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Nocturnal Frontal Lobe Epilepsy and Febrile Seizures: genetic and molecular aspects

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A Edda, Federico, Michele
e a tutti coloro che mi vogliono bene.

ABSTRACT

Idiopathic epilepsies are common and devastating neurological disorders in which genetic background and physiopathological mechanisms underlying the clinical phenotype are not fully characterized yet. These diseases are assumed to have a strong genetic component, being monogenic or oligo/polygenic with different recurrence risks in the same family. However, even in monogenic epilepsy, additional genes and environmental factors may modulate its expression, thus resulting in incomplete penetrance and variable phenotype. Ethiology, phenotypic manifestations and prognosis are indeed highly heterogeneous. Idiopathic epilepsies represent about 30-40% of all epilepsies in childhood and 20% in adults. Most of them are complex diseases: patients may shift from one phenotype to another during their lifetime and parents affected by one form may have children suffering from another epileptic syndrome.

The identification of genes responsible for distinct epilepsy syndromes or influencing the risk for epilepsy has important implications, for both research and clinical purposes.

In this work we studied the genetic bases of two different epilepsies: nocturnal frontal lobe epilepsy (NFLE/ADNFLE) and febrile seizures (FS/GEFS+).

In the case of the NFLE/ADNFLE phenotype, we performed a mutational screening of known genes, including CRH and its promoter, in a sample of both sporadic and familial patients. The study allowed the identification of: an already known mutation in the CHRNA4 gene (p.Ser284Leu) originated *de novo* in one NFLE patient; three unknown variants in the CRH promoter in both sporadic and familial patients which we demonstrated to not cosegregate with the disease; one unknown missense mutation in the coding portion of the CRH gene (p.Pro30Arg) in one ADNFLE patient. By functional *in vitro* analysis we demonstrated that the p.Pro30Arg causes impairment in the production and release of the CRH hormone. This impairment could be related to an altered capability of patients to respond quickly to stress agents. Finally, by analyzing candidate genes encoding the orexin system we demonstrated an unlikely role of this system in the

pathogenesis of ADNFLE: none of the patients has mutations in the three genes.

In the study of FS/GEFS+ phenotype, the role of the SCN1A gene was evaluated. Several intronic and exonic polymorphisms were detected. In the case of unknown intronic variants, an in silico analysis revealed that these variations do not introduce or remove any splicing sites. Interestingly, we found in a patient two missense mutations: the already known p.Thr297Ile and the unknown p.Arg1525Gln. These two variants co-segregated with the pathology being present in all affected individuals and in two obligate carriers. Owing to the location of both mutations in important regions of the sodium channel, we are now testing the hypothesis of their causative role in the pathogenesis of this family's disease. The study will allow the evaluation of the effect of these mutations (considered either singly or in conjunction with the other) on the activation/inactivation properties of the sodium channel in the presence/absence of the β-1 accessory subunit.

Chapter 1:

INTRODUCTION

1.1 EPILEPSY

1.1.1 SEIZURES

Epilepsy is a devasting neurological disorder characterized by the occurrence of recurrent seizures. An epileptic seizure is defined as an excessive burst of abnormally synchronized neuronal activity affecting small or large neuronal networks that results in clinical manifestations that are sudden, transient, and usually brief (Tamber MS et al., 2012). There are few studies evaluating the incidence of epilepsy in world population. In developed countries, the incidence of epilepsy (recurrent unprovoked seizures) ranges from 24 to 53 per 100,000 person-years (Aicardi et al., 2007).

Electroencephalogram (EEG) is the main diagnostic tool for epilepsy diagnosis. EEG records the electrical activity of brain neurons through different electrodes usually placed on the scalp. Epileptic patients show electric abnormalities, even without perceptible symptoms, that are different from normal neural oscillations observed in healthy patients. (Kandel et al., 2003). Specific imaging tests, such as Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT), are used for general localization and for guiding intracranical electrode placement or to localize the epileptogenic focus during the ictal state.

Epileptic seizures are divided in two categories:

- Partial seizures
- Generalized seizures

Partial seizures originate from a small group of neurons called epileptic focus, and affect only a single brain hemisphere. Partial seizures are divided in: simple (without consciousness alteration), complex (with consciousness alteration), and partial seizures that evolve in generalized seizures involving both the brain hemispheres. Symptoms are different and strictly related to the epileptic focus localization. Epileptic focus is composed by neurons with a synchronous increasead excitability called paroxysmal depolarizing shift

(PDS). PDS is the intracellular correlate of interictal spikes and consists in an overt depolarization that last tens of milliseconds and can be so large that it leads to sodium-spike inactivation.

The mechanisms for generation of interictal spikes derive from a basic and ubiquitous operation of neocortical and hippocampal networks: the activation of brief periods dominated by synaptic excitation between pyramidal cells followed by a period dominated by synaptic inhibition and/or activation of intrinsic hyperpolarizing conductances. PDS is mediated by Calcium which causes the opening of voltage gated sodium channels for the propagation of action potential (Kandel et al., 2003).

Generalized seizures affect both cerebral hemispheres, and they are divided, according to the effect on the body, in:

- Absence seizures
- Myoclonic seizures
- Clonic seizures
- Tonic seizures
- Tonic-clonic seizures
- Atonic seizures.

Among these groups, the most typical is that of absence seizures which are commonly observed in childhood (between 4 and 10 years old). These seizures are brief (10 seconds) but they occur frequently, usually more than 10/die, and consist in a sudden loss of awareness and arrest of any motor activity. Absences show specific electrical patterns in EEG.

Tonic-clonic seizures are the most common generalized phenotype. These seizures begin suddenly and they are divided in two stages: an initial tonic phase (30 seconds) with loss of consciousness and subsequent clonic phase (1-2 minutes) with violent muscle contractions. Tonic-clonic generalized seizures affect the whole brain and produce abnormal electrical activity at the frontal, temporal and occipital sites (Kandel et al., 2003).

Table 1. Classification of seizures[a]
Generalized seizures
Tonic–clonic (in any combination)
Absence
Typical
Atypical
Absence with special features
Myoclonic absence
Eyelid myoclonia
Myoclonic
Myoclonic
Myoclonic atonic
Myoclonic tonic
Clonic
Tonic
Atonic
Focal seizures
Unknown
Epileptic spasms

[a]Seizure that cannot be clearly diagnosed into one of the preceding categories should be considered unclassified until further information allows their accurate diagnosis. This is not considered a classification category, however.

Table 1.1: Classification of seizures (Berg et al., 2010).

1.1.2 CLASSIFICATION:

The classification of epileptic seizures is still largely based on clinical observations and expert opinions. The International League Against Epilepsy (ILAE) first published a classification system in 1960. The last official update for seizures was published in 1981, and the last official update for the epilepsies was in 1989 (Berg et al., 2010) (Table 1.1).

By definition, epilepsy is diagnosed after a patient has had two or more unprovoked seizures. With the development of new diagnostic technologies (e.g. modern Neuroimaging and genomic strategies) and of scientific advances, it is necessary to develop a new epilepsy classification, that would be constantly updated (Berg et al., 2010; Berg et al., 2011). For these reasons the Commission on Classification and Terminology of the ILAE decided to break with the nearly century old concepts and language of the ILAE classification systems and to propose some new alternative concept and terminology (Berg et al., 2010; Berg et al., 2011).

This chapter will focus both on the currently accepted standard (based on the 1981 and 1989 reports) and the recent recommendations.

FOCAL(PARTIAL)/GENERALIZED EPILEPSY: generalized and focal were poor terms for characterizing many of the encephalopathic conditions that occur in infants and young children and for some of the neurodegenerative

disorders of later life. For epilepsies, it's better to abandon these terms as overall classification categories, because there are many cases for which this classification is not significant. ''Generalized'' and ''focal'' may be useful in characterizing some forms of epilepsy, especially as the networks involved in those epilepsies become better understood (Berg et al., 2011).

CLASSIFICATION BASED ON ETIOLOGY:

- Genetic causes: this class includes all epilepsies with a recognized genetic cause. This group includes both epilepsies in which the gene and the mechanism were identified and those in which segregation studies showed evidence of a genetic basis but the gene/genes have not been identified yet. Evidences of a genetic cause don't exclude the possibility of environmental factors contributing to the epileptic phenotype.
- Structural-Metabolic causes: this class groups altogether epilepsies caused by external or environmental causes (e.g. structural lesions, malformations) and epilepsies caused by internal pathologic processes (e.g. tumors, neurodegenerative disorders, autoimmune disorders).
- Unknown cause: epilepsies for which the cause is still unknown.

Even if changes were made in epilepsy classification, the electroclinical diagnosis was not affected, because the diagnosis doesn't depend on classification. An electroclinical syndrome, however, is a complex of clinical features, signs, and symptoms that together define a distinctive, recognizable clinical disorder. Every epileptic disorder is identifiable on the basis of a typical age of onset, specific EEG patterns and seizure types and a specific diagnosis could be put forward only analyzing all these aspects.
A list of electroclinical syndromes arranged by age at onset is reported below. (Berg et al., 2010).

ELECTROCLINICAL SYNDROMES ARRANGED BY AGE AT ONSET:

Neonatal period

- Benign familial neonatal epilepsy (BFNE)
- Early myoclonic encephalopathy (EME)
- Ohtahara syndrome

Infancy:

- Epilepsy of infancy with migrating focal seizures
- West syndrome
- Myoclonic epilepsy in infancy (MEI)
- Benign infantile epilepsy
- Benign familial infantile epilepsy
- Dravet syndrome
- Myoclonic encephalopathy in nonprogressive disorders

Childhood:

- Febrile seizures plus (FS+) (can start in infancy)
- Panayiotopoulos syndrome
- Epilepsy with myoclonic atonic (previously astatic) seizures
- Benign epilepsy with centrotemporal spikes (BECTS)
- Autosomal-dominant nocturnal frontal lobe epilepsy (ADNFLE)
- Late onset childhood occipital epilepsy (Gastaut type)
- Epilepsy with myoclonic absences
- Lennox-Gastaut syndrome
- Epileptic encephalopathy with continuous spike-and-wave during sleep (CSWS)
- Landau-Kleffner syndrome (LKS)
- Childhood absence epilepsy (CAE)

Adolescence – Adult:

- Juvenile absence epilepsy (JAE)
- Juvenile myoclonic epilepsy (JME)
- Epilepsy with generalized tonic–clonic seizures alone
- Progressive myoclonus epilepsies (PME)
- Autosomal dominant epilepsy with auditory features (ADEAF)
- Other familial temporal lobe epilepsies

Less specific age relationship:

- Familial focal epilepsy with variable foci (childhood to adult)
- Reflex epilepsies

1.1.3 TREATMENT:

The pharmacological treatment of epileptic seizures strives for maximum seizure control along with preservation of cognitive functions and improvement of quality of life (QOL) (Witt et al., 2012).

Antiepileptic drugs exert their anticonvulsant effects by interfering with brain processes that involve structures that are also involved in learning, memory, and emotional behavior. Thus, modulation of ion channels, neurotransmitters, second messengers, and other processes by antiepileptic drugs, although helpful in controlling seizures, can interfere with normal brain function in undesired ways (Sankar et al., 2004).

In general, most antiepileptic drugs exert their action by attenuating excitatory currents, typically inward cationic currents. Classic antiepileptic drugs, such as phenytoin and carbamazepine, and other agents, such as felbamate, lamotrigine, topiramate, oxcarbazepine, and zonisamide, have been demonstrated to attenuate voltage-gated sodium channels in a use-dependent manner. Such an effect has also been demonstrated with valproic acid at high concentrations. To our knowledge, this effect of antiepileptic drugs has not been specifically attributed to significant cognitive or behavioral effects. However, each of the antiepileptic drugs mentioned above may have other distinctive pharmacologic features that can contribute to their overall effects (Sankar et al., 2004).

A large number of antiepileptic drugs exert their effects by augmenting GABA-ergic inhibition. This is varyingly accomplished by acting directly at the postsynaptic $GABA_A$ receptor site to allosterically influence the chloride current (barbiturates, benzodiazepines, and perhaps also felbamate), by antagonizing neuronal and glial reuptake of GABA (tiagabine), or by interfering with the metabolic breakdown of GABA (vigabatrin) (Rho et al., 1999; Sankar et al., 2004) (Figures 1.1 and 1.2).

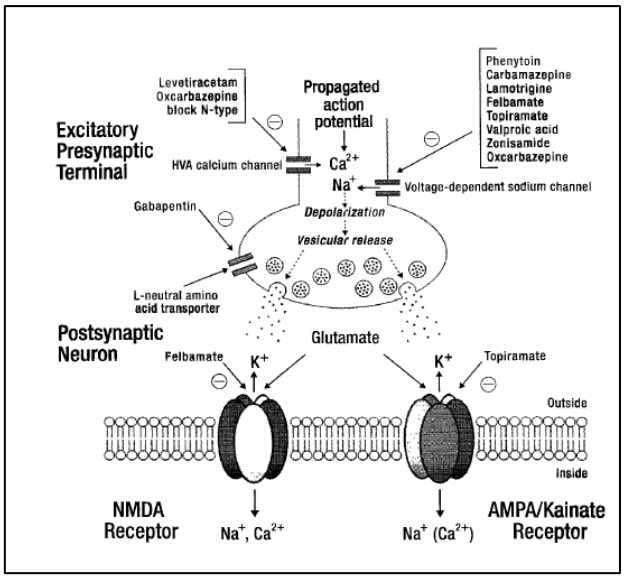


Figure 1.1: Mechanism of action of AED at Excitatory presynaptic terminal (Sankar et al.,2004).

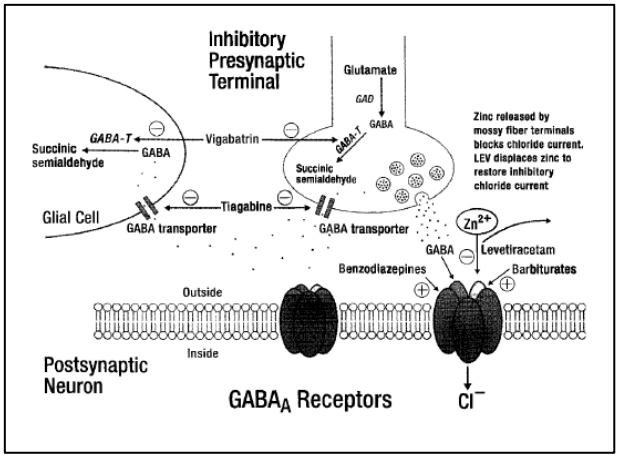


Figure 1.2: Mechanism of action of AED at Inhibitory presynaptic terminal (Sankar et al.,2004).

An appropriate diagnosis together with proper selection and utilization of currently available antiepileptic drugs (AEDs) is necessary for therapeutic success in the management of epilepsy. With the range of drugs currently available, there are immense opportunities for patient-tailored drug therapy. However, the management of epilepsy is primarily based on optimum use of AEDs with the choice of drugs varying considerably among

physicians and across countries. The choice is primarily based on evidence of efficacy and effectiveness for the individual's seizure type, but other patient-specific factors, including age, sex, childbearing potential, adverse-effect profile, comorbidities, and concomitant medications are also needed to be considered (Das et al., 2012).

Traditional AEDs (bromide, benzodiazepines, phenobarbital) are more frequently associated with adverse cognitive effects than phenytoin, valproic acid ore carbamazepine (Witt et al., 2012). Newer generation AEDs, as lamotrigne, levetiracetam and tigabine appear to have superior cognitive profiles (French et al., 2004).

The effects and the mechanisms of action of AEDs are listed in Tables 1.2 and 1.3.

Overview of affected domains by different antiepileptic drugs.

Antiepileptic agent	Affected domains		
	Attention	Memory	Language
Carbamazepine (CBZ)	↓	↓	
Clobazam (CLB)	↓	0	↓
Felbamate (FBM)	(↓)		
Gabapentin (GBP)	↓	0	0
Lamotrigine (LTG)	0	0	0
Levetiracetam (LEV)	0	0	
Oxcarbazepine (OXC)	↓/↑	0	
Phenobarbital (PB)	↓	↓	↓
Phenytoin (PHT)	↓	↓	
Tiagabine (TGB)	0	0	0
Topiramate (TPM)	↓	↓	↓
Valproic acid (VPA)	↓	↓	0
Vigabatrin (VGB)	0	0	0
Zonisamide (ZNS)	(↓)		(↓)

↓, negative effect; ↑, positive effect; ( ): possible effect; 0: no deficits.

Table 1.2: Effects of AED (Witt et al., 2012).

Drugs	Mechanism of action
Vigabatrin	Irreversibly inhibits GABA-T
Tiagabine	Inhibitor of GAT-1
^Lamotrigine	Inhibits the release of excitatory neurotransmitter glutamate. It also inhibits the voltage-sensitive Na^+ channels (VDSC); Blockade of α4β2-nAChR
Carbamazepine, oxcarbazepine, eslicarbazepine	Stabilize the inactivated state of VDSC
^Felbamate, fluorofelbamate	Inhibits NMDA receptor. Also potentiates GABA-mediated inhibition and blocks VDSC
Carisbamate, rufinamide, losigamone, soretolide, valrocemide	Yet to be ascertained
^Topiramate	Selectively blocks excitatory synaptic transmission mediated by GluR5 kainate receptors; also acts at VDSC
Levetiracetam, seletracetam, brivaracetam	Interacts with the synaptic vesicle protein 2A
^Zonisamide	Block sodium channels and reduce voltage dependent T-type Ca^{2+} currents; also modulates dopaminergic, GABA ergic, and serotonergic systems
^Lacosamide	Enhances slow inactivation of VDSC and modulates CRMP-2
Ganaxolone	Positive allosteric modulation of the $GABA_A$ receptor
Remacemide	Potent Na^+ channel blocker and non-competitive NMDA channel antagonist.
Retigabine	It is a KCNQ K^+ channel opener that involves opening of neuronal Kv7.2 (KCNQ2) voltage activated K^+ channels
^Safinamide	Antagonize the Ca^{2+} and Na^+ channels; also reversibly inhibit MAO-B
Stiripentol	It is positive allosteric modulator of $GABA_A$ receptor
Talampanel, perampanel	Non-competitively blocks AMPA receptor
Pregabalin	It binds potently to the α2-γ subunit, an auxiliary protein associated with voltage-gated Ca^{2+} channels

^ Indicates multiple mechanisms.

Table 1.3: Main AED and mechanism of action (Das et al., 2012).

1.2 NOCTURNAL FRONTAL LOBE EPILEPSY (NFLE)

1.2.1 HISTORY

Nocturnal frontal lobe epilepsy (NFLE) started its history has a parasomnia named Nocturnal Paroxysmal Dystonia owing to the absence of evident ictal anomalies during scalp EEG recordings of affected patients (Lugaresi et al., 1986). However, several aspects of its phenotype, such as the stereotypy of motor behavior, the short length of manifestations and the good response to carbamazepine, suggested an epileptic origin of the disease. In 1990, Nocturnal Paroxysmal Dystonia was firmly recognized as an epileptic disease; in fact, epileptic anomalies were found in several cases (Tinuper et al., 1990). Two different classifications have been proposed to describe the semiology of epileptic seizures in NFLE.

The first classification was introduced in 1998 (Oldani et al., 1998) and provides 3 different kinds of seizures:

- ✓ Minor attacks: dystonic and repetitive movements of the legs, continuous pelvic thrusting or swinging, repetitive scratching or rubbing the nose, facial grimacing with vocalization

- ✓ Typical major attack: sudden elevation of head and trunk, fear expression, vocalization or screaming, followed by complex motor activity (sometimes feeling of breath stuck in the throat)
- ✓ Prolonged attacks: bizarre sequence of movements (cycling the legs, rocking the pelvis, choreoatetoid movements), jumping out of the bed, twisting around as a dance ('wandering or deambulation'), sometimes generalized tonic-clonic seizures.

The second classification, derived from the videopolygraphic study of 100 consecutive cases, was proposed later in 1999 (Provini et al., 1999) and identified four kinds of seizures:

- ✓ Paroxysmal arousals: repetitive seizures with short duration (3-5 sec) which come back every 20-30 sec for an extended period of the NREM stage 2 sleep and are characterized by bilateral involvement, resembling a sudden awakening, stereotyped movements of the trunk with elevation of head. The patient opens the eyes, sits in bed, may have vocalizations and a fearful expression. The EEG shows K-complexes before episodes and slow-wave (SW) activity in the frontal area.
- ✓ Hypermotor seizures: sudden awakening from NREM sleep with complex and violent movements of the body with distonic or diskinetic aspects. It occurs with kicks, movements, pedalage, vocalizations. They are longer than paroxysmal arousals, the semiology is similar among patients and even in a single patient attacks are extremely stereotyped and identical in one night and from a night to another.
- ✓ Asymmetric bilateral tonic seizures: sudden asymmetric tonic-dystonic postures of arms and legs which are kept in such positions for few seconds long. The patient is conscious but he cannot speak.
- ✓ Epileptic nocturnal wanderings: they start as asymmetric bilateral tonic seizures which then proceed with a prolonged phase (1-2 min) characterized by wanderings and complex motor activity. They mimic nocturnal wandering episodes in which the patient jumps, shouts, tries to exit the room and has a frightened expression of the face.

1.2.2 Clinical Aspects and Diagnostic Procedures:

NFLE is a partial epilepsy characterized by a wide spectrum of stereotyped motor manifestations, mostly occurring during NREM sleep. NFLE appears to be rare but is probably underdiagnosed since semiological similarities plus nonspecific surface EEG findings make it difficult to distinguish NFLE from parasomnias (Zucconi et al., 2000). The onset of NFLE is usually in infancy or in childhood, with a possible persistence in adulthood. NFLE is characterized by repetitive attacks with predominantly motor component, high frequency per night, inter-night repetition, stereotypy of the episodes, starting in childhood and persisting into young adulthood (Zucconi et al., 2000). Since 1985, a familial clustering of NFLE had already noted in several reports. In 1994 and 1995, Scheffer et al. (1995) reported five families with NFLE inherited as an autosomal dominant trait and introduced the term of autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE). A family history of possible nocturnal frontal lobe seizures is found in about 25% of NFLE cases, while a family history of nocturnal episodes that fit the diagnostic criteria for NREM parasomnias is reported for the 40% of NFLE patients. Neurologic examination in NFLE/ADNFLE is usually normal. Affected individuals are typically of normal intelligence, although rare families with ADNFLE and intellectual disability and/or psychiatric disorders (i.e. depression, personality disorder, paranoid schizophrenia) have been reported. Neuroimaging studies (CT scan, MRI) show normal results in NFLE patients. However, they should be performed in these patients for excluding a rare symptomatic form. The main differentiating features characterizing NFLE/ADNFLE are:

- ✓ several attacks per night at any time during the night;
- ✓ brief duration of the attacks;
- ✓ stereotyped motor pattern.

Nocturnal video-polysomnography (video-PSG) is now considered the gold-standard for the diagnosis. Most of the seizures appears during NREM sleep with preponderance in NREM Stage 2 (>60%). Rarely they emerge from REM sleep (Provini et al., 1999). In some cases, the motor attacks (especially paroxysmal arousals) show a periodicity (every 20 seconds-2

minutes). A recent study showed that the interobserver reliability of diagnosis of NFLE, based on videotaped observation of sleep motor phenomena, is not completely satisfactory (Vignatelli et al., 2007). Owing to the presence of muscle artifacts, some studies showed that EEG during the attacks is uninformative in almost half the cases. However, rhythmic theta or delta waves, sharp waves predominantly in the frontal regions, attenuation of the background activity, and, in a minority of cases, classic spike-and-wave activity or small-amplitude fast activity may be recorded during ictal or interictal EEG. A burst of delta activity may frequently precede or be simultaneous with the episode. Arousal fluctuations, expressed by periodic delta bursts, are commonly related to the occurrence of both epileptic and non-epileptic (confusional arousals, sleep terrors, or sleep-walking) attacks (Parrino et al., 2006); thus, PSG without videorecording may be poorly informative. It has been postulated that the complexity of the motor behavior in NFLE reflects a different duration and propagation of the discharge within the frontal lobe. Some authors confirmed this hypothesis by studying NFLE patients with intracerebral recording techniques: seizures started within the supplementary motor area (Vignatelli et al., 2007). Deep electrode recordings showed that sometimes sleep-related seizures similar to those observed in NFLE may arise from temporal lobe rather than from orbito-frontal zones. In particular, this is the case of seizures characterized by affective symptoms, agitated and deambulatory behaviors. They may involve large neuronal networks with sometimes emergence from the frontal zone (orbito-frontal, anterior cingulated) but also with spreading to temporal limbic cortices. Autonomic changes, such as tachycardia, tachypnea or respiratory abnormalities and irregularities, and electrodermal changes, are frequently observed during the attacks.

As reported by Tinuper et al. (2007), distinguishing nocturnal seizures originating from the frontal lobe from paroxysmal non-epileptic sleep disorders is often difficult and sometimes impossible on clinical grounds alone, even for experienced epileptologists and sleep medicine physicians.

