency, freshly dissociated MGE cells mainly differentiated into INs, with only 0.7% of GFAP$^+$cells (Table 1). Thus, the anticonvulsant effects of GF-neurospheres seems to be glial-mediated, while freshly dissociated MGE cells appear more appropriates for cell-therapy treatment of epilepsy, targeting the inhibitory circuitry [119]. In this study only short-term effects were examined, and cell differentiation, senescence and survival were not assessed on the long-term.

On the whole, these studies indicate that grafted embryonic MGE cells are effective in reducing seizure frequency, duration and severity, as well as in alleviating behavioral co-morbidities. However, for clinical application cells sources must be standardized, well characterized, quality-controlled and unlimited in supply. For these reasons and for strong ethical issues, human embryonic MGE-cells cannot be routinely exploited. To overcome this limit, human iPSC have been tested as a valid and promising alternative. hiPSC-derived GABAergic INs were shown to be able to migrate and integrate into dysfunctional circuitry in mice with pilocarpine-induced TLE, and to generate inhibitory postsynaptic responses in host hippocampal neurons [93]. Two weeks post transplantation, grafted cells were found to primarily cluster near the injection site, with nearly 80% of cells expressing GABA, and a large fraction of these scoring positive for Nkx2.1, NeuN and Lhx6 (Table 1). Four months post-injection hiPSC-derived INs had migrated and integrated in the host circuitry, without significant differences in the total number of surviving cells. At this time-point, 80% of the grafted cells were *bona-fide* GABAergic INs (expressing Nkx2.1, Sox6, Lhx6 and GABA) and scored positive for the subtype-specific markers SST, CB, PV, CR, NPY and VIP (Table 1). All transplanted cells showed reduced positivity for glial markers, suggesting a proper IN differentiation. Overall, the approach suppressed seizures and ameliorated behavioral abnormalities, providing a first evidence of hiPSC application in the management of intractable epilepsy [93]. Similar results were recently published using hiPSC-derived MGE-like progenitor cells transplanted into the hippocampi of KA-treated rats [112]. The authors found that the grafted cells proliferate immediately after grafting, then migrate into DG, CA1 and CA3 subfields, were they differentiated in GABAergic INs with high efficiency (80%) and integrate into the host network forming synapses with excitatory neurons. The grafting resulted in reduction of aberrant neurogenesis, and a significative reduction of seizure frequency, duration and severity, as well as anhedonia and cognitive disfunctions [112].These effects, however, were analysed only in the short-term.

In summary, current data show that MGE-derived cells efficiently migrate and differentiate into GABAergic INs, able to functionally integrate in the host brain network when transplanted into the hippocampus of various models of epilepsy. The overall effect is a reduction of seizures occurrence and severity, associated with increase inhibitory activity. We should note, however, that most of the published studies observe cell and phenotypes in the short-term, while long-term analyses are very limited. Furthermore, all studies adopt a pharmacological induction of SE, which is difficult to relate this to epilepsy in human, characterized by spontaneous seizures caused by a combination of genetic and environmental factors.

Neocortex: The epileptic brain is characterized by a disfunction in excitatory-inhibitory balance that also involves the neocortex [50]. Thus, several studies focused on the effects of cell grafting directly into the cortex and the effect of this procedure on cortical circuity. The main results are summarized in table 2.

In an early study, MGE progenitor cells were dissociated from embryonic MGE and bilaterally transplanted into the cortex of wild-type mice; one month after transplantation cells were found to be migrated and dispersed in the host cortex and differentiated into GABAergic INs and their subclasses, the largest proportions being PV+ and SST+ [110] (Table 2). Grafted cells, specifically starting from 7-10 days after grafting, acquired intrinsic membrane properties of immature INs and 30-40 days after transplantation an increased inhibition of II/III layers pyramidal neurons was shown, while not changes in inhibition by host INs. This potentiation of inhibition involves postsynaptic GABA receptors and provides evidence that grafted cells integrate in brain circuits, receive inputs from host brain neurons and form synaptic contacts onto their dendrites primarily targeting excitatory pyramidal neurons [110].

