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Genetic alterations in epilepsy: the cases of Unverricht-Lundborg disease, Fragile X and B1 null mutation

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# ABSTRACT

## *Genetic alterations in epilepsy: the cases of Unverricht-Lundborg disease, Fragile-X and B1 null mutation*

Genetic mechanisms are estimated to underlie about 40% of epilepsies and may present with different manifestations and syndromes. In this thesis, three different cases of genetic implications in epilepsy have been studied: Unverricht-Lundborg disease (ULD), a monogenic Progressive Myoclonic Epilepsy in which disruption of the cystatin B gene results in the manifestation of the pathology; Fragile X, the most frequent genetic mental retardation disease which associates to epileptic seizures in about 25% of the cases; and involvement of the bradykinin system in epilepsy, a possible cause of latent hyper-excitability.

The study on ULD revealed increased brain excitability in mice deficient for cystatin B, the genetic defect associated with the pathology. This hyperexcitability seems to be related to a decreased inhibitory cell number and/or to the activation of microglia. Our findings on Fragile-X reveal that, after a chemically induced epileptic event (pilocarpine status epilepticus), active synapses accumulate Fmr1 mRNA and its protein, FMRP. This phenomenon may account for an involvement in activity-dependent synaptic plasticity and epileptogenesis. Finally, we studied bradykinin involvement in brain excitability, and found that electrical (kindling) and chemical (kainate) epileptic insults exacerbate a latent tissue hyper-excitability in B1 null mice.

These findings are examples of how genetic alterations may alter brain function, directly or indirectly leading to epilepsy manifestations. In depth, investigation of the different aspects of the genetic mechanisms of epilepsy is very important to identify key factors that, in the end, may allow discovering new therapeutic targets and new treatments. Moreover, study of the basic mechanisms underpinning pathological events may also shed light on the normal brain function.



## RIASSUNTO

### *Genetic alterations in epilepsy: the cases of Unverricht-Lundborg disease, Fragile-X and B1 null mutation*

Si stima che intorno al 40% dei casi di epilessia abbiano un'etiologia genetica. Le epilessie genetiche possono manifestarsi con una elevata varietà di sintomi e differenti forme di crisi. In questa tesi abbiamo studiato tre aspetti di questo fenomeno: la patologia di Unverricht-Lundborg (ULD), un esempio di epilessia monogenetica dovuta a mutazioni del gene per la cistatina B; la sindrome dell'X Fragile (FX), la più diffusa forma di ritardo mentale dovuta a cause genetiche (la mutazione del gene *Fmr1*), che si associa a fenomeni epilettici nel 25% dei casi; ed infine il coinvolgimento delle bradichinine nell'epilessia, una possibile causa di iper-eccitabilità latente e quindi una possibile classe di geni predisponenti alla malattia.

Gli studi sull'ULD hanno dimostrato una maggiore eccitabilità tissutale dei topi KO per la cistatina B, probabilmente riconducibile ad una perdita di neuroni inibitori e/o all'attivazione della microglia. Nello studio sull'FX, l'analisi molecolare dell'insulto epilettico da pilocarpina ha dimostrato che le sinapsi attive accumulano l'mRNA di *fmr1* e il suo prodotto proteico, FMRP, implicando un loro ruolo nella plasticità sinaptica attività-dipendente e nell'epilettogenesi. Infine, abbiamo studiato il coinvolgimento della bradichinina nell'eccitabilità cerebrale, e abbiamo scoperto che la delezione del gene codificante il recettore B1 per le bradichinine comporta una ridotta resistenza alle crisi indotte da kindling e da kainato, espressione di una latente iper-eccitabilità.

I dati raccolti in questa tesi rappresentano esempi di come le alterazioni genetiche possano, direttamente o indirettamente, alterare la funzione del cervello e causare manifestazioni di tipo epilettico. Lo studio approfondito delle alterazioni genetiche in epilessia è importante ai fini di identificare bersagli molecolari idonei per lo sviluppo di nuovi farmaci o terapie adeguate. Non solo: lo studio dei meccanismi che sottendono eventi patologici può anche aiutarci a spiegare il normale funzionamento del cervello.