There are three orders of troubles in differentiating epileptic seizures from non-epileptic sleep-related events on the basis of clinical history. Firstly, the behavioral patterns encountered in NREM arousal parasomnias and NFLE

may be similar. Secondly, the semiological subjective elements are often absent in all types of motor events during sleep. Furthermore, a reliable description of motor events occurring during the night is often difficult to collect from a witness or sleep partner. Finally, the available standard criteria for nocturnal motor episodes are unreliable, as in the case of several parasomnias, or are still lacking, as in the case of NFLE (Tinuper et al., 2007). Some authors (Derry et al., 2009) have recently described the semiological features of NREM arousal parasomnias in detail in order to identify features that can be used to reliably distinguish parasomnias from NFLE. They evaluated 120 events (57 parasomnias, 63 NFLE seizures) from 44 subjects: elemental clinical features strongly favoring parasomnias included interactive behavior, failure to wake after the event, and indistinct offset (all $P<0.001$). Although sleep stage at onset was discriminatory (82% of seizures occurred during stage 1 or 2, with 100% of parasomnias occurring from stage 3 or 4), ictal EEG features were less useful. Recently, some authors emphasized the commonly shared semiological features of some frontal seizures and parasomnias (Tassinari et al., 2009). Such similar features might be explained by the activation of the same neuronal networks (so-called ‘‘central pattern generators’’ or CGP). These produce the stereotypical rhythmic motor sequences (in other words, behaviors) that are adaptive and species-specific (such as eating/alimentary, attractive/aversive, locomotor, and nesting habits). CGP are located at the subcortical level (mainly in the brainstem and spinal cord) and, in humans, are under the control of the phylogenetically more recent neomammalian neocortical structures. Although shared semiological behaviors in NFLE and parasomnias indicate that semiology alone does not always lead directly to the diagnosis, nocturnal video-PSG remains, as reported previously, a mandatory procedure in the diagnostic process of NFLE. However, video-PSG is an expensive procedure and not universally available. A new scale, the Frontal Lobe Epilepsy and Parasomnia (FLEP) scale, has been proposed recently as a tool for distinguishing NFLE from parasomnias, especially NREM arousal parasomnias, such as sleep-walking and night terrors (Derry et al., 2006). In the FLEP scale, responses favoring epilepsy score positively and those favoring parasomnias score negatively (Table 1.4). The FLEP scale could be a useful tool in such an algorithm, helping the physician to better

address simple clinical impressions, sometimes suggesting the need for a video-PSG, and other times making it possible to avoid these investigations (Manni et al., 2008). In particular, the items about ‘‘recall’’ and ‘‘clustering’’ of the events throughout the night may increase the likelihood of mistaking REM sleep behavior disorders for seizures.

1.2.3 TREATMENT

NFLE is considered a relatively benign clinical entity because seizures occur during sleep and, in most of the cases, a positive response to antiepileptic drugs is observed. According to literature data, in about two-thirds of NFLE patients, carbamazepine was demonstrated to be efficacious at low doses (200–600 mg/bedtime) in greatly reducing seizure frequency and complexity (Oldani et al., 1998; Provini et al., 1999). Other reported treatments for NFLE include the following: (1) topiramate (dose range 50–300 mg/bedtime) evaluated in 24 patients with a mean age of 29 years (seizure free=25%; reduction in at least 50% of seizures=62.5%; weight loss in six cases; speech dysfunction in two cases) (Oldani et al., 2006); (2) oxcarbazepine (dose range 15–45 mg/kg/day) in eight children aged 4–16 years (complete seizure control in all cases; transient diplopia in one case; mild somnolence in one case) (Raju et al., 2007); (3) transdermal nicotine patch in one case (Willoughby et al., 2003). The positive effect of a nicotine patch on seizures could be related to the possible presence of mutations in the nicotinic acetylcholine receptors (nAChRs) described in NFLE. Moreover, a significant association between tobacco use and seizure control and, on the contrary, a persistence of seizures in nonsmokers has been demonstrated in a group of ADNFLE patients (Brodtkorb et al., 2006). About 30% of NFLE cases in larger samples are resistant to antiepileptic drugs and in particular this is observed for patients with more frequent and complex attacks. Surgical treatment may be an indication in these non-responder patients who usually present sleep fragmentation, non-restorative sleep, and important daytime sleepiness. An accurate presurgical evaluation, including invasive EEG recording, is mandatory for resective surgery in drug-resistant and severe forms of NFLE (Nobili et al., 2007).

THE FRONTAL LOBE EPILEPSY AND PARASOMNIAS (FLEP) SCALE		
Clinical Features		**Score**
Age onset		
At what age did the patient their first clinical event?	<55y	0
	≥55y	-1
Duration		
What is the duration of a typical event	<2 min	+1
	2-10 min	0
	>10 min	-2
Clustering		
What is the typical number of events to occur in a single night?	1 or 2	0
	3-5	+1
	>5	+2
Timing		
At what time of night do the events most commonly occur?	Within 30 min of sleep onset	+1
	Other times (including if no clear pattern identified)	0
Symptoms		
At the events associated with a define aura?	Yes	+2
	No	0
Does the patient ever wander outside the bedroom during the events?	Yes	-2
	No (or certain)	0
Does the patient perform complex, directed behaviours (eg, picking up objects, dressing) during events?	Yes	-2
	No (or uncertain)	0
Is there a clear history of prominent dystonic posturing, tonic limb extension, or cramping during events?	Yes	+1
	No (or uncertain)	0
Stereotypy		
Are the events highly stereotyped or variable in nature?	Highly stereotyped	+1
	Some variability/uncertain	0
	Highly variable	-1
Recall		
Does the patient recall the events?	Yes, lucid recall	+1
	No or vague recollection only	0
Vocalization		
Does the patient speak during the events and, if so, is there subsequent recollection of this speech?	No	0
	Yes, sounds only or single words	0
	Yes, coherent speech with incomplete or no recall	-2
	Yes, coherent speech with recall	+2
TOTAL SCORE		

Table 1.4: FLEP scale.

1.2.4 GENETICS OF ADNFLE/NFLE:

Both familial and sporadic forms of NFLE were reported, even though these forms show no differences from a clinical point of view (Combi et al., 2004). The familial form of the disorder accounts for at least a third of cases and, until a few years ago, it was considered a Mendelian disorder with autosomal dominant inheritance and incomplete penetrance (range 29–100%) and thus named ADNFLE (autosomal dominant NFLE).

The phenotypic expression among generations appeared to be simpler than the one observed for other types of idiopathic epilepsies that are characterized by the coexistence of different epileptic phenotypes even in a single pedigree (in ADNFLE, only two families were reported showing this kind of overlapping) (Picard et al., 2000). ADNFLE is the first idiopathic epilepsy to have its genetic bases reported: in 1995, the first mutation was identified in a very large Australian family (Steinlein et al., 1995).

Until now, five loci have been associated with the disease (Marini et al., 2007; Heron et al., 2012), showing the presence of locus heterogeneity for this disorder. In particular, ENFL1 was detected on chromosome 20q13, ENFL2 on chromosome 15q24, ENFL3 on chromosome 1q21, ENFL4 on chromosome 8p21, and recently a new locus on chromosome 9q34.3 (Heron et al., 2012). Only for the ENFL2 locus has the underlying gene not been yet identified, whereas for the three loci ENFL1, ENFL3, ENFL4, mutations have been detected in genes coding for the alpha4, beta2, and alpha2 subunits of the neuronal nicotinic acetylcholine receptor respectively. As far as ENFL1 locus is concerned, five different mutations have been detected in the CHRNA4 gene coding for the alpha4 subunit of the neuronal nAChR. Four are missense mutations (p.S248F, p.S252L, p.T265I, and p.R308H), whereas one is an insertion of a leucine (p.259InsL). Altogether, the *CHRNA4* mutations have been found in only 11 unrelated patients (Table 1.5). In all these patients, *CHRNA4* mutations were present in the heterozygosis state, consistent with an autosomal dominant transmission pattern. In the ENFL3 locus, five missense mutations have been detected in the CHRNB2 gene, coding for the beta2 subunit of the nACh receptor, in seven unrelated patients (Table 1.5). In 2006, a molecular genetic study of a large Italian family revealed a heterozygous missense

mutation in the TM1 domain of the nAChRs alpha2-subunit gene (CHRNA2), which maps within the ENFL4 locus (Table 1.5). However, CHRNA4, CHRNB2, and CHRNA2 mutations remain a rare cause of ADNFLE as the majority of families, and nearly all sporadic cases, were negative for these mutations (Phillips et al., 1998; Combi et al., 2004; 2005; 2009; De Marco et al., 2007).

The absence of mutations in the CHRNA2 gene as well as in *CHRNA4* and *CHRNB2* in the majority of NFLE patients suggests that they are all minor loci for the disease and that the major locus responsible for NFLE, if any, seems to be still waiting to be discovered. A genome-wide linkage analysis with the purpose of identifying candidate genes identified two new putative loci, one on chromosome 3p23-p21 and one on chromosome 8q13-q21, in an Italian family, suggesting that the inheritance of the disease could be better explained by an oligogenic than a monogenic model in the majority of cases (Combi et al., 2005). In the 8q13-q21 locus, two candidate susceptibility variations (g.-1470C>A and g.-1166G>C) affecting the promoter of the CRH gene coding the corticotropin releasing hormone were detected in a total of seven unrelated NFLE patients (Combi et al., 2005; 2008).

Very recently, a genome-wide linkage analysis identified a new locus on chromosome 9q34.3 corresponding to a 2.36 Mb region at the telomeric end of the q arm of chromosome 9 that contains 99 genes including two genes coding for ion channels, *KCNT1* and *CACNA1B*, and one coding for a glutamate receptor *GRIN1* (Heron et al., 2012). Whole exome sequencing analysis performed on two affected family members identified one unknown missense mutation (p.Arg928Cys) in *KCNT1*. Sanger analysis performed on affected individuals of 3 different families identified additional 3 missense mutations in *KCNT1*: p.Tyr796His, p.Arg398Gln, p.Met896Ile. PolyPhen-2 and SIFT prediction showed a damaging effect of all these mutations with the exception of p.Arg398Gln that is predicted to be tolerated. KCNT1 gene encodes for a sodium-activated potassium channel expressed in brain, not widely expressed in the cortex but found in neurons of the frontal cortex (Heron et al., 2012). All the four mutations were detected in the C-terminal region: one located in the NAD^+ binding site, while two near NAD^+ binding-site. Affected individuals with an

aminoacid change located in or around this binding site resulted to have a more severe phenotype than patients with a mutation in the c-terminal domain but far from NAD^+ binding site. Mutations in *KCNT1* cause a severe form of ADNFLE/NFLE with an earlier age of onset, intellectual disabilities and psychiatric features different from those observed in patients with mutations in nAChR (Heron et al., 2012).
The limited number of families with a defined genetic cause so far reported makes phenotype–genotype correlations difficult. Moreover, the variability of expression of symptoms observed even among members of the same family with the same genetic defect emphasizes the need to understand the multiple influences responsible for the phenotype in each individual.

1.2.5 NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR (NACHR) AND NFLE:

Neuronal nicotinic acetylcholine receptors (nAChRs) belong to a family of ligand-gated ion channels widely distributed in both central and peripheral nervous systems (Gotti et al., 2009). The α4β2 is the predominant receptor in mammalian brain. Several functional studies have been performed to analyze *in vitro* the pathogenetic role of all ADNFLE mutations detected in nAChR subunits. In particular, it was demonstrated that they all have a gain of function effect. It was concluded that either an increased acetylcholine sensitivity or a shift in the proportion between low-sensitivity and high-sensitivity receptors is the common trait linking together all mutations so far identified in *CHRNA4* or *CHRNB2* (Steinlein et al., 2010). It was also reported that some but not all ADNFLE mutations display an abnormal desensitization profile (De Fusco et al., 2000). Another important effect observed for several mutations is a reduction in Ca^{2+} dependence of the response to ACh. This can explain the occurrence of epileptic seizures during sleep with a modulation of Ca^{2+} that enhances more the inhibitory than the excitatory transmitter release during sleep spindles (Rodrigues-Pinguet et al., 2003; 2005). The *in vitro* functional studies inform on the altered functionality of mutated receptors, but they are not sufficient to understand how these receptors interact with transmission in the neuronal circuits. *In vivo* experiments in mouse models carrying mutations in the α4 and β2 subunits of the nAChR demonstrated that they had a highest

GENE	AA CHANGE	PROTEIN POSITION	N° AFFECTED INDIVIDUALS	EFFECT
CHRNA2	I279N	Exon5/TM1	1	Increased sensitivity to ACh (Aridon et al. 2006)
CHRNA4	S248F	Exon 5/TM 2	4	Acceleration of desensitization; decreased Calcium permeability (Marini et al. 2007, McLellan et al. 2003, Saenz et al. 1999, Steinlein et al. 2000)
	S252L	Exon 5/TM 2	5	Acceleration of desensitization (Cho et al. 2003, Hirose et al. 1999, Phillips et al. 2000, Rozycka et al. 2003, Sansoni et al. 2012)
	259insL	Exon 5/TM 2	1	Increased affinity to ACh (Steinlein et al. 1997)
	T265I	Exon 5/TM 2	1	Increased ACh sensitivity (Leniger et al. 2003)
	R308H	Exon 5/intracellular loop 2	1	NA (Chen et al. 2009)
CHRNB2	V287L	Exon 5/TM 2	1	Retardation of desensitization; gain of function (De Fusco et al. 2000)
	V287M	Exon 5/TM 2	2	Increased affinity to ACh; gain of function (Diaz-Otero et al. 2001, Phillips et al 2001)
	L301V	Exon 5/TM 3	1	Increased ACh sensitivity (Hoda et al. 2001)
	V308A	Exon 5/TM 3	2	Increased ACh sensitivity; gain of function (Bertrand et al. 2005)
	I312M	Exon 5/TM 3	2	Increased ACh sensitivity (Bertrand et al. 2005, Cho et al. 2008)
	V337G	Exon 5/intracellular loop	1	NA (Liu et al. 2011)
KCNT1	R398Q	C-Terminal Domain	4	NA (Heron et al. 2012)
	Y796H	C-Terminal domain adiacent NAD^+ binding site	4	NA (Heron et al. 2012)
	M896I	C-Terminal domain in NAD^+ binding site	1	NA (Heron et al. 2012)
	R928C	C-Terminal domain adiacent NAD^+ binding site	6	NA (Heron et al. 2012)

Table 1.5: Known mutations in genes associated with ADNFLE. (Ferini-Strambi et al., 2012).

susceptibility to seizures and showed abnormal cortical EEG patterns. This demonstrated that mutations in nAChR could be responsible for epileptic seizures (Klaassen et al., 2006; Teper et al., 2007). In addition, mutant nicotinic receptors are responsible for abnormal formation of neuronal circuits and alteration of network assembly in the developing brain leading to epilepsy.

1.2.6 Corticotropin Releasing Hormone (CRH) and NFLE

The CRH hormone is a 41-amino acid peptide widely distributed throughout the central nervous system; it acts as a neurotransmitter or neuromodulator in extrahypothalamic circuits to integrate a multisystem response to stress that controls numerous behaviors such as locomotor activity, anxiety, food intake, sexual behavior, sleep, arousal and learning (Combi et al., 2005). Response to stress is mediated by the hypotalamic-pituitary-adrenal axis (HPA axis). Activation of this pathway by stressors causes the hypotalamic production and release of CRH, which stimulate pituitary CRH receptors. CRH enhances the production and secretion of: propiomelanocortin (POMC), peptides melanocyte stimulating hormone (MSH), ACTH and endorphin (Slominski et al., 2000). In the adrenal cortex ACTH causes the release of cortisol, a powerful anti-inflammatory factor that counteracts the effect of stress. The systemic response to stress is showed in Figure 1.3 (Slominski et al., 2000).

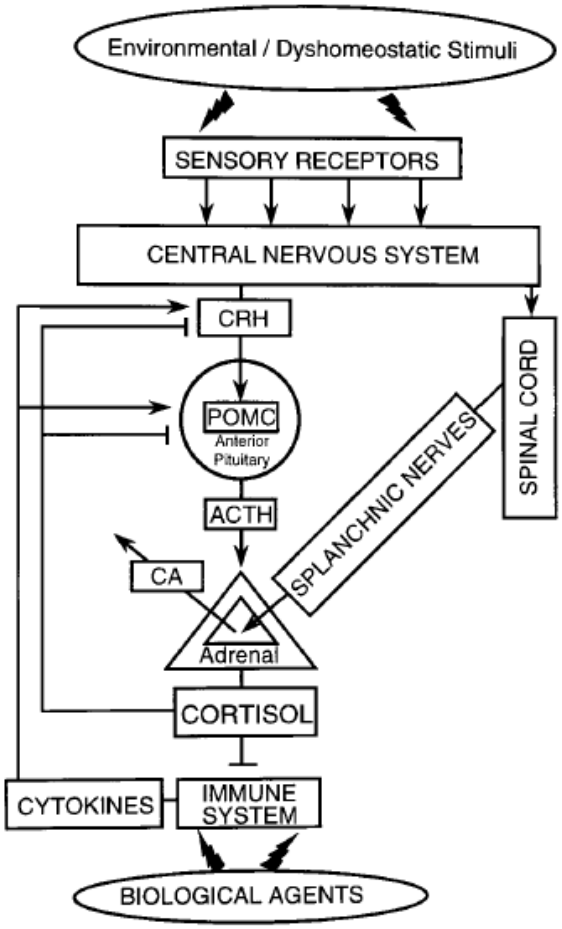


Figure 1.3: Systemic response to stress (Slominski et al., 2000).

The CRH gene is composed of two exons separated by an intron. Exon 1 encodes most of the 5'-untraslated region in the mRNA, while exon 2 contains the information for the preprohormone sequence and the 3'-untraslated region. The translation of exon 2 generates the 196 amino acid prepro-CRH. The starting 26 amino acids represent the signal peptide (Slominski et al., 2000). After removal of the signal peptide and C-terminal amidation, pro-CRH has a molecular size of about 19 kDa. The Pro-CRH contains two potential cleavage sites, CS1 (AA 124-125) and CS2 (AA 151-152). Endoproteolytic processing of pro-CRH within the trans-Golgi network and secretory granules generates the final 41aa-long protein with a molecular mass of 4,7 kDa.

The enzymes involved in this proteolytic process are the PC1 and PC2 convertases (Brar et al., 1997).

CRH is produced predominantly in the paraventricular nucleus (PVN) of the hypothalamus and delivered into portal capillaries converging in the anterior lobe of the pituitary. In addition, autonomic neurons of the PVN projecting to the brain stem and spinal cord supply CRH to the simpathoadrenal system and, thorough neurons projecting to the pituitary, CRH is involved in osmotic regulation not connected with stress. CRH is also involved in the functional modulation of the immune system, reproductive and cardiovascular system (Slominski et al., 2000).

It was previously reported that this hormone promotes wakefulness and impairs sleep in a dose-dependent way (the higher the level of CRH, the poorer the sleep continuity) (Terzano et al., 1992) and that overexpression of the CRH enhances REM sleep (Kimura et al., 2010). Moreover, it was demonstrated that high levels of CRH are correlated with a high sigma activity and an altered delta activity, which was found to be altered in patients with mutations in the CRH promoter (Antonijevic et al., 2010). Altered CRH levels could modify the sigma activity, thus increasing the susceptibility to seizures as well as to abnormal sleep spindles timing. A role of the thalamus could explain why there are no interictal scalp EEG abnormalities in NFLE (Picard et al., 2007; Crespel et al., 1998). The human EEG shows two types of spindles: one of 12 Hz in the frontal region and one of 14 Hz centroparietally. Interestingly, the power of frontal spindles is reported to be greatest in young children (Nakamura et al., 2003), and this

could be finally related with the onset of the disease in childhood. Moreover, the involvement of CRH, which has a much higher proconvulsant effect in young people (Baram et al., 1991) could be related to the fact that a complete remission of the disease was reported for some patients.
An *in vitro* functional analysis demonstrated that both identified variations in CRH promoter modify the downstream level of expression introducing the question if misregulation of CRH levels could be one of the factors involved in the pathogenesis of the disease.
A genotype-phenotype correlation was observed in mutated patients by evaluating the cycling alternating pattern rates which resulted higher compared with those of either age-matched controls or patients with no mutations in the CRH promoter. These cycling alternating pattern rates demonstrate that in these patients there is a very high level of sleep fragmentation which could be related to an altered expression of CRH protein (Combi et al., 2005).

1.3 FEBRILE SEIZURES

1.3.1 CLINICAL ASPECTS

Febrile seizures (FSs) are relatively common and represent most childhood seizures. Studies in the developed nations indicate that 2–5% of all children will experience an FS before 5 years of age. In Japanese population, the incidence rate is 6–9%. FSs are not a true epileptic disease but a special syndrome characterized by seizures and fever ranging from 6 months to 6 years. The prognosis is generally very good, but people who experienced FS have a higher risk of developing spontaneous afebrile seizures, which define epilepsy when they recur (Nakayama et al., 2006). Febrile seizures can be classified as either simple or complex. A simple febrile seizure is isolated, brief, and generalized. Conversely, a complex febrile seizure is focal, multiple (more than one seizure during the febrile illness), or prolonged, lasting either more than 10 or 15 minutes (Shinnar et al., 2002).
Most febrile seizures are simple. In a study on 428 children with a first febrile seizure, at least one complex feature was noted in 35% of children, including focality (16%), multiple seizures (14%), and prolonged duration

(>10 minutes, 13%). Five percent of the total group experienced a seizure lasting more than 30 minutes (i.e., febrile status epilepticus). Only 21% of the children experienced seizures either prior to or within 1 hour of the onset of fever; 57% had a seizure after 1 to 24 hours of fever, and 22% experienced their febrile seizure more than 24 hours after the onset of fever (Shinnar et al., 2002).

In population[24]
First- or second-degree relative with history of FS
Neonatal nursery stay of >30 days
Developmental delay
Attendance at day care
Two of these factors → 28% chance of at least 1 FS
In children with a febrile illness[25]
First- or second-degree relative with history of FS
Height of temperature

FS = febrile seizure.

Table 1.6: Risk factor for first Febrile Seizures (Shinnar et al., 2002).

A case-control study identified as significant independent risk factors for first febrile seizures the followings: height of temperature, history of febrile seizures in a first- or in a higher degree relative and gastroenteritis, as the underlying illness had a significant inverse (i.e., protective) association with febrile seizures (Berg et al., 1995). In a more recent study, Shinnar et al. (2002) identified risk factors for first febrile seizures in population and in children with a febrile illness.

Children with multiple risk factors have a 28% chance to develop a FS. The same authors examined also the risk factors for developing epilepsy after FS. Following a single simple FS, the risk of developing epilepsy is not substantially different than the risk in the general population (Shinnar at al., 2002). The cause of FS probably relates on both genetic and environmental factors. The environmental factor is, of course, the fever; this is likely to be due to an underlying infection, predominantly viral. Specific investigation at

the time of a seizure should be directed with the purpose of diagnosing the underlying infection (Cross, 2012).

1.3.2 TREATMENT

The majority of febrile seizures are brief, lasting in less than 10 minutes, and no intervention is necessary. Rectal diazepam or diazepam gel has been shown to be effective in terminating febrile seizures and it is the therapy of choice for intervention outside the hospital. Families with children at high risk for, or with a history of, prolonged or multiple febrile seizures and those who live far from medical care are excellent candidates to have rectal diazepam or diazepam gel readily available in their homes (Shinnar et al., 2002).

Antipyretics: There are little evidences suggesting that antipyretics could reduce the risk of recurrent febrile seizures. It should be noted that children, in whom febrile seizures occur at the onset of the fever, have the highest risk of recurrent febrile seizures.

Benzodiazepines: Diazepam, given orally or rectally at the time of onset of a febrile illness, has demonstrated a statistically significant, yet clinically modest, ability to reduce the probability of a febrile seizure.

Barbiturates: Intermittent therapy with phenobarbital at the onset of fever is ineffective in reducing the risk of recurrent febrile seizures. Surprisingly, it is still fairly widely used for this purpose. Phenobarbital, given daily at doses that achieve a serum concentration of 15 µg/mL or higher, has been shown to be effective in reducing the risk of recurrent febrile seizures in several well-controlled trials. However, in these studies, a substantial portion of children had adverse effects, primarily hyperactivity, which required discontinuation of therapy.

Valproate: Daily treatment with valproic acid is effective in reducing the risk of recurrent febrile seizures in both human and animal studies. However, it is very rarely used since children considered most often for prophylaxis (young and/or neurologically abnormal) are also the ones at highest risk for fatal idiosyncratic hepatotoxicity (Shinnar et al., 2002).

There is no evidence that preventing febrile seizures will reduce the risk of a subsequent epilepsy onset. One rationale for starting chronic antiepileptic therapy in children with febrile seizures is to prevent the development of future epilepsy. In different studies comparing effects of the use of placebo compared with treatment (with daily phenobarbital or diazepam at the

onset of fever), it was demonstrated that treatment significantly reduced the risk of FS recurrence, but the risk of developing epilepsy was no lower in the treated group than in the control population (Shinnar et al., 2002).

1.3.3 GENETICS

Febrile seizure is a complex and heterogeneous disease in which genetic factors contribute significantly to the etiology. A positive family history can be observed in 25-40% of FSs patients (Nakayama et al., 2006). Polygenic inheritance is usual, although in a minority of families an autosomal dominant inheritance was reported. Siblings have a 25% risk, with high concordance in monozygotic twins. Until now eleven loci have been associated with FSs (Table 1.7), but rarely the underlying gene has been identified.

LOCUS		GENE	REF	REF OMIM
FEB1	8q13-q21	NA	Wallace et al.,1996	121210
FEB2	19p13.3	NA	Johnson et al., 1998	602477
FEB3A	2q23-q24	SCN1A	Mantegazza et al., 2005	604403
FEB3B	2q24.3	SCN9A	Peiffer et al.,1999	603415
FEB4	5q14.3	MASS1	Nakayama et al.,2000/2002	604352
FEB5	6q22-q24	NA	Nabbout et al.,2002	609255
FEB6	18p11.2	IMPA2	Nakayama et al.,2004	609253
FEB7	21q22	NA	Hedera et al.,2006	611515
FEB8	5q34	GABRG2	Audenaert et al.,2006	611277
FEB9	3p24.2-p23	NA	Nabbout et al.,2007	611634
FEB10	3q26.2-q26.33	NA	Dai et al.,2008	612637
FEB 11	8q12.1-q13.2	CPA6	Salzman et al., 2012	614418

Table 1.7: Loci associated with Febrile Seizures.

