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GENEESKUNDE

master in de biomedische wetenschappen: klinische moleculaire wetenschappen

Masterproef

Role of EFHC1 in the control of tangential migration in the developing rat brain

Promotor :
Prof. dr. Jean-Michel RIGO

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prof. dr. THIERRY GRISAR
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Masterproef voorgedragen tot het bekomen van de graad van master in de biomedische wetenschappen , afstudeerrichting klinische moleculaire wetenschappen

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List of abbreviations

BT	basal telencephalon
CP	cortical plate
CSJ	corticostriatal junction
DAPI	4',6-diamidino-2-phenylindole
DCX	doublecortin
DiI	1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate
E	embryonic day
EEG	electroencephalogram
EFHC1	EF-hand domain containing 1
EFHC2	EF-hand domain containing 2
EGFP	enhanced green fluorescent protein
GABA	gamma-aminobutyric acid
GFP	green fluorescent protein
GGE	genetic generalized epilepsy
hEFHC1	human EF-hand domain containing 1
ILAE	International League Against Epilepsy
IZ	intermediate zone
JME	juvenile myoclonic epilepsy
LGE	lateral ganglionic eminence
MAP	microtubule-associated protein
MGE	medial ganglionic eminence
MRI	magnetic resonance imaging
NCx	neocortex
rEFHC1	rat EF-hand domain containing 1
shRNA	short hairpin RNA
Str	striatum
SVZ	subventricular zone
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
VZ	ventricular zone

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Abstract

Juveniele myoclonus epilepsie (JME) is de meest voorkomende genetisch gegeneraliseerde epilepsie (GGE), maar de pathofysiologie van deze ziekte is nog steeds onbekend. EFHC1 is een interessant kandidaatgen in de zoektocht naar de oorzaak van JME omdat mutaties in dit gen gedetecteerd werden bij verschillende niet-verwante families met JME wereldwijd. Onze onderzoeksgroep heeft onlangs aangetoond dat het uitschakelen van de expressie van EFHC1 in de cortex van rattenembryo's door middel van ex vivo en in utero electroporatie leidt tot een significante verstoring van de radiale migratie van projectieneuronen. Deze bevindingen suggereren dat EFHC1 een rol speelt in de nucleokinese van migrerende neuronen. Om dit te bewijzen onderzochten we of EFHC1 ook betrokken is bij de tangentiële migratie van interneuronen in de hersenen van rattenembryo's. Hiertoe werd de expressie van EFHC1 in het basale telencephalon (BT) gemoduleerd met behulp van ex vivo en focale electroporatie. Zogenaamde «loss of function» en «gain of function» experimenten werden uitgevoerd door respectievelijk gebruik te maken van short hairpin RNA (shRNA) en overexpressie van EFHC1. Directionele ex vivo electroporatie bleek niet geschikt te zijn voor het bestuderen van de tangentiële migratie. Met behulp van focale electroporatie konden de tangentieel migrerende neuronen echter wel specifiek beïnvloed worden, vandaar dat deze laatste techniek gebruikt werd in alle hierop volgende experimenten. EFHC1 knockdown in het BT veroorzaakte een significante verstoring van de tangentiële migratie in vergelijking met de negatieve controle. Een rescue experiment toonde aan dat dit effect te wijten was aan EFHC1 loss of function. EFHC1 overexpressie had echter geen effect op het migratieproces van interneuronen tijdens de hersenontwikkeling. Een terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay demonstreerde ten slotte dat EFHC1 knockdown geen apoptose induceert in het BT. De resultaten van dit onderzoeksproject tonen aan dat EFHC1 een rol speelt in de tangentiële migratie van interneuronen. Bovendien kunnen ze ook de observatie van «microdysgenesis» en overexcitatie in de hersenen van JME patiënten verklaren. Als deze hypothese klopt, dan is JME eerder een ontwikkelingsstoornis in plaats van een «channelopathy», een aandoening van de ionenkanalen in de celmembranen. Er is echter verder onderzoek nodig om te kunnen bevestigen dat EFHC1 inderdaad een rol speelt in de nucleokinese van migrerende neuronen en dat het geobserveerde defect in migratie niet te wijten is andere gebeurtenissen tijdens de corticogenese die het migratieproces voorafgaan.

Juvenile myoclonic epilepsy (JME) is the most common genetic generalised epilepsy (GGE), but its underlying pathophysiological mechanism is still unknown. *EFHC1* is an interesting causative candidate gene for JME as mutations in this gene have been linked to several unrelated affected families all over the world. Our research group recently demonstrated that EFHC1 impairment in the rat developing neocortex by *ex vivo* and *in utero* electroporation causes a significant disruption of radially migrating projection neurons. These findings suggest that EFHC1 plays a role in the nucleokinesis of migrating neurons. In order to prove this, we examined whether EFHC1 also affects the tangential migration of inhibitory interneurons in the developing rat brain. This was studied by modulating its expression in the basal telencephalon (BT) using *ex vivo* and focal electroporation. Both loss of function experiments using short hairpin RNA (shRNA) and gain of function experiments overexpressing EFHC1 were performed. Directional *ex vivo* electroporation resulted only in the labelling of radially migrating neurons in the neocortex. Focal electroporation, however, succeeded in targeting specifically tangentially migrating interneurons and was therefore used in all subsequent experiments. EFHC1 knockdown in the BT caused a significant disruption of tangentially migrating neurons compared with the control condition. A rescue experiment demonstrated that this effect was due to EFHC1 loss of function. Overexpression of EFHC1, in contrast, had no effect on the migratory process of developing interneurons. Furthermore, a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay revealed that EFHC1 knockdown does not induce apoptosis in the BT. Taken together, these results indicate that EFHC1 is involved in the tangential migration of interneurons originating from the BT. This could explain the observed microdysgenesis and hyperexcitability in JME patients. This also suggests that, instead of being a new form of channelopathy, JME could be considered as a developmental disorder. However, future experiments have to reveal whether EFHC1 indeed plays a role in the nucleokinesis of migrating neurons and whether the observed effect is not due other steps in corticogenesis preceding the migratory process.

1 Introduction

Epilepsy is the second most common neurological disorder affecting 50 million people worldwide (1). In fact, the term «epilepsy» does not refer to one disease, but to a diverse family of syndromes characterized by recurring epileptic seizures caused by synchronized electrical discharges of central neurons. The International League against Epilepsy (ILAE) Commission has classified all types of epilepsy that have been described until now. Based on the origin of the seizures, epileptic syndromes can be focal or generalized, whereas based on their underlying etiology they are classified as structural-metabolic (including cortical malformations, brain tumors and strokes), genetic or unknown (2). It is presumed that approximately 50% of all epilepsies are genetic GGEs because of their family history, and so is the type of epilepsy discussed in the following section of this paper (1, 2).

1.1 Juvenile myoclonic epilepsy

JME represents 10-30% of all epilepsy cases based on hospital reports (3-5), with an incidence of 1 per 100,000 and a prevalence that varies from 10 to 20 per 100,000 (3). However, exact figures may be higher as the disease is still often under-diagnosed (4). JME is principally characterized by myoclonic seizures that correspond to abrupt involuntary jerks of mainly the upper limbs. Generalized tonic-clonic seizures are also frequently observed in patients and sometimes absences are one of the recurring symptoms (3). Seizures usually occur shortly after awakening and are triggered by alcohol, sleep deprivation or psychological stress. They typically appear for the first time during adolescence between the age of 12 and 18 years old (3, 5). Furthermore, a patient's electroencephalogram (EEG) shows typically diffuse bilateral, symmetric, synchronous spike-wave and polyspike-wave complexes during actual myoclonias (4). JME patients require lifelong treatment in order to become seizure-free, as seizures nearly always return after withdrawal of their medication. Anticonvulsants such as valproic acid are the first choice of treatment, but unfortunately 15% of JME patients are still resistant to current therapies (3, 4).

Neuroimaging studies are usually normal in patients with JME (3-5). However, many independent research groups have recently observed subtle abnormalities in the cerebral structure of several patients with JME with the help of volumetric measurements and voxel-based analyses of magnetic resonance imaging (MRI) studies. They reported an increase in cortical grey matter in the mesial frontal lobe and an atrophy of the hippocampus, the thalamus and the corpus callosum (6-8). Additionally, post-mortem analysis of the brains of JME patients has identified the presence of

microscopic malformations called «microdysgenesis», characterized by dystrophic neurons in the grey matter and an abnormal cortical architecture (9). All these findings have been interpreted as a manifestation of developmental disturbances at a cellular level and may reflect changes of neuronal connectivity in the epilepsy brain (10, 11).

A genetic contribution to JME has long been established. There is a positive family history in 17-66% of all studied epilepsy patients (3-5), but the exact mode of inheritance is still unclear as there have been reports of autosomal dominant, autosomal recessive, multifactorial and complex models (4, 12). So the JME phenotype depends most likely on the interaction of various identified genes with other genetic, epigenetic or environmental factors (3).

Up to now, 15 chromosome loci (EJM1a-e, EJM2 … EJM11) have been genetically linked to JME by genome-wide linkage studies or by linkage analysis that tests specific candidate regions (13). With the help of mutation analyses, two possible causative genes have been identified. The first one, *GABRA1*, is localized at locus EJM5 and encodes the α_1-subunit of the $GABA_A$ receptor (14). Though, the contribution of this gene to JME seems to be small as mutations in this gene have been identified in only one separate family so far. The second one, *EFHC1* at locus EJM1a, is an interesting possible causative gene for JME as mutations in this gene have been described in several unrelated families with JME from all over the world (15-21). Currently, EFHC1 mutations are even the most common «cause» of JME, accounting for 9% of all cases (13). Moreover, the fact that *EFHC1* encodes a non-ion channel protein brings a new point of view to the pathophysiology of JME and genetic epilepsies in general, as defects in genes coding for ion channels have long been thought to be the only genetic cause of epilepsy (22). This is why several epilepsy syndromes are often called «channelopathies» in literature. However, more and more research groups believe that, regardless defects in ion channels, also defects in processes taking place during brain development may underlay several epilepsy phenotypes, including JME (15, 23-25).

1.2 EFHC1

1.2.1 Gene and protein characteristics

The *EFHC1* gene was discovered by Suzuki *et al.* in 2004 (15). It is located at the chromosomal region 6p12.3 between the markers D6S1960 and D6S1024 and spans 72 kb (15, 26). It consists of 11 exons encoding a 70 kDa protein of 640 amino acids (Fig. 1, Isoform A). EFHC1 transcripts undergo alternative splicing of exon 4, resulting in a C-terminally truncated protein of 278 amino acids (Fig. 1, Isoform B). The EFHC1 protein consists of three tandemly repeated DM10 domains of unknown function and one EF-hand domain at the C-terminus (Fig. 1). This EF-hand is a Ca^{2+}-binding domain

with a helix-loop-helix topology, much like the spread thumb and forefinger of the human hand . This also explains how the gene received its name, as EFHC1 stands for «EF-hand domain containing 1».

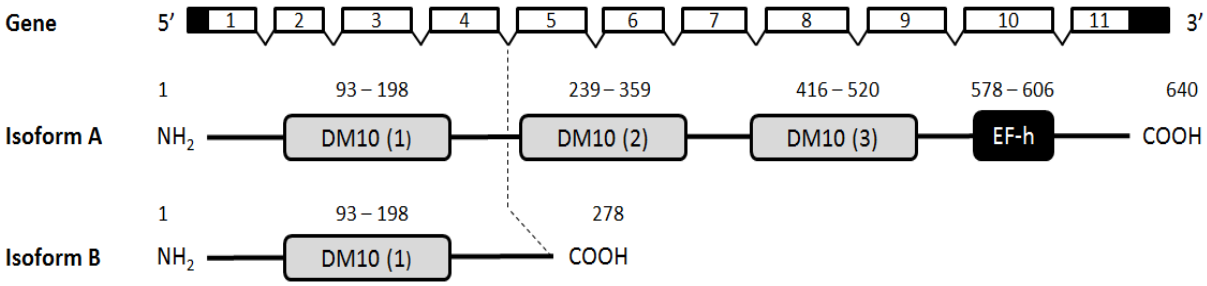

Figure 1. Schematic illustration of the *EFHC1* gene and both protein forms encoded by this gene. Isoform B is the consequence of alternative splicing in exon 4, resulting in a truncated form of the protein. The dotted line indicates the alternative splicing site.