Chapter I

# **Epilepsy**



# GENERALITIES

## *Epilepsy and its definition*

Through centuries, epilepsy has been described and defined in different ways based on knowledge and superstition of the different ages. Several times epilepsy was associated to divinity, in a good or bad fashion, charging this disease of a magical aura that sometimes survive even to date. Hippocrates, around the 4<sup>th</sup> century B.C., was among the first describing epilepsy as an ordinary, and not having magical properties, pathology, which origins from the brain. Since then, several other definitions and approaches alternated in time to describe and treat this pathology, resulting in the most peculiar and sometimes ridiculous ways to face the problem.

First clinically defined by John Hughlings Jackson as “*an occasional sudden, excessive, rapid and local discharge of grey matter*”, epilepsy, mainly due to its semiology, is still difficult to properly define.

The International League Against Epilepsy and the International Bureau for Epilepsy define epilepsy as “**a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures and by the neurobiologic, cognitive, psychological, and social consequences of this condition. The definition of epilepsy requires the occurrence of at least one epileptic seizure**” (Fisher et al, 2005).

Therefore, the term “epilepsy” indicates a brain disorder characterized predominantly by recurrent and unpredictable interruptions of normal brain function, called seizures. Epilepsy is not a singular disease entity but a variety of disorders reflecting underlying brain dysfunction that may result from many different causes. This pathology is not one condition but is a diverse family of disorders, having in common an abnormally increased predisposition to seizures, that are defined as “**transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain.**” (Fisher et al, 2005). Discussion about a few points of this new definition of epilepsy is raging, mainly related to: 1) abolishment of the concept of “unprovoked seizure”; 2) absence of the “two seizures” criteria; 3) lack of clear clinical definition of the “enduring alteration in the brain that increases the likelihood of future seizures” (Ahmed et al, 2005; Beghi et al, 2005; Gomez-Alonso et al, 2005).

Therefore, debate on a proper statement which will exhaustively define epilepsy is still a central point in the scientific community, because of its importance in terms of medical, legislative and education communication (Fisher et al, 2005).

## *Epidemiology*

About 1% of world population is estimated to be affected by epilepsy (prevalence), which makes this disease one of the most common neurological disorders. Age specific incidence of epilepsy is higher in children (0-14 years) and in elderly (>60 years) than in adult individuals (15-59 years) (respectively mean incidence rates of epilepsy are: 82,2/100000 per year – 39,7/100000 per year – 34,7/100000 per year; total incidence rate is 47,4/100000 per year). Incidence rate for age-specific epilepsy distribution is significantly different. A non significant trend to higher incidence of epilepsy in males compared to females has been reported showing respectively mean incidence rates of 50,7/100000 per year and 46,2/100000 per year (Kotsopoulos et al, 2002; McHugh and Delanty, 2008). Incidence seems to be higher in males due to the greater exposure to risk factors that cause symptomatic epilepsies. Generally, the median seizure-specific incidence rates are higher for partial (34,4/100000 per year) than for generalized seizures (19,6/100000 per year), but this difference is not significant. The incidence of epilepsy appears to differ from country to country and to change over time. Indeed the incidence rate results to be different in industrialized countries (40-70/100000) compared to the resource-poor countries (100-190/100000) where incidence tends to double. Moreover, in this case, higher rates are found in children and adults than in the elderly. Incidence can change also over time as demonstrated by two studies by Hauser and Kurland, 1975 and Houser et al, 1993 and by Forsgren, 1990 and Forsgren et al, 1996. In these reports, incidence for epilepsy changes with age, doubling for elderly individuals over the young in Hauser studies, and showing a higher incidence for unprovoked seizures in adults in Forsgren studies. Several factors may be involved in this pattern, such as improved antenatal and prenatal care for children, and an increase in life expectancy in the elderly, associated with an increased risk for causes of epilepsy common in old age (for a review see Kotsopoulos et al, 2002; McHugh and Delanty, 2008; Sander, 2003)

Epidemiology terms:

**Prevalence:** In epidemiology, the prevalence of a disease in a statistical population is defined as the total number of cases of the disease in the population at a given time, or the total number of cases in the population, divided by the number of individuals in the population. It is used as an estimate of how common a condition is within a population over a certain period of time. It helps physicians or other health professionals understand the probability of certain diagnoses and is routinely used by epidemiologists, health care providers, government agencies, and insurance companies.