1.4 GENETIC (GENERALIZED) EPILEPSY WITH FEBRILE SEIZURE PLUS

1.4.1 CLINICAL ASPECTS

Genetic epilepsy with febrile seizures plus (recently changed from "Generalized Epilepsy with febrile seizures plus") is a familial epilepsy syndrome whose diagnosis is based on the presence of at least two family members showing phenotypes consistent with the GEFS+ spectrum (Fig. 1.4) (Scheffer et al., 2005). GEFS+ is a dominantly inherited epilepsy characterized by febrile seizures in childhood progressing to generalized epilepsy in adults (Meisler et al., 2005). GEFS+ is distinguished by many phenotypes showing a predisposition to seizures with fever but this predisposition is not universal. GEFS+ families may just show one phenotype within them such as FSs, but more typically, they show a pattern of phenotypic heterogeneity (Scheffer et al., 2009). The most common phenotypes include FS and FS+, in which FS persist beyond 6 years of age or are associated with afebrile, mostly generalized or more rarely partial, seizures. More severe epilepsy phenotypes such as myoclonic–atonic epilepsy or SMEI have also been described within GEFS+ families (Nakayama, 2009).

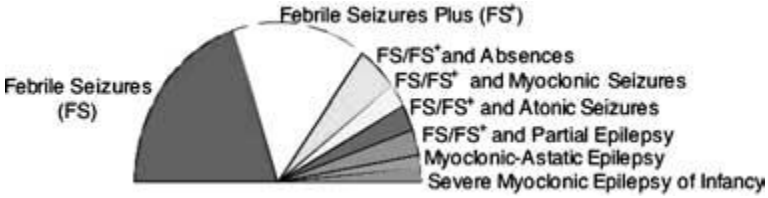


Figure 1.4: Spectrum of different phenotypes found in GEFS+ families.

1.4.2 TREATMENT

GEFS+ is usually a relatively mild epilepsy syndrome. Seizures are typically well controlled by treatment with anti-epileptic drugs and no cognitive impairment is observed. Drugs commonly used to treat GEFS+ seizures are:
Benzodiazepines: a study has shown that the use of anticonvulsants in patients can drastically reduce the febrile attacks (Verrotti at al., 2004);

Barbiturates: phenobarbital prevents recurrent febrile attacks. To be effective, however, it must be administered daily and maintained within the therapeutic range (Farwell et al., 1990);
Valproate: it is rarely used and it prevents febrile attacks with the same effectiveness of phenobarbital, but with severe side effects including liver toxicity, especially in children under the age of two years old, weight loss, thrombocytopenia and gastrointestinal disorders (Camfield et al., 1980).

1.4.3 GENETICS

Genes associated with GEFS+ have been identified in large autosomal dominant families but mutations have been only found in a minority of GEFS+ families overall. GEFS+ most commonly shows complex inheritance where several genes are involved possibly together with an environmental contribution. Many GEFS+ families have been recognized throughout the world, but in the majority of them the molecular bases have not been identified yet (Scheffer et al., 2009). Complex inheritance is suggested not only by genetic analyses but also by the observation that different phenotypes are frequently found within one family (Scheffer et al., 1997). The identified loci are listed in Table 1.8; in some cases the underlying gene has not been identified yet.
The majority of GEFS+ mutations were found in genes encoding subunits of either voltage-gated or ligand-gated ion channels, confirming GEFS+ as a clinical entity. In particular, the neuronal sodium channel type I has had mutations reported in two different subunit genes: rare mutations have been described in the auxiliary β-1 subunit gene, *SCN1B*, while a lot of mutations have been found in the α-1 subunit gene, *SCN1A*. The β-1 subunit has a role in modulating channel gating kinetics and it lies on either side of the alpha pore-forming subunit. Moreover, mutations have been detected in two $GABA_A$ receptor's subunits: the γ-2 subunit encoded by the GABRG2 gene, in which mutations were found in families with GEFS+ alone and in other kindred with childhood absence epilepsy as well; the delta subunit (GABRD gene) in which an unconfirmed paper reported a variant detected in a small family with GEFS+ showing functional changes (Scheffer et al., 2009). Additional mutations associated with GEFS+ were reported in SCN2A and SCN9A genes (Sugawara et al., 2001; Singh et al., 2009) coding

for two different voltage gated sodium channels (type II and type IX, respectively).

LOCUS		GENE	REF	REF OMIM
GEFS1	19q13.1	SCN1B	Wallace et al.,1998	604233
GEFS2	2q24.3	SCN1A SCN2A	Escayg et al.,2000 Sugawara et al., 2001	604403
GEFS3	5q34	GABRG2	Baulac et al., 2001	611277
GEFS4	2p24	NA	Audenaert et al.,2005	609800
GEFS5	1p36.33	GABRD	Dibbens et al.,2004	613060
GEFS6	8p23-p21	NA	Baulac et al.,2008	612279
GEFS7	2q24.3	SCN9A	Peiffer et al., 1999, Singh et al.,2009	613863
GEFS8	6q16.3-q22.31	NA	Poduri et al., 2009	613828

Table 1.8: Loci associated with GEFS+.

1.4.4 Voltage-gated sodium channel and nav1.1

Voltage-gated sodium channels (VGSCs) play essential roles in normal neurologic function, especially in initiation and firing of action potentials. It is, therefore, not surprising that gene variations can have effects, and even potentially devastating consequences, on the nervous system. Indeed, sodium channel mutations are the most important currently recognized cause of genetic epilepsies (Oliva et al., 2012). At resting membrane potentials these channels are normally in the closed state. With mild membrane depolarization, they open to allow the inward flow of sodium, causing a further rapid depolarization that underlies the rising phase of the action potential (AP). Following this opening, a rapid inactivation (on a millisecond time scale) stops the flow of sodium and channels enter a closed state and are unavailable for opening. The subsequent opening of voltage-gated potassium channels causes a slow membrane repolarization

that causes the sodium channels to recover from inactivation and once again become available for opening (Oliva et al., 2012).

The sodium channel α-subunit consists of a highly processed 260-kDa protein that encompasses four homologous domains termed I–IV (Fig. 1.5). Within each domain, there are six transmembrane segments called S1–S6. A hairpin-like P-loop between S5 and S6 forms part of the channel pore, and the intracellular loop that connects domains III and IV forms the inactivation gate. Channels in the adult CNS are associated with accessory β1, β2, β3, or β4 subunits. Each β subunit consists of a single transmembrane segment, an extracellular immunoglobulin (Ig)–like loop, and an intracellular C-terminus. A β2 or β4 subunit is covalently linked to the α subunit by a disulfide bond, while a β1 or β3 subunit is noncovalently attached. The voltage dependence, kinetics, and localization of the α subunits are modulated by interactions with the β subunits (Escayg et al., 2010).

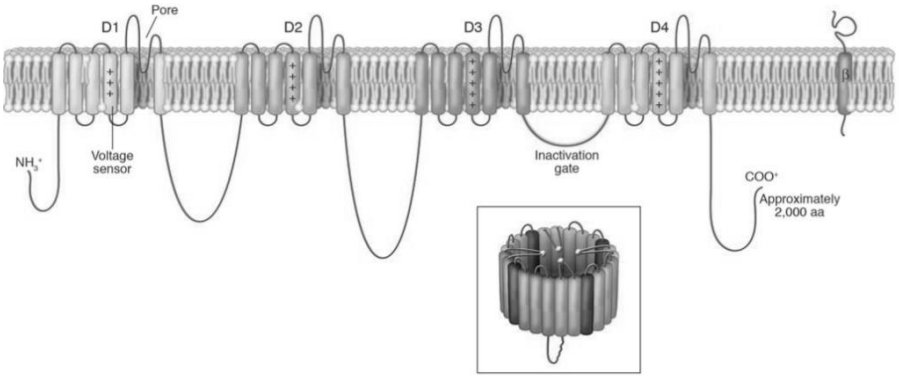


Figure 1.5: Nav1.1 protein structure (Meisner et al., 2005).

At least 9 of the 10 Nav1 channel α-subunit genes of the mammalian genome are expressed in the nervous system, the exception being the muscle-specific Nav1.4, Nav1.7, Nav1.8, Nav1.9, and Nax, which are expressed predominantly in the peripheral nervous system, Nav1.5 expressed in adult cardiac and embryonic skeletal muscle. The remaining α subunits (Nav1.1, Nav1.2, Nav1.3, and Nav1.6) are expressed at high levels in the brain.

The different subtypes exhibit different subcellular localization; Nav1.1 and Nav1.3 are predominantly localized to the neuronal soma and to proximal

dendrites, where they control neuronal excitability through integration of synaptic impulses to set the threshold for action potential initiation and propagation to the dendritic and axonal compartments (Vacher et al., 2008).
Nav1.1 is encoded by SCN1A, an 81-kb gene on the long arm of chromosome 2 (2q24.3). SCN1A is part of a cluster of voltage-gated sodium channel genes that is home to SCN2A, SCN3A, SCN7A, as well as SCN9A, which encode Nav1.2, Nav1.3, Nax, and Nav1.7, respectively. Organized into 26 exons, the Nav1.1 open-reading frame blueprints the instructions for a protein incorporating between 1976 and 2009 amino acids. The variance in length stems from alternative splice junctions at the end of exon 11 that produce a full-length isoform or two shortened versions thereof, from hereon referred to as Nav1.1[-33] and Nav1.1[-84] based on the number of base pairs deleted (Lossin et al., 2009).
Until now more than 300 missense mutations in the SCN1A gene have been described.

DNA screenings of GEFS+ patients in large families led to identification of several mutations in the SCN1A gene. Functional effects of GEFS+ mutations were first studied by expression in non-neuronal cells and whole-cell voltage-clamp analysis. These studies revealed that the effects of SCN1A mutations are either loss or gain of function, and the effect depends on the aminoacid change that alters the biophysical properties of Nav1.1 channel (Catteral et al., 2012).
Studies of GEFS+ mutations in families with variable disease penetrance revealed that loss of function resulted from folding and/or trafficking defects that prevented channel expression in the absence of β subunits and that reduced expression significantly in the presence of β subunits. Remarkably, these GEFS+ mutations can also be partially rescued by treatment with anti-epileptic drugs, which apparently stabilize the mutant channels by contributing their binding energy to stabilization of the correctly folded protein. These results indicate that loss-of-function effects can result from changes in biophysical properties and/or defects in folding and cell surface expression (Catterall et al., 2012).

Chapter 2:

MATERIAL AND METHODS

2.1 SAMPLE COMPOSITION AND INCLUSION CRITERIA

ADNFLE:

Since several years our group is engaged in the study of both clinical and genetic aspects of autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) as well as of different forms of additional idiopathic epilepsies.

In particular, a sample composed by 39 families and 30 sporadic cases affected by Nocturnal Frontal lobe epilepsy has been already collected. An extensive clinical and video-polysomnographic analysis of these patients complaining repeated abnormal nocturnal motor and/or behavioral phenomena was performed by several experts in the field, mainly Prof Luigi Ferini-Strambi (Sleep disorder centre, Università Vita-Salute San Raffaele, Milano) and Dr. Lino Nobili (Centre of Sleep Medicine, Centre for Epilepsy Surgery "C. Munari", Department of Neuroscience Niguarda Hospital, Milano) who tightly collaborates as external clinical consultants. The study was approved by the Ethical Committee of the Istituto Scientifico H. San Raffaele and the Niguarda Hospital Milan, and all patients signed an appropriate informed consent form and then underwent the following study protocol: (i) physical and neurological examinations; (ii) detailed sleep interview with parents or the bed partner; (iii) EEG studies during wakefulness; (iv) video-EEG studies after sleep deprivation; (v) nocturnal video-polysomnography (after an adaptation night to the laboratory) including EEG monitoring (at least eight bipolar leads positioned according to the International 10–20 System), electrooculogram, submental electromyography, ECG and, in most cases, electromyography of arms and legs and abdominal and/or thoracic respiratory movements. The patients were monitored overnight with a video (split-screen system) and the recordings were analyzed to detect abnormal behavior and/or motor activity. The nocturnal repetitive motor activity was carefully analyzed and classified according to duration, semiology and complexity of motor behavior as previously described (Oldani et al., 1998).

Pedigrees' analysis of the large cohort of families (39) was consistent with autosomal dominant transmission with reduced penetrance (about 81%)

(Oldani et al., 1998). Pedigrees of the available families were previously reported (Oldani et al., 1998; Tenchini et al, 1999).
A subset of 15 families (see references Oldani et al., 1998; Tenchini et al, 1999, pedigrees 3, 4, 5, 7, 10, 12, 15, 18, 20, 30, 32, 34, 35, 36, 37) was compliant and consequently was further analyzed from a genetic point of view. In 9 additional families genetic material was obtained for only the proband. Involvement of the CHRNA2, CHRNA4 and CHRNB2 genes, coding for α2, α4 and β2 subunits of the neuronal nicotinic acetylcholine receptor (nAChR), was investigated in all probands and sporadic cases. Segregation and linkage analysis of intragenic *CHRNA4* and *CHRNB2* markers, together with DNA sequencing of the three genes excluded their involvement in the pathogenesis of NFLE in this sample (Combi et al., 2004; 2009; Ferini-Strambi et al., 2003; Tenchini et al., 1999).
A group of 115 healthy control subjects, selected for absent clinical history of common diseases and epilepsy, also was considered. All individuals were adult, and the sex ratio was 1:1.

FS/GEFS+:

Affected children with a familial history for epilepsy as well as sporadic cases of FS syndromes were selected from the database of all cases collected by the Epilepsy Unit of the Infantile Neuropsychiatric Department of San Gerardo Hospital in Monza, Italy. We have considered as 'familial epilepsy' the occurrence of epilepsy in at least two members of the same family including the proband. Individuals with different forms of idiopathic generalized and focal epilepsy and individuals with febrile seizures were enrolled in the study. Patients showing symptomatic epilepsy were excluded.
In all patients, complete diagnostic evaluation was performed, inclusive of: 1) collection of the detailed medical history of epileptic disorder in proband and relatives (pregnancy and delivery, type of seizures and age of onset, seizure susceptibility to fever, drugs resistance); 2) complete neurological examination and neurophysiological studies; 3) review of all available medical records (neurological examination reports, pharmacological treatments, CT and MRI scans, EEG studies).

For each proband the pedigree was extended as much as possible. We collected the medical history and reviewed available medical records to define the epileptic syndrome in relatives; if necessary we performed new clinical examinations.
Patients' parents or patients themselves (>18 years old) signed an informed consent form and the study was approved by the Ethical Committee of S. Gerardo Hospital.
A group of 100 healthy control subjects, selected for absent clinical history of common diseases and epilepsy, also was considered. All individuals were adult, and the sex ratio was 1:1.

2.2 BLOOD COLLECTION AND GENOMIC DNA EXTRACTION

Venous blood was collected in BD Vacutainer® Blood Collection Tubes containg K_2 EDTA (spray-dried) 5.4mg and genomic DNA was extracted using the Wizard genomic DNA purification kit (Promega, Madison, WI, USA) that is designed for the isolation of gDNA from white blood cells. The extraction protocol is based on a four-step process. The first step in the purification procedure lyses the cells and the nuclei. For isolation of gDNA from white blood cells, this step involves lysis of the red blood cells in the Cell Lysis Solution, followed by lysis of the white blood cells and their nuclei in the Nuclei Lysis Solution. An RNase digestion step has been included at this time. The cellular proteins are then removed by a salt-precipitation step, which precipitates the proteins but leaves the high molecular weight genomic DNA in solution. Finally, the genomic DNA is concentrated and desalted by isopropanol precipitation and resuspended in nuclease free water. DNA purified with this system is suitable for a variety of applications, including amplification, digestion with restriction endonucleases and membrane hybridizations (e.g., Southern and dot/slot blots).
The DNA yield depends on the quantity of white blood cells present in the sample; however, it is expected a typical DNA yield of 5-15ug from 300ul of starting material.

2.3 POLYMERASE CHAIN REACTION (PCR) AND DNA SEQUENCING

Polymerase chain reactions (PCRs) were performed directly on 50-100ng of genomic DNA in a 25µL volume. Each reaction was performed using the PCR Master Mix (Promega, Madison, WI, USA) which is a premixed, ready-to-use solution containing Taq DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. The purchased 2x mixture contains 50units/ml of Taq DNA polymerase supplied in a proprietary reaction buffer (pH 8.5), 400µMdATP, 400µM dGTP, 400µM dCTP, 400µM dTTP, 3mM $MgCl_2$. PCRs were carried out on Mastercycler Ep Gradient thermomodules (Eppendorf, Milano, Italy) under standard conditions. Primers (Sigma St. Louis, Mo, USA) were designed on the basis of the known genomic sequence of each gene using the *Oligo 6.0* software (Molecular Biology Insights Inc, Cascade, CO). The sequence of each primer used in mutation screening analysis is listed in Tables 2.1, 2.2, 2.3, 2.4 and 2.5

For mutation detection, primers were designed to amplify the flanking intronic regions, allowing the sequencing of the whole exon and exon/intron boundaries. The specific PCR conditions for each primer couple are available on request.

Sequencing was carried out directly on purified PCR products. Sequence analysis was carried out on both strands using of the BigDye Terminator Cycle Sequencing kit v1.1 and an automated ABI-3100 DNA sequencer (Applied Biosystems, Foster City, CA). ChromasPro v1.34 (Technelysium Ltd.) software was used for mutation detection.

In case of identification of a nucleotide variation in a patient, its presence was tested in all available DNAs from the relatives and in the control sample.

PCR PRIMERS designed on gDNA sequences: SCN1A gene			
EXON ID	PRIMER Forward	PRIMER Reverse	bp
1	TCATGGCACAGTTCCTGTATC	GCAGTAGGCAATTAGCAGCAA	589
2	TGGGGCACTTTAGAAATTGTG	TGACAAAGATGCAAAATGAGAG	391
3	GCAGTTTGGGCTTTTCAATG	TGAGCATTGTCCTCTTGCTG	314
4	AGGGCTACGTTTCATTTGTATG	TGTGCTAAATTGGAATCCAGAG	421
5	CAGCTCTTCGCACTTTCAGA	TCAAGCAGAGAAGGATGCTGA	307
6	AGCGTTGCAAACATTCTTGG	GGGATATCCAGCCCCTCAAG	477
7	ATTGGGACTTTCTAGGAGG	AAATTAGTTGGCTGTTATC	1041
	F1TACTATAAGTTGCCTATATTC	/	/
8	GAAATGGAGGTGTTGAAAATGC	AATCCTTGGCATCACTCTGC	581
9	TTGAAGCCACCACTTAGTGAA	TTAATTCCTCATACAACCACC	372
10	TCTCCAAAAGCCTTCATTAGG	TTCTAATTCTCCCCCTCTCTCC	544
11	TCCTCATTCTTTAATCCCAAGG	GCCGTTCTGTAGAAACACTGG	670
12	GTCAGAAATATCTGCCATCACC	GAATGCACTATTCCCAACTCAC	372
13	TGGGCTCTATGTGTGTGTCTG	GGAAGCATGAAGGATGGTTG	543
	F1ACTTGAAGAATCCAGGCAGA	R1CTACTGTAAGCACATTATTG	232
14	TACTTCGCGTTTCCACAAGG	TGCTATGCAAGAACCCTGATTG	435
15	ATGAGCCTGAGACGGTTAGG	ATACATGTGCCATGCTGGTG	544
16	TGCTGTGGTGTTTCCTTCTC	TGTATTCATACCTTCCCACACC	652
17	TCTTCCCCTTATTCAATCTCT	ATTGGGCAGATATAATCAAA	456
18	TTATGGAAGCAGAGACACT	GCATTGGATACTAAGACAA	305
19	TCTGCCCTCCTATTCCAATG	GCCCTTGTCTTCCAGAAATG	445
20	AAATAAAGGAAGAATAGCCA	TCAAGAGAGGCCTATTTC	642
21	TGAACCTTGCTTTTACATATCC	ACCCATCTGGGCTCATAAAC	579
22	TGTCTTGGTCCAAAATCTGTG	TTGGTCGTTTATGCTTTATTCG	283
23	CCCTAAAGGCCAATTTCAGG	ATTTGGCAGAGAAAACACTCC	378
24	GAGATTTGGGGGTGTTTGTC	GGATTGTAATGGGGTGCTTC	600
25	CAAAAATCAGGGCCAATGAC	TGATTGCTGGGATGATCTTG	483
26	F CGCATGATTTCTTCACTGGTTG	AGAAAATTCCAACAGATGGGTTC	1157
	F2TGAGATGTTCTATGAGGTTTG	GTCATAGGAAGGTGGACAAG	
	F3TGCTTTTACAAAGCGGGTTC	GTTTGCTGACAAGGGGTCAC	592

Table 2.1: PCR primers for the SCN1A gene.

PCR PRIMERS designed on cDNA sequences: SCN1A gene				
F-SCN1A cDNA		R-SCN1A cDNA		bp
1	GTGGATAGCGGTTTGACTC	1	GTATTGAATAAAGGGAAGG	601
2	GCCCAAAGCCAAATAGTGACT	2	TTCTGTCTGAGCGTATTTG	642
3	TGTGGACCTGGGCAATGTC	3	GAAGAAGCAGAACAGAAAGAG	706
4	CTAATGACTCAGGACTTCT	4	GAGAGCCGTAGAGATTCCTTG	693
5	AGGCAGCAACGGCAACTG	5	GTGAGGTTTCCTTGGTTGGTG	588
6	CAACCTGAGTCAGACCAGTAG	6	TAATAAAGATCATCGGCAAT	763
7	GACGCTTAGCCTGGTAGAAC	7	TCATACAGCAGAAATTGGGAA	693
8	CGCCAGTGATTGTCAACT	8	GACGTGTTTCCGAATAGTTG	878
9	ACGGAAGACTTTAGTAGTGA	9	AATGTAGGATTTGGGTATC	883
10	AATGCCTTGGGTTACTCAG	10	TATTTCGTGTCCCCTACCCTG	999
11	ATGTAGGATTTGGGTATCTCT	11	TCCTGCGTTGTTTAACAT	769
12	CCCTACCCTGTTCCGAGTG	12	CAATCTGCCACAACCAAACA	644
13	TGGAGAACTTCAGTGTTGCTA	13	ATGAGCAAGAAGGCAAAGATG	654
14	AGGTGGGGCTAATCTTCTT	14	CAACGCCATATTTCTATTTAC	928

Table 2.2: PCR primers for the SCN1A cDNA.

PCR PRIMERS designed on reference gDNA sequences			
CHRNA2			bp
EX 5	A2ex5F1new	ACTTGGTTCTTCGGGTTC	552
	A2ex5R1new	AATGCACAGCGTGATCTT	
	A2ex5F2new	GCTGCCGCTCTTCTACAC	794
	A2ex5R2new	GAAAGTGGGCCTTCACGA	
EX6	A2ex6 F	GCCCCTGTCACTGTGTGCTGT	631
	A2ex6 R	TGGCTGTTCTCCCTGGCACTTTG	
CHRNA4			bp
EX1	CHRNA4EX1-F1	TCCCCGGCTCAGGCACGTC	731
	CRHNA4EX1-R1	GAATGCGGAGGGCTGGGAA	
EX2	CHRNA4EX2-F1	GTGGTGCGGTCAAGGTGCT	806
	CRHNA4EX2-R1	CACCTTCGGAAGCAAAGAA	
EX 3-4	CHRNA4EX3-4F1	CCCGTCCACCATATCTTG	863
	CHRNA4EX3-4-R1	CTATGGCACCTGGACACAG	
EX5	CHRNA4EX5-F1	TGTGGACGTGGGCATGGGT	1558
	CHRNA4EX5-R2	CCCGGCTCCTGGATTACACACC	
	CHRNA4EX5-F2	GGGTGTGGACGTGGGCATG	
	CHRNA4EX5-R1	GGTTCCGTCTGGGTCAGAG	
	CHRNA4EX5-F3	ACGCACACCATGCCCACCT	
	CHRNA4EX5-R3	TACTGGATGCTCCGAGACC	
EX6	CHRNA4EX6-F3	TGGGAGGTGGAGAAGCTGTT	427
	CHRNA4EX6-R2	AAGCAGCTCCACACTCGGTC	

Table 2.3: PCR primers for CHRNA2 and CHRNA4 genes.

PRIMERS designed on reference gDNA sequences			
CHRNB2			bp
EX1	CHRNB2EX1-F1	ACGCACACCCTACCACGGCAGA	523
	CHRNB2EX1-R1	CCACCTTCCGGCCTAGCA	
EX2	CHRNB2EX2-F	TGTAGGGAGATACTGGTTGG	497
	CHRNB2EX2-R	ACTTACGCTTACCTGGGTCA	
EX3	CHRNB2EX3-F	GTGGTGAGTAGAGGTCCCAG	474
	CHRNB2EX3-R	ACTTACGCTTACCTGGGTCA	
EX4	CHRNB2EX4-F	ACTGTACCTTGGCCTAGCTGT	482
	CHRNB2EX4-R	GGCTGTCCACGCTGTACCTCA	
EX5	CHRNB2EX5-F	GAGGAAGGAACGCTTAGGCCAG	1069
	CHRNB2EX5-R	TGGCAGACCCCATATCTCACGC	
	CHRNB2EX5-5CF	CACCATCAACCTCATCATCC	/
	CHRNB2EX5-F2	GTCTTCCTGGAGAAGCTGCC	/
	CHRNB2EX5-5BR	GAAGACAAGGATGGCTAGCG	/
	CHRNB2EX5-R1	GCTTCTCCAGGAAGACGACC	/
	CHRNB2EX5-R2	CCTCGCTCCGCATGTGGT	/
	CHRNB2EX5-R3	CGCGGTTGACGAAGCAC	/
	CHRNB2EX5-5DR	GGTCCCAGCCTGCGGCACT	/
EX6	CHRNB2EX6-F	CGTTTGTCTCCCATCCTGC	264
	CHRNB2EX6-R	TGGCAGGGTGAAAGAGCATG	
CRH			bp
EX1	CRH-F1	ACACCCCTCTCCTGATGCTT	1123
	CRH-R2	TTCTGCCCTTGCTGCCTTAT	
	CRH-R1	GCTGACGCTCTCTTGACA	/
	CRH-F2	GTCACCAAGAGGCGATAA	/
EX2	CRH-F3	CTCCTTGCTGGCTGTGTAA	1075
	CRH-R4	CTTGCTGTGCTAACTGCTC	
	CRH-F4	GCCTGGGGAACCTCAACA	1080
	CRH-R5	CAACCTCAGAGAAGTAGTC	
	CRH-R3	AGTCCTGCTGGTGGCTCT	/
	CRH-F5	TCCGTTTCCAGGTGTTTA	/
	CRH-RT-F	GGGAACCTCAACAAGAGCCC	116
	CRH-RT-R	AACACGCGGAAAAAGTTGGC	

Table 2.4: PCR primers for CHRNB2 and CRH genes.