EFHC1 is highly conserved in a variety of organisms including human, chimpanzee, mouse, rat, dog, cow, chicken, zebrafish, fruitfly and even green alga. A BLAST search with the human EFHC1 protein (hEFHC1) sequence as a query showed an identity of 83% with the rat protein (rEFHC1, also known as Efhc1). *EFHC1* has one paralog, *EFHC2*, which also has been shown to be associated with JME (27).

The distribution of EFHC1 mRNA has been verified by quantitative RT-PCR, northern blot analysis and in situ hybridization (15, 28-30). Both EFHC1 transcripts were found to be expressed in various tissues, including the brain. Interestingly, higher mRNA levels could be detected at embryonic stages than in adult stages (15, 28, 31). Immunostainings confirmed these observations (29, 30). The majority of EFHC1-positive cells in the brain cortex corresponded to neurons, although also a few glial cells were stained (15, 29).

1.2.2 Mutations in EFHC1 cause juvenile myoclonic epilepsy?

Table 1 gives an overview of all mutations in EFHC1 that have been identified so far in six different cohort studies. These mutations are all considered to be pathological instead of being neutral polymorphisms as they were not observed in unrelated healthy controls (15-20). They are relatively more common in Hispanic JME populations from California, Mexico, and Honduras and in Caucasians from Italy and Austria compared with populations from Japan and Tenessee (13). Eleven of the 14 mutations are missense mutations, meaning that the nucleotide change results in an amino acid substitution. These mutations may possibly alter the conformation and hence the function of the protein. Also one nonsense mutation was identified in isoform B. The remaining two mutations are deletions situated respectively in the promoter region and coding sequence of isoform B. The first deletion may affect the promoter function whereas the second causes a frameshift mutation.

Table 1. Overview of all currently identified mutations in the *EFHC1* gene. The nucleotide changes and the subsequent amino acid substitutions (if applicable) are listed in the first two columns, followed by the location in the gene sequence and the publications that reported these mutations.

Nucleotide change	Amino acid change	Location	Publications
-364-362del.GAT	n/a	5' promoter region	Medina *et al.* 2008
229C>A	P77T	Exon 1 of isoform A	Suzuki *et al.* 2004
520A>G	I174V	Exon 3 of isoform A	Stogmann *et al.* 2006
628G>A	D210N	Exon 5 of isoform A	Suzuki *et al.* 2004
662G>A	R221H	Exon 5 of isoform A	Suzuki *et al.* 2004 Ma *et al.* 2006
685T>C	F229L	Exon 5 of isoform A	Suzuki *et al.* 2004 Annesi *et al.* 2007
755C>A	T252K	Exon 5 of isoform A	Medina *et al.* 2008
757G>T	D253Y	Exon 5 of isoform A	Suzuki *et al.* 2004
776G>A	C259Y	Exon 5 of isoform A	Stogmann *et al.* 2007
1057C>T	R353W	Exon 6 of isoform A	Annesi *et al.* 2007
1180G>T	A394S	Exon 7 of isoform A	Stogmann *et al.* 2007
1523C>G	T508R	Exon 9 of isoform A	Medina *et al.* 2008
789del.A	Frameshift V264fsx280	Exon 4 of isoform B	Medina *et al.* 2008
829C>T	Q277X	Exon 4 of isoform B	Medina *et al.* 2008

1.2.3 Unraveling the function of EFHC1

It is important to identify the physiological function of EFHC1 to understand its role in the pathophysiology of JME. It is for example still not known whether the disease phenotype results from a gain or loss of function of the mutant protein. Moreover, whether or not both splice variants – isoform A and B – are involved in the etiology of JME is yet to be established (22). Several research groups, including ours, tried to unravel the function of EFHC1 and came up with their own hypothesis of how EFHC1 mutations can cause JME (15, 23, 24, 32, 33).

In their seminal paper, Suzuki *et al.* demonstrated that EFHC1 overexpression induces apoptosis of neurons *in vitro* through its association with an R-type voltage-dependent calcium channel ($Ca_v2.3$) (15). This pro-apoptotic effect was partially reversed in cells expressing the pathologically mutated forms of EFHC1 described in their article (15). Therefore, they hypothesized that the decrease in apoptosis by mutations in EFHC1 leads to a diminished elimination of unwanted neurons during brain

development and hence an increased density of neurons in the cortex, resulting in hyperexcitable circuits. This would explain the generation of seizures in JME patients.

Another research group reported that Rib72, an ortholog of EFHC1 found in *Chlamydomonas renhardtii*, plays a role in the organization and regulation of flagella motility (32). Two years later, they demonstrated that the mouse ortholog of EFHC1 is an axonemal protein that is widely conserved among organisms with motile cilia and flagella (25). It was confirmed that EFHC1 was expressed in cilia located on ependymal cells lining the ventricles of the brain (29, 30). These cilia are responsible for the movement of cerebrospinal fluid. Sawamoto *et al.* reported that the flow of this cerebrospinal fluid creates a chemical gradient within the subventricular zone (SVZ) of the neocortex (NCx) and is required for the correct migration of neuroblasts in the adult mouse brain (34). These findings imply that defects in EFHC1 may indirectly lead to a disturbed neuronal migration and consequently a state of hyperexcitability in the brain (33).

Our research group revealed that the EFHC1 protein is a microtubule-associated protein (MAP) that associates with the centrosome and the mitotic spindle through the first 45 amino acids of its N-terminus (23, 35). As the protein sequence at the N-terminus shows no obvious homology with the microtubule-binding domains (MTBD) of other MAPs, EFHC1 has probably a unique function. Furthermore, we demonstrated that loss of function of EFHC1 in *in vitro* experiments disrupts mitotic spindle organization, impairs M phase progression, induces microtubule bundling and increases apoptosis (23). Moreover, we demonstrated that EFHC1 impairment in the rat developing cortex by *ex vivo* and *in utero* electroporation causes a significant disruption of radial migration of projection neurons by affecting different steps during corticogenesis: (i) division, cell cycle exit and survival of cortical progenitors, (ii) organization of radial glia scaffolding and (iii) locomotion of post-mitotic neurons (23).

Preliminary data from our research group showed that EFHC1 mutations discovered by Suzuki *et al.* do not hinder the protein's ability to co-localize with the centrosome and the mitotic spindle, but induced the formation of aggregates and disrupt cell division. They also seemed to impair radial migration during brain development. These results indicate that mutations in EFHC1 could be responsible for abnormal cell division and neuronal migration during brain development. These defects may clarify the observed microdysgenesis and the existence of hyperexcitable circuits in JME patients.

1.3 Tangential migration in the developing brain

1.3.1 Origin and route of tangentially migrating interneurons

Neuronal migration is important for the formation of the cortex and defects in this process may result in subtle abnormalities in neuronal positioning in the cortex. The direction of cell migration in the developing brain can be determined from the orientation of the leading processes of the neurons. Based on this orientation and on their origin, there are two major types of neuronal migration in the developing brain of mammals: radial and tangential (36, 37).

Radially migrating neurons give rise to excitatory projection neurons that produce and respond to the neurotransmitter glutamate. They originate in the ventricular zone (VZ) of the pallium and use radial glial cells as guides to migrate towards the pial surface. Tangentially migrating neurons, on the other hand, are principally inhibitory interneurons characterized by the production of gamma-aminobutyric acid (GABA) and their responsiveness to this neurotransmitter. Although they only represent 20-35% of the total neuron population in rodent brains (37, 38) they are very important for conserving the balance between excitation and inhibition in the cortex. Unlike radially migrating neurons, they are independent of radial glia, which means they can change their cellular partners and direction of migration (38). This explains for example why GABAergic interneurons turn from tangential to radial migration at the end of their journey to reach their final position in the cerebral cortex (39). However, the molecules and mechanisms that control the migratory process of these neurons in the developing brain are still poorly understood (40). Most tangentially migrating cells are born in the ventricular zone (VZ) of the BT and more precisely in the medial and lateral ganglionic eminences (MGE, LGE), structures protruding into the lateral ventricles (Fig. 2).

The generation of tangentially migrating cells begins around embryonic day 13 (E13) in the rat brain and continues throughout embryonic development and probably even shortly after birth (41). At early embryonic stages (rat E13-14; Fig. 2A), they originate mainly from the MGE (39, 41, 42). Most articles agree that MGE cells follow mainly a superfacial route, passing the developing striatum (Str), to reach the marginal zone (MZ) and the subplate (36, 41, 43). Kriegstein *et al.* showed that the cells already disperse to all cortical layers, with prominent bands in the lower intermediate zone (IZ) and SVZ (39).

At mid-embryonic stages (rat E14-16; Fig. 2B), the peak of migration is reached with interneurons still arising primarily from the MGE (43). Nevertheless, the MGE starts to regress in favor of the LGE that eventually will occupy almost the whole extension of the BT (41). Most cells evade the striatum and follow a deep route towards the cortex (36), whereas a few of them enter the striatum or migrate still superficially (43). Once they have crossed the corticostriatal junction (CSJ), interneurons seem to

follow two different routes: some of them are directed to the MZ and others to the lower IZ and SVZ (36, 41, 42). The IZ/SVZ is believed to be the main corridor through which cells enter the cortex (39).

Finally, at late embryonic stages (rat E16-17; Fig. 2C), both the LGE and MGE contribute to the generation of tangentially migrating interneurons (36, 43). MGE-derived cells follow a deep route and migrate preferably through the lower IZ and subplate (41, 43) whereas LGE-derived cells also enter the SVZ (39). At E17, the MGE has completely disappeared and both ganglionic eminences are restructured as a consistent elevation of tissue called the BT (41).

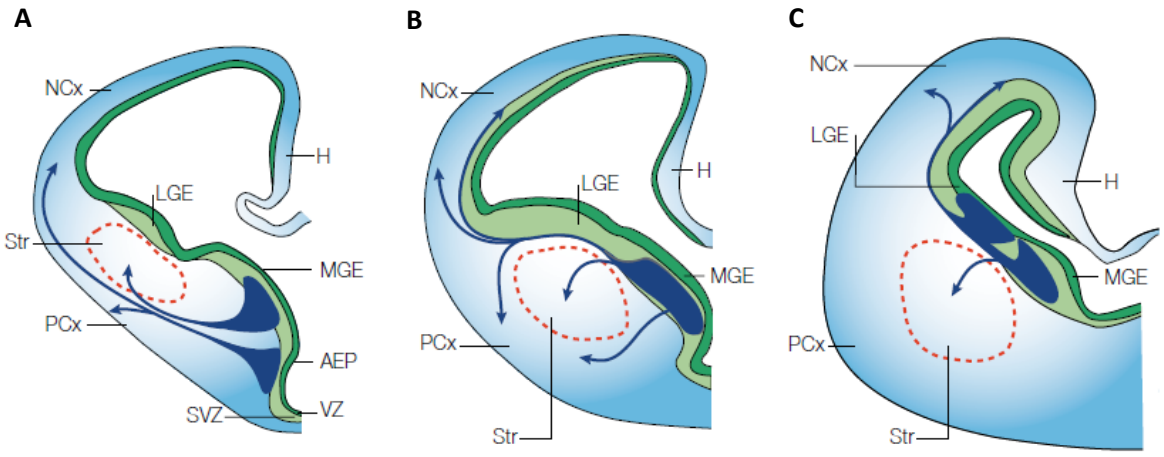

Figure 2. Coronal brain sections showing the route and origin of tangentially migrating interneurons during different stages of brain development. (A) At early embryonic stages (E13-14), cells originate principally from the MGE and follow a superfacial route. **(B)** At mid-embryonic stages (E14-16), cells still mainly arise from the MGE and enter the cortex via two different migratory streams within the MZ and the IZ/SVZ respectively. **(C**) At late-embryonic stages, tangentially migrating neurons are generated in both the MGE and LGE and migrate mainly into the lower IZ, subplate and SVZ. Drawings adopted from Marìn *et al.* (43). LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; SVZ, subventricular zone; VZ, ventricular zone

1.3.2 Mechanisms and molecules involved in tangential migration

A wide range of molecules has been proposed to play a role the migratory process of interneurons, including factors involved in chemoattraction/-repulsion, cell adhesion, cell motility and cytoskeletal dynamics (43, 44). The fact that interneurons can switch from tangential to radial migration, however, indicates that the pathways involved in both types of migration are not necessarily exclusive (36). Time-lapse videos have revealed that cells from both types of migration move forward by means of nucleokinesis, a saltatory process existing of two repetitive steps: (i) extension of the leading process and movement of the centrosome into this leading process and (ii) translocation of the nucleus towards the centrosome and retraction of the trailing process (Fig. 3A) (45-47).