Mathematically prevalence can be defined as follows: the ratio between the number of individuals in the population with the disease at a given time (a) divided by the sum of individuals in the population with the disease at a given time (a) and individuals in the population without the disease at a given (b).

Prevalence=  $a/(a+b)$

**Incidence:** Incidence is a measure of the risk of developing some new condition within a specified period of time. Although sometimes loosely expressed simply as the number of new cases during some time period, it is better expressed as a proportion or a rate with a denominator.

**Incidence proportion** (also known as **cumulative incidence**) is the number of new cases within a specified time period divided by the size of the population initially at risk. For example, if a population initially contains 1,000 non-diseased persons and 28 develop a condition over two years of observation, the incidence proportion is 28 cases per 1,000 persons, i.e. 2.8%.

The **incidence rate** is the number of new cases per unit of person-time at risk. In the same example as above, the incidence rate is 14 cases per 1000 person-years, because the incidence proportion (28 per 1,000) is divided by the number of years (two). Using person-time rather than just time handles situations where the amount of observation time differs between people, or when the population at risk varies with time. Use of this measure implicitly implies the assumption that the incidence rate is constant over different periods of time, such that for an incidence rate of 14 per 1000 persons-years, 14 cases would be expected for 1000 persons observed for 1 year or 50 persons observed for 20 years.

**Incidence** should not be confused with **prevalence**, which is a measure of the total number of cases of disease in a population, rather than the rate of occurrence of new cases. Thus, incidence conveys information about the risk of contracting the disease, whereas prevalence indicates how widespread the disease is.

## Treatment

Epilepsy treatment is symptomatic, mainly aiming to control seizure recurrence, and only surgery can be sometimes curative. No currently available drug is capable of preventing epileptogenesis. Anti-epileptic drugs (AEDs), better if called anticonvulsants, target different biological substrates such as voltage-gated Na-channel (e.g. Phenytoin and its derivatives), GABA system (e.g. Barbiturate or Benzodiazepine) and voltage-gated Ca-channels (ethosuximide) (Tab 1).

Table 1: Anti-Epileptic Drugs

YEAR	NAME	MECHANISM OF ACTION	TREATMENT	ADMINISTRATION	SIDE EFFECTS
1857	<i>Bromide (Potassium bromide)</i>		Frequently used as sedatives in the 19th and early 20th century, bromide ion is antiepileptic, and bromide salts are still used as such, particularly in veterinary medicine		Loss of appetite, nausea/emesis, lethargy, propensity to sleep during the daytime, depression, loss of concentration and memory, confusion, headache, and Bromism Acne-form dermatitis and other forms of skin disease may also be seen, as well as mucous hyper secretion in the lungs. Asthma and rhinitis may worsen. Rarely, tongue disorder, aphten, bad breath, and constipation occur.
1912	<i>Phenobarbital</i>	Regulate Chloride permeability reducing excitatory effects of neurotransmitters	Still used as AED. It is used to treat generalized convulsive seizures, myoclonus and partial seizures. It is also used in childhood febrile seizures	50-300mg once a day	Sedation and hypnosis are the principal side effects of phenobarbital. Central nervous system effects like dizziness, nystagmus and ataxia are also common. In elderly patients, it may cause excitement and confusion while in children, it may result in paradoxical hyperactivity.