PRIMERS designed on reference gDNA sequences		
CRH promoter		bp
CRH PROM F2	ATCACAGTGCCTTCAGCC	3600
CRH PROM R1	ACCAATCCACAACTGCTC	
CRH PROM F1	CCTTTCACCCTCCATACT	/
CRH PROM F3	CTGGTGGGAGGTAACTGA	/
CRH PROM F4	CACAGCAAGGACTCAACA	/
CRH PROM F5	CTGTCAACATTTCTCTCC	/
CRH PROM F6	GATAGTATTTGGAGGTGA	/
CRH PROM R2	AAGCAGCAGCCAGCCAGT	/
CRH PROM R3	*CAGTTACCTCCCTCCACCAG*	/
CRH PROM R4	CAGCTTGGATGTCAATCTG	/
CRH PROM R5	CACCTCCAAATACTATC	/

Table 2.5: Primers for the CRH promoter.

2.4 MICROSATELLITES

Microsatellites, or STR (short tandem repeats), are tandem repeats of a short DNA sequence of 1-4 nucleotides in length. They are distributed throughout the genome of eukaryotes and prokaryotes. Caused by slippage mutations which are produced during the DNA replication, microsatellites are highly variable in the number of repeats within and among individual population. Microsatellites are then used as genetic markers for their heterogeneity within a population

Microsatellite loci can be scored by PCR amplification followed by electrophoresis on an automated sequencer to separate alleles which differ in length as a result of differences in the number of repeat units. In the present work, amplified products (Table 2.6) were separated using an automated multicapillary ABI-3100 DNA sequencer and analyzed by Genescan 3.1 software (Applied Biosystems). GeneScan software completely automates the entire process of baselining, peak detection, peak sizing, and size calling of DNA fragments. Using an internal-lane size standard that is co-electrophoresed with each sample, GeneScan software automatically sizes the PCR products and normalizes the differences in electrophoretic mobility between gel lanes or injections. Internal lane size standards are fluorescently-labeled DNA ladders characterized by a uniform spacing of size standard fragments that ensures precise size calling throughout the size-calling range.

In microsatellite analysis, one of the primers used in PCR is fluorescently-labeled with a fluorochrome different from that of the standard.

MICROSATELLITE MARKER ID	FLUORESCENT DYE	FORWARD PRIMER	REVERSE PRIMER	PCR PRODUCT SIZE
D1S199	FAM	GGTGACAGAGTGAGACCCTG	CAAAGACCATGTGCTCCGTA	94-116
D1S207	FAM	CACTTCTCCTTGAATCGCTT	GCAAGTCCTGTTCCAAGTCT	142-170
D1S2868	FAM	AGGTATAATCTGCAATAAAAAACTT	AAAGTAAAACAATATGAAGCCAC	144-154
D1S413	FAM	AGTTTTAAGGAAACCTGCCA	GACAATACTTGAACAGATTGGG	276
D1S238	FAM	CTACCATCTCACTTTCTCCTTCC	TAAGCAAATGACAGAGAGTCATCC	170
D3S1614	NED	CCTCTTCCAAGATATGTGTGA	AAGCCTTGACCCTGAACTTG	150
D4S1534	NED	CCATGTGTGTATTCAGTTTCAGCC	TAGACCAGCCCAAGGTAGAGGAG	169
D3S1263	NED	CTGTTGACCCATTGATACCC	TAAAATCACAGCAGGGGTTC	231-249
D3S1285	NED	ATTTAGAAAACCCATACAGCATGGC	TCTGTTCATCACAGGGGTAGCATC	232-242
D4S1597	NED	GAAATAGTAACAGCAGCATGG	TGACTATGAGGGTAGATGACAG	150
D6S462	HEX	TGGTTCTCTCACTCTCTCAATC	AATCCACTGGAGTTGGGG	144
D5S2115	HEX	GGCACTCATGCTGCACT	GTAAGCCCCTGGCTCCT	251-277
D5S418	HEX	TGTTACTAGAGATGCATAGGGG	GTTTTCAGTTAGAGCATTCTGC	307
D5S428	HEX	CACACATACACACACTCATACAC	GGAGCATTTTAGTAGATATTCACAG	233
D5S630	HEX	CTCTTCGCTCTTCTTTCTCC	TCACTGCTTTACCTTTCAGTG	141

Table 2.6: Microsatellites analyzed in the present work.

2.5 CLONING

Cloning of DNA fragments into vectors was carried out with Quick ligation Kit (New England Biolabs, Ipswich, England) that enables ligation of cohesive end or blunt end DNA fragments. In particular, 50ng of empty linearized vector was combined with a 3-fold molar ratio of insert (10ul total volume), both properly digested with specific enzymes. After the addiction of T4 ligase, the ligation-mix was incubated at 25°C for 5 minutes. Quick ligation products were then used to transform competent cells as described below.

2.6 SITE DIRECTED MUTAGENESIS

In vitro site-directed mutagenesis was performed with Stratagene's QuikChange II XL site-directed mutagenesis kit specifically optimized for large constructs, which allows site-specific mutation in any double-stranded plasmid. The reaction consists in 3 steps: denaturation of DNA template, annealing of mutagenic primers and extension with PfuUltra DNA polymerase. After digestion with DpnI (to eliminate parental methylated

DNA), 2ul of the resulting mix were used for transformation of competent cells.

MUTAGENESIS PRIMERS	
SCN1A-EX6-MUT F	GAAAAGAATATAATTGTGAATTATAATGGTAC
SCN1A-EX6-MUT R	CCATTATAATTCACAATTATATTCTTTTCTATC
SCN1A-EX24 MUT F	AGCCTATACCTCAACCAGGAAAC
SCN1A-EX24 MUT R	TGTTTCCTGGTTGAGGTATAGGC
CRH-MUT-F	GCCGCGGGCGGGTCCCGGG
CRH-MUT-R	CCCGGGACCCGCCCGCGGC

Table 2.7: Primers used in mutagenesis experiments.

2.7 PLASMID CONSTRUCTS AND EXPRESSING VECTORS

In the present work, several mammalian expression vectors were used for transient transfection.

CRH constructs:

An IMAGE full length human CRH cDNA clone (IRAUup969F078D) cloned into pOTB7 plasmid was purchased by Source Bioscience LifeSciences (Nottingham, UK).

Quick Change II XL Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) was used to introduce the CRH mutation (c. 89C>G). The cDNA was completely resequenced after mutagenesis to confirm the presence of the desired mutation and to exclude the introduction of other undesired DNA variations.

Wild type and mutant cDNAs were then subcloned into the pcDNA3.1 vector (Fig. 2.1), previously cut with EcoRI and XhoI. DNA sequencing confirmed the expected sequence of all constructs.

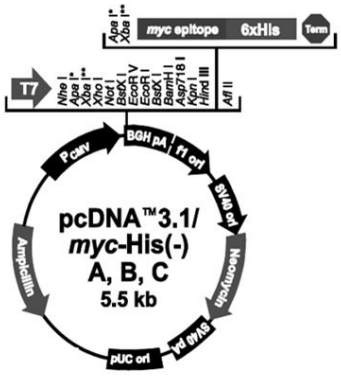


Figure 2.1: pcDNA3.1 map.

SCN1A constructs:

The cDNA of the human Nav1.1 Na^+ channel α subunit (hNav1.1) was provided by Dr. Jeff Clare (GlaxoSmithKline, Stevenage, Herts, U.K.) and encodes the shorter splice variant isoform of 1998 amino acids (Schaller et al., 1992), which could be the predominant Nav1.1 variant expressed in brain (Schaller et al., 1992). hNav1.1 has a high rearrangement rate when propagated in bacteria. We subcloned hNav1.1 cDNA into the plasmid pCDM8 (Mantegazza et al., 2005) because in our experience it was able to stabilize the cDNA of several Na^+ channel α subunits (Mantegazza et al., 2005). We introduced mutations by means of Quick Change XL site directed mutagenesis kit (Stratagene) using pCDM8-hNav1.1 as template. Plasmids containing hNav1.1 were propagated in TOP10/P3 or MC1061/P3 *E.Coli* bacteria (Invitrogen) grown at 28°C for >48h in order to minimize the rearrangements, and the entire coding sequence was sequenced after each propagation.

The cDNA of human voltage gated Na^+ channel β1 subunit was provided by Dr. Al George (Vanderbild University, Nashville, TN); we subcloned the coding sequences of the two subunits into the bicistronic plasmid pIRES-YFP (Clontech) in order to express with the same plasmid both the protein of interest and the fluorescent protein as reporter (Rusconi et al., 2007). Plasmid maps are shown in Figures 2.2, 2.3 and 2.4.

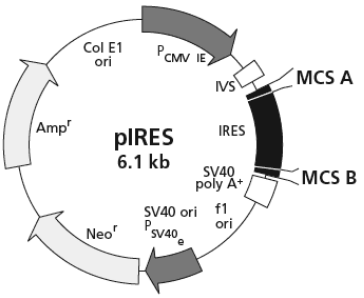


Figure 2.2: pIRES-YFP map.

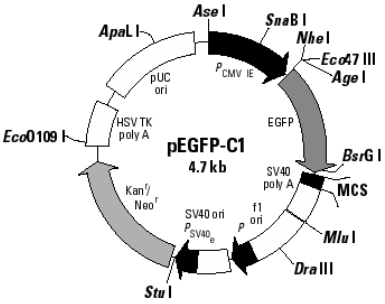


Figure 2.3: pEGFP-C1 which has been used empty in cotransfection with Nav1.1 alone without β-1 subunit.

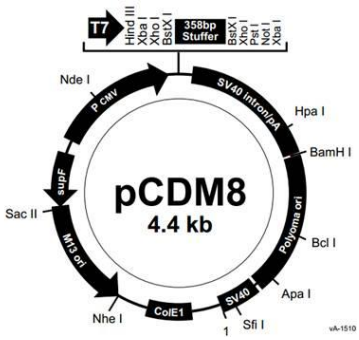


Figure 2.4: pCDM8 map.

2.8 BACTERIAL TRANSFORMATION AND PLASMID RECOVERY

Transformations were performed with different competent cells on the basis of the plasmid to be amplified. Cells used were: JM109 (Promega, Madison, USA), DH5α (NEB, Ipswich, England), Top10/P3 (Life Technologies, Carlsbad, USA). After a transformation using heat-shock standard protocols, cells were seminated on LB-agar plates in the presence of specific antibiotics (Ampicillin: final concentration 100µg/ml; Tetracycline: final concentration 12,5µg/ml, Chloramphenicol: final concentration 27µg/ml) and allowed to grow at 37°C.
Plasmids were purified using the QIAGEN Plasmid Maxiprep kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions and resuspended in water.

2.9 CELL CULTURE AND TRANSFECTION

Two cell lines were used.
Neuro-2a cells: isolated originally from a mouse neuroblastoma, Neuro-2a cells produce large quantities of microtubular protein which is believed to play a role in a contractile system which is responsible for axoplasmic flow in nerve cells. N2a cells could differentiate into a neuron-like morphology, expressing many neural markers. Differentiation is induced using low serum medium (1-2%) and is also confluency-dependent. Cultures were carried out in DMEM containing 10% fetal bovine serum (FBS), 100U/ml penicillin, 100µg/ml streptomycin and 8mM glutamine.

tsA-201 cells: is a transformed human kidney (Hek 293, Sigma catalogue no. 85120602) cell line stably expressing an SV40 temperature-sensitive T antigen. The cell line has been reported to produce high levels of recombinant proteins. Cultures were carried out in DMEM HAM F12 containing 10% Fetal bovine Serum (FBS), 100U/ml Penicillin, 100µg/ml Streptomicyn.
All cell cultures were maintained in 5% CO_2 humidified atmosphere at 37°C (Thermo Scientific, Waltham, MA, USA).

Transient transfections were performed using the XtremeGENE 9 DNA Transfection reagent (Roche, Mannheim, Germany) and TransfectGene Transfection reagent (Immunological Sciences, Rome, Italy) according to the manufacturer's instructions.

XtremeGENE9: cells were plated at a density of 6,5 x 10^5 cells per 94mm plate. Briefly, 5ug of each expression vectors (pCDNA3.1-CRHWt, pCDNA3.1-CRHP30R) were transfected using a 3:1 ratio between X-tremeGENE 9 and DNA. Transfections were performed 24h after plating and all procedures were according to the manufacturer's standard protocol.

TransfectGene: cells were plated at a density of 3 x 10^5 cells per 35mm plate. Briefly, 4µg of each expression vector (pCDM8-SCN1AcDNA-R1525Q, pCDM8-SCN1AcDNA-T297I and pCDM8-SCN1AcDNA-T297I-R1525Q) were cotransfected with pIRES-SCN1B or pEGFP empty, using a 2:1 ratio between TransfecteGene and DNA.

2.10 TOTAL RNA EXTRACTION FROM CULTURED CELLS

Total RNA was extracted from cultured cells by means of the RNeasy mini kit (QIAGEN Hilden, Germany). The RNeasy procedure represents a well-established technology for RNA purification. This technology combines the selective binding properties of a silica-based membrane with the speed of microspin technology. A specialized high-salt buffer system allows up to 100µg of RNA longer than 200 bases to bind to the RNeasy silica membrane. Biological samples are first lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate–containing buffer, which immediately inactivates RNases to ensure purification of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to an RNeasy Mini spin column, where the total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA is then eluted in 30–100µl water.

2.11 cDNA SYNTHESIS AND REAL-TIME QUANTITATIVE PCR

Synthesis of first-strand cDNA was carried out using Quantitec Reverse Transcription kit (QIAGEN Hilden, Germany), using 1μg of total RNA as template. The first-strand cDNA was used as a template for real-time PCR using a human CRH specific primer pair (Fw 5'-GGGAACCTCAACAAGAGCCC-3' and Rv 5'AACACGCGGAAAAAGTTGGC-3') and SYBR Green technology (Applied Biosystem). β-actin was used as housekeeping gene (Fw 5'-CGACAGGATGCAGAAGGAG-3', Rv 5'-ACATCTGCTGGAAGGTGGA-3'). The relative expression levels were calculated with the $2^{-[\Delta C(t)]}$.

2.12 IMMUNOFLUORESCENCE AND CONFOCAL ANALYSIS

Neuro2A cells were plated onto coverslips (2.5×10^4 cells/coverslip) and grown for 24h before transfection. Cells were transfected with X-Treme GENE 9 (Roche Mannheim, Germany) and constructs coding for the wild type or the mutated form of human CRH using a 3:1 ratio. 24 and 48h after transfection, cells were fixed for 20min in 3% (w/v) paraformaldehyde in PBS and quenched for 30min with 50mM NH_4Cl in PBS. Permeabilization was carried out by incubating the cells in the presence of 0.3% (w/v) saponin in PBS (7min for 3 times). Cells were then doubly stained with anti-CRH rabbit polyclonal antibody (Source Biosciences, 1:200). Cells were also incubated with anti-GM130 mouse polyclonal antibody (BD Biosciences, 1:250) for Golgi visualization.

After extensive washes, cells were incubated with Alexa-488 anti-rabbit conjugated antibody (1:200) and Alexa-555 anti-mouse conjugated antibody (1:500). All antibodies were from Invitrogen (Carlsbad, CA, USA). Incubations and washes were carried out at room temperature in PBS, 0.3% saponin. At the end cells were incubated for 15min with the nuclear marker TO-PRO-3 iodide 1:300 (Molecular Probes, Invitrogen UK Ltd Paisley, England). Confocal microscopy was performed using a Leica Mod. TCS-SP2 (Leica Microsystem). Image processing was performed with Leica Confocal Software (LCS) and Adobe Photoshop Software. Confocal microscopy

images were collected under the same conditions in order to compare fluorescence intensities among different images.

2.13 CELL EXTRACTS AND CELL FRACTIONATION

Cell extracts: cells were washed in cold phosphate-buffered-saline solution and then lysed with Sample Buffer 1x (5x: 250mM TrisHCl pH 6,8, 10% SDS, 30% Glycerol, 5% β-mercaptoethanol, 0,02% bromophenol blue). The extracts were passed through a syringe needle and then denatured at 100°C for 5 minutes. Cell extracts were then used in Western blot analysis.

Cell fractionations: subcellular fractionations were carried out using the Subcellular Protein Fractionation Kit for Cultured Cells (Thermo Scientific) according to the supplier's instructions. The kit allows the separation and preparation of cytoplasmic, membrane, nuclear soluble, chromatin-bound and cytoskeletal protein extracts from mammalian cultured cells. The first reagent added to a cell pellet causes selective cell membrane permeabilization, releasing soluble cytoplasmic contents. The second reagent dissolves plasma, mitochondria and ER/Golgi membranes but does not solubilize nuclear membranes. After recovering the intact nuclei by centrifugation, a third reagent yields the soluble nuclear extract. A second nuclear extraction with micrococcal nuclease is performed to release chromatin-bound nuclear proteins. The recovered insoluble pellet is then extracted with the final reagent to isolate cytoskeletal proteins.
Extracts obtained were then used to perform Western blot analysis.

2.14 PROTEASOME INHIBITION

Neuro2a cells were treated with MG132 (Sigma St. Louis, Mo, USA), a potent membrane-permeable proteasome inhibitor. Briefly, Neuro2a cells were cultured in DMEM as described above; 21 hours after transfection with pCDNA3.1-CRH and pCDNA3.1-CRHP30R cells were treated for 3 hours with 20μM MG132. Control cells (WT and MUT) were not treated. Cells

were then washed in a cold phosphate-buffered-saline solution and then lysed as described above. Extracts were then used to perform Western blot analysis.

2.15 SDS PAGE-WESTERN BLOT

SDS-PAGE and Western-blot were carried out by standard procedures. PVDF Immobilon™ P (Millipore Billerica, MA, USA) membranes were blocked for 30min in PBS, containing 5% (w/v) dried milk. Membranes were probed overnight in PBS containing 5% dried milk with anti-CRH rabbit polyclonal antibody (1:800) (Source Biosciences, Nottingham, UK). As a control we used anti-α-Tubulin mouse antibody (1:10000) in TBS-T (50mM Tris-HCl, 150mM NaCl, pH 7.5, 0,1% Tween20) containing 5% dried milk and anti-calnexin rabbit antibody (1:2000) in PBS containing 1% dried milk. Membranes, probed with mouse antibodies, were incubated for 1h with an anti-mouse horseradish peroxidase-conjugated IgG (1:10000) (Cell Signalling Technology, Danvers, MA, USA) in PBS, 0.1% (v/v) Tween20 containing 1% (w/v) dried milk, while membranes probed with rabbit antibodies were incubated for 1 h with an anti-rabbit horseradish peroxidase-conjugated IgG (1:10000) (Cell Signalling Technology) in PBS containing 5% (w/v) dried milk. Detection of antibody binding was carried out with ECL (Amersham GE Healthcare, Uppsala, Sweden), according to the manufacturer's instructions. Protein levels were quantified by densitometry of scanned not saturated X-ray films using the NIH Image-based software Scion Image (Scion Corporation).

2.16 ELISA

Enzyme-linked immunosorbent assay (ELISA) is a method used to detect the presence of an antigen in a sample. We used two methods to confirm our data: indirect ELISA and Sandwich ELISA.

Indirect ELISA: consists in a five steps protocol:

1) well coating with serial dilution of standard antigen (for standard curve) and with the sample for 2 hours at room temperature;
2) after removal of coating solution and three extensive washes with PBS-Tween 0,05%, add blocking buffer (PBS1x-BSA 1%) for 2 hours at room temperature;
3) wash the plate twice (PBS1x-Tween 0,05%) and add diluted primary antibody (rabbit anti-CRH 1:800 in PBS-BSA1%) over-night at 4°C;
4) wash the plate four times (PBS1x-Tween 0,05%) and add diluted secondary antibody (anti-rabbit horseradish peroxidase-conjugated 1:5000 in PBS-BSA1%) for 2 hours at room temperature;
5) wash the plate four times (PBS1x-Tween 0,05%) and add specific substrate (TMB Sigma St. Louis, Mo, USA) for 30min. Stopped the reaction with an equal volume of H_2SO_4 2M and read the plate at 450nm.

Sandwich ELISA: this method measures the amount of antigen between two layers of antibodies. We used a specific Human CRH kit (Sunred Biological Technology, Shangai, China) to detect the amount of CRH in cell culture media. The microtiter plate provided in this kit has been pre-coated with an antibody specific to CRH. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for CRH and Streptavidin conjugated to Horseradish Peroxidase (HRP). After five extensive washing steps, chromogenic solutions are added and incubated for 10 minutes at 37°C. Only those wells that contain CRH, biotin-conjugated antibody and enzyme-conjugated Streptavidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a stop solution. The kit has a sensitivity of 0.327ng/L and a detection range of 0.5ng/L-150ng/L.

The color change for both methods is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of CRH in the samples is then determined by comparing the O.D. of the samples to the standard curve. Our samples were composed by media collected from cultures of cells transfected with the vector expressing either the wt or the mutant CRH.

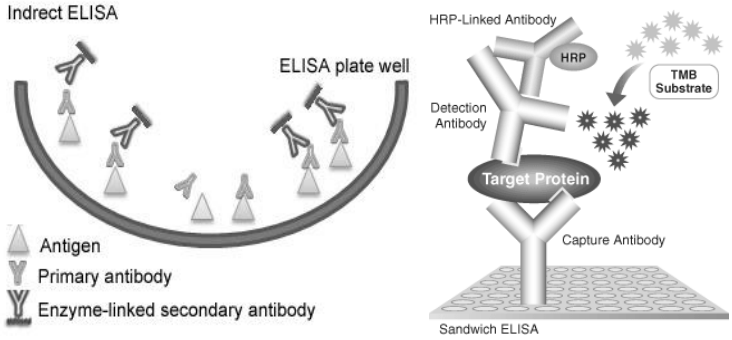


Figure 2.5: ELISA methods. On the left: indirect ELISA; on the right: Sandwich Elisa.

2.17 STATISTICAL ANALYSIS

Statistical analyses were performed by two-way ANOVA with genotype (either mutant or wild type), time and their interaction as predictors. In no case the removal of the non-significant interaction term altered the significance of main terms. We therefore present the results of the full models only. Robustness of the results to possible deviations from the assumptions of ANOVA test was checked by a randomization procedure (unrestricted resampling of observations for the main terms, unrestricted sampling of residuals for the interaction term, 5000 resamples in both cases; see Manly, 1997). Results from the randomization procedure always confirmed those of parametric tests and were therefore not reported for brevity. Post-hoc tests were performed by the Tukey method. All the analyses were performed by R 2.15.1 (R Core Team, 2012).

2.18 BIOINFORMATIC TOOLS:

Detected nucleotide variations were searched in NCBI (http://www.ncbi.nlm.nih.gov/) and Ensembl databases (http://www.ensembl.org/). Prediction analyses of the effects of the detected nucleotide variations were performed with: Polyphen2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.jcvi.org/) and Spliceview (http://zeus2.itb.cnr.it/~webgene/wwwspliceview_ex.html).

Chapter 3:

RESULTS AND DISCUSSION

3.1 NOCTURNAL FRONTAL LOBE EPILEPSY (NFLE/ADNFLE)

3.1.1 SEARCH FOR MUTATIONS IN nACHR SUBUNIT GENES IN A GROUP OF PATIENTS NOT PREVIOUSLY TESTED

Neuronal nicotinic acetylcholine receptor was historically the first protein complex whose mutations were found in NFLE patients. In particular, until now 12 mutations affecting genes coding for different subunits (α2, α4 and β2) have been associated with the pathogenesis of the disease (Ferini-Strambi et al., 2012). However, these mutations account for a minority of patients and the existence of additional loci was demonstrated by our group (Combi et al., 2004).

In a large cohort of patients belonging to our sample, a mutation screening of the three genes encoding these subunits was previously performed and no mutations were detected.

The first aim of the present work was then to complete this study by extending it to all available samples. In particular, the mutational screening was performed for 9 sporadic patients (NFLE) and for the proband of a newly enrolled family showing ADNFLE. The coding region, the exon/intron boundaries and the UTRs of the CHRNA4, CHRNB2 and CHRNA2 genes were directly sequenced on purified PCR-amplified DNA fragments obtained for each patient. The resulting sequences were aligned to reference sequences specific for each gene and analyzed by means of the ChromasPro software. Neither pathological mutations nor polymorphisms were detected in both the CHRNA2 and CHRNB2 genes.

In the CHRNA4 gene, the study allowed the identification of a pathological mutation in one sporadic patient (NFLE2) as well of several silent variants (the latter are listed in Table 3.1). The identified pathological mutation and the relevant case report are described in detail in the following paragraph. Silent variations resulted to be already known and present in the dbSNP database.