Nucleokinesis appears to require many of the same cytoskeletal and signalling molecules involved in cell mitosis (45), with both microtubule and actin networks operating synergistically. A system of longitudinal microtubules provides stability to the growing leading process and plays a vital role in the association of the centrosome during nucleokinesis (Fig. 3B). Moreover, MAPs have been shown to stabilize these microtubules trough their MTBDs (45, 46). This makes clear that proper regulation of microtubule dynamics by MAPs is necessary for neuronal migration. The MAPs Lis1 and doublecortin (DCX) have already been shown to be involved in neuronal migration and even in the pathogenesis of neurological disorders (48, 49).

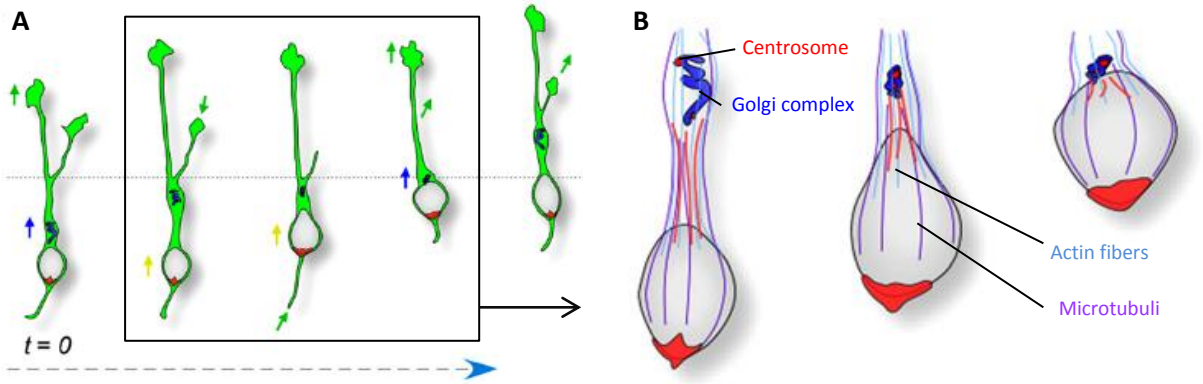

Figure 3. Nucleokinesis in tangentially migrating neurons. (A) The migratory cycle of interneurons illustrating the two different phases during nucleokinesis: (i) extension of the leading process and movement of the centrosome into this leading process (blue arrows) followed by (ii) translocation of the nucleus towards the centrosome and retraction of the trailing process (yellow arrows). **(B)** A high magnification schema of the nucleus and the cytoskeleton during nucleokinesis. Both microtubule (red/purple) and actin networks (light blue) are involved in this process, as well as the centrosome (red) and golgi apparatus (ark blue). Drawing adapted from Valiente *et al.* (50).

1.4 Hypothesis and objectives

Although JME is the most common GGE, its underlying pathophysiological mechanism is still unknown. As mentioned before, *EFHC1* is an interesting possible causative gene for JME as mutations in this gene have been described in several unrelated families with JME all over the world, but not in healthy controls (15-20). Recently, our research group has demonstrated that EFHC1 is involved in the radial migration of excitatory projection neurons during brain development (23). These and other findings from our lab suggest that JME is a developmental disorder instead of a channelopathy. In order to prove this hypothesis, we want to link EFHC1 to the tangential migration of inhibitory interneurons, i.e. cells important for maintaining a proper balance between excitation and inhibition in the brain. We hypothesise that if the migration of these interneurons is disturbed, this will lead to an imbalance between excitation and inhibition in the cortex, resulting in hyperexcitability and hence epilepsy. Moreover, this may explain the observation of microdysgenesis in the brain of JME patients.

The aim of this research project is to investigate whether EFHC1 affects the tangential migration of interneurons in the developing rat brain. The role of EFHC1 in this process will be studied by modulating its expression in the rat BT at E17 using *ex vivo* and focal electroporation. Both loss of function experiments using shRNA and gain of function experiments by overexpressing EFHC1 will be performed. Furthermore, we will test whether EFHC1 knockdown leads to apoptosis of the cells in the BT by means of a TUNEL assay. The results of this project will not only contribute to research on the physiological function of EFHC1, they will also improve our understanding of JME and epilepsy in general.

2 Materials & methods

2.1 Animals

Pregnant Wistar rats were ordered from the Central Animal Facility of the University of Liège at gestational day 17 (E17). All rats were housed under standard conditions and were treated according to the guidelines of the Belgian Ministry of Agriculture in agreement with European Community Laboratory Animal Care and Use regulations. Pregnant females were deeply anesthetized with isoflurane and then euthanatized by decapitation in order to collect their embryos.

2.2 Expression plasmids

All plasmids were already constructed by researchers from the GIGA-Neurosciences department and verified by sequencing. They all were produced by an *E. coli* DH5α strain and carry the ampicillin resistance gene. They were purified with the EndoFree Plasmid Maxi Kit (Qiagen) according to the manufacturer's protocol and quantified using a NanoDrop micro-volume spectrophotometer (Isogen Life Science). The name of the plasmid's insert has been used in all sections of this paper when referring to a specific plasmid. The different conditions presented in Table 1 were used to determine whether EFHC1 plays a role in the tangential migration of interneurons. For every condition, green fluorescent protein (GFP) or enhanced green fluorescent protein (EGFP) fused to EFHC1 was injected in order to label the electroporated cells.

Table 2. Plasmid combinations and characteristics of the plasmids used for each condition.

Condition	Plasmid	Insert	Restriction site	Final conc.
Negative control 1	pCAGGS	GFP	*EcoR*I – *Bgl*II	0.5 µg µl^{-1}
Negative control 2	pCAGGS	GFP	*EcoR*I – *Bgl*II	0.5 µg µl^{-1}
	mU6pro	scrambled shRNA	*Bbs*I – *Xba*I	1.5 µg µl^{-1}
EFHC1 knockdown	pCAGGS	GFP	*EcoR*I – *Bgl*II	0.5 µg µl^{-1}
	mU6pro	rEFHC1 shRNA	*Bbs*I – *Xba*I	1.5 µg µl^{-1}
Rescue experiment	pCAGGS	EGFP-hEFHC1	*Kpn*I – *Pme*I	2.5 µg µl^{-1}
	mU6pro	rEFHC1 shRNA	*Bbs*I – *Xba*I	1.5 µg µl^{-1}
EFHC1 overexpression	pCAGGS	EGFP-hEFHC1	*Kpn*I – *Pme*I	2.5 µg µl^{-1}

2.3 *Ex vivo* electroporation

Ex vivo electroporation was carried out as described previously (23). Briefly, E17 embryos were removed from the uterus and dissected in cold 0.1 M phosphate buffered saline (PBS) containing 0.25 mM glucose (PBS/glucose). Heads were isolated and placed in L15 medium (Invitrogen) supplemented with 33 mM glucose, 2.6 mM Na HCO_3 and 1% (v/v) penicillin/streptomycin. Then 1-3 µl of plasmid solution (Table 1-2) dissolved in PBS with 2 mg ml^{-1} Fast Green (Sigma) was injected into one lateral ventricle using a pulled glass micropipette and a microinjector (Femtojet, Eppendorf). Electroporation was carried out by placing the heads between tweezer-type electrodes (Nepa Gene). Electrodes were placed at about 30°C from the brain's horizontal plane (51) in order to target the cells in the MGE (Fig. 4). An electroporator (ECM 830, BTX) was used to generate five square electric pulses (55 V, 50 ms) with an interval of 1 s. Then brains were dissected in cold PBS/lucose and embedded into liquid 3% (w/v) low-melting agarose at 37°C (Bio-Rad). After 1 h of incubation on ice, coronal vibratome slices of 300 µm (VT1000S, Leica) were made and transferred onto sterilized MilliCell culture plate inserts (0.4 µm pore size, Millipore). Slices with a visible MGE were cultured for four days in semidry conditions at 37°C and with 5% CO_2 in plastic petri dishes containing Neurobasal medium (Invitrogen) supplemented with 2% (v/v) B27, 1% (v/v) N2, 1% (v/v) penicillin/streptomycin and 1% (v/v) glutamine. Half of this culture medium was renewed every day except for the first day after the electroporation.

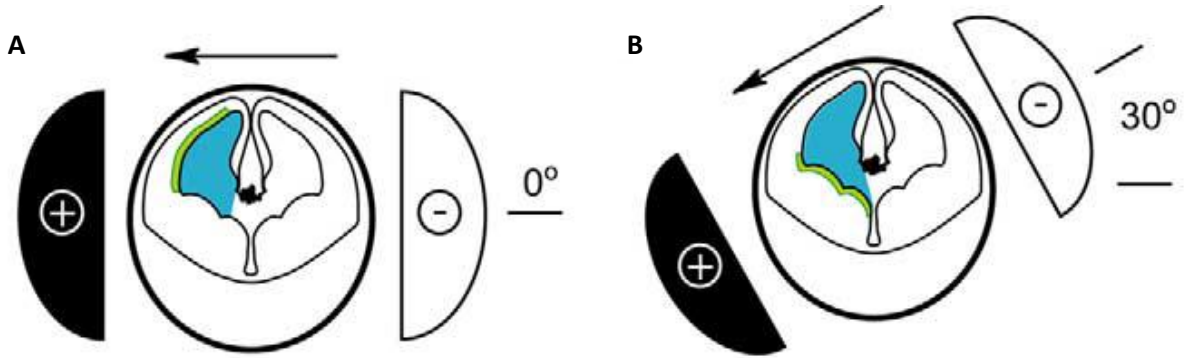

Figure 4. Schematic drawing of *ex vivo* electroporation. Electroporation takes place after plasmid injection in one of the ventricles (blue), targeting specifically **(A)** radially migrating neurons originating from the SVZ of the neocortex (green) and **(B)** tangentially migrating neurons born in the MGE and LGE (green). Drawing adopted from Borrell *et al.* (51).

2.4 Focal electroporation

E17 brains were embedded in 3% (w/v) low-melting agarose in PBS (37°C, Bio-Rad) and incubated on ice for 1 h after being dissected in cold PBS/glucose. The embedded brains were cut coronally into 300 µm slices with a vibratome (VT1000S, Leica) and transferred onto Nuclepore Track-Etched membranes (0.08 µm pore size, Whatman). Slices with their supporting membranes were placed

onto a 1% (w/v) low-melting agarose gel in PBS covering a petridish square platinum plate electrode (Nepa Gene). Then 0.5 µl of plasmid solution (Table 2) dissolved in PBS with 2 mg ml^{-1} Fast Green (Sigma) was injected into the MGE region of the brain slice with a pulled glass micropipette and a microinjector (Femtojet, Eppendorf). A different plasmid solution was injected into the BT of each hemisphere in order to compare the tangential migration under two different conditions within one brain slice. An agarose column was punched with a glass Pasteur pipette from a 1% (w/v) low-melting agarose gel in PBS and attached to a cover square platinum plate electrode (Nepa Gene). After adding 1 drop of PBS to the injected MGE region, the column was placed onto this region (Fig. 5) and square electric pulses (100 V, 10 ms) were passed five times with an interval of 1 s using an electroporator (ECM 830, BTX). The electroporated slices, together with their membranes, were transferred to wells containing Neurobasal medium (Invitrogen) supplemented with 2% (v/v) B27, 1% (v/v) N2, 1% (v/v) penicillin/streptomycin and 1% (v/v) glutamine. Slices were cultured for three days in semidry conditions in an incubator at 37°C and with 5% CO_2.