In a subsequent study, hiPSC-derived MGE-like cells were grafted into the cortex of newborn SCID (severe combined immunodeficient) mice, and monitored thereafter [92]. The results show that these MGE-like cells survived in the brain after 2, 4 and 7 months, with an average surviving rate of 5%, 3% and 8% respectively. Two months post-injection grafted cells were found to proliferate and to express the immature neuroblast marker DCX+, while at 3 and 7 months post-injection cells had migrated in the cortex. By 7 months post-injection, the grafted cells were seen to express NeuN, a marker of more mature neurons. From a functional point of view, mature hiPSC-derived INs fired subtype-specific trains of action potentials, received synaptic inputs, generated GABAergic-exclusive synaptic output, and functionally integrated into the rodent cortex [92].

Three main types of animal models of cortical epilepsy have been used to test the efficacy of cell-based strategies: a) genetically induced, b) induced by maximal electroshock (MES), c) induced by pharmacological treatment with pentylenetetrazole (PTZ).

a) the most used genetic model of cortical epilepsy is the *Kv1.1/Kcna1* mutant mouse strain; these animals lack a shaker-like potassium channel and mimic a neuronal ion channelopathy associated with epilepsy in humans [110]. Freshly dissociated MGE progenitors were grafted into the cerebral cortex of P2 *Kv1.1/Kcna1* mutant mice resulting in a reduced frequency and duration of spontaneous EEG seizures 2 months after transplantation. Almost 65% of grafted cells differentiated in GABAergic INs, and expressed the subtype-specific markers PV, SST, NPY and CR [110].

b) the maximal electroshock (MES) model relies on the application of an alternating current causes a generalized tonic–clonic seizures [120,121]. Adopting this model, freshly dissociated MGE progenitors transplanted into the brain cortex 2 months after MES-induced seizure were shown to protect against tonic seizures and reduce the mortality rate [120]. On the contrary, cells dissociated from the MGE but then maintained and expanded *in vitro* as floating neurospheres showed strikingly different results in the MES model: unlike freshly dissociated MGE cells, neurosphere-expanded progenitors had no effects on seizures frequency [121], despite both experimental groups used a comparable number of cells injected bilaterally in the cortex. Thus, in the MES model transplantation of precursors dissociated directly from MGE is more efficient in reducing frequency and intensity of tonic seizures.

From a cellular point of view, in both studies, two months after injection grafted cells efficiently survived, migrated and differentiated into INs. However, the migration sites are different between these studies: Calcagnotto and colleagues found grafted cells in cortex, hippocampus, striatum, subventricular zone and corpus callosum, while Paiva and colleagues found INs throughout the brain parenchyma (piriform cortex, fimbria, and ventricular zone). Moreover, Paiva and colleagues examined only the PV+ cells, while Calcagnotto and colleagues evaluated the morphology and the expression of more IN markers including NPY+, CR+ and PV+ cells [120,121].

c) The pharmacological model based on treatment with pentylenetetrazole (PTZ) induces clonic or tonic–clonic seizures, by acting on GABA-A receptors and impairing local inhibition. Grafting of MGE progenitors maintained as neurospheres in the neocortex of PTZ-treated animals resulted in a protective effect from seizure [120,121]. These grafted cells showed a high rate of survival and of PV+ differentiation as compared to the same cells transplanted into mice that underwent MES [121].

Based on the available results, it can be concluded that neural progenitors maintained as neurospheres are not ideal, as compared to fresh isolated or *in vitro*-expanded progenitors from MGE. However, this seriously hampers the applications in human therapy, due to the limited availability of fetal tissues.