CHRNA4 EXON ID	VARIATION	dbSNP ID	GENOTYPE									
			NFLE 1	NFLE 2	NFLE 3	NFLE 4	NFLE 5	NFLE 6	NFLE 7	NFLE 8	NFLE 9	FAM PA
EXON1	c.229 G>A P-Leu17Leu	rs79739740	GG	GG	GA	GA	GG	GG	GG	GG	GG	GG
EXON5	c.639T>C P-Asp213ASp	rs1044393	CC	CC	CC	CC	CC	CC	CC	TC	CC	TC
	c.678T>C P-Cis226Cis	rs1044394	CC	CC	CC	CC	CC	CC	CC	CC	CC	TC
	c.851 C>T p.Ser284Leu	rs28931591	CC	CT	CC	CC	CC	CC	CC	CC	CC	CC
	c.1209 G>T p.Pro403Pro	rs2229959	TT	TT	TT	TT	TT	TT	TT	GT	GT	GT
	c.1227 T>C p.Cis409Cis	rs2229960	CC	CC	CC	CC	CC	CC	CC	CC	CC	TC
	c.1629 C>T p.Ser543Ser	rs1044396	CT	CC	TT	TT	CT	TT	CC	CT	CT	CC
	c.1659G>A p.Ala553Ala	rs1044397	GA	GG	AA	AA	GA	AA	GG	GA	GA	GG

Tab 3.1: Nucleotide variations identified in the CHRNA4 gene. The genotype at each position is indicated for each tested individual.

3.1.2 CHRNA4 P.SER284LEU MUTATION DETECTION

In the NFLE2 sporadic case, a *CHRNA4* c.851C>T mutation (numbering referred to ENST00000370263.4 sequence), resulting in a p.S284L substitution, was detected by sequencing the exon 5 of the gene. The patient was heterozygous for the mutation (dbSNP:rs28931591). The electropherogram showing the region encompassing the mutation is shown below (Fig. 3.1).

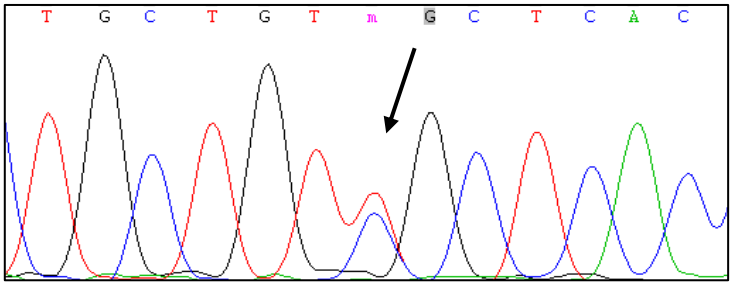


Figure 3.1: Sequence electropherogram of part of the exon5 region of the CHRNA4 gene, showing the identified c.851C>T mutation. The arrow indicates the position of the mutated nucleotide.

To verify the origin of the mutation, blood samples were collected from healthy parents of the NFLE2 case and the region encompassing the mutation was sequenced. The study showed that this variant is absent in both parents (Fig. 3.2). Segregation of highly polymorphic microsatellite markers spread across the human genome was in concordance with

paternity and confirmed that the mutation was originated *de novo* in the patient (Fig. 3.3).

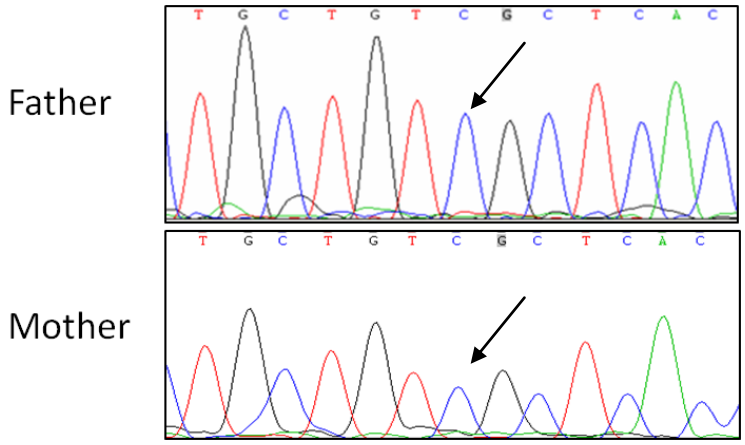


Figure 3.2: Sequence electropherograms of part of the exon5 region of the CHRNA4 gene encompassing the c.851C>T mutation. The top panel shows the sequence of the father, while at bottom the sequence of the mother is shown. Arrows indicate the c.851 nucleotide.

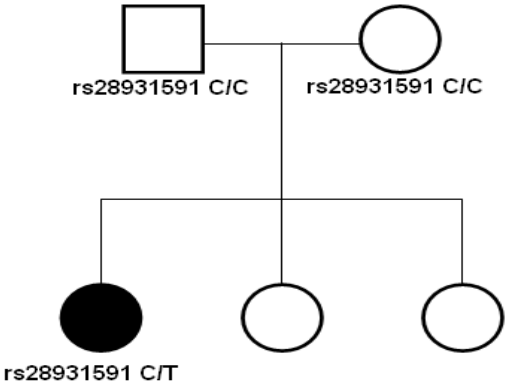


Figure 3.3: Pedigree of the NFLE2 case.

The NFLE2 patient is a right-handed 25-year-old woman of Italian origin and with a negative family history for epilepsy and parasomnias (she was the first of 3 daughters in their twenties and her parents and siblings were clinically unaffected). From age 7 the patient had nocturnal episodes while

asleep characterized by a sudden elevation of the trunk generally associated with dystonic posturing of both arms. She had no recall of the episodes, but was immediately oriented at the end of seizures. Seizures, since the beginning of epilepsy, occurred almost every night and could last from ten to more than sixty seconds. She never complained of subjective manifestations both during sleep and during wakefulness. Seizures did not respond to different drugs in monotherapy or in association. Neurologic examination and MRI were normal. No intellectual disabilities were observed. Video-EEG analysis of the ictal manifestations confirmed the anamnestic description of the episodes and showed the occurrence of ictal frontal rhythmic slow waves, more evident on the right side. The identified mutation was previously reported in three ADNFLE families of different origins (Japanese, Polish and Korean) and in a Lebanese woman initially diagnosed as sporadic case who subsequently had an affected son (Hirose and Kurahashi, 2010; Phillips et al, 2000; Cho et al., 2003). However, the phenotype of the present NFLE case appears slightly different from the one previously reported for p.S284L mutation: in fact, all previous cases showed mental retardation or intellectual disabilities (Phillips et al, 2000; Cho et al., 2003) while a normal intellect and good school performance (second level degree of school) were documented in the present case.

This report confirms that serine 284 in the CHRNA4 is a hot point for *de novo* mutation and adds new data useful in the disease's genetic analysis. Moreover, the study describes the first Italian NFLE case with a CHRNA4 mutation adding new data concerning the opportunity of performing both genetic testing and counseling for sporadic cases. All up to now performed studies by our group on the role of the CHRNA4 gene in NFLE allows to calculate a rate of success in mutations detection of about 3.6% for Italian sporadic cases. Considering that in ADNFLE families, where a genetic basis is certain, mutations have been detected in only 10% of cases, a 3.6% rate in sporadic patients appears to be higher than the one expected. It is worthwhile to note that the patient with the CHRNA4 mutation has a typical form of NFLE and she doesn't show particular distinctive clinical aspects. This suggests that clinical data are generally no helpful in evaluating the usefulness of a genetic test for each single patient.

3.1.3 SEARCH FOR NEW PATHOLOGICAL MUTATIONS IN THE CRH GENE

In the 8q13-q21 locus, two candidate susceptibility variations (g.-1470C.A and g.-1166G.C) affecting the promoter of the CRH gene coding the corticotropin-releasing hormone were detected in a total of seven unrelated NFLE patients (Combi et al., 2005; 2008). These nucleotide variations were reported to co-segregate with the disease and to affect the gene expression, thus suggesting a possible role in the disease pathogenesis (Combi et al., 2005; 2008).

Several studies performed on rats have been reported showing a relation between altered levels of CRH and seizures. In particular, picomolar amounts of CRH induce severe and prolonged seizures within minutes. CRH can increase the frequency of spontaneous excitatory postsynaptic current and in the presence of some level of excitability, CRH can lead to hyperexcitability. Moreover, CRH has a much greater proconvulsant potency during the first period of life compared with during adulthood, and this observation, might explain the juvenile onset of this epilepsy (Combi et al., 2005).

The second aim of the present study was then to search for new mutations in the coding sequence of the gene for a group of patients not previously tested and to extend, for the whole patient cohort with no mutation detected in known genes, the mutational screening to the whole promoter region (which has never been considered in previous works).

Results are reported in the following paragraphs.

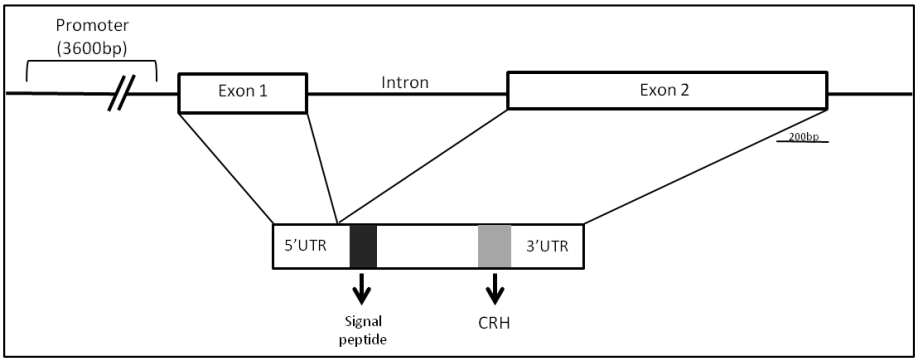


Figure 3.4: Schematic map of the CRH gene and its transcript.

Mutational screening of the CRH coding sequence for a group of patients not previously tested

We performed a mutation screening of the CRH gene in a recently enrolled Italian family showing a typical form of ADNFLE without psychiatric comorbidities (thus excluding an involvement of the KCNT1 gene) and in 9 sporadic patients. All these patients have never been tested for mutation in ADNFLE/NFLE known genes. The analysis allowed the identification of a new missense mutation in the familial case and of a known silent polymorphism (dbSNP: rs6159) in three sporadic cases (Table 3.2).

Exon	VARIATION	dbSNP ID	1	2	3	4	5	6	7	8	9	FAM PA
CRH ex2	c.89C>G p.Pro30Arg	NA	CC	CC	CC	CC	CC	CC	CC	CC	CC	CG
	c.288A>C p.Gly96Gly	**rs6159**	AC	AA	AC	AA	AA	AA	AA	AC	AA	AA

Tab 3.2: Nucleotide variations detected in the CRH coding sequence.

CRH p.PRO30ARG mutation detection

The novel mutation, a C>G transversion at cDNA position 89 (c.89C>G) was found in heterozygosity in the proband of Family PA (Fig. 3.5) and it leads to a non-conservative Pro to Arg change at position 30 (p.Pro30Arg).

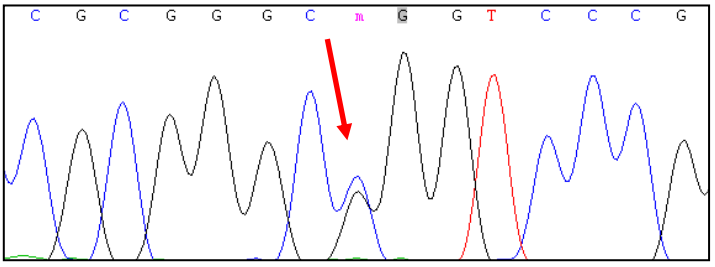


Figure 3.5: Sequence electropherogram of part of the exon2 region of the CRH gene, showing the identified c.89C>G mutation. The arrow indicates the mutation position.

The mutation was not present in 100 ancestry-match control samples and it was also not found in public databases. The aminoacid change in CRH was predicted to be pathogenic (PolyPhen2) being the affected Proline a highly evolutionary conserved aminoacid (see below).
Due to these results, a depth study of the clinical history of the PA family as well as a functional *in vit*ro analysis of the effects of this variant on the cells ability to correctly produce and secrete the CRH was performed. These studies and their results are reported in the following sections.

CLINICAL DATA OF FAMILY PA:

From age 10 the patient had recurrent nocturnal episodes, characterized by a sudden elevation of his head and trunk, frequently associated with bimanual and bipedal motor activity. Episodes occurred every night, more frequently in the first third of the night, lasting from 15 to more than 60s. Sometimes (4-5 / month) after the arousal, he would get out of the bed and start wandering around, jumping and making puppet-like movements with his arms. He had no memory of the episodes. Neurological examination and magnetic resonance imaging (MRI) were normal. Video-polysomnography showed two episodes characterized by an arousal with a sudden elevation of head and trunk and tonic/dystonic posture of the arms: one episode was from stage 2 NREM sleep, and the second one from Slow Wave Sleep. Sleep EEG showed ictal rhythmic slow activity over frontal areas. The patient has been treated by levetiracetam; with the dose of 1,000mg at bedtime, the nocturnal seizures were greatly reduced in frequency and complexity.
The sleep EEG recording of the patient is shown in the Figure 3.6.
The proband's father, now deceased, was affected by Parkinson's disease and REM Sleep Behavior Disorders while the mother was healthy.
The sister of the patient experienced similar nocturnal episodes from age 11: until age 28 the episodes occurred almost every night (2-3 times/night) and afterwards the frequency was reduced (1-2 episodes/month). She had twin daughters one of which affected by spina bifida and carrier of two known MTHFR polymorphisms: the C677T originated *de novo* and the A1298C inherited from the mother (II-3) who is a carrier of this SNP as well as all her brothers (II-1, II-2, II4) and her mother (I-2).

The pedigree of the family is shown below (Fig. 3.7).

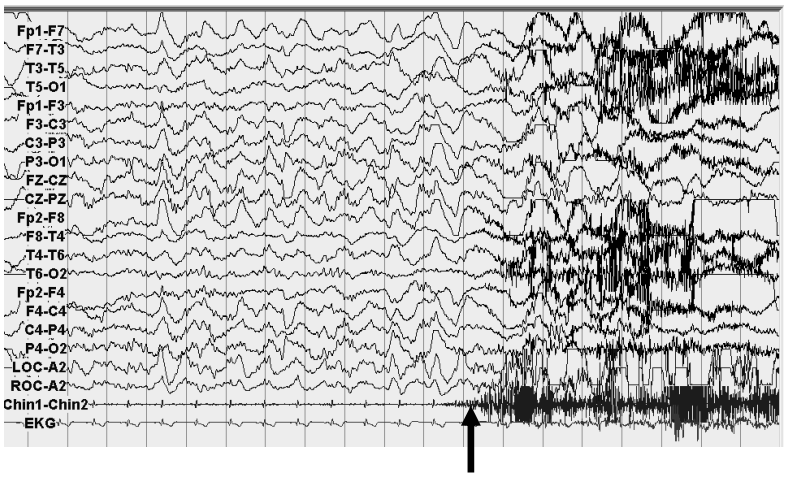


Figure 3.6: Sleep EEG recording for the proband of family PA showing the beginning of the clinical manifestation (indicated by the arrow). In this figure a violent and prolonged attack is shown.

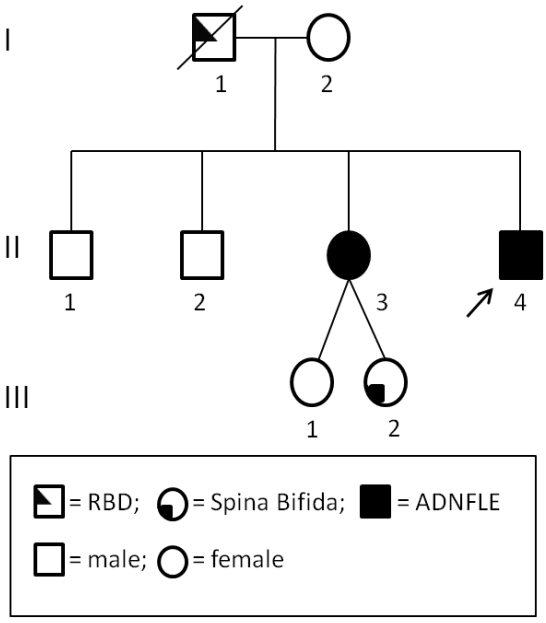


Figure 3.7: Pedigree of family PA. The arrow indicates the proband.

Unfortunately, only the mother (I-2) and the sister affected by ADNFLE (II-3) were collaborative and accepted to participate to the mutational screening of the CRH gene. The p.Pro30Arg mutation was found in the affected sister of the proband in heterozygous state, while it was absent in the healthy mother (Fig. 3.8). This suggests that the mutation was not originated *de novo* in our proband while it was probably inherited from the dead father, who suffered from sleep disorders. There is then a cosegregation of the mutation with ADNFLE and, more generally, with sleep disturbances.

The impossibility to test the two healthy brothers was a limit of the segregation study. However, even if one of them (or even both) would be a carrier of the mutation we would have not been able to exclude the involvement of p.Pro30Arg in the pathogenesis of the disease due to the well known reduced penetrance of the disease.

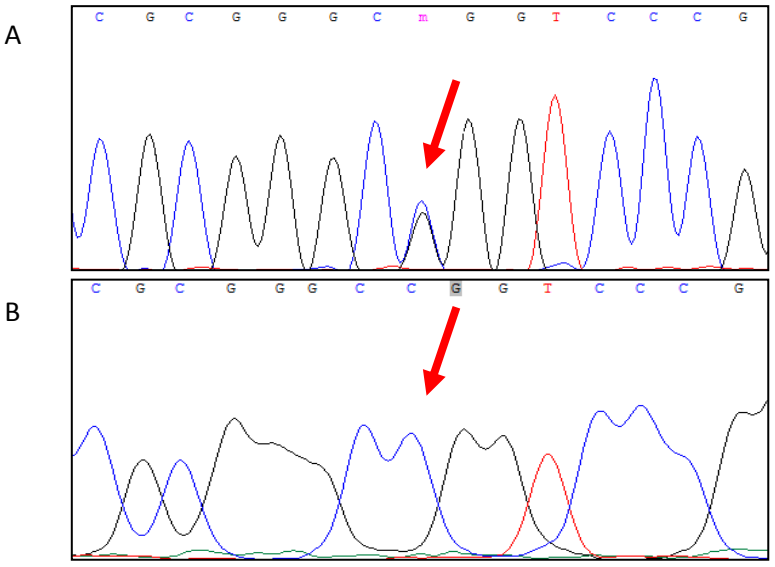


Figure 3.8: Sequence electropherograms of the CRH gene region encompassing the newly identified mutation obtained for relatives of the Family PA proband. A) Electropherogram of the affected sister showing the c.89C>G transversion; B) electropherogram of the healthy mother showing a wild-type sequence. Arrows indicate the position of the mutated nucleotide.

Functional characterization of the novel p.Pro30Arg

The human corticotropin releasing hormone is firstly synthesized as a (h)preproCRH precursor of 21 kDa composed by 196-amino acids that must be correctly processed, during its translocation towards the cell membrane, to release the active peptide. The latter is composed by 41-amino acids corresponding to the C-terminal region of the precursor and in particular to the region between the 153 and the 194 amino acids (Brar et al., 1997). The N-terminal region of the (h)preproCRH contains the signal peptide which is composed by the first 26 amino acids. This pre-sequence is cut directly in the rough endoplasmic reticulum giving rise to the CRH pro-hormone (from aminoacid 27 to 196). A successive endoproteolytic processing of pro-CRH within the trans-Golgi-network and secretory granules leads to the release of the mature hormone.

The identified mutation is located at position 30, therefore without the signal peptide but it resides in a well conserved domain of the pro-sequence region (Fig. 3.9). Moreover, the mutation doesn't affect the two known cleavage sites (CS1 at position 124-125 and CS2 at position 151-152) (Ahmed et al., 2000).

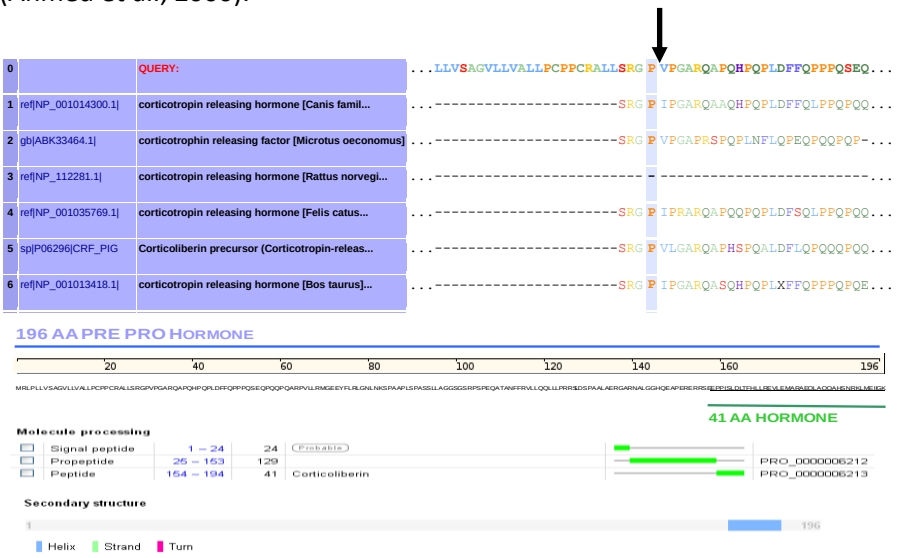


Figure 3.9: Structural features of the CRH protein. Conserved aminoacids (the arrow indicates Proline at position 30) as well as secondary structures are shown.

Owing to the mutation location, we performed an in silico analysis using several bioinformatic tools aimed at predicting its effect on the functionality of the protein. In particular, we firstly used two equivalent tools: Polyphen2 (http://genetics.bwh.harvard.edu/pph2/) and SIFT (http://sift.jcvi.org/www/SIFT_chr_coords_submit.html). The pPro30Arg was predicted to be probably damaging by both algorithms. In Figure 3.10 is reported, as an example, the result of the prediction performed by means of Polyphen2.

Query

Acc number	Position	AA_1	AA_2	Description
P06850	30	P	R	RecName: Full=Corticoliberin; AltName: Full=Corticotropin-releasing hormone; AltName: Full=Corticotropin-releasing factor; Short=CRF; Flags: Precursor; LENGTH: 196 AA

Prediction

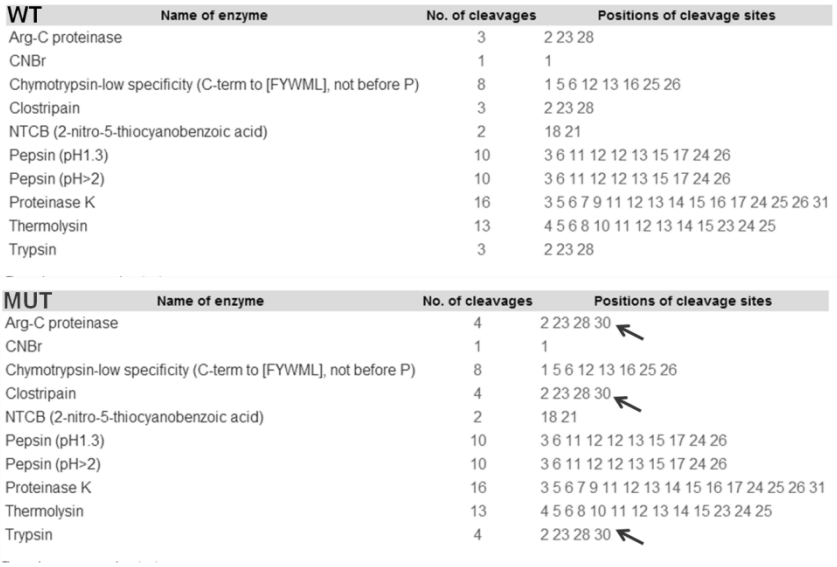
This variant is predicted to be probably damaging

Prediction	Available data	Prediction basis	Substitution effect	Prediction data
probably damaging	FT alignment	alignment	N/A	PSIC score difference: 2.325
Remarks				
Substitution in proven PROPEP region				

Figure 3.10: Polyphen2 prediction of the effect of the p.Pro30Arg mutation on the protein functionality.

Moreover, to verify the possibility that the mutation could introduce new cleavage site in the pro-sequence region, we used the PeptideCutter tool (http://web.expasy.org/peptide_cutter/) which predicts the potential cleavage sites in a protein sequence. Results showed that this substitution introduces three new putative cleavage sites for different preoteases (Arg-C Proteinase, Clostripain, Trypsin), as shown in the Figure 3.11.

All these data suggested a possible pathological role for the identified mutation, thus we decided to carry out an *in vitro* functional analysis to verify this hypothesis. Several experiments were then performed. The detailed experimental plan and results obtained in each step of the study are described in following paragraphs.



WT

Name of enzyme	No. of cleavages	Positions of cleavage sites
Arg-C proteinase	3	2 23 28
CNBr	1	1
Chymotrypsin-low specificity (C-term to [FYWML], not before P)	8	1 5 6 12 13 16 25 26
Clostripain	3	2 23 28
NTCB (2-nitro-5-thiocyanobenzoic acid)	2	18 21
Pepsin (pH1.3)	10	3 6 11 12 12 13 15 17 24 26
Pepsin (pH>2)	10	3 6 11 12 12 13 15 17 24 26
Proteinase K	16	3 5 6 7 9 11 12 13 14 15 16 17 24 25 26 31
Thermolysin	13	4 5 6 8 10 11 12 13 14 15 23 24 25
Trypsin	3	2 23 28

MUT

Name of enzyme	No. of cleavages	Positions of cleavage sites
Arg-C proteinase	4	2 23 28 30
CNBr	1	1
Chymotrypsin-low specificity (C-term to [FYWML], not before P)	8	1 5 6 12 13 16 25 26
Clostripain	4	2 23 28 30
NTCB (2-nitro-5-thiocyanobenzoic acid)	2	18 21
Pepsin (pH1.3)	10	3 6 11 12 12 13 15 17 24 26
Pepsin (pH>2)	10	3 6 11 12 12 13 15 17 24 26
Proteinase K	16	3 5 6 7 9 11 12 13 14 15 16 17 24 25 26 31
Thermolysin	13	4 5 6 8 10 11 12 13 14 15 23 24 25
Trypsin	4	2 23 28 30

Figure 3.11: Prediction of the putative cleavage sites of the (h)preproCRH in the presence (top panel) or absence (bottom panel) of the p.Pro30Arg mutation obtained using PeptideCutter.