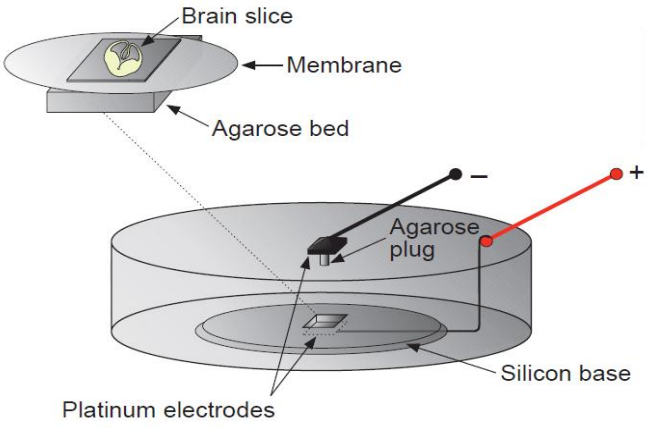

Figure 5. Schematic representation of focal electroporation on coronal brain slices. The agarose plug has to make contact with the injected MGE region before electric pulses are generated. Drawing adopted from Stühmer *et al.* (52).

2.5 Tissue processing

Cultured brain slices were successively fixed in 4% paraformaldehyde in PBS for 30 min at 4°C, rinsed in PBS and cryoprotected in 20% (w/v) sucrose in PBS overnight at 4°C. The agarose was removed from the slices before embedding them into Neg-50 frozen section medium (Richard-Allen Scientific). Coronal sections of 18 µm were cut using a cryostat (HM 560 M, Microm) and collected on glass slides (SuperFrost Plus, Menzel-Gläser).

2.6 Immunohistochemistry

Immunostainings were performed in order to amplify the fluorescence signal produced by GFP or EGFP. A rabbit polyclonal antibody to GFP (Invitrogen) and a fluorescein-conjugated AffiniPure donkey anti-rabbit IgG (Jackson ImmunoResearch) were respectively used as primary and secondary antibodies. Air dried cryostat sections were washed in PBS for 5 min at room temperature and then blocked in PBS with 0.1% (v/v) Triton X-100 and 1.5% (w/v) nonfat dried milk for 1 h at room temperature. Brain sections were incubated overnight at 4°C with the primary antibody diluted 1:2000 in PBS with 0.1% (v/v) Triton X-100 and 1.5% (w/v) nonfat dried milk. After 3 times 5 min of washing in PBS, the secondary antibody diluted 1:500 in the same blocking solution was added to the sections for 1 h at room temperature. All sections were washed 3 more times 5 min in PBS and finally 5 min in MilliQ water before coverslips were mounted in Vectashield hard set mounting medium with 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories). Brain sections were analyzed with an inverted fluorescence microscope (IX81, Olympus) coupled to a laser scanning confocal system (Fluoview FV1000, Olympus). Nuclei stained with DAPI were visualized in blue using a 405 nm laser line whereas cells labeled with GFP or EGFP were visualized in green using a 488 nm laser line.

2.7 TUNEL assay

A TUNEL assay was carried out to detect apoptotic cells using the In Situ Cell Death Detection Kit, TMR red (Roche Applied Science). Briefly, brain slices were permeabilized 2 min with ice cold and freshly prepared 0.1% (v/v) Triton X-100 in 0.1% (w/v) sodium citrate and subsequently rinsed 2 times 5 min with PBS. Then sections were incubated with 100 µl of TUNEL reaction mixture for 60 min at 37°C in a humidified atmosphere and in the dark. The TUNEL reaction mixture consisted of enzyme solution (Roche Applied Science) diluted 1:10 in label solution (Roche Applied Science). After incubation, all slices were washed 3 times 5 min with PBS and another 5 min in MilliQ water. Negative controls were incubated with 100 µl of label solution instead of the TUNEL reaction mixture. Positive controls were treated with 500 U ml^{-1} DNase I grade I (Roche Applied Science) for 10 min at room temperature to induce DNA strand breaks, prior to the incubation with TUNEL reaction mixture. A 561 laser line was used to visualize the apoptotic cells under an inverted fluorescence microscope (IX81, Olympus) coupled to a laser scanning confocal system (Fluoview FV1000, Olympus).

2.8 Quantitative analysis

In order to determine the percentage of GFP^+ cells that has migrated tangentially into the cortex, each brain slice was divided into two regions i.e., the neocortex and the striatum by drawing a line at

the place of the CSJ. The amount of cells in both regions of the brain was counted using the JAVA-based image processing program ImageJ (NIH). For quantification of apoptosis, each brain slice was divided into the same two regions. The amount of GFP^+ or $EGFP^+$ cells and double-positive GFP^+-$TUNEL^+$ or $EGFP^+$-$TUNEL^+$ cells was quantified in the Str using also the JAVA-based image processing program ImageJ.

2.9 Statistical analysis

This study was performed blind in order to increase the reliability of the results. Statistical analysis was carried out using GraphPad Prism software. All values are presented as mean ± SEM, with n being the sample size of each condition. Because of the small sample size in all experiments, both parametric and non-parametric statistical hypothesis tests were used. An unpaired Student's t-test and a Mann-Whitney test were used to determine a difference between only two conditions. A one-way ANOVA and a Krustall Wallis test, respectively followed by a Bonferroni and a Dunn's post hoc test, were used in order to determine if there is a difference in tangential migration under all different conditions. Differences were considered as being significant if $p < 0.05$. Asterisks indicate the level of significance: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

3 Results

3.1 Directional *ex vivo* electroporation fails to target tangentially migrating neurons

A previous study directed by Borrell *et al.* described how to perform an MGE-directed *in utero* electroporation to target specifically gene expression in interneurons i.e., by placing the electrodes at 30°C from the brain's horizontal plane (Fig. 4) (51). In order to study the role of EFHC1 in tangential migration, this electrode placement was adapted to *ex vivo* electroporation, a technique that is currently used in our laboratory to study radial migration (23). Several E17 embryos from three different mother rats were electroporated *ex vivo* after injection of a plasmid encoding GFP into one of the ventricles. However, instead of the MGE, parts of the VZ of the neocortex were electroporated and so radially instead of tangentially migrating GFP^+ cells could be observed after 4 days in culture (Fig. 6). This indicates that directional *ex vivo* electroporation is not the method of choice for this research project.

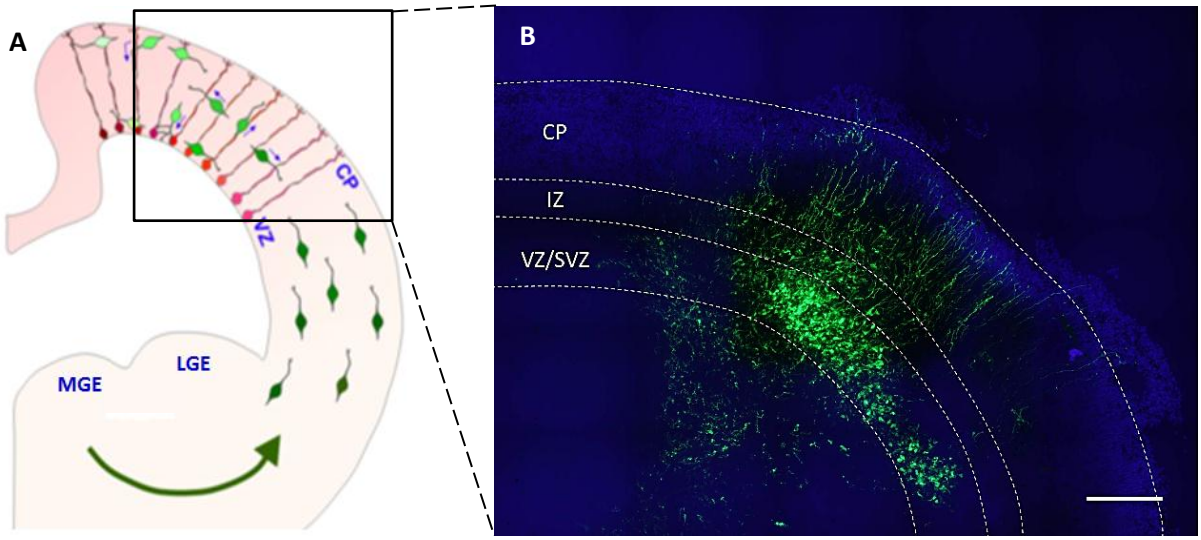

Figure 6. Directional *ex vivo* electroporation targets radially migrating neurons. (A) Schematic drawing of radially (red) and tangentially (green) migrating neurons in an embryonic coronal brain slice. **(B)** E17 coronal brain slice four days after *ex vivo* electroporation of a plasmid encoding GFP with electrodes placed at 30°C from the brain's horizontal plane. Electroporated cells (green) are located in the VZ/SVZ of the neocortex instead of the MGE. CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone. Scale bar = 100 µm.

3.2 Focal electroporation targets specifically tangentially migrating neurons

As the technique proposed by Borrell *et al.* did not produce the desired results, another technique called focal electroporation was tested on E17 brain slices. The procedure used in this research project is slightly adapted from the one proposed by Stühmer *et al*. (Fig. 5) (52). In most brain slices, the MGE and LGE were not visible anymore as a separate entity, but were converted into one structure, called the BT, of which the lower part corresponds to the former MGE and the upper part to the former LGE (41).

Injection of a plasmid encoding GFP into the BT followed by focal electroporation resulted in the observation of GFP^+ cells coming from the BT and migrating tangentially into the neocortex in approximately 10% of all brain slices after three days in culture (Fig. 7). All fluorescently labelled cells seem to enter the cortex via the two different routes that have been described before (39). Most cells clearly migrate through the SVZ and lower IZ whereas a smaller population of interneurons could be detected in the MZ. Moreover, it seems that some cells have already switched from tangential to radial migration. These observations indicate that focal electroporation is an appropriate technique to target and label specifically tangentially migrating neurons born in the BT.

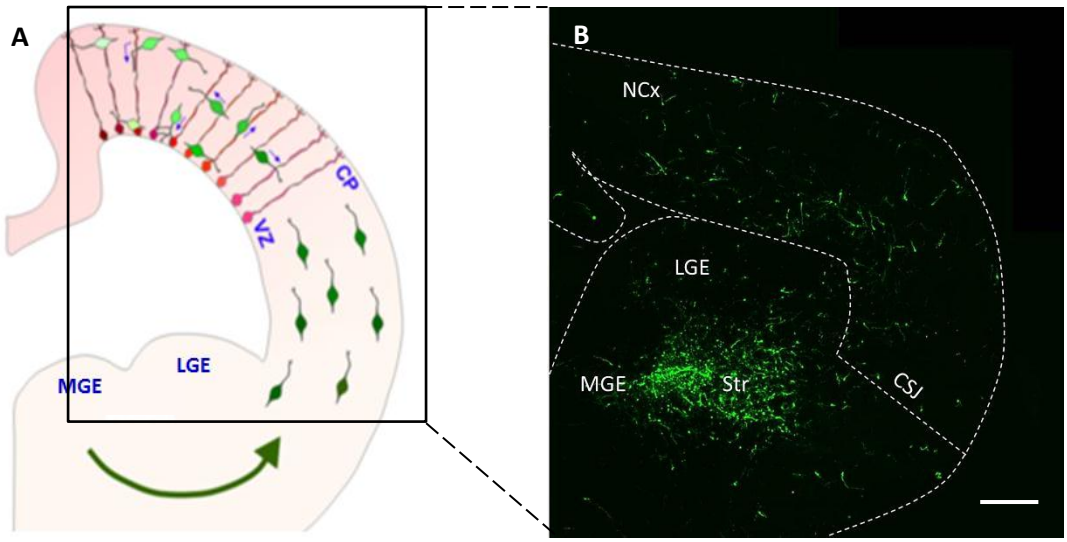

Figure 7. **Focal electroporation targets specifically tangentially migrating neurons. (A)** Schematic drawing of radially (red) and tangentially (green) migrating neurons in an embryonic coronal brain slice. **(B)** Confocal picture showing tangentially migrating GFP^+ cells (green) in an E17 coronal brain slice three days after focal electroporation and injection of a plasmid encoding GFP. CSJ, corticostriatal junction; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; Str, striatum. Scale bar = 200 µm.