1938	<b>Phenytoin</b>	Regulate sodium channel permeability	Effective in treating partial and generalized convulsive seizures, particularly partial convulsive seizures.	100-600mg per day, per os. Intravenous administration is used when patient is on status epilepticus.	At therapeutic doses, phenytoin produces nystagmus, At toxic doses, patients experience sedation, cerebellar ataxia, and ophthalmoparesis, as well as paradoxical seizures. Idiosyncratic side effects are rash and severe allergic reactions. Phenytoin may cause atrophy of the cerebellum when administered at chronically high levels. Phenytoin predispose patients to megaloblastic anemia and gingival hyperplasia. Phenytoin is a known teratogen and suggested to be carcinogenic. Phenytoin has been known to cause Drug-induced Lupus and other dangerous or even fatal skin reactions. (such as Stevens-Johnson syndrome and toxic epidermal necrolysis)
1952	<b>Primidone</b>	Methabolized by the liver in Phenobarbital has its same mechanism of action	See Phenobarbital	250-1000mg, two or three times per day.	See Phenobarbital
1955	<b>Ethosuximide</b>	Primary thalamic regulation of Calcium channel permeability	Used for absence seizures.	500-2000mg	CNS common side effects are drowsiness, mental confusion, insomnia, nervousness, headache, euphoria, ataxia, hiccups, impaired, concentration, irritability, hyperactivity, loss of taste, night terrors. Gastrointestinal side effects are dyspepsia, vomiting, nausea, cramps, constipation, diarrhea, stomach pain, loss of appetite, weight loss, gingival hyperplasia, swelling of tongue. Other side effects involves Genitourinary Hematopoietic Integumentary Ocular apparatus.
1963	<b>Barbexalone</b>	Regulate excitability by modulation of chloride channels. When associated to phenobarbital has awakening properties exerted by the propylexidine component.	Treatment of generalized convulsive seizures and partial seizures.	25-200mg	See Phenobarbital. Better tolerated than Phenobarbital.
1966	<b>Carbamazepine</b>	Regulate sodium channel permeability	Drug of choice for partial seizures. Used also in partial and generalized convulsive seizures.	400-2400mg	Its common side effects include drowsiness, headaches and migraines, motor coordination impairment and/or upset stomach. Carbamazepine decrease tolerance to alcohol. Less common side effects include cardiac arrhythmias, blurry or double vision and/or the temporary loss of blood cells or platelets and in rare cases can cause aplastic anemia. Additionally, carbamazepine may exacerbate pre-existing cases of hypothyroidism, there are also reports of auditory side effects.
1972	<b>Valproate</b>	Potenziate GABA inhibitory activity and modulate sodium channel permeability.	Effective for all kind of seizures, it represent the drug of choice for primary generalized seizures and for benign epilepsy with partial seizures	600-3000mg	Common side effects are dyspepsia and/or weight gain. Less common are fatigue, peripheral edema, dizziness, drowsiness, hair loss, headaches, nausea, sedation and tremors. Valproic acid also causes hyperammonemia, which can lead to brain damage. Rarely, valproic acid can cause blood dyscrasia, impaired liver function, jaundice, thrombocytopenia, and prolonged coagulation times. Valproic acid may also cause acute haematological toxicities cognitive dysfunction and reversible pseudo-atrophic brain changes. It is known as a teratogenic agent.
1974	<b>Clonazepam</b>	Increases GABA action	Treatment for partial seizures.	0.5-8mg used intravenously in status epilepticus with dosage evaluated patient by patient.	Common side effects are drowsiness, impairment of cognition and judgment, irritability and aggression, psychomotor agitation, lack of motivation, impaired motor function, cognitive impairments. Occasional and rare side effects are serious dysphoria, Thrombocytopenia, serious psychological and psychiatric side effects, induction of seizures or increased frequency of seizures, personality changes, psychosis, incontinence, paradoxical behavioural disinhibition