EXPRESSION OF WILD-TYPE AND P.PRO30ARG CRH PRECURSOR IN NEURO2A CELLS

To evaluate the effect of the identified missense mutation in the production and secretion of CRH, Neuro2A cells, which express only a basal level of endogenous CRH and which are reported to be able to correctly process the prohormone to the mature protein (Brar et al., 1997), were transiently transfected separately with the wild-type and the mutant plasmids (see Material and Methods chapter for detailed information on the constructs). Cells lysates were prepared 24h after transfection and the CRH precursor content was measured by SDS-Page Western blot and densitometry. Alpha-tubulin was used as a loading control. Results are shown in Figure 3.12.
These experiments indicated a lower intracellular protein level in cells transfected with plasmid containing the mutated cDNA as compared to that measured in cells expressing the wild-type form.

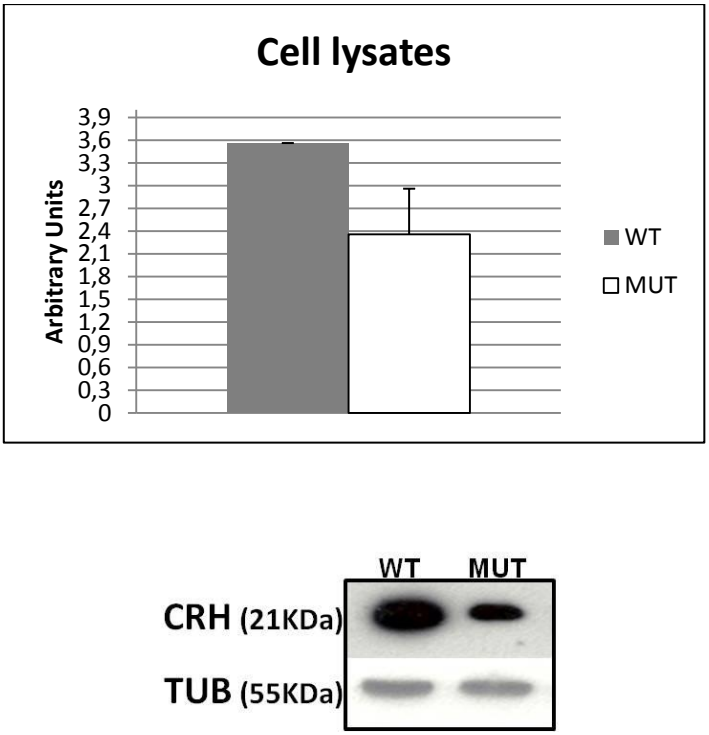


Figure 3.12: Analysis of the ability to express CRH in Neuro2A cells transiently transfected with wild-type or mutant construct for preproCRH. Top: Densitometric analysis of CRH immunoreactive proteins in cell lisates of the Neuro2A cells. Each data point represents the mean ± S.E.M. (n=3) and protein content is expressed in arbitrary units. Bottom: Western-Blot image of one experiment as an example.

To verify that this reduction in mutant protein levels was not related to an altered gene expression, we performed a quantitative real time PCR to measure transcript levels. In particular, the total mRNA was extracted from cultured cells transfected either with the wild-type or the mutant construct at 24, 48 and 72 hours after transfection and then each sample was retro-transcribed and PCR-amplified. The β-actin was used as housekeeping gene. Results are shown in Figure 3.13.

No significant differences in gene expression were detected between cells expressing CRH-WT and cells expressing CRH-p.Pro30Arg. An obvious decrease in expression levels could be seen after 24h in all samples owing to the fact that the performed transfections were transient. These results

were expected due to the location of the here reported mutation which maps in a region not involved in gene expression regulation.

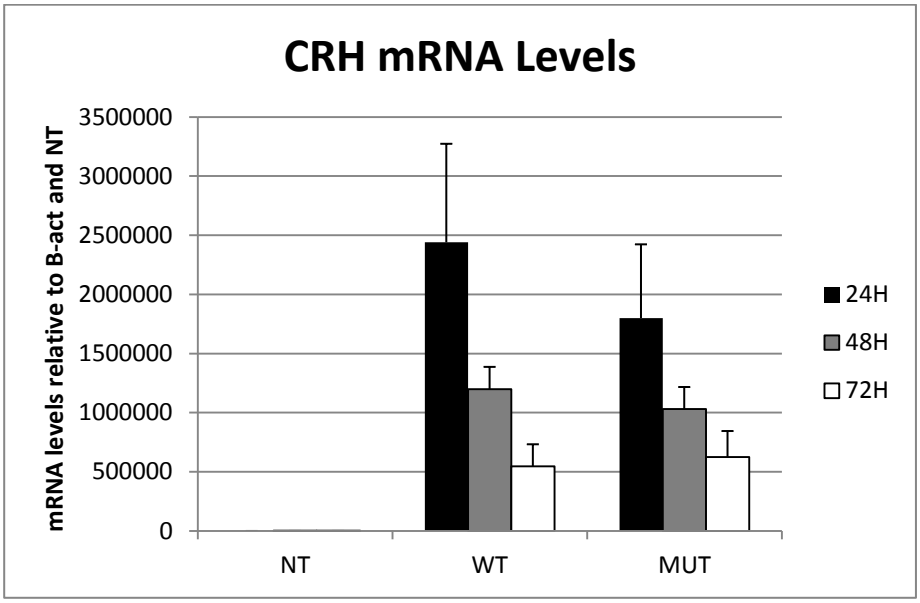
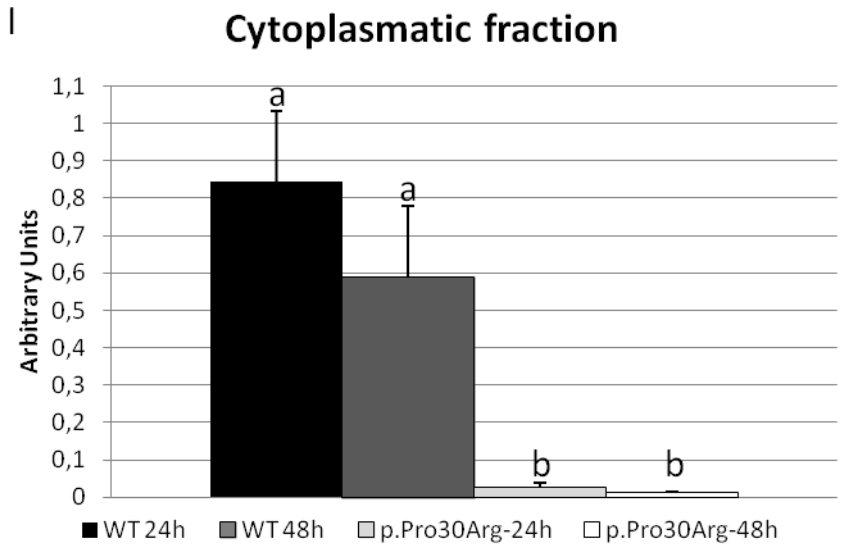


Figure 3.13: CRH levels of expression detected by realtime quantitative PCR in not transfected (NT) or transfected cells at three different times: 24h, 48h and 72h. Each data point represents the mean ± S.E.M. (n=3) of mRNA levels normalized to the basal CRH expression in Neuro2A cells (NT values) and to a housekeeping control gene (β-Actin).

SUBCELLULAR FRACTIONATION IN NEURO2A CELLS

To test if the reduction in protein level was generally distributed overall the cell or related to a particular subcellular location, CRH precursor contents in extracts from cytoplasm, membrane, nuclei and cytoskeleton fractions were measured 24h and 48h after transfection. Cells fractionation was performed as described in the Material and Methods chapter and protein levels were assessed by means of Western blot and densitometric analysis. Each experiment was replicated three times. Results are reported in Figures 3.14, 3.15, 3.16.



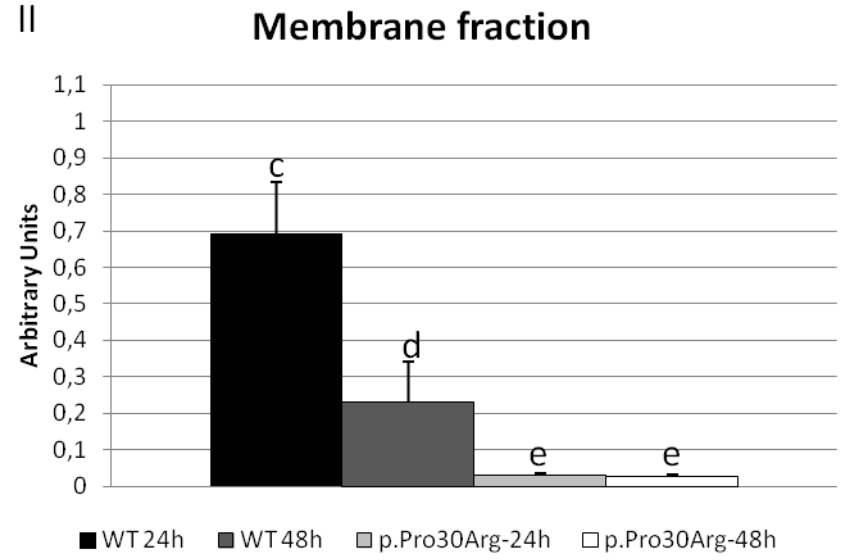
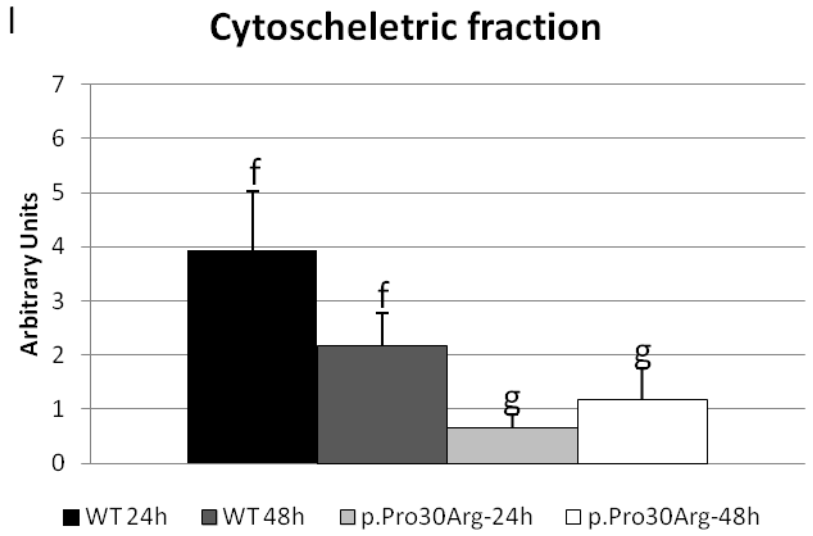


Figure 3.14: Densitometric analysis of CRH immunoreactive proteins in cytoplasmatic and in membrane subcellular fraction of the Neuro2A cells. Each data point represents the mean ± S.E.M. (n=3) and protein content is expressed in arbitrary units. Bars with different letters indicate significant differences. (I): a≠b p=0,033. (II): c≠d p=0,020; c≠e p=0,002.



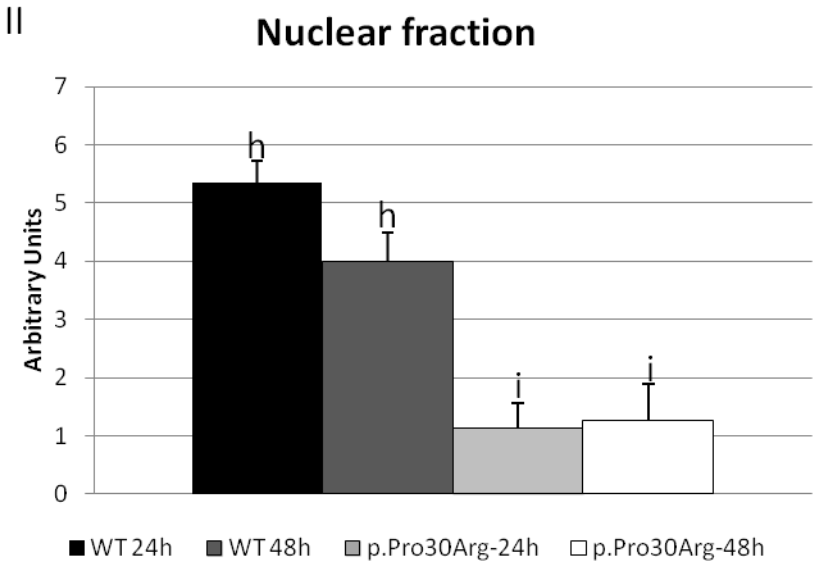


Figure 3.15: Densitometric analysis of CRH immunoreactive proteins in nuclear and cytoscheletric subcellular fractions of the Neuro2A cells. Each data point represents the mean ± S.E.M. (n=3) and protein content is expressed in arbitrary units. Bars with different letters indicate significant differences. (I): f≠g p=0,014. (II): h≠i p<0,001.

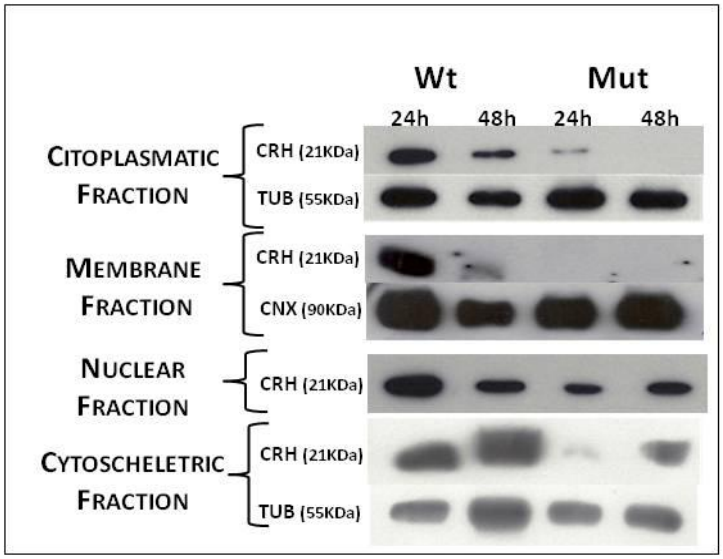


Figure 3.16: Western blot image of one fractionation experiment: for each subcellular fraction both the CRH precursor's and the control's bands are shown.

Briefly, statistical analyses showed a significantly lower level of CRH-precursor in extracts from all above mentioned fractions of cells transfected with the mutant construct in respect to the wild-type, independently of time ($F_{1,8} \geq 6.646$, $P \leq 0.033$). Moreover, cells expressing the mutant or the wild-type form showed different patterns of variation between 24h and 48h in the protein levels of the membrane fraction (effect of the genotype by time interaction: $F_{1,8} = 6.618$, $P = 0.033$). In particular, post-hoc tests indicated that cells expressing the wild-type CRH precursor had significantly higher protein levels than those expressing the mutant form in the membrane fraction 24h after transfection, while their level decreased significantly between 24h and 48h, when it did not differ significantly from that of the mutant form.
In more details:

-*Cytoplasmatic fraction*: cells expressing the wild-type had significantly higher CRH-precursor levels than those expressing the mutant form ($F_{1,8}=6.646$, $P=0.033$), independently of time, while the effect of time or of

the time by genotype interaction on protein levels was not significant ($F_{1,8}$=0.248, P=0.632 and $F_{1,8}$=0.193, P=0.672, respectively). Randomization tests confirmed these results.

-*Membrane fraction:* CRH-precursor levels significantly differed between genotypes ($F_{1,8}$=23.874, P=0.001) and times ($F_{1,8}$=6.898, P=0.030). In addition, cells expressing the mutant and the wild-type form showed different variation in protein levels between 24h and 48h (effect of the genotype by time interaction: $F_{1,8}$=6.618, P=0.033). Randomization tests confirmed these results. Post-hoc tests (Tukey methods) showed that at 24h cells expressing the wild-type CRH precursor had significantly higher protein levels than those expressing the mutant form (t=5,274, P=0.002), while this was not true at 48h (t=1.636, P=0.360). Moreover, protein levels for cells expressing the mutant construct did not change significantly between 24h and 48h (t=-0.038, P>0.999), while they decreased significantly for cells expressing the wild-type construct (t=-3.676, P=0.020);

-*Cytoscheletric fraction:* cells expressing the wild-type had significantly higher CRH-precursor levels than those expressing the mutant form ($F_{1,8}$=9.683, P=0.014), while the effects of time or of the time by genotype interaction were not significant ($F_{1,8}$=0.788, P=0.401 and $F_{1,8}$=2.715, P=0.138, respectively). Randomization tests confirmed these results.

-*Nuclear fraction* (also including the nuclear envelope): cells expressing the wild-type had significantly higher CRH-precursor levels than those expressing the mutant form ($F_{1,8}$=48.837, P<0.001), while the effects of time or of the time by genotype interaction were not significant ($F_{1,8}$=1.523, P=0.252 and $F_{1,8}$=2.180, P=0.178, respectively). Randomization tests confirmed these results.

All results so far reported in our functional *in vitro* analysis of the p.Pro30Arg highlighted its possible role in altering the ability of the cell to promptly produce the mature hormone.
To explain the reduction in protein levels among the two different genotypes, two possible hypotheses could be put forward: the process of

translation on ribosomes of the mutant mRNA is impaired or the mutant protein is somehow degraded more than the wild-type form. The first hypothesis appears to be less convincing owing to the fact that the mutation is not at the 5' end of the mRNA and is located far from the translation starting codon. Conversely, the *in silico* analysis of the mutation effects argued in favor of the second hypothesis owing to the fact that the mutation resulted to introduce new putative cleavage sites. Moreover, the half-life of the CRH precursor is very brief thus we could postulate that the mutant protein could be not promptly processed in the rough endoplasmic reticulum and in Golgi apparatus and this delay could result in a higher level of protein degradation. This delay in post-translational modifications in the presence of the p.Pro30Arg could be related to the identified difference in the membrane fraction's patterns of protein levels: cells expressing the wild-type protein are able to produce and secrete the CRH more quickly than those expressing the mutant form.

PROTEASOME INHIBITION IN NEURO2A CELLS EXPRESSING WILD-TYPE AND P.PRO30ARG CRH PRECURSOR

Owing to the absence in literature of studies concerning the intracellular degradation of the CRH precursor, we decided to test if the reduced protein levels were caused by a proteasome-mediated degradation. We then used a potent proteasome-inhibitor (MG132) to treat our transfected cells. Results of the Western blot and densitometric analysis are shown in Figure 3.17.

Results showed that the MG132 treatment caused a drastic decrease of the CRH-precursor level in cells expressing CRH-WT, while this effect was reduced in cells expressing CRH-p.Pro30Arg.

By the way, our data demonstrated that CRH-mutant precursor (as well as the wild-type) was not degraded by the proteasome, because the treatment with a potent inhibitor caused a significant decrease in protein levels and not the expect increase.

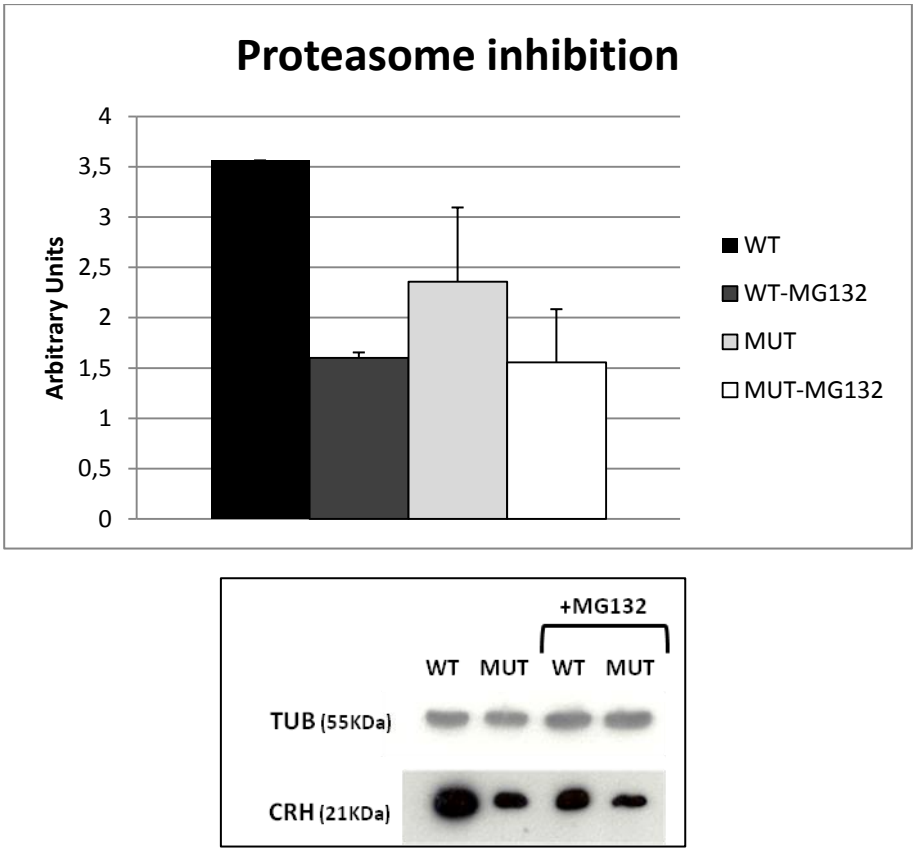


Figure 3.17: Top: Densitometric analysis of CRH immunoreactive proteins in cellular lysates of the Neuro2A cells. Each data point represents the mean ± S.E.M. (n=3) and protein content is expressed in arbitrary units. Bottom: Western blot image of one experiment. Both the CRH precursor's and the control's bands are shown. WT: cells expressing the wild-type CRH-precursor and not treated; MUT: cells expressing the mutant CRH-precursor and not treated; WT-MG132: cells expressing the wild-type CRH-precursor and treated with MG132 (20µM 3h); MUT-MG132: cells expressing the mutant CRH-precursor and treated with MG132 (20µM 3h).

SUBCELLULAR LOCALIZATION BY FLUORESCENCE AND CONFOCAL MICROSCOPY

Due to our hypothesis of a delayed processing of the CRH precursor in the presence of p.Pro30Arg mutation, we performed immunofluorescence experiments to investigate a possible increase in colocalization of the mutant protein with the Golgi apparatus in respect to that observed in cell expressing the wild-type protein. Cells were transiently transfected with the

mutant or the wild-type construct and the experiments were performed (as described in the Materials and Methods chapter) at 48h from the transfection. The choice of performing the experiment at 48h was to allow the possible formation of protein deposits in the Golgi. Results are shown In Figure 3.18.

A difference in CRH intracellular distribution was observed. In particular, a higher co-localization with the Golgi apparatus was observed in cells expressing the CRH-p.Pro30Arg-precursor protein (yellow spots in the merge panel) compared to those expressing the wild-type protein. The immunofluorescence experiments allowed assessing a transfection efficiency of approximately 30% for both constructs.

These results were consistent with our hypothesis of a difficulty to promptly process the mutant CRH precursor during its translocation towards the membrane and, in particular, during its permanence in the Golgi apparatus.

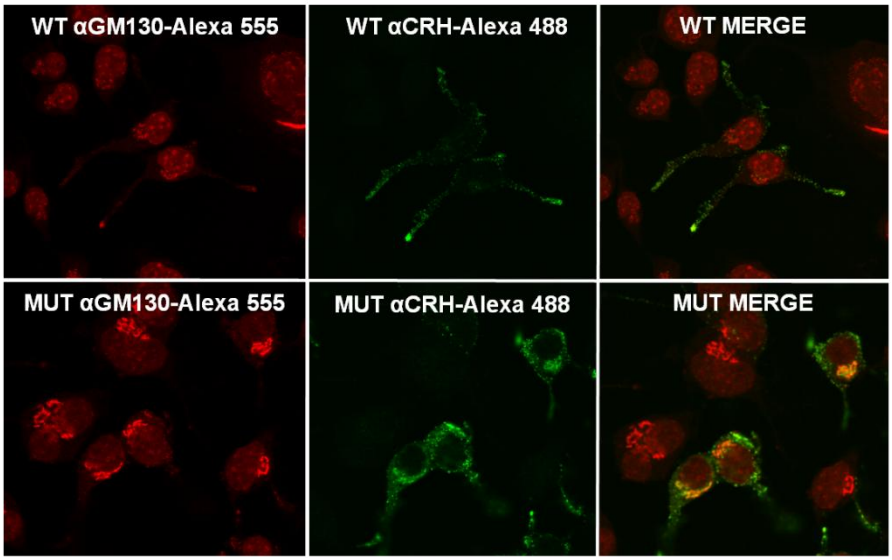


Figure 3.18: Subcellular localization of CRH detected by fluorescence and confocal microscopy. CRH primary antibody is linked to the secondary antibody Alexa-488 (green), while anti-Golgi antibody is linked to Alexa-555 (red).