3.3 EFHC1 knockdown causes a significant disruption of tangentially migrating neurons

To determine whether EFHC1 is involved in the tangential migration of interneurons, its expression was knocked down by focal electroporation of an shRNA directed to rEFCH1 (rEFHC1 shRNA) into one hemisphere of each brain slice. Scrambled shRNA was used as a negative control in the other hemisphere of the same brain slice. A plasmid encoding GFP was added to both solutions in order to label the electroporated cells. The shRNA was already validated previously (23). After three days in culture, electroporated cells could be detected in the neocortex of both hemispheres, but the percentage of GFP^+ cells seemed to be higher in the control condition compared with EFHC1 knockdown (Fig. 8A-B).

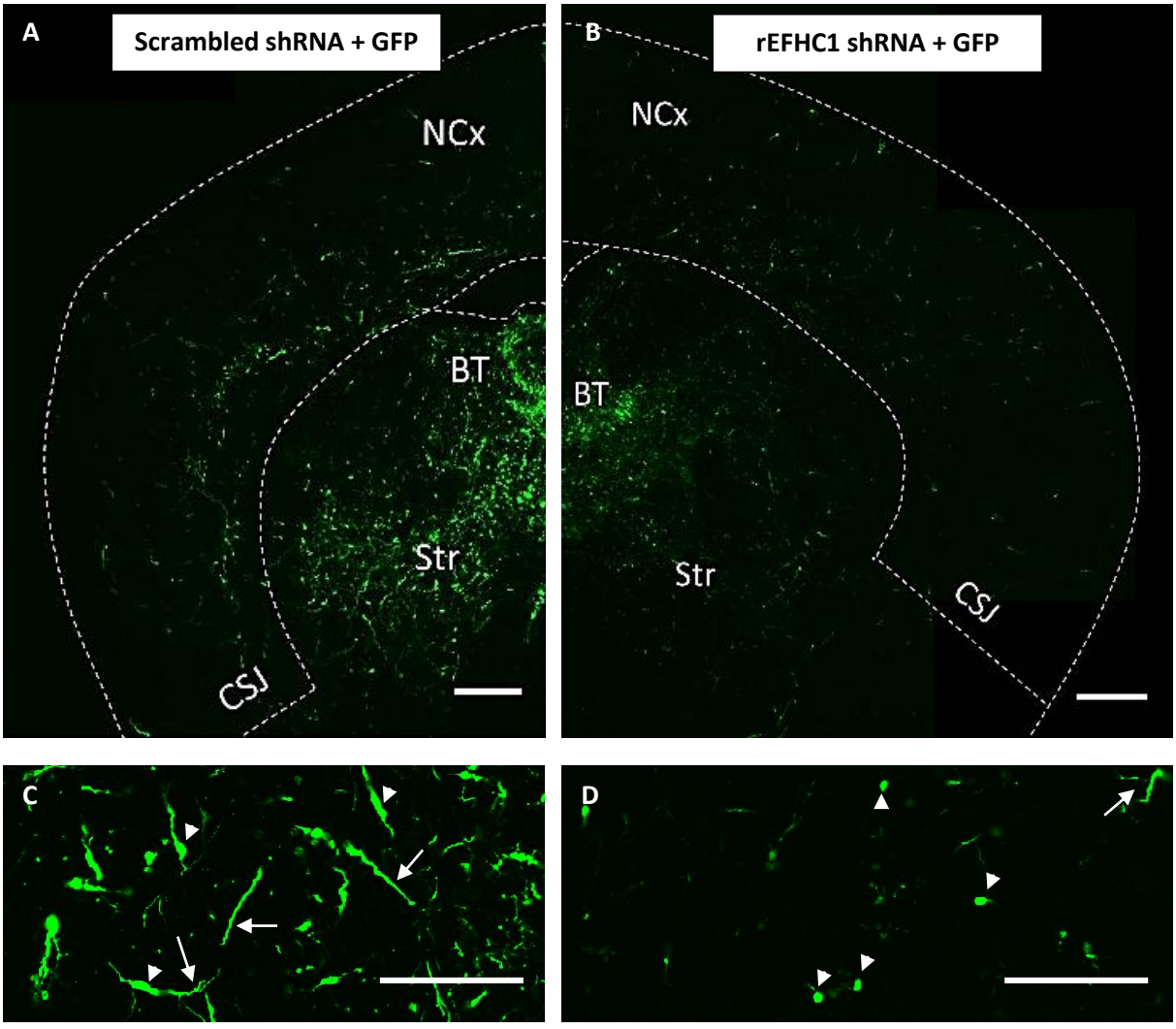

Figure 8. Distribution of GFP^+ cells in an E17 coronal brain slice three days after focal electroporation in the BT of **(A)** scrambled shRNA + GFP and **(B)** rEFHC1 shRNA + GFP in the BT. Magnification of GFP^+ cells in the neocortex after electroporation of **(C)** scrambled shRNA + GFP showing elongated nuclei (arrowheads) and long leading processes (arrows) and GFP^+ cells electroporated with **(D)** rEFHC1 shRNA + GFP showing round-shaped cells (arrowheads) with very short

leading processes (arrows). BT, basal telencephalon; CSJ, corticostriatal junction; NCx, neocortex; Str, striatum. **(A-B)** Scale bar = 200 µm; **C-D)** Scale bar = 100 µm**.**

Both hemispheres were not always equally electroporated, but this confounding factor was taken into account by quantifying the amount of GFP^+ cells both in the striatum and neocortex and by expressing results as percentage of cells in both regions. Most labelled cells in the neocortex electroporated with scrambled shRNA were bipolar or multipolar, with elongated nuclei, several leading processes orientated in roughly the same direction and a short trailing process (Fig. 8C). These are typical characteristics of migrating interneurons (45, 47, 50). Though, in the hemispheres electroporated with rEHFC1 shRNA, the leading processes seemed to be shorter and did not always pointed in the same direction. Also more cells with round-shaped nuclei could be observed (Fig. 8D).

Quantifications were carried out on several brain slices (n = 12) from embryos coming from five different mother rats (Fig. 9). A t-test and a Mann-Whitney test confirmed that there is indeed a significant decrease of the percentage of GFP^+ cells in the neocortex ($P < 0.001$) after knockdown of EFHC1 (15.60 ± 2.15%) compared with the negative control (35.07 ± 2.66%) (Fig. 9). So, the decrease in GFP^+ cells in the neocortex after rEFHC1 shRNA injection is probably due to a defect in tangential migration, suggesting a role of EFHC1 in this process.

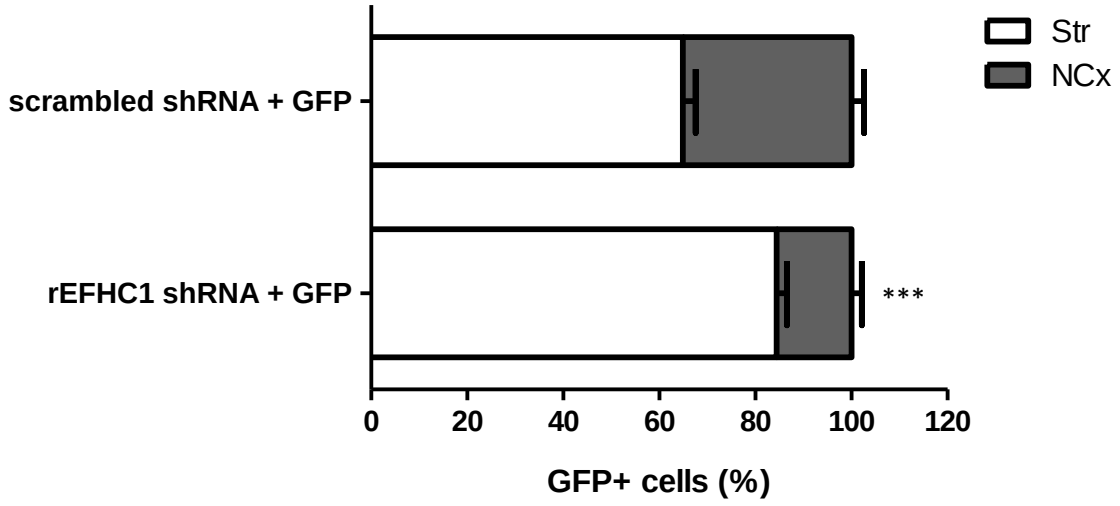

Figure 9. Quantification of GFP^+ cells in the striatum and neocortex of coronal E17 brain slices three days after electroporation of scrambled shRNA + GFP (n = 12) in one hemisphere and rEFHC1 shRNA + GFP (n = 12) in the other hemisphere of each brain slice. Str, striatum; NCx, neocortex. Error bars show SEM. *** $P < 0.001$.

3.4 The decrease of GFP+ cells in the cortex is specifically due to EFHC1 loss of function

A rescue experiment was carried out to confirm the specificity of the rEFHC1 shRNA and to prove that the relative decrease of GFP^+ cells in the neocortex is indeed due to EFHC1 loss of function. This was done by knocking down the expression of the endogenous rEFHC1 and by overexpressing the wildtype hEFHC1 simultaneously, so by injecting rEFHC1 shRNA together with EGFP-hEFHC1. The other hemisphere of each brain slice was injected with rEFHC1 shRNA and GFP (Fig. 10) in order to compare both conditions on the same brain slice. For this experiment, GFP^+ cells were quantified in the striatum and neocortex in eight slices (n = 8) from embryos from four different rats (Fig. 11).

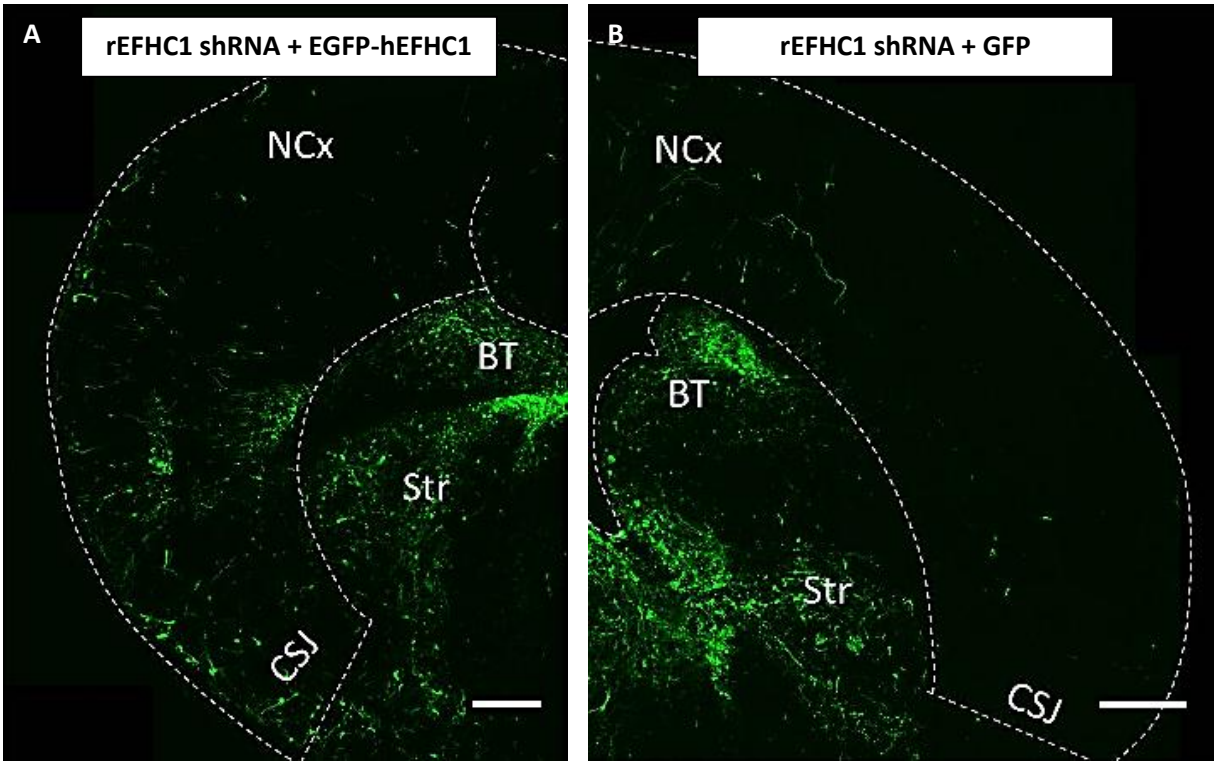

Figure 10. Distribution of **(A)** $EGFP^+$ and **(B)** GFP^+ cells in an E17 coronal brain slice three days after focal electroporation in the BT of **(A)** rEFHC1 shRNA + EGFP-hEFHC1 and **(B)** rEFHC1 shRNA + GFP. BT, basal telencephalon; CSJ, corticostriatal junction; NCx, neocortex; Str, striatum. Scale bar = 200 µm.