1988	<b>Acetazolamide</b>	Inhibits CO <sub>2</sub> catabolism increasing its concentration in the CNS. This modify the membrane excitability and the modulation of buffer function of glial cells.	Useful for all kind of seizures, but more often used for absence seizures.	250-750mg in association with other AEDs.	Common side effects include numbness and tingling in the fingers and toes, taste alterations (parageusia), frequent urination, and blurred vision, but this usually disappears shortly after stopping the medication. Acetazolamide also increases the risk of developing calcium oxalate and calcium phosphate kidney stones.
1989	<b>Felbamate</b>	Reduces Glycine action	Used for the treatment of partial seizures and secondarily generalized	1200-3600mg in association with other AEDs.	Side effects include decreased appetite, vomiting, insomnia, nausea, dizziness, somnolence, and headache. Many patients report increased alertness with the drug. Two rare but very serious effects include aplastic anemia and hepatic (liver) failure.
1989	<b>Vigabatrin</b>	Potenziate GABA action blocking its catabolic enzyme.	Used for the treatment of partial seizures and secondarily generalized	1000-3000mg, in association with other AEDs.	CNS side effects are somnolence, headache, dizziness, nervousness, depression, memory disturbances, diplopia, aggression, ataxia, vertigo, hyperactivity, vision abnormalities, confusion, insomnia, impaired concentration, personality disorder. 11% of children become hyperactive. Some patients develop psychosis during the course of vigabatrin therapy. Other rare CNS side effects include anxiety, emotional lability, irritability, tremor, abnormal gait, and speech disorder. Gastrointestinal side effects are abdominal pain, constipation, vomiting, and nausea.
1991	<b>Lamotrigine</b>	Regulate sodium channels the effects of reducing the excitatory neurotransmitters	Used for the treatment of partial seizures and secondarily generalized it is also effective for treatment of many kinds of primary or idiopathic generalized seizures	50-600mg, in association with other AEDs.	Common side effects include headaches, dizziness and insomnia. Other side effects may include acne and skin irritation, vivid dreams or nightmares, night sweats, body aches and cramps, muscle aches, dry mouth, fatigue, memory and cognitive problems, irritability, weight changes, hair loss, changes in libido, frequent urination, nausea, and other side effects. In very rare cases, Lamotrigine has been known to cause the development of a dangerous rash called Stevens-Johnson syndrome (or SJS).
1993	<b>Gabapentin</b>	Increases inhibitory activity of GABA	Used for the treatment of partial seizures and secondarily generalized	900-2400mg, in association with other AEDs.	Most common side effects include dizziness, drowsiness, and peripheral edema (swelling of extremities). Children 3–12 years of age were observed to be susceptible to mild-to-moderate mood swings, hostility, concentration problems, and hyperactivity. Rare side effects are represented by cases of hepatotoxicity. Gabapentin should be used carefully in patients with renal impairment due to possible accumulation and toxicity. It has a carcinogenic potential.
1995	<b>Topiramate</b>	Modulate voltage dependent sodium channels. Potentiate inhibitory activity of GABA and reduces excitatory activity of AMPA-Kainate neurotransmitters	Used for the treatment of partial seizures and secondarily generalized it is also effective for treatment of many kinds of primary or idiopathic generalized seizures	200-600mg, in association with other AEDs.	The most often reported side effects are tiredness, pins and needles in the fingers and toes, dizziness, lowered sense of feeling in the skin, difficulty with language, nausea, diarrhoea, indigestion, dry mouth, weight loss, decrease in appetite, drowsiness, forgetfulness, difficulty with concentration or attention, difficulty in sleeping (insomnia), anxiety, mood swings, depression, changes in taste and vision disorders. Rare side effects are blood clots, blurring of vision and eye pain, reduced sweating and metabolic acidosis
1998	<b>Tiagabine</b>	Increases GABA action maintaining GABA at synaptic level for longer time.	Used for the treatment of partial seizures and secondarily generalized	30-50mg, in association with other AEDs.	Its side effects include confusion, difficulty speaking clearly/stuttering, mild sedation, and, in doses over 8 mg, paresthesia in the body's extremities, particularly the hands and fingers.

2000	<b>Oxcarbazepine</b>	See Carbamazepine	See Carbamazepine	600-3000mg	Oxcarbazepine causes dizziness, drowsiness, blurred or double vision, fatigue and may cause headaches, nausea, vomiting and stomach pain, diarrhoea, constipation and dry mouth. It can also cause hyponatremia. Concentration loss is also reported to be a frequent side effect. Skin sensitivity to sunlight also may increase.
2001	<b>Levetiracetam</b>	Binds synaptic sites of CNS and modulates neuronal burst inhibiting epileptic discharges.	Used for the treatment of partial seizures and secondarily or idiopathic generalized epilepsies	500-1000mg, two or three times per day	Side effects include: hair loss; pins and needles sensation in the extremities; anxiety and psychiatric symptoms ranging from irritability to depression; and other common side effects like headache and nausea. It is generally well tolerated[6] but may cause sleepiness, weakness, dizziness, and infection. In children, the most common side effects are sleepiness, accidental injury, hostility, irritability, and weakness.
2001	<b>Clobazam</b>	Increases GABA action	Effective on partial seizures	10-40mg, in association with other AEDs.	Common side effects are ataxia, somnolence, diplopia, dysarthria and rarely gelastic seizures and urticaria rashes