SECRETION OF CRH IN CELL CULTURE MEDIUM

All up to now reported results suggested an impairment in the ability of cells expressing a CRH-mutant precursor to correctly transfer to the cell membrane a properly processed hormone, thus causing a delay in protein secretion as well as a probably reduction in the amount of secreted protein (the latter due to the high degradation). We then decided to evaluate levels of CRH released in the culture medium by cells expressing the two different proteins (the wild-type and the mutant).

The CRH protein level in the culture medium was evaluated by ELISA at 24h and 48h after transfection. The ELISA analysis was performed using two different and independent methods (see Material and Methods chapter). Both methods allowed drawing the same conclusions.

Results of one of these experiments are shown in Figure 3.19 as an example.

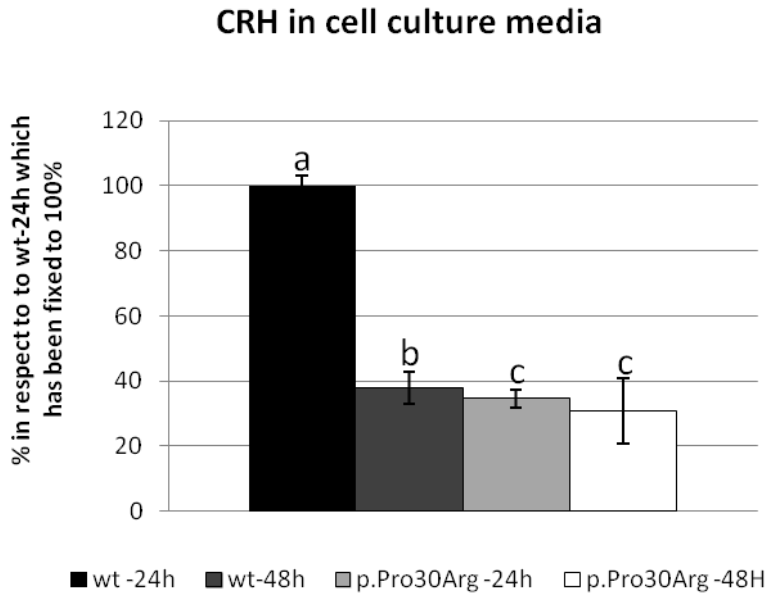


Figure 3.19: Levels of secreted CRH protein measured by indirect ELISA. The ability of cells to secrete the CRH hormone was evaluated by measuring the protein level in cultured media of cells transfected either with the wild-type or the mutant construct at 24h or 48h after the transfection. Each data point represents the mean ± S.E.M. (n=2) and protein content is expressed as % in respect to the mean percentage value of wt 24h which has been fixed as 100%. Bars with different letters indicate significant differences: a≠b p=0,005; a≠c p=0,004.

A significant difference in protein levels between cells transfected with the two construct were observed only at 24h. In particular, at that time protein levels resulted to be significantly lower in media of cells transfected with the CRH-p.Pro30Arg construct with an observed reduction of about 70% ($F_{1,4}$=37.391, P=0.004). A huge reduction (about 60%) in the amount of released CRH at 48h compared to that measured at 24h was observed for cells expressing the CRH-Wt (t=7.403, P=0.005). This difference in secretion levels at different times was instead not observed for cells expressing the CRH-p.Pro30Arg (t=0.459, P=0.954).

These results demonstrated that levels of secreted CRH were significantly lower for cells expressing the mutant CRH at 24h after the transfection while an apparent recovery could be seen at 48h when no significant differences were measured among cells expressing the two different forms. A possible explanation of this recovery could be that, while the wild-type protein is mainly secreted at 24h, only a reduced amount of the mutant protein (which is less abundant in the cell and "blocked" in the Golgi apparatus) is able to be processed and released rapidly. Instead, in the presence of the mutation there is a delayed in this process, thus the majority of the mutant protein is secreted later (in our experiments at 48h), when we could measure the sum of both the delayed mutant protein produced in the first day after the transfection and the protein produced and released in the second day. This addiction effect masks the intrinsic differences in secretion levels of the two population of cells transfected with different plasmids. It is worthwhile to note that the released mature hormone is the same in the two cases in respect to the protein structure. The mutation resides outside the C-terminal domain that produces the mature CRH, thus the incorrect amino acid is intracellularly removed.

In conclusion, overall the reported results suggest an impairment in the ability to promptly produce and release the hormone in the presence of the p.Pro30Arg mutation in the pro-sequence. This impairment, which is however partially mitigated in our patients by the fact that the mutation was always found in heterozygosity, could be related to a altered capability of patients to respond quickly to stress agents and this would result in an impaired HPA axis cascade as well as an impairment in the CRH-mediated sleep/arousal cycle regulation.

Although a functional effect of the mutation was demonstrated by our results, a direct role of the p.Pro30Arg in NFLE pathogenesis has still to be proved. This could be done only by the identification of new ADNFLE families with the mutation cosegregating with the disease or by the development and study of specific transgenic mouse models.

Mutational screening of the CRH promoter in the whole patient cohort.

To increase our knowledge on the role of the CRH promoter in the pathogenesis of the disease, we sequenced the known promoter region of the CRH gene in all patients (both familial and sporadic cases) where previously performed mutational analysis excluded the involvement of the nAChRs genes.

The known CRH promoter is a 3600 bp long region and it contains several response elements which are especially located between the -600bp and -25bp positions. In particular, in this region the followings regulatory elements were reported: MTF1RE (metal response element-binding transcription factor), HRE (hormone response element), EcRE (ecdysone regulatory element), nGRE (a negative glucocorticoid response element), YY1RE (ying yang 1 response element that was reported to have no obvious effect unless the other elements are not functioning), CRE (cAMP response element that directly mediates the response to cAMP but its action is influenced by interactions with the other previously mentioned elements), CDXARE (caudal type homeobox protein response element), GRR (glucocorticoid responsive region) and the TATA box. The CDXA and the -213 to -99 bp glucocorticoid responsive region (GRR) appear to act as second cAMP response elements (King and Nicholson, 2007).

The sequencing study in patients affected by nocturnal frontal lobe epilepsy allowed the identification of several known polymorphisms as well as of 3 unknown variants (Table 3.3).

Additional studies were performed to assess the role of the three unknown variants in the pathogenesis of the disease. Studies and results for each variant are reported below.

Position	Nucleotide Variation	Sample		homozygosis	Heterozygosis	dbSNP ID
		Sporadic	Familial			
g.- 3531	C>G	2	7	3	6	Rs 5030877
g.- 3509	C>A	1	1	0	2	Rs 7839698
g.- 3371	T>G	1	3	0	4	Rs 5030875
g.- 3203	delT	1	4	0	5	NA
g.- 2353	T>C	2	7	2	7	Rs 5030876
g.- 2264	A>G	1	3	0	4	Rs 7818110
g.- 1693	T>G	1	1	0	2	NA
g.- 1485	G>A	2	4	2	4	Rs 6999780
g.- 1296	C>T	3	0	0	3	NA
g.- 684	T>C	2	7	3	6	Rs 3176921

Tab 3.3: Nucleotide variations identified in the CRH promoter. For all detected variations, the number of sporadic and familial cases where they were found as well as the homo/heterozygous state are reported.

CRH g.- 3203 delT nucleotide variant

The variant was detected in heterozygosis in four familial and one sporadic cases. Among them, one patient belongs to a compliant ADNFLE family (Fig. 3.20). This family was analyzed to test the presence of cosegregation among the variant and the phenotype. The study allowed to exclude an involvement of this new variant in ADNFLE owing to the fact that the mother (II-16), who is the parent from which the proband inherited the disease, did not showed the variant.

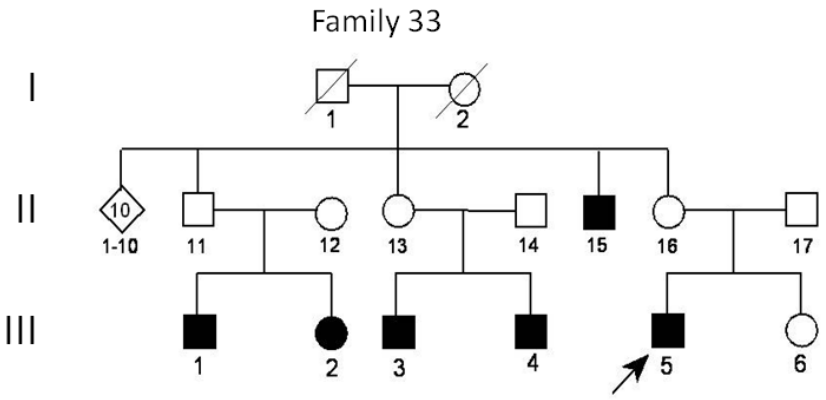


Figure 3.20: Pedigree of Family 33. The arrow indicates the proband resulted to be heterozygous for the g.-3203 delT variant.

CRH G.- 1693 T>G NUCLEOTIDE VARIANT

This SNP was found in one familial and one sporadic cases, both heterozygous for the variation. To verify its role in the pathogenesis of the disease, a segregation analysis was performed for the familial case. The pedigree of the relevant family and results of the analysis are shown in Figure 3.21. In particular, the variation was detected in the CRH promoter region of the affected mother while it was absent in the healthy brother. This suggested a possible role of the nucleotide variant in the disease.
Owing to this possible association and to the fact that the variant was not previously reported in literature and thus no data on its population frequency were available, the promoter of 115 healthy controls was sequenced, allowing an estimation of the mutated allele frequency of 5%. Control individuals were selected by means of an absent clinical history for the more common diseases and, in particular, for epilepsy. All individuals were adult and the sex ratio was 1:1. The mutated allele frequency observed in the patients (2.32%) was near a half than the one calculated in the control sample. This finding excluded a role of the variant both in the pathogenesis and in the individual susceptibility to NFLE.

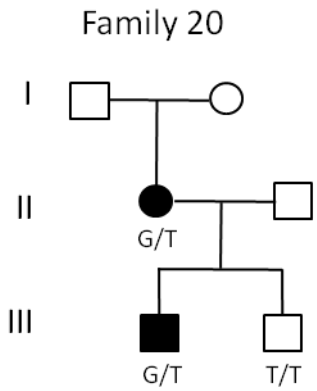


Figure 3.21 : Pedigree and results of the segregation analysis of the g.- 1693 T>G SNP in Family 20. The arrow indicates the family proband.

CRH G.- 1296 C>T NUCLEOTIDE VARIANT

The variant was found only in a sporadic case therefore it was not possible to perform a segregation analysis. To evaluate the allele frequencies, the promoter of 115 healthy controls was sequenced, allowing an estimation of the mutated allele frequency of 2.69% compared with an allele frequency in affected individuals of 3.5%. This difference resulted not statistically significant thus excluding a role of this variant in the pathogenesis of the disease.

3.1.4 STUDY OF GENES ENCODING THE OREXIN SYSTEM AS CANDIDATE FOR ADNFLE IN A GROUP OF PATIENTS WITHOUT MUTATIONS IN ALL KNOWN GENES

Due to the fact the several patients resulted negative to all mutational screening of known ADNFLE genes, we searched for new candidate genes. This part of the project was performed in collaboration with several European groups working on the genetic bases of this epilepsy. By a literature survey we identified as candidate genes those encoding proteins of the orexin system. Literature data are briefly here reported.

The orexin/hypocretin and the cholinergic systems work in parallel in the context of arousal induction from sleep, as a specific activation of both these systems precedes arousal. Orexin neurons may activate the cholinergic cells of tegmental mesopontine nuclei responsible for arousal-generating EEG desynchronization (Kilduff et al., 2000; Burlet et al., 2002). They were shown, like cholinergic neurons, to discharge before the onset of cortical EEG activation concomitant to the transition from sleep to waking (Lee et al., 2005). Orexin has one specific physiological role: it anticipates the return or the increase of muscular activity; the co-release of acetylcholine and orexin thus allows arousal from sleep with concomitant cortical activation and the presence of postural muscle tone. In contrast to acetylcholine, which is involved in the transition from non-REM sleep to either waking or to REM sleep, orexin is actively involved only in the transition from non-REM sleep to waking - when released, it prevents the transition to REM sleep. Loss-of-function defects of the orexin system may induce narcolepsy, with episodes of loss of muscle tonus and inability to move for a few tens of seconds at the time of awakenings from sleep (Lee

et al., 2005). However, while mutations in the orexin receptor and peptide have been found to induce narcolepsy in animal models, only one mutation in the gene encoding orexin has been identified in a single patient with early onset narcolepsy (Peyron et al,. 2000). The frequent decrease in orexin levels in the cerebrospinal fluid (CSF) of the patients demonstrates a decreased orexin neurotransmission, and an HLA-associated autoimmune-mediated destruction of orexin-containing neurons in the lateral hypothalamus has been hypothesized (Nishino et al., 2000).

We then postulated that some forms of ADNFLE could constitute the clinical counterpart of narcolepsy being caused by gain-of-function anomalies of the orexin system.

We analyzed 21 probands came from different European family searching for variants in three genes of the orexin system: *HCRT, HCRTR1* and *HCRTR2*. The single preproorexin gene (*HCRT*) encodes the two orexin peptides, orexin-A and orexin-B, which bind to two receptors encoded by the *HCRTR1* and *HCRTR2* genes. All exons (*HCRT*: 2 exons; *HCRTR1*: 7 exons; *HCRTR2*: 7 exons) were amplified by PCR from genomic DNA, by standard techniques, and sequenced on both strands.

No potentially pathological variants were identified in all three genes (Table 3.4). Known benign polymorphisms of *HCRTR1* and *HCRTR2* were identified, at frequencies similar to that of the general population.

Although the absence of detectable mutations in the three tested genes in 21 patients does not formally exclude an involvement of the orexin system in the pathophysiology of ADNFLE, it does make it improbable. Further investigation consisting in measures of orexin in the CSF of ADNFLE patients could support the absence of involvement of the orexin system in ADNFLE.

Patient ID	*HCRTR1*				*HCRTR2*		*HCRT*
Genetic variant	c.111C>T p.R37R rs1056526	c.652G>A p.G167S -	c.780C>T p.R260R rs76500934	c.1222A>G p.I408V rs2271933	c.922A>G p.I308V rs2653349	c.942A>G p.A314A rs41403545	All
Population Frequencies	0.347/0.653	0.995/0.005	NA	0.292/0.708	0.117/0.883	NA	-
A62 24365	T+T	=	=	G+G	G+G	=	=
A64 24366	C+T	=	=	=	A+G	=	=
A65 24367	C+T	=	=	G+G	A+G	=	=
A72 24368	C+T	=	=	A+G	G+G	=	=
A77 24369	T+T	=	=	G+G	G+G	=	=
A78 24370	=	=	=	=	G+G	=	=
A88 24371	C+T	=	=	A+G	A+G	=	=
A89 24372	T+T	=	=	G+G	G+G	A+G	=
M 24374	=	=	=	=	A+G	=	=
CIII.2 24375	=	=	=	=	G+G	=	=
I 24376	=	=	=	=	G+G	=	=
K 24377	C+T	=	=	A+G	A+G	=	=
013-016-24862	=	=	=	=	A+G	=	=
8 24379	C+T	=	=	A+G	G+G	=	=
60 24380	C+T	=	=	A+G	A+G	=	=
222 24381	C+T	=	C+T	A+G	G+G	=	=
A8 24382	=	=	=	=	G+G	=	=
Y4 24383	T+T	G+A	=	G+G	A+G	=	=
R06 24384	T+T	=	=	A+G	G+G	=	=
N3 24385	T+T	=	=	A+G	G+G	=	=
D1 24386	=	=	=	=	A+G	=	=

Tab 3.4: Nucleotide variations identified in the orexin genes.

3.2 FEBRILE SEIZURES (FS) AND GENETIC EPILEPSY WITH FEBRILE SEIZURE PLUS (GEFS+)

Previously reported studies on the genetic basis of genetic epilepsy with febrile seizures plus (GEFS+) demonstrated that mutations in genes encoding voltage-gated sodium channels are the most common cause. In particular, the NaV1.1 channel (encoded by the SCN1A gene) is the most frequent target of mutation. Only recently an involvement of this gene has been suggested also for febrile seizures (FS), a common disease of the pediatric age that sometimes persists after six years old. In the latter case the patient FS phenotype becomes a GEFS+ phenotype. We decided to evaluate the role of the SCN1A gene in a group of FS patients. During the study two patients reached the six years old age and where then classified as GEFS+.

3.2.1 SAMPLE COMPOSITION

The sample was composed by 2 sporadic FS cases and 7 familial cases selected from a large cohort of epileptic children. The 7 probands were all originally affected by FS but two became GEFS+ during the study. Probands' families showed two or more members affected by different forms of both generalized and focal idiopathic epilepsies. Pedigrees of families are shown in Figure 3.22.

Patients' neuroradiological study (CT scan or T1W, T2 W, T2 FLAIR MRI) as well as neurological examination and psychomotor development were normal in all cases.

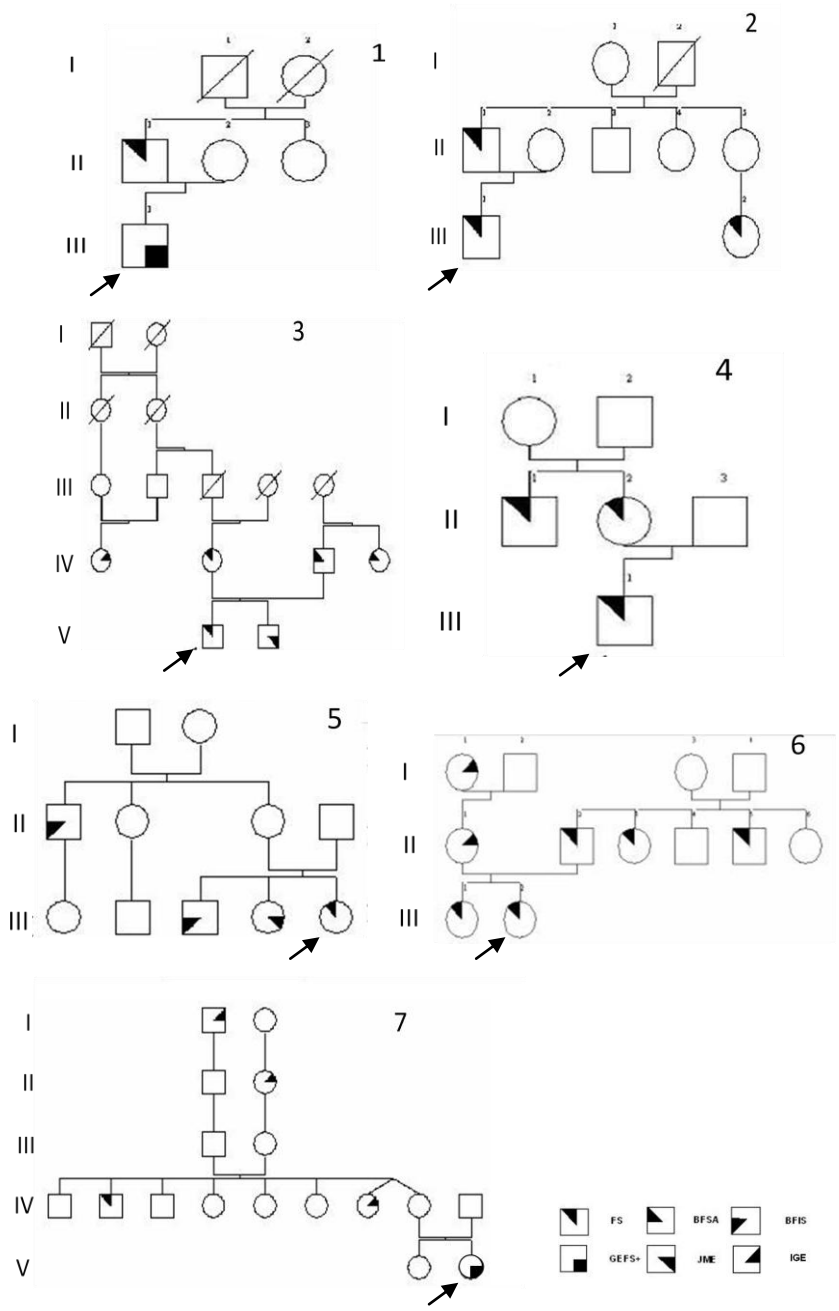


Figure 3.22: Pedigrees of families in which the molecular analysis was performed. GEFS+: genetic epilepsy with febrile seizures plus; FS: febrile seizures; IGE: idiopathic generalized epilepsy: JME: juvenile myoclonic epilepsy; BFSA: benign focal seizures of adolescence; BFIS: benign familial infantile seizures.

3.2.2 SCN1A GENE SEQUENCING AND MUTATION ANALYSIS

The gene was sequenced searching for mutations in the coding portion and in the exon / intron boundaries. We restricted our analyses to this part of the SCN1A gene because mutations located outside the coding region and associated with an epileptic phenotype have never been found.

The SCN1A gene (81Kb) is mapped on the long arm of chromosome 2 (2q24.3). The coding region of this gene is divided into 25 very small exons followed by a final large exon (exon 26) which covers about 30% of the whole cDNA (Fig.3.23).

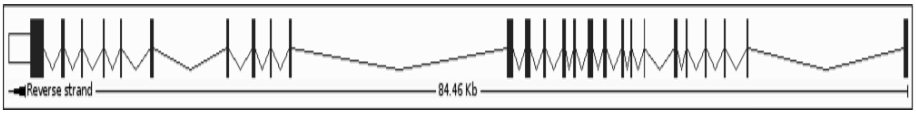


Figure 3.23: SCN1A gene structure

The mutational screening allowed the identification of several known variations as well as a number of unknown nucleotidic changes (Table 3.5).

With regard to the newly identified nucleotide variations, three out of four were located in intronic regions and were detected by the sequencing of intron/exon boundaries of the relevant exonic sequence. An in silico analysis, performed by means of online software (i.e. SpliceView and HMMGene), revealed that these variations do not introduce or remove any splicing sites thus we did not perform additional study on them. The remaining new variant was located in an exonic region. We decided to study in deep this new variant as well as the already known missense mutations and the 5'UTR variation which were detected in our patients.
Results of the depth study are reported in the following sections.

5'UTR Variant:

In one proband (Family 4, patient III-1) we identified a 5'UTR polymorphism at position c.-84C>G, recently reported in one SMEI patient (Depienne et al., 2009). A segregation analysis of this variant in the relevant family was performed demonstrating that the variant did not cosegregate with the disease being absent in the affected uncle (Figure 3.24).

			PROBANDS								
LOCALI-ZATION	cDNA POSITION/AA VARIATION	DBSNP ID	F. 1 III-1 GEFS+	F.2 III-1 FS	F.3 V-1 FS	F.4 III-1 FS	F.5 III-5 FS	F.6 III-2 FS	F.7 V-2 GEFS+	SPO-1 FS	SPO-2 FS
5'UTR	c.-84C>G	NA (Depienne et al. 2009)	CC	CC	CC	CG	CC	CC	CC	CC	CC
Intr 4	c.603-106T>G	rs3812719	GG	GG	GG	GG	GG	TT	GG	TT	GG
	c.603-91G>A	rs3812718	GG	GG	AA	GG	GG	AA	GG	AA	GG
EX6	c.890C>T p.Thr297Ile	rs121918771	CC	CC	CC	CC	CC	CC	CT	CC	CC
Intr 6	c.965-21C>T	rs994399	CC	CC	CC	CC	CC	CC	CC	TT	CT
	c.965-113A>T	NA	AA	AA	AA	AA	AA	AA	AA	AA	TT
Intr 7	c.1028+21T>C	rs1542484	TT	TT	TT	TT	TT	TT	TT	CC	CC
	c.1029-68T>C	rs1461193	CC	TT	TT	CC	TT	TT	TT	TT	CC
Intr 8	c.1170+75C>A	rs11690962	AA	CC		CC	CC	CA	CA	AA	CC
	c.1170+112C>T	rs11690959	TT	CC	TT	CC	TT	TT	TT	TT	CC
EX9	c.1212A>G p.Val404Val	rs7580482	GG	GG	GG	GG	GG	GG	GG	GG	AG
Intr 9	c.1377+52G>A	rs6432861	AA	GA	AA	GG	AA	AA	GA	GG	GG
	IVS10-61delC	NA	/	/	/	/	/	/	/	delC	/
Intr 10	c.1663-47G>T	rs6753355	GG	GG	GG	TT	GG	GG	GT	GG	GT
EX13	c.2292T>C p.Val764Vak	rs6432860	CC	CC	CC	TT	CC	CC	CC	CC	CT
Intr 13	c.2416-37A>C	rs2126152	CC	CC	CC	AA	CC	CC	CA	AA	AC
	c.2416-72G>A	rs490317	GG	GG	GG	AA	GG	GG	GA	GG	GG
Intr 15	c.2947-41T>C	rs7601520	TT	TT	TT	CC	TT	TT	TC	TT	TT
	IVS15+56A>G	NA	AA	AA	AA	AA	AA	AA	AA	GG	AA
EX16	c.3199A>G p.Thr1067Ala	rs2298771	AA	AA	AA	GG	AA	AA	AG	AA	AG
EX24	c.4574G>A p.Arg1525Gln	NA	GG	GG	GG	GG	GG	GG	GA	GG	GG

Tab 3.5: Variations detected by sequencing the SCN1A gene.

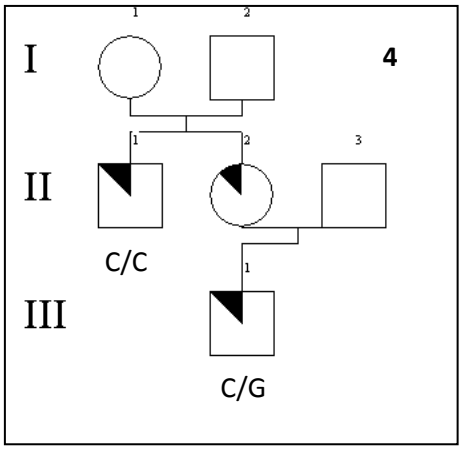


Figure 3.24: Segregation analysis of the c.-84C>G variant.