After quantification, a significant difference ($P < 0.001$) could be detected in the percentage of GFP^+ cells in the neocortex after EFHC1 knockdown (13.16 ± 1.42%) compared with the rescue experiment (35.64 ± 3.41%) (Fig. 11). Moreover, the percentage of GFP^+ cells in the neocortex after performing the rescue experiment was not significantly different from that of the scrambled shRNA (t-test; $P = 0.8971$). Taken together, these data indicate that the decreased tangential migration after focal electroporation of rEFHC1 shRNA is specifically due to EFHC1 loss of function as the injection of hEFHC1 is able to restore the migration defect.

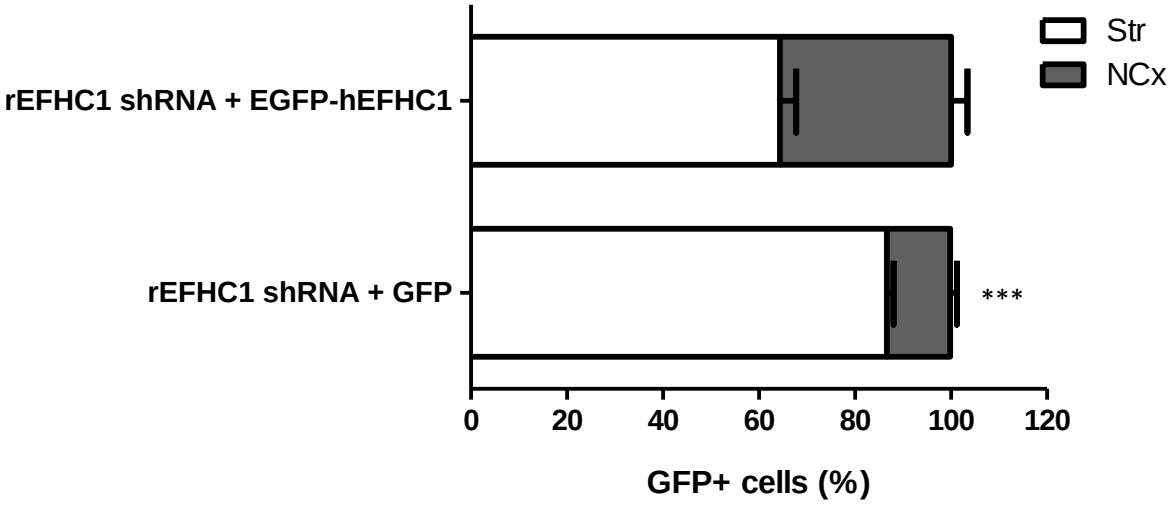

Figure 11. Quantification of GFP$^+$ cells in the striatum and neocortex of coronal E17 brain slices (n = 8) three days after electroporation of rEFHC1 shRNA + GFP (n = 8) in one hemisphere and rEFHC1 shRNA + EGFP-hEFHC1 (n = 8)in the other hemisphere of each brain slice. Str, striatum; NCx, neocortex. Error bars show SEM. *** P < 0.001.

3.5 EFHC1 overexpression does not influence tangential migration

Previous studies from our research group have shown that overexpression of the EGFP-hEFHC1 protein in the VZ/SVZ of the neocortex has no significant effect on radial migration (23). However, this doesn't necessarily mean that the same is true for tangentially migrating interneurons. To determine whether EFHC1 overexpression influences the tangential migration of interneurons, a plasmid encoding EGFP-hEFHC1 was injected into the BT of one hemisphere of each brain slice. GFP alone was used as a negative control and injected into the opposite hemisphere of the same brain slice (Fig. 12). Embryos from four pregnant rats were used for this experiment and 15 slices (n = 15) were included into the statistical analysis (Fig. 13).

Although the amount of EGFP$^+$ cells is lower than the amount of GFP$^+$ cells in all brain slices (Fig. 12), the percentage of electroporated cells in the cortex (36.08 ± 1.57% vs. 33.09 ± 1.56% respectively) and striatum (63.92 ± 1.57% vs. 66.91 ± 1.56% respectively) was not significantly different (t-test; P = 0.4914) between both conditions (Fig. 13). All together, these results demonstrate that EFHC1 overexpression has no effect on the tangential migration of interneurons in the developing brain. Moreover, the percentage of GFP$^+$ cells in the cortex of both negative controls (GFP and scrambled shRNA + GFP) was not significantly different (t-test; p < 0.8613). This proves that the scrambled shRNA has no effect on the tangential migration.

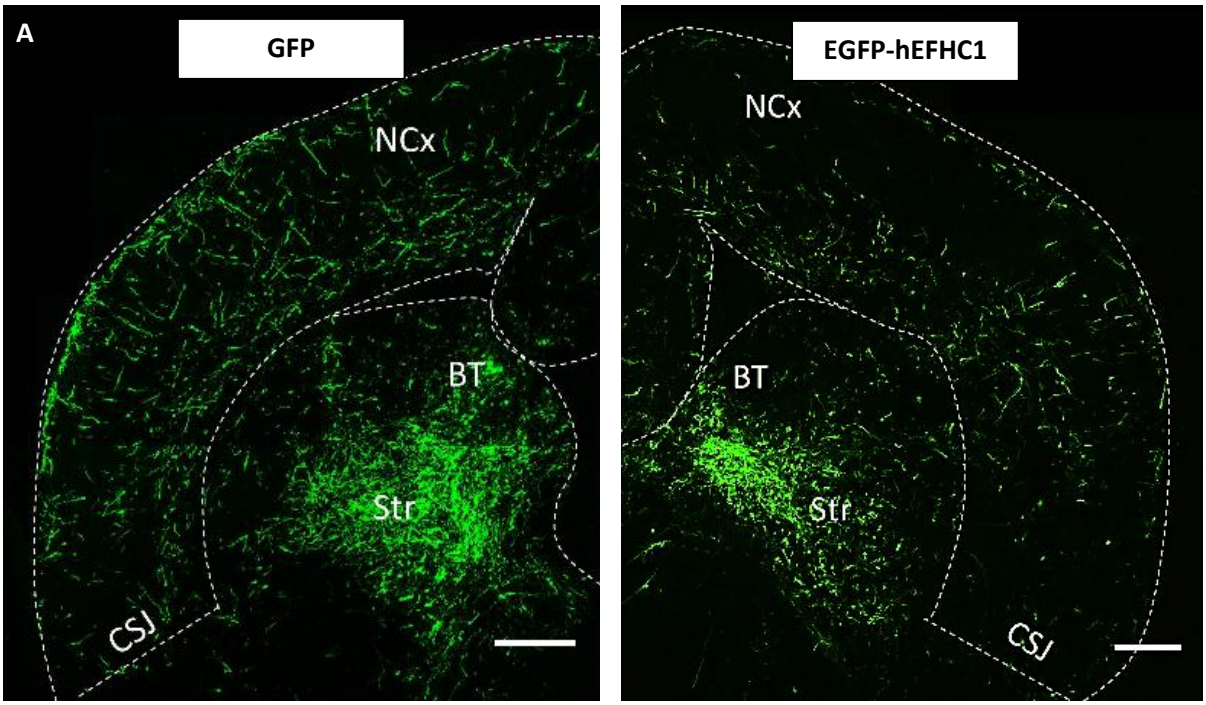

Figure 12. Distribution of **(A)** GFP^+ and **(B)** $EGFP^+$ cells in an E17 coronal brain slice three days after focal electroporation in the BT of **(A)** GFP and **(B)** EGFP-hEFHC1. BT, basal telencephalon; CSJ, corticostriatal junction; NCx, neocortex; Str, striatum. Scale bar = 200 µm.

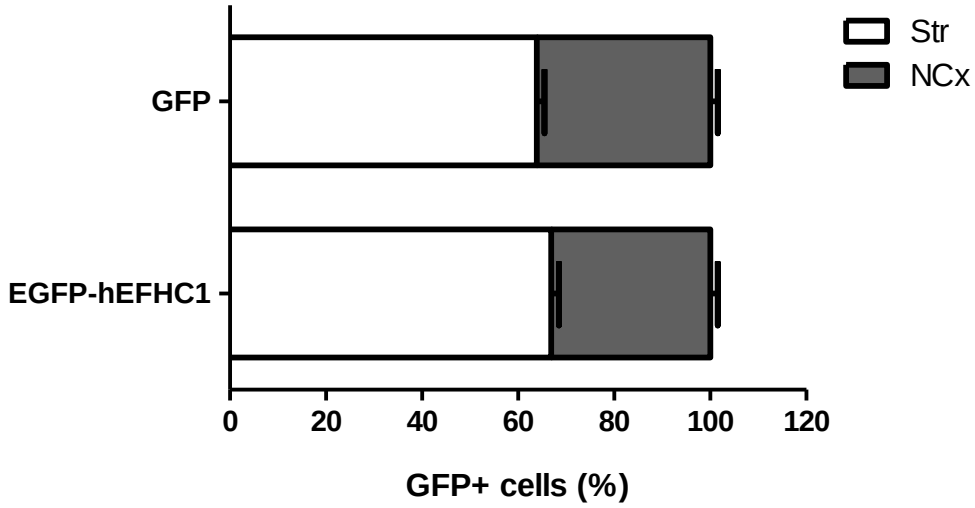

Figure 13. Quantification of GFP^+ cells in the striatum and neocortex of coronal E17 brain slices three days after electroporation of GFP (n = 15) in one hemisphere and EGFP-hEFHC1 (n = 14) in the other hemisphere of each brain slice. Str, striatum; NCx, neocortex. Error bars show SEM.

If all data from the last three experiments are finally compared to each other, it becomes clear that only EFHC1 knockdown causes a significant decrease ($P < 0.001$) of GFP^+ cells in the cortex compared with all other conditions (Fig. 14), suggesting a role for EFHC1 in tangential migration.