The choice among the different AEDs depends on the semiology of the disease and the management of the treatment is correlated to the containment of seizures: some patients reach the goal by a monotherapy, some others, unfortunately, require polytherapy with the use of two or more AEDs. Balance between control of seizures and side effects of AEDs is another important point that physicians must take into consideration when starting a treatment for epilepsy. Medication administration is sometimes life-long lasting, some other times it could be slowly withdrawn, depending on recurrence of seizures when drugs are stopped. In spite of the numerous AEDs available, several patients are refractory to pharmacological treatments and continue to experience seizures. Indeed, of those who develop epileptic seizures 47% will be controlled with the first AED prescribed, 32% with the second AED, and 9% with the third. Fourth and subsequent AEDs have at most a 5% chance of bringing remission (Duncan, 2007). This leaves around 7% of individuals that have no chances to control their seizures by pharmacological approaches. Therefore it is of central importance to develop new drugs able to contain seizures or even to prevent epileptogenesis. This latter goal could be reached, not only by empirical experimentation of new chemical compounds, but also by increasing our knowledge of the complex basis of the pathology. When drug treatments result ineffective and patients experience refractory seizures, other medical approaches may be applied. These alternative treatments include surgery, vagus nerve stimulation, ketogenic diet, avoidance therapy.

Epileptic surgery is a delicate tool that does not apply to any kind of epileptic patient. Best candidate for surgery are those individuals that continue to experience seizures despite the fact they are under pharmacological treatment. Focal and very localized epileptic lesions are the target for surgery and only epilepsy with these anatomical characteristics must be taken into consideration when surgery is to apply. Surgery follows after a full assessment of the epileptic conditions. Video-



electroencephalogram monitoring, neuropsychological examination, structural magnetic resonance imaging, functional imaging tests and intracranial electrode recordings are tools used to identify the type of epilepsy, determine the severity of the seizures, and locate the epileptogenic zone (Villanueva et al, 2007). The ictogenic area will be the one dissected from the remaining presumed healthy brain tissue. Mapping the epileptic area must be done with care in order to verify which will be the important areas that might be involved in resection and, if no impairment is forecasted in the dissectible loci, the patient will undergo surgery. Surgery usually reaches 90% of success in healing focal epilepsy. Best surgical results are achieved when small epileptic lesions are dissected, but improvement in the quality of life in partially successful surgery should also be considered a good achievement.

Intractable epilepsy may also be treated by Vagus Nerve Stimulation (VNS). VNS is an alternative treatment for patients refractory to drug therapy and/or in which surgery could not be applied or resulted ineffective. Chronic refractory partial and secondarily generalized seizures are examples of epileptic syndromes that can be treated by VNS. VNS consists of a stimulating device implanted under the skin, which sends electrical impulses to the left side of the vagus nerve in the neck. Its mechanism of action is still on debate. Vagus, the tenth cranial nerve, arises from the medulla and carries both afferent and efferent fibres. The afferent vagal fibres connect to the nucleus of the solitary tract which in turn projects connections to other locations in the central nervous system. Little is understood about exactly how vagal nerve stimulation modulates mood and seizure control but proposed mechanisms seem related to an increase of thalamic synaptic activity. This effect seems to be brought about by release of epinephrine through the nucleus coeruleus and/or involvement of nucleus tractus solitarius, the structure that receives the C fibres of the Vagus nerve. The nucleus tractus solitarius is related to several areas involved in seizures onset (Milby et al, 2008; Ghanem and Early, 2006). Pilot study results demonstrated significant reduction in the frequency, intensity, and duration of seizures with chronic, intermittent VNS (Utman et al, 1993; Penry and Dean, 1990; Uthman et al, 1990; Wilder et al, 1991; Murphy et al, 1995; Labar et al, 1998). Follow up studies report a mean of 50-60% success for VNS when associated to normal pharmacological treatment (Ardesch et al, 2007; Abubakr and Wambacq, 2008; Milby et al, 2008). Indeed, patients experience a reduction of seizure frequency and duration. VNS seems also to progressively increase its effectiveness in seizures containment in time (Ardesch et al, 2007; Milby et al, 2008). Very few are the side effects of this practice, comprising hoarseness of voice, cough and sometimes weight loss; most of them are very mild and bearable for patients.