Exonic Variants

We identified 3 nucleotide variations resulting in an aminoacid change: p.Thr297Ile (exon 6), p.Thr1067Ala (exon 16) and p.Arg1525Gln (exon 24).

The p.T1067A was an already known missense mutation (Wallace et al., 2001), with a reported allelic frequency of 29,5% in GEFS+ patients and of 60% in healthy individuals. Despite the aminoacidic substitution, this mutation was classified as a benign polymorphism (Wallace et al., 2001).

The other missense mutations, one of which previously unknown, were both identified in a heterozygous state in the same proband (Family 7, proband V-2).

SCN1A-P.THR297ILE

This missense mutation, identified in a GEFS+ patient (originally enrolled as FS)(Figure 3.25), was previously reported in SMEI (Severe Myoclonic Epilepsy of Infancy) patients (Nabbout et al., 2003; Kanai et al., 2004; Mancardi et al., 2006).
A segregation analysis was performed within the family, and results are shown in Figure 3.26. In particular, a cosegregation with the disease was confirmed owing to the fact that the mutant allele was present in all compliant patients (IV-2 FS, IV-7 IGE) and in two healthy individuals (III-2 and IV-8) who however were obligate carriers. The mutation penetrance was therefore incomplete.

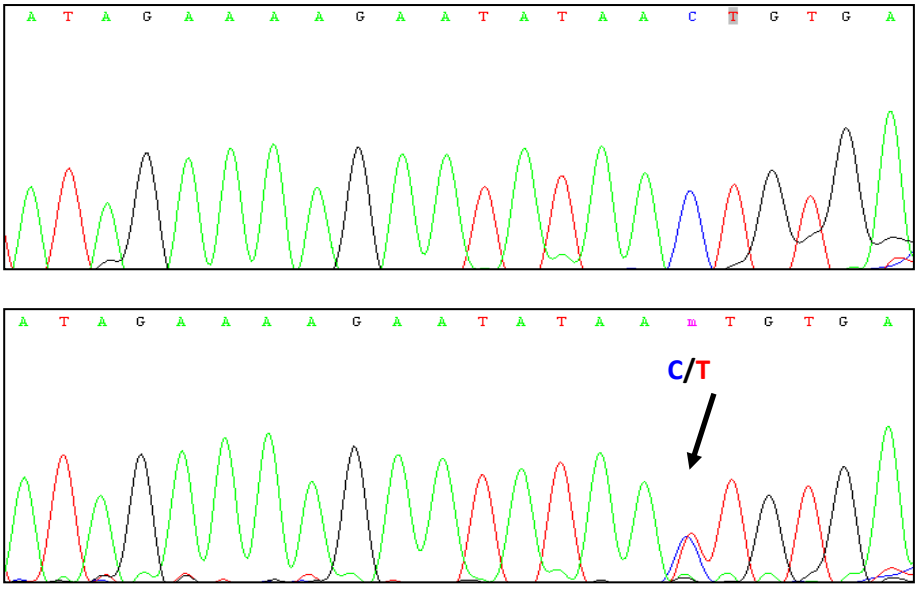


Figure 3.25: Sequence electropherograms of the exon6 region of the SCN1A gene encompassing the c.890C>T variation. The relevant wild-type sequence (top part of the figure) also is shown. The arrow indicates the position of the mutated nucleotide.

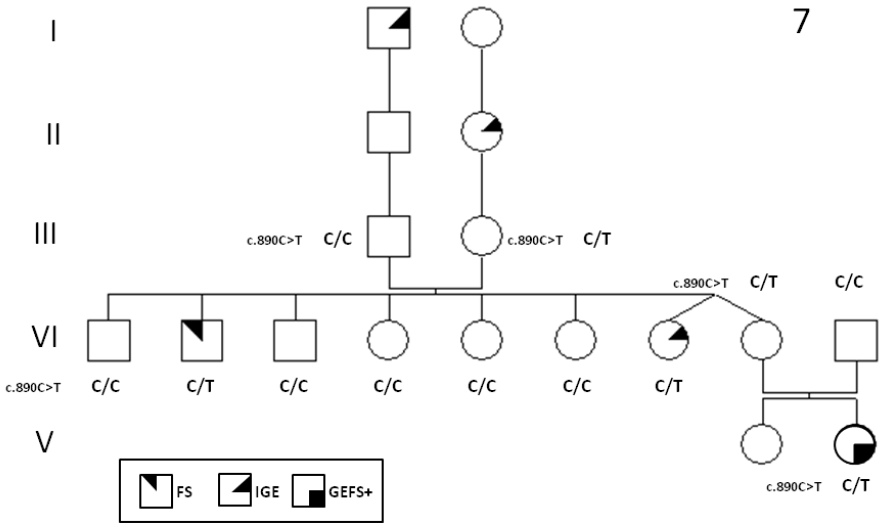


Figure 3.26: Segregation analysis of the c.890C>T mutation in Family 7.

The Nav1.1 protein is composed of four homologous domains (D1-D4), each containing six transmembrane segments (S1 through S6). The p.Thr297Ile is located in the linker between S5 and S6 segments of the first domain (D1), as shown in Figure 3.27.

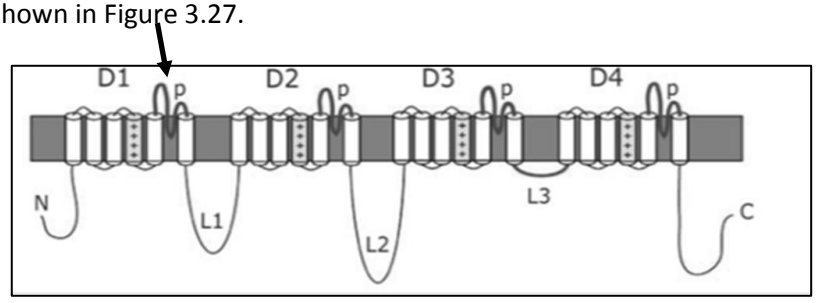


Figure 3.27: Nav1.1 protein structure and localization (arrow) of the p.Thr297Ile mutation.

The four homologous domains are pseudosymmetrically arranged around a central pore whose structural constituents determine the selectivity and conductance properties of the channel. Especially, S5 and S6 segments and the S5-S6 linker constitute the functional pore. These segments are also considered to play important roles in ion selectivity and gating kinetics. In general, mutations in the pore regions may produce more severe channel

dysfunctions, including activation/inactivation dysfunctions, abnormal voltage dependency or altered ion selectivity (Kanai et al., 2004).
Since this mutation has never been studied from a functional point of view, we performed a bioinformatic analysis using Polyphen-2, in order to predict its possible effect on the channel functionality (Fig. 3.28).

Query

Acc number	Position	AA_1	AA_2	Description
P35498	297	T	I	RecName: Full=Sodium channel protein type 1 subunit alpha; AltName: Full=Sodium channel protein type I subunit alpha; AltName: Full=Voltage-gated sodium channel subunit alpha Nav1.1; AltName: Full=Sodium channel protein brain I subunit alpha; LENGTH: 2009 AA

Prediction

This variant is predicted to be benign

Prediction	Available data	Prediction basis	Substitution effect	Prediction data
benign	alignment	alignment	N/A	PSIC score difference: 1.350

Details

SEQUENCE FEATURES OF THE SUBSTITUTION SITE

Region	Site	Feature table	Critical sites
N/A	N/A	show FT fields for P35498	N/A

PSIC PROFILE SCORES FOR TWO AMINO ACID VARIANTS

Score1	Score2	|Score1-Score2|	Observations	Diagnostics	Multiple alignment around substitution position
+1.125	-0.225	**1.350**	1	precomputed	Sequences: all Flanks: 25 Show alignment

MAPPING OF THE SUBSTITUTION SITE TO KNOWN PROTEIN 3D STRUCTURES

Database	Initial number of structures	Number of structures
PQS	24	0

Figure 3.28: Results of p.Thr297Ile analysis with Polyphen-2.

This variant is predicted to be benign, but given that this mutation seems to cosegregate with the disease and that it was previously reported in a severe epileptic phenotype, we are now performing in vitro functional studies to assess its effect on the physiological properties of the Nav1.1 channel.

SCN1A-P.ARG1525GLN

This missense mutation, identified in heterozygosity in patient V-2 (Family 7), was unknown and resulted in an Arginine to Glutamine change at protein position 1525.
Electropherograms of the exon 24 region encompassing the mutation are shown in Figure 3.29.

A segregation analysis was performed within the family and, as for the p.Thr297Ile mutation, this variation was detected in all compliant patients (IV-2 FS, IV-7 IGE) and in the two previously mentioned obligate carriers (III-2 and IV-8) (Fig. 3.30).

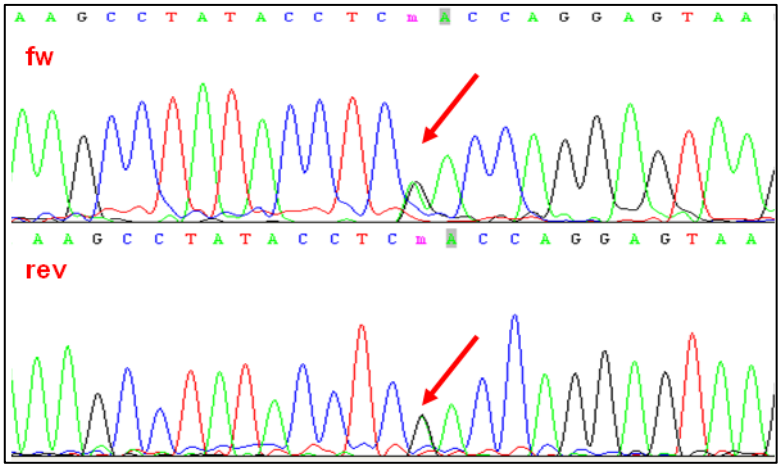


Figure 3.29: Electropherograms of the exon 24 region encompassing the c.4574G>A variation. The electropherograms of both strands are shown.

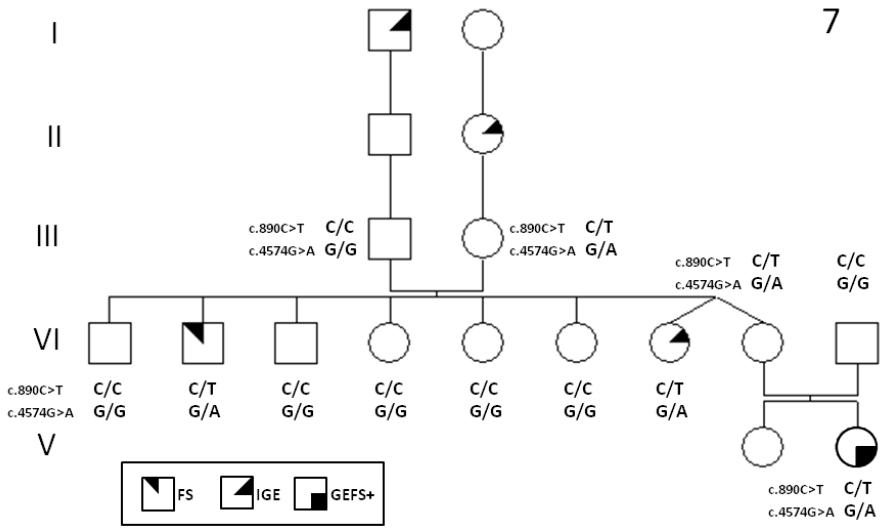


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Figure 3.30: Pedigree of Family 7 in which we performed segregation analysis of the two variants identified: c.890 C>T which leads to p.Thr297Ile aminoacidic change and c.4574G>A which leads to p.Arg1525Gln aminoacidic change.

The c.4574G>A variation was located in the L3 linker which connects the S6 segment of Domain3 to the S1segment of Domain4 (Figure 3.31). This short intracellular loop is very important because constitutes the inactivation gate (Kanai et al., 2004).

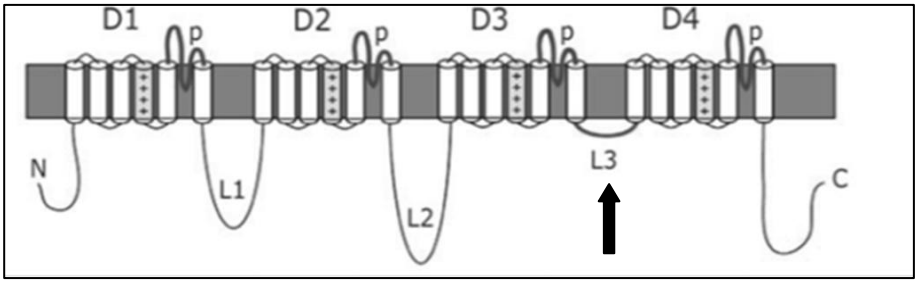


Figure 3.31: Nav1.1 protein structure and localization (red arrow) of the p.Arg1525Gln mutation.

Sodium channels exist in 3 states. In Figure 3.32 the activation/inactivation states of the Nav1.1 are shown.

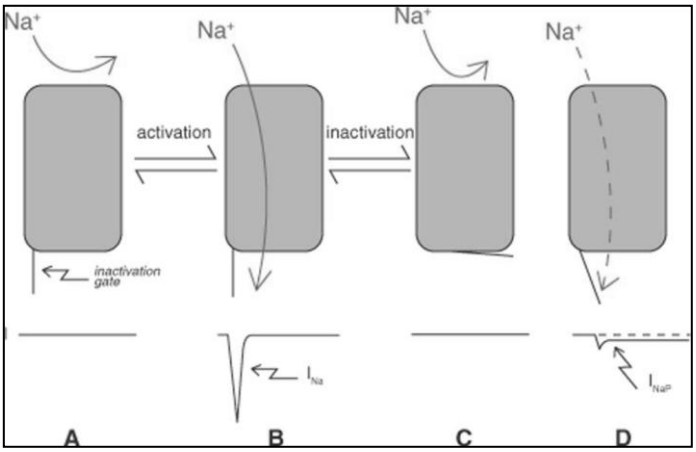
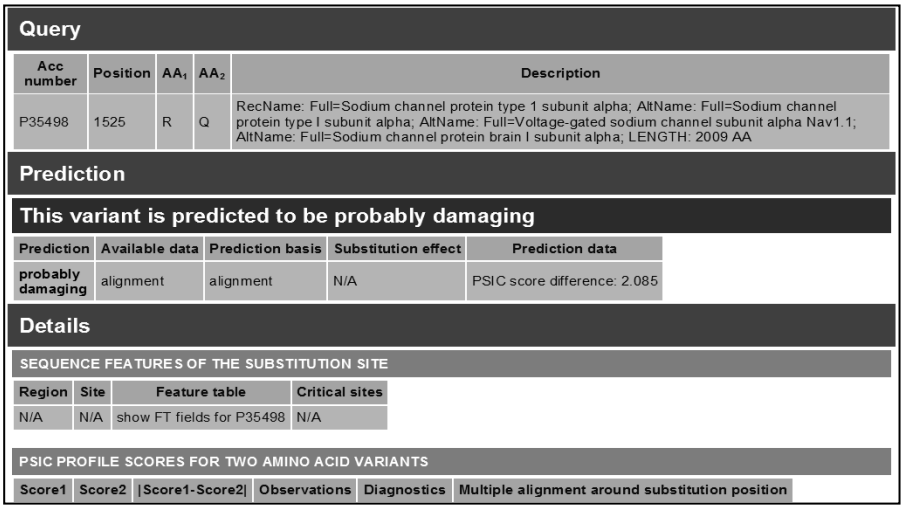


Figure 3.32: Schematic diagram of the activation states of voltage-gated sodium channels (Stafstrom, 2009).

When the channel is closed (Fig. 3.32 A), no sodium ions (Na^+) can pass through the channel and no sodium current (INa) flows. When the channel is open (Fig. 3.32 B), Na^+ ions pass through the channel and sodium current (INa) flows into the neuron. Soon after the activation, the inactivation gate closes, disallowing further Na^+ flow (Fig 3.32 C). On membrane

repolarization the channel reverts to its closed state and becomes available for further depolarization and repeat of the cycle. If the inactivation process is compromised, for example due to a mutation affecting the inactivation gate, some Na^+ ions can pass through the channel (Fig. 3.32 D) causing a persistent sodium current (Stafstrom, 2009).

We performed an in silico analysis using Polyphen-2 to predict the effect of the p.Arg1525Gln mutation (Fig. 3.33).



Query

Acc number	Position	AA_1	AA_2	Description
P35498	1525	R	Q	RecName: Full=Sodium channel protein type 1 subunit alpha; AltName: Full=Sodium channel protein type I subunit alpha; AltName: Full=Voltage-gated sodium channel subunit alpha Nav1.1; AltName: Full=Sodium channel protein brain I subunit alpha; LENGTH: 2009 AA

Prediction

This variant is predicted to be probably damaging

Prediction	Available data	Prediction basis	Substitution effect	Prediction data
probably damaging	alignment	alignment	N/A	PSIC score difference: 2.085

Details

SEQUENCE FEATURES OF THE SUBSTITUTION SITE

Region	Site	Feature table	Critical sites
N/A	N/A	show FT fields for P35498	N/A

PSIC PROFILE SCORES FOR TWO AMINO ACID VARIANTS

Score1	Score2	|Score1-Score2|	Observations	Diagnostics	Multiple alignment around substitution position

Figure 3.33: Results of p.Arg1525Gln bionformatic analysis with Polyphen-2.

The p.Arg1525Gln is predicted to be probably damaging.

We are now performing *in vitro* functional studies to assess its effect on the physiological properties of the Nav1.1 channel.

In particular, we are now performing transient transfections in tsA-201 cells using several constructs that we have prepared (see Material and Methods chapter) followed by patch-clamp whole-cell experiments. The experimental plan is to compare firstly the properties of each mutant channel to the wild-type channel in the presence/absence of the accessory β-1 subunit. Then, since p.Thr297Ile and p.Arg1525Gln were detected on the same chromosome of Family 7 proband, we will analyze the effect of their co-presence on the channel properties to verify if they could interact each other to modulate the channel regulation.

Chapter 4:

CONCLUDING REMARKS

Idiopathic epilepsies are common and devastating neurological disorders in which genetic background and physiopathological mechanisms underlying the clinical phenotype are not fully characterized yet. These diseases are assumed to have a strong genetic component, being monogenic or oligo/polygenic with different recurrence risks in the same family. However, even in monogenic epilepsy, additional genes and environmental factors may modulate its expression, thus resulting in incomplete penetrance and variable phenotype. Ethiology, phenotypic manifestations and prognosis are indeed highly heterogeneous. Idiopathic epilepsies represent about 30-40% of all epilepsies in childhood and 20% in adults. Most of them are complex diseases: patients may shift from one phenotype to another during their lifetime and parents affected by one form may have children suffering from another epileptic syndrome.

The identification of genes responsible for distinct epilepsy syndromes or influencing the risk for epilepsy has important implications, for both research and clinical purposes. The discovery of new genes and their effects may improve our knowledge of the processes underlying seizure susceptibility, therefore potentially leading to the discovery of new treatments. On the other hand, genetic testing is useful in clinical practice, usually to support the diagnosis of a specific epileptic entity. In some cases, genetic testing may influence the choice of drugs and overall treatment, whereas in other cases it may have little clinical utility in terms of therapeutic approach (Michelucci et al., 2012).

Since the discovery, in 1995, of the first mutation underlying idiopathic epilepsy, the majority of genes associated with this condition have been shown to encode either voltage-gated or ligand-gated ion channels subunits suggesting that idiopathic epilepsies are, at least in part, channellopathies. The story is becoming increasingly complex as more genes are discovered and phenotype–genotype correlations are drawn. For example, a precise mutation of a Na^+ channel can be associated with epilepsy syndromes that vary from benign self-limited disorders, such as febrile seizures, to severe epilepsies with intractable seizures and intellectual disability. By contrast,

mutations in different genes cause syndromes that are indistinguishable clinically (Scheffer et al., 2003).

In this work we studied two idiopathic epilepsies: Nocturnal Frontal Lobe Epilepsy and Febrile Seizures / Genetic Epilepsy with Febrile Seizures Plus.

As regarding to ADNFLE, in this work we performed a mutational screening of the known genes, including CRH and its promoter, in a sample of both sporadic and familial patients. The study allowed the identification of: an already known mutation in the CHRNA4 gene (p.Ser284Leu) originated *de-novo* in one NFLE patient; three unknown variants in the CRH promoter in both sporadic and familial patients which we demonstrated to not cosegregate with the disease; one unknown missense mutation in the coding portion of the CRH gene (p.Pro30Arg) in one ADNFLE patient. The latter is the first mutation described in the coding region of the CRH gene associated to ADNFLE.

The p.Pro30Arg, located in the hormone pro-sequence region, was found in heterozygosis in two affected individuals of the same family and we demonstrated by functional *in vitro* analysis that it causes an impairment in the ability to produce and release the hormone. This impairment could be related to an altered capability of patients to respond quickly to stress agents and this would result in an impaired HPA axis cascade as well as impairment in the CRH-mediated sleep/arousal cycle regulation. Although a functional effect of the mutation was demonstrated by our results, a direct role of the p.Pro30Arg in NFLE pathogenesis has still to be proved. This could be done only by the identification of new ADNFLE families with the mutation cosegregating with the disease or by the development and study of specific transgenic mouse models.

By analyzing genes encoding the orexin system (*HCRT*, *HCRTR1*, *HCRTR2*) in a group of 21 European patients without ADNFLE mutations in all known genes, we finally demonstrated an improbable role of this system in the pathogenesis of disease: none of the patients has mutations in the three genes. However, further investigation consisting in orexin dosage in the CSF

of ADNFLE patients must be performed to definitely exclude an involvement of the orexin system in the disease.

The present work strengths the importance of a mutation screening of all known genes as well as the whole CRH gene in patients affected by NFLE/ADNFLE adding new data on mutations' frequencies. Commonly, genetic testing is not now routinely performed for patients affected by this disease. This is due to several causes: the disease is considered relatively benign and, in the majority of cases, patients are responsive to common pharmacological therapies; the high cost of the genetic test could be not supported by the national health system; the rate of success in the identification of an underlying mutation is very low.

All these considerations are true. However, it's worthwhile to note that ADNFLE is one of the rare simple idiopathic epilepsies thus a better comprehension of its pathophysiology will be useful in the subsequent genetic analysis of idiopathic epilepsies with a complex mode of inheritance. It's therefore very important to know the frequency of mutations in known genes (and this could be done only by performing a lot of mutation screenings) as well as to search for new genes responsible for ADNFLE that should exist owing to the absence of mutations in the majority of patients. The finding of new gene/s may help in defining the list of the main genes, responsible for epilepsies and in understanding how the different genes participate in the pathophysiological process. Moreover, it could provide a rational diagnostic strategy in order to perform a clear differential diagnosis (and, thus, a correct therapy) between nocturnal frontal lobe epilepsy and parasomnias. As a matter of fact, the nocturnal video-polysomnography (even when repeated) is not diagnostic in all cases. As a consequence, about 15-20% of these patients do not receive a definite diagnosis. Finally, the possibility of identifying, through a genetic screening, patients suffering from an epileptic syndrome (NFLE) could also provide an economic benefit, due to the high cost of nocturnal video-polysomnography.

Understanding the role of genetic variants in human health and disease is crucial in modern biology and medicine. The International HapMap Project

and, more recently, the 1000 Genomes Project are progressively unveiling the map of human genome variation at the scale of the human population, generating a flood of interesting data. Smaller research projects focused on disease-causing mutations also contribute through the development of new fruitful approaches. One of the current trends in large and small scale projects is exome sequencing. The rationale is that the clear majority of allelic variants known to underlie Mendelian disorders disrupt protein-coding sequences. Restricting sequencing to exons decreases the sample size to 2-5% of that of the whole genome, thus saving time and money, while allowing the identification of missense and nonsense mutations, of small insertions and deletions (indels), as well as of splice donor and acceptor site variants. The future approach of our study on NFLE genetic bases will be then to search for a common genetic background among ADNFLE patients so far collected by our group, and this will be done by exome sequencing. Recently, we performed a pilot exome sequencing study on a group of unrelated ADNFLE patients that highlighted several candidate genes. SNPs in these genes will be analyzed in the future by resequencing the relevant region in all family members to test their cosegregation with the disease and all positive genes will be then analyzed in the whole patient cohort.

As far as febrile epileptic phenotypes, we evaluated the role of SCN1A gene in a group of FS patients, among which two patients became GEFS+ during the study.

In this work several intronic and exonic polymorphisms were detected. In the case of unknown intronic variants, we performed an in silico analysis which revealed that these variations do not introduce or remove any splicing sites. Interestingly, we found in a patient two missense mutations: one already known and one unknown. These two variants co-segregated with the pathology being present in all affected individuals and in two obligate carriers. The already known mutation (p.Thr297Ile) was located in an important region (the S5-S6 linker of the first domain) which is involved in the functional pore constitution. The unknown mutation (p.Arg1525Gln) was located in the inactivation gate of the channel and was predicted to be

probably damaging. Owing to their location in important regions of the sodium channel, we are now testing the hypothesis of a causative role of these mutations in the pathogenesis of this family's disease. In particular, we have already prepared several vectors to be used in transient transfection experiments in tsA-201 cells followed by patch-clamp whole-cell experiments. The study will allow the evaluation of the effect of these mutations (considered either singly or in conjunction with the other) on the activation/inactivation properties of the sodium channel in the presence/absence of the β-1 accessory subunit.

Moreover, we plan to perform exome sequencing studies in FS/GEFS+ patients negative for mutations in the SCN1A gene in order to find new pathological mutations/genes, as above described for ADNFLE.

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