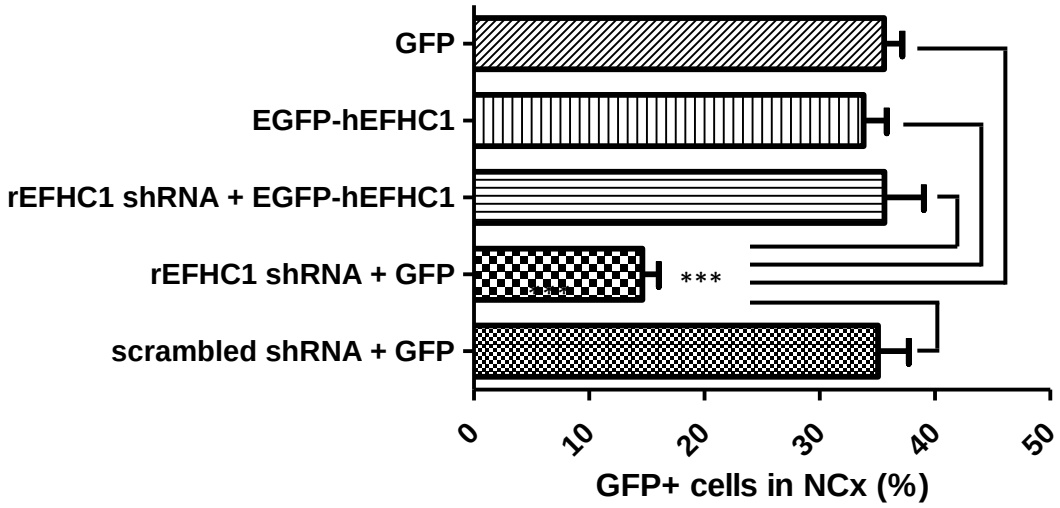

Figure 14. EFHC1 knockdown disrupts tangential migration. Percentage of GFP+ or EGFP$^+$ cells in the neocortex after focal electroporation of scrambled shRNA + GFP (n = 12), rEFHC1 shRNA + GFP (n = 20), rEFHC1 shRNA + EGFP-hEFHC1 (n = 8), EGFP-hEFHC1 (n = 14) and GFP (n = 15). NCx, neocortex. Error bars show SEM. *** $P < 0.001$

3.6 EFHC1 knockdown does not induce apoptosis in the BT and striatum

It was previously demonstrated that there is a significant increase in apoptosis in the VZ/SVZ of the neocortex of brain slices with impaired EFHC1 function (23). Therefore, A TUNEL assay was carried out to reveal whether the knockdown of EFHC1 in the BT and striatum induces apoptosis. Three independent TUNEL assays were performed on a total of eleven brain slices (n = 11) from embryos from five different rats electroporated with scrambled shRNA and rEFHC1 shRNA. Both a positive and negative control was included to exclude false positive and/or false negative results (Fig. 15C-D). Positive controls were first treated with a DNase I solution to induce DNA strand breaks. Negative controls were incubated without the enzyme solution. The enzyme terminal deoxy-nucleotidyl transferase in this solution catalyzes the addition of dUTPs from the label solution at nick ends in the DNA. For each TUNEL assay, there was no sign of TUNEL$^+$ cells in the negative control (Fig. 15C) whereas almost all cells were TUNEL$^+$ in the positive control (Fig. 15D).

Confocal pictures taken from the BT and striatum revealed only a few TUNEL$^+$ GFP$^+$ cells (arrows) in both conditions (Fig. 15A-B). Surprisingly, both the t-test and Mann-Whitney test revealed that the percentage of apoptotic GFP$^+$ cells in the hemispheres electroporated with scrambled shRNA (6.67 ± 1.45%) was not significantly different from that in the hemispheres electroporated with rEFHC1 shRNA (6.77 ±2.55%) (Fig. 16). Thus, these data do not confirm those obtained previously in our lab as they suggest that EFHC1 impairment does not induce apoptosis in the BT of organotypic brain slices.

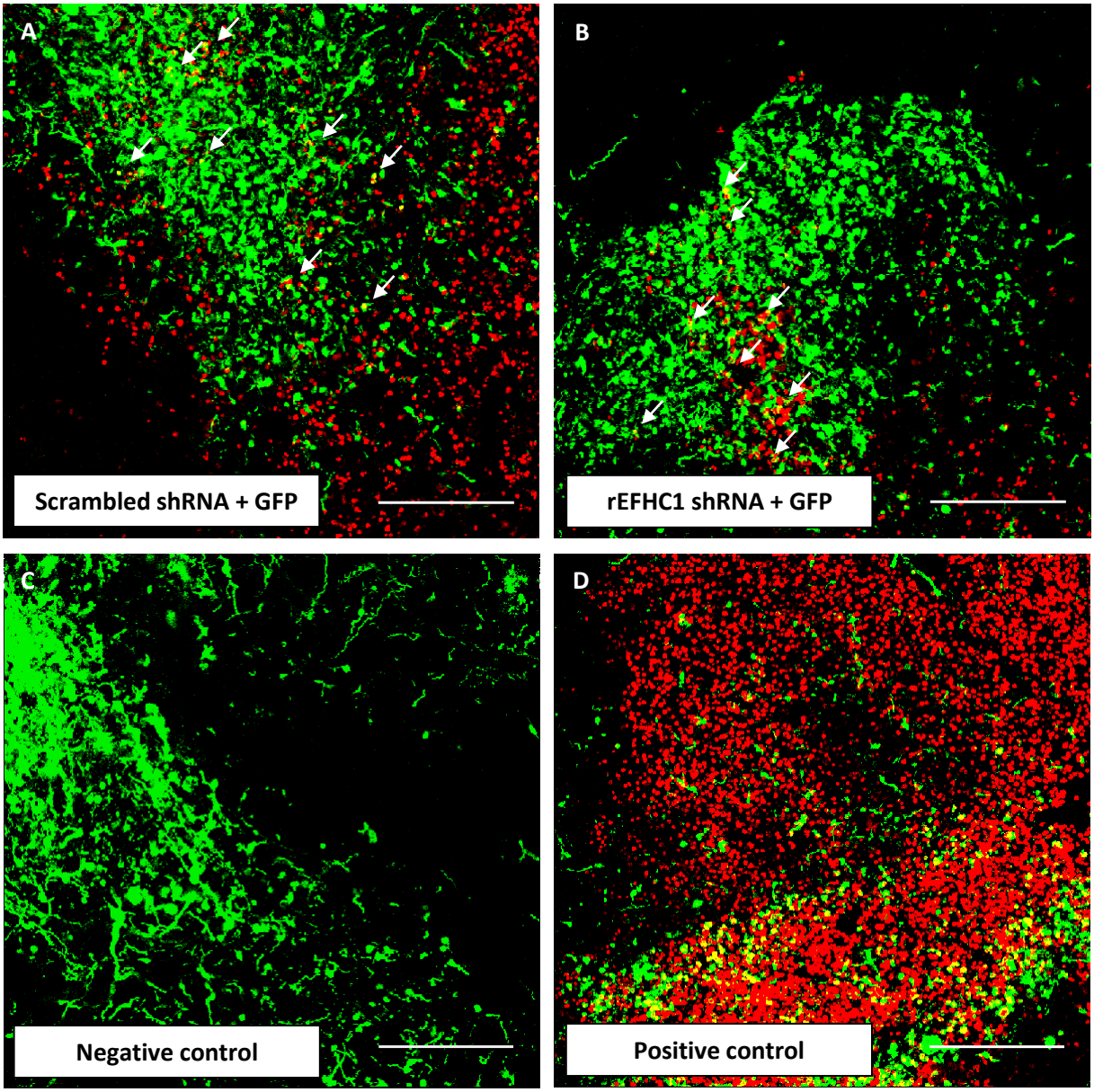

Figure 15. EFHC1 knockdown with shRNA does not lead to an increase of apoptosis. BT and striatum region of a coronal E17 brain slice electroporated with **(A)** scrambled shRNA + GFP and **(B)** rEFHC1 shRNA + GFP and consequently subjected to a TUNEL assay. **(C)** Negative control and **(D)** positive control. GFP^+ cells, green; $TUNEL^+$ cells, red; apoptotic GFP^+ cells, yellow (arrows). Scale bar = 100 µm.

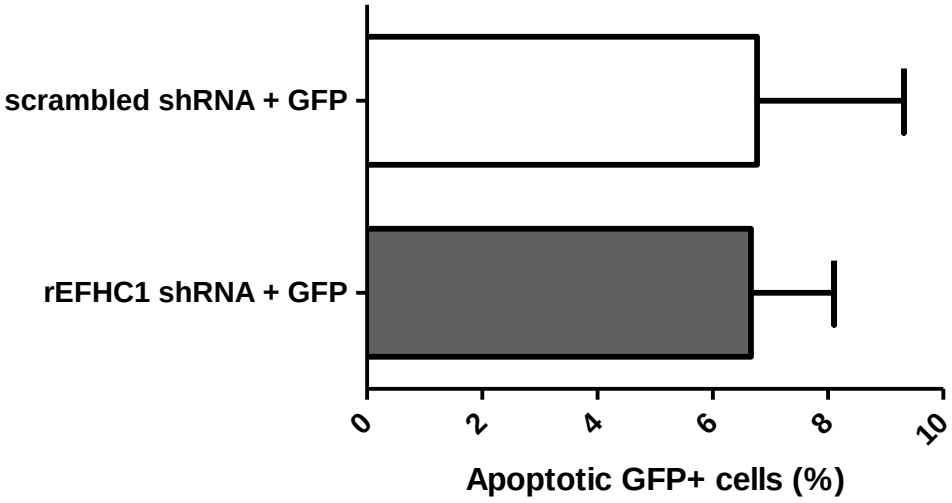

Figure 16. Quantification of apoptotic GFP^+ cells from TUNEL assays in the BT and striatum region of coronal E17 brain slices three days after electroporation with scrambled shRNA + GFP (n = 11) in one hemisphere and rEFHC1 + GFP (n = 11) in the other hemisphere of the each brain slice. Error bars show SEM.

4 Discussion

4.1 *Ex* vivo and focal electroporation: critical evaluation

Most articles studying tangential migration have only been published within the last ten years from now, as radial migration has long been thought to be the only type of migration contributing to the assembly of the cortex. Though, the number of research groups in this field remains limited. One of the reasons may be that the techniques to examine tangential migration still need to be optimized or require a lot of practice. The first experiments were performed on embryonic brain slices after placement of tracers such as 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate (DiI) crystals in the MGE region of the BT (41, 44). A more recent approach to study tangential migration is slice transplantation, a technique in which a labeled MGE explant from one slice is grafted on the MGE region of a host slice (43, 47, 48). Next to this, also conditional knockout mice (53) and co-cultures of MGE explants on dissociated cortical cell progenitors followed by time-lapse video microscopy are being used (47). A last possibility to identify the molecules and mechanisms involved in tangential migration is to perform gain and loss of function studies using *ex vivo*, *in utero* or focal electroporation (49, 51, 52).

Because our research group was already familiar with *ex vivo* electroporation experiments, this technique was chosen to study the effect of EFHC1 on tangential migration in the developing rat brain. Tweezer-type electrodes were placed according to the instructions from Borrell *et al.* (51) in order to target the progenitor cells in the BT (Fig. 4). However, after several attempts this technique did not work as only radially instead of tangentially migrating neurons could be targeted (Fig. 6). This proves that it is very difficult to place the electrodes exactly at 30°C from the horizontal plane of the embryo's brain. Moreover, Borrell *et al.* performed this technique on E12-E14 mice embryos instead of on E17 rat embryos.

Focal electroporation is a relatively new technique that has only been used by very few research groups so far. Therefore, it was first of all necessary to test whether this technique was also useful for this project, especially after the disappointing results of directional *ex vivo* electroporation. The method for focal electroporation described in this report is adopted from Stühmer *et al.* (52). The only difference is that here the plasmid solution was first injected into the brain slices instead of dipping the agarose column into this solution (Fig. 5). By doing so, not only the cells at the top layer of the brain slice but also cells deeper in the tissue could be electroporated.

The low success rate of focal electroporation (10%) can be due to several factors. Most slices were lost in one of the steps following focal electroporation (fixation, cryopreservation, cryostat sections

and immunohistochemistry) as they are very fragile and consequently get easily damaged. Furthermore, it is important to emphasize that the ventricles of most brain slices closed after making vibratome sections and so the BT and neocortex touched each other. This complicated the injection into the right region and sometimes led to the electroporation of cells in the SVZ of the neocortex. At last, analysis under the confocal microscope revealed that some slices were not electroporated at all as there was no sign of GFP^+ cells. This was probably because the agarose column did not make adequately contact with the brain slice.

A more precise focal electroporation (and hence a higher success rate) may be obtained by designing a construct expressing GFP or EGFP under the control of a promoter that is specifically activated in interneurons. MGE progenitors express genes that are involved in the specification of interneurons, including several transcription factors but also chemokine receptors, molecules involved in cell cycle progression, enzymes etc. (40). Especially *Dlx* genes have been proved to be good interneuron markers as they are expressed in the vast majority of tangentially migrating interneurons throughout all stages of development (40, 43). So, insertion of a *Dlx* promoter into the GFP and/or EGFP construct is probably the best option to label specifically tangentially migrating interneurons. This option was already considered at the beginning of the research project, but unfortunately there was too little time to construct such plasmids.