Concluding remarks and perspectives

The reported studies show that MGE-derived cells efficiently migrate and differentiate into GABAergic INs, able to functionally integrate in the host brain network when transplanted into hippocampus or cortex of various models of epilepsy. The overall effect is a reduction of seizures occurrence and severity. Electrophysiological recordings reveal that exogenous INs functionally integrate in the host circuity providing increased inhibitory activity, although not all authors investigated this aspect. Importantly, the

anticonvulsant efficacy of grafted cells seems to largely depend on the mode of collecting and expanding these cells: whether from primary dissociated MGE embryonic tissues [110,115,118-120], from neurosphere-expanded MGE progenitors [119,121], from differentiated hIPSC [92,93,112], or from hNSC [31]. Among these, the use of dissociated MGE cells seems to be the most valid and applied strategy. Studies on differentiation and transplantation of hiPSC-derived cells are likely to lead to significant improvements in the next few years.

Concerning the translation to the human setting, one issue that has to be taken into account is the animal model of epilepsy or TLE that have been used so far. Models of epilepsy induced by drugs, although easily standardized and quantifiable- and thus adopted in scientific studies –only partially and imprecisely represent human conditions. In alternative, genetic models in rodents (knock-out or transgenic), in which congenital chronic epileptic syndromes result from a known inherited genetic lesion, have been introduced [65]. Although these are certainly closer to reality, the issue that emerges is the existence of numerous, rare epileptic syndromes, with quite distinct and peculiar phenotypic manifestations. Each genetic model will only represent a single and specific human condition, certainly not the full spectrum and not the majority of conditions, which are rather sporadic or with multigenic risk determinants.

Most studies have demonstrated survival and differentiation of the grafted cells in the short- or medium-term (2-6 months), but not in the long-term; with the exception of two studies that prolonged the analyses to 12 months [117,118]. Information on long-term cell survival, differentiation and circuit integration is the next important step, in order to better evaluate the translation value of these experimental therapies in view of future clinical applications and to understand if they have a transient short-term effect or a long-term efficacy. In the perspective of clinical trials, a transient therapeutic effect is clearly insufficient.

The follow up of the grafted progenitors or the immature INs, monitoring their proliferation, stability of the differentiated phenotype, senescence and apoptosis, is another key task. In the rodent brain cells labeled with fluorescent markers can be used for *in vivo* imaging, and single neurons can be visualized and subjected to neuro chemical and electrophysiological analyses. The PV+ and SST+ neurons are the most studied ones, however the repertoire of IN subtypes is much wider, and the contribution and/or involvement of each of these in seizure is not fully known. Upon grafting, we are currently unable to monitor all IN subtypes, and consequently their survival, senescence and differentiation is not really known. It is likely that we are still missing some important contribution, and this could also be the reason for discrepancies in the results from lab to lab.

Likewise, which and how each IN subtype participates and contributes to epilepsy is not fully understood. Although PV+ neurons appear to be more directly implicated and are observed after grafting, this could simply reflect the fact that they are mostly studied as compared to other types. If we knew better which types participate and how, we could adopt in vitro procedures that can be able to lead and expand that kind of progenitors, avoiding unnecessary mix of cells. A deeper knowledge of the main actors in the epilepsy's seizure context would be necessary to develop a better and more specific therapeutic approach. This could in principle be done using optogenetic methods, which these consents to activate or inihibit specific subpopulation of neurons *in vivo* with unprecedented time-resolution [60].

In parallel, strong efforts in basic and applied research on neural progenitor cells are needed to fully unravel the differentiation trajectory of each IN subtypes in vitro [11,86] and to optimize expansion and differentiation protocols aimed to obtain the specific IN subclass required, reliably and reproducibly. One important advancement will be the development of human cell models of seizure, either deriving hiPSC from patients carrying known mutation in epilepsy–relevant genes, or introducing the same mutations via genome editing (CRISPR-cas9). These cells could then be used in research (set up mixed cultures of excitatory and inhibitory neurons or mixed cortical and basal brain organoid). Once validated, these cells could open to way to screening efforts for the search of novel compounds.

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Author contributions

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