Ketogenic diet represent another side treatment for intractable refractory epilepsy and for epilepsy at large. The efficacy of the diet is independent of the type of seizure and is effective for both

generalized and partial seizures at varied ages. This therapy is characterized by a diet high in fat and low in carbohydrates and protein. In these conditions, energy is retrieved from fatty acid oxidation and high concentration of ketone bodies (such as  $\beta$ -hydroxybutyrate, acetoacetate, and acetone) are generated. Ketones are then used as nutrients for the body and even for the brain. The use of ketones as source of energy in the brain seems to directly or indirectly affect neurotransmitter biology leading to a possible, even if yet unknown, mechanism of action. High percentage of good outcome is reported for this treatment, also reviewing patients in seizure free conditions of after one year of treatment. Recent findings attributed to ketogenic diet a potential effectiveness in preventing epileptogenesis (for review see: Bough and Rho, 2007; Freeman et al, 2007; Hartman et al, 2007). Avoidance therapy is a therapy applicable only to reflex seizures (see below “classification”). It consists in putting patients in the condition to avoid the precipitating phenomenon of their seizures. Main precipitating phenomena are related to sensory organs and comprise: music and noise, light and dark, touching feelings, tastes and sometimes emotional involvement. This very simple approach is usually successful, leading patients to experience very few seizures.

## *Classification*

Epilepsy is classified by 5 main criteria:

- Aetiology (cause of the first seizures)
- Semiology (manifestation of the seizures)
- Triggering event(s)
- Syndromic symptoms
- Brain anatomic focus-foci (focal/partial or generalized seizures)

Considering aetiology, epilepsy is classified as *idiopathic* or *secondary and probably symptomatic* (definition preferred to *criptogenic* in the last revision of the terms). Each of these comprehends generalized or partial seizure types (Tab. 2).

The term *idiopathic* describes a syndrome that is only epilepsy, with no underlying structural brain lesion or other neurological signs or symptoms. These are presumed to be genetic and are usually age-dependent (Engel, 2006). Attention must be posed on the correct definition of "idiopathic," which means a disorder onto itself, *sui generis*, and not aetiology unknown.

Epilepsies described as *secondary* are related to a known or a suspected disorder of the CNS which is the trigger of the symptoms. Known causes of secondary epilepsies are: head traumas, tumors,

viral and bacterial encephalopathies, anoxia, etc. The probably symptomatic (formerly cryptogenic) epilepsies are referred to epilepsies of unknown nature which are usually suspected to be related to a specific disorder of the brain (Engel, 2006).

The definition of partial (or focal) seizures refers to a specific brain-localized-origin of the seizures. Generalized seizures, instead, refers to a general involvement of both brain hemispheres in the origin of the seizures.

Table 2: Classification by aetiology of the disease.

Idiopathic	Partial seizures type	Benign Familiar Epilepsy in Infancy
		Childhood benign epilepsy with central or temporal burst
		Childhood benign epilepsy with occipital burst
		Other form of idiopathic epilepsy with partial seizures
	Generalized seizures type	Benign myoclonic epilepsy in infancy
		Childhood absence epilepsy (Pyknolepsy)
		Juvenile myoclonic epilepsy
		Juvenile absence epilepsy
Secondary and Probably symptomatic epilepsy syndrome	Generalized seizures type	Generalized epilepsy with tonic-clonic seizures
		Early suppression-burst encephalitis in infancy
		West syndrome
		Lennox-Gastaut syndrome
	Partial seizures type	Progressive epileptogenic encephalitis
		Severe childhood myoclonic epilepsies
		Mesial temporal epilepsy
		Other epilepsy defined by their focus of origin

By semiology, epilepsy is classified in *self limited seizure types* and *continuous seizure types* and each of these are subdivided in *generalized* and *focal* seizures (Tab. 3) (Engel, 2006). Self limited seizures usually end in a reasonable limit of time; on the contrary, continuous seizures are characterized by repetition of the ictal phenomenon for a long period of time with no remission between seizures (i.e. status epilepticus).

Table 3: Classification by semiology (Epileptic seizure types)

Self limited seizure types	Generalized seizures	Tonic-clonic seizures (includes variations beginning with a clonic or myoclonic phase)	
		Clonic seizures	Without tonic features
			With tonic features
		Typical absence seizures	
		Atypical absence seizures	
		Myoclonic absence seizures	
		Tonic seizures	
		Spasms	
		Myoclonic seizures	
		Eyelid myoclonia	Without absences
			With absences
Myoclonic atonic seizures			

	Focal seizures	Negative myoclonus		
		Atonic seizures		
		Focal sensory seizures	With elementary sensory symptoms (e.g., occipital and parietal lobe seizures)	
			With experiential sensory symptoms (e.g., temporo parieto occipital junction seizures)	
		Focal motor seizures	With elementary clonic motor signs	
			With asymmetrical tonic motor seizures (e.g., supplementary motor seizures)	
			With typical (temporal lobe) automatisms (e.g., mesial temporal lobe seizures)	
			With hyperkinetic automatisms	
			With focal negative myoclonus	
			With inhibitory motor seizures	
			Gelastic seizures	
			Hemiclonic seizures	
			Secondarily generalized seizures	
Continuous seizure types	Generalized status epilepticus	Generalized tonic-clonic status epilepticus		
		Clonic status epilepticus		
		Absence status epilepticus		
		Tonic status epilepticus		
		Myoclonic status epilepticus		
	Focal status epilepticus	Epilepsia partialis continua of Kojevnikov		
		Aura continua		
		Limbic status epilepticus (psychomotor status)		
		Hemiconvulsive status		

Adapted from Engel (2001) with permission.

By definition, a reflex epilepsy syndrome is a syndrome in which all epileptic seizures are precipitated by sensory stimuli. Reflex seizures that occur in focal and generalized epilepsy syndromes that are also associated with spontaneous seizures are listed on the basis of seizure types. Isolated reflex seizures can also occur in situations that do not necessarily require a diagnosis of epilepsy. Seizures precipitated by other special circumstances, such as fever or alcohol withdrawal, are not reflex seizures (Engel, 2006). Principal stimuli considered as precipitating circumstances of reflex epilepsy syndromes are reported in Tab. 4.

Table 4: Precipitating stimuli for reflex seizures

Precipitating stimuli for reflex seizures	Visual stimuli	Flickering light: color to be specified when possible
		Patterns
		Other visual stimuli
	Thinking	
	Music	
	Eating	
	Praxis	
	Somatosensory	
	Proprioceptive	
	Reading	
	Hot water	
Startle		

Adapted from Engel (2001) with permission.

An epilepsy syndrome is a complex of signs and symptoms that define a unique epilepsy condition with different aetiologies (Engel, 2006). This must involve more characteristics than just the seizure type such as: age of onset, progressive nature, interictal EEG, associated interictal signs and symptoms, pathophysiologic mechanisms, anatomic substrates, etiological categories and genetic basis (Engel, 2006). The different syndromes are summarized in Tab. 5. In this table are also reported syndrome conditions which, by their nature, must not be considered as epilepsy but as convulsive episodes.

Classification of epileptic syndromes is a difficult matter, considering that a full description of each syndrome is sometimes far from being complete and, for some of them, only a few characteristics have been reported. Including an epilepsy syndrome in an official classification gives it the status of a recognized syndrome. On the other hand, excluding poorly described syndromes from an accepted classification could lead to a reduced investigation in that direction, failing to uncover important features of scarcely reported syndromes (Engel, 2001). For these reasons the reported table has signalled few syndromes as in definition development.

Another important case is raised by syndromes of Idiopathic Generalized Epilepsy (IGE), only eight