4.2 EFHC1 is involved in the tangential migration of interneurons

The data presented in this paper showed that EFHC1 knockdown causes a significant disruption of tangentially migrating neurons in the neocortex in contrast with the control condition (Fig. 8-9). Moreover, they demonstrate that the cells electroporated with rEFHC1 shRNA + GFP had an abnormal morphology, with shorter leading processes and round-shaped instead of elongated nuclei (Fig. 8C-D). A rescue experiment confirmed that these effects were due to EFHC1 loss of function (Fig. 10-11). However, no significant difference could be detected between the control hemispheres and those in which EFHC1 was overexpressed (Fig. 12-13). These results indicate that EFHC1 plays a role in the tangential migration of interneurons as the migratory process was only disturbed when EFHC1 was knocked down.

The relative number of GFP^+ cells in the neocortex was decreased with approximately 50% after electroporation with rEFHC1 shRNA + GFP compared with the negative control (Fig. 9). It is normal that not all tangentially migrating GFP^+ cells were equally affected by the EFHC1 knockdown, as this depends on the amount of plasmid incorporated into each cell. Previous experiments showed already that only 70% of the gene expression can be silenced with the rEFHC1 shRNA used in this

project (23). Moreover, we have to take into account that probably not all cells were transfected with both GFP and rEFHC1 shRNA as the incorporation of these plasmids occurs randomly. This means that some GFP^+ cells have only taken up GFP and, as a consequence, behave like the cells observed in the negative controls. For this reason, it is also impossible to exclude whether EFHC1 is only a mediating factor or an essential regulatory factor for tangential migration.

Finally, quantifications revealed that the absolute amount of $EGFP^+$ cells was always lower than the amount of GFP^+ cells in the striatum of the brain slices (Fig. 10 and Fig.12). This difference could be due to differences in transcription and/or translation efficiency between the large EGFP-hEFHC1 construct (~100 kDa) and the GFP construct alone (~30 kDa). However, it is also possible that the presence of EGFP-hEFHC1 and hence the overexpression of the wildtype EFHC1 protein in the BT induces apoptosis. This possibility needs to be verified by performing TUNEL assays in the near future.

4.3 Is EFHC1 involved in the nucleokinesis of migrating neurons?

These and previous results from our research group strengthen the hypothesis that EFHC1 plays a role in nucleokinesis of migrating neurons during development. A first indication is that the aspect of the migrating GFP^+ neurons was slightly different after EFHC1 knockdown compared with control conditions. They seemed to have shorter leading processes and their nuclei were rather round-shaped instead of being elongated (Fig. 8C-D). Furthermore, we have previously demonstrated that EFHC1 impairment affects the radial migration of excitatory projection neurons in the neocortex (23). It is known that these radially migrating cells move forward by means of nucleokinesis (45), a process that also has been described in tangentially migrating interneurons (47, 50). This suggests that the molecular pathway involved in both types of migration is the same or that at least some regulatory factors are common. Also the fact that interneurons can switch from tangential to radial migration indicates that the pathways involved in both types of migration are not necessarily exclusive (36).

Several articles report that the centrosome plays a key role in the coordination of the microtubules during the nucleokinesis of migrating neurons (36, 37, 46). Different kinds of MAPs are known to mediate the interactions between the centrosome and microtubules during this process. A few years ago, our research group revealed that EFHC1 interacts directly with α-tubulin through a unique MTBD and so could be one of these MAPs (35). Moreover, it was demonstrated that EFHC1 associates with the centrosome of dividing cells and that impairment of this protein leads to a disturbed radial migration (23). All this information led us to the hypothesis that EFHC1 impairment causes a disruption of the normal function of the centrosome and, as a consequence, to defects in

the nucleokinesis. Of course, the hypothesis that loss of EFHC1 affects nucleokinesis still needs to be proved. Time-lapse videos will have to clarify whether EFHC1 plays a role in nucleokinesis, as they can provide more information about the speed of migration, the migratory route, the branching of the leading processes, the movement of the nucleus etc.

4.4 EFHC1 and apoptosis in the BT and striatum

The morphology of cells electroporated with rEFHC1 shNRA + GFP was different compared with the other conditions that were analyzed in this project. The round-shaped nuclei resembled very much the typically picnotic nuclei of apoptotic cells (Fig. 8C-D). Apoptosis of precursor cells and immature neurons in the embryonic brain was first recognized already one century ago. Today, the cellular processes controlling apoptosis during brain development are extensively described, with particularly significant roles for bcl-X, bax, caspase-9 and caspase-3 (54). However, it is still not clear whether there are differences in apoptotic activity and in the expression of its regulators between different brain regions.

A TUNEL assay was carried out in order to determine whether the observed round-shaped cells were indeed undergoing apoptosis. However, this was not the case. No significant difference could be detected between the percentage of apoptotic GFP^+ cells in the BT and striatum after focal electroporation with rEFHC1 shRNA + GFP (6.77 ±2.55%) and scrambled shRNA (6.67 ± 1.45%) (Fig. 15- 16). These results do not correspond to previous experiments demonstrating a significant increase in apoptosis in the VZ/SVZ of the neocortex after impairment of EFHC1 (23). In these experiments, almost all apoptotic cells were dividing precursor cells. Dividing progenitors of interneurons are located in the VZ/SVZ of the BT. However, because the border between the VZ/SVZ of the BT and the striatum is not clear, both regions were included for quantification. As a consequence, not only dividing progenitor cells, but also postmitotic cells were taken up in the quantification of apoptosis. So, the observation of shorter leading processes and round-shaped nuclei is maybe due to a defect in the centrosome, which in turn causes a default of microtubule elongation in the leading processes during nucleokinesis.

4.5 Perspectives

The results obtained during this project confirm that EFHC1 can regulate neuronal migration during cortical development and that disruption of its function could lead to developmental defects that in turn could lead to JME. In the near future, time-lapse experiments have to be carried out first in order to confirm our hypothesis that EFHC1 plays a role in the nucleokinesis of migrating neurons.

They will also prove that the observed effect of EFHC1 on tangential migration is indeed due to a defect in the migratory process itself instead of being just a consequence of default in other steps of corticogenesis preceding migration. It is also necessary to determine whether EFHC1 overexpression in the basal telencephalon induces apoptosis or not, because today the answer to this question is controversial. Our research group demonstrated that overexpression of the EGFP-hEFHC1 protein in the VZ/SVZ of the neocortex by means of *ex vivo* electroporation has no significant effect on radial migration and did not induce apoptosis (23). In contrast, others claim that EFHC1 overexpression *in vitro* induces neuronal cell death through its association with an R-type voltage-dependent calcium channel ($Ca_v2.3$) (15). So, by performing TUNEL assays on embryonic brain slices in which EFHC1 is overexpressed, more information concerning this topic will be obtained. Furthermore, these experiments may exclude the possibility that the lower amount of $EGFP^+$ cells in the BT and striatum, compared with the amount of GFP^+ cells in this region, is due to an increased apoptotic activity.

The data obtained in our laboratory using *ex vivo* electroporation suggest that EFHC1-knockout mice should present strong developmental defects. However, these mice are viable with a normal outward appearance and fertility (55). Therefore, it would be interesting to perform a rescue experiment with EFHC2 after EFHC1 knock down. EFHC1 and its paralog EFHC2 have a similar protein structure (three DM10 domains and one EF-hand motif) and for this reason they are believed to have similar physiological functions. This rescue experiment will reveal whether both paralogs have overlapping functions and whether EFHC2 could, at least, partially take over EFHC1's functions, as this is often the case in knockout mice when paralogs do exist (56).

Because all the pathological mutations reported to date are missense mutations whose effects on EFHC1 function remain unknown, it will be of great interest to test their influence on tangential migration by means of focal electroporation. This objective was already set before the start of this project, but unfortunately there was not enough time left anymore to perform the experiments. In conclusion, knowledge of the consequences of EFHC1 mutations linked to the JME phenotype will not only further improve our understanding of the mechanisms involved in the disease's etiology, it will also enable us to improve the diagnosis, to develop better and safer drugs and to discover new therapies. On the long run, it may even allow genetic counseling and, who knows, the prevention of epilepsies.

5 Conclusion & synthesis

There are currently around 40 different types of epilepsy (2), with each type presenting its own unique combination of seizure types, typical onset of age, EEG diagnosis and treatment. Although most of these epilepsies can be controlled with anticonvulsants, none of them can be cured. JME is one of those epilepsies, accounting for 10-30% of all cases (3-5). Its underlying disease mechanism remains unknown, but studies using linkage analysis indicate that *EFHC1* is an interesting candidate gene that may contribute to the JME phenotype. However, the physiological function of the protein it encodes remains unknown until today.

In this report, we provide evidence that EFHC1 plays a role in the tangential migration of inhibitory interneurons, as EFHC1 loss of function not only caused a significant decrease of tangentially migrating cells in the neocortex, but also altered the morphology of these cells. Previous results from our research group demonstrated already that EFHC1 is a MAP that associates with the centrosome to regulate cell division and other processes taking place during corticogenesis. These results confirm the hypothesis that EFHC1 is involved in the nucleokinesis of both tangentially and radially migrating neurons, as the centrosome plays a key role in this process (Fig. 16). Moreover, they suggest that JME is a developmental disorder instead of a channelopathy. According to our hypothesis, a disturbed tangential migration will lead to an improper distribution of interneurons in the neocortex, which in turn will lead to an imbalance between excitation and inhibition in the cortex (Fig. 16).

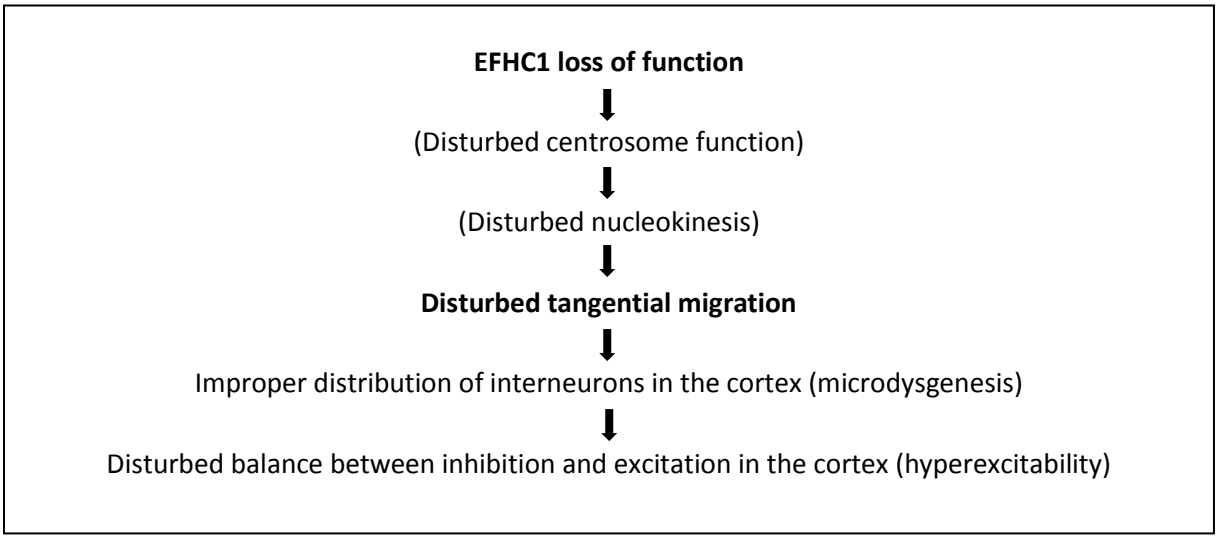

Figure 16. Hypothesis proposed in order to relate our results to JME. The steps depicted in bold are the ones that have been linked to each other at the end of this research project. The ones between brackets are the missing links that will be proved in the near future.

In conclusion, the results from this project could explain the observed microdysgenesis and hyperexcitability in JME patients. However, time-lapse videos have to reveal whether EFHC1 indeed plays a role in the nucleokinesis of migrating neurons. Moreover, it is still rash to say that the decrease in tangential migration is not due to other steps in corticogenesis preceding the migratory process. It is also important to test the effect of the previously described pathological mutations in EFHC1 on the tangential migration. Preliminary results from our research group revealed that these mutations give rise to aggregates and disrupt cell division. Therefore, it is very likely that also the migratory process of interneurons will be disturbed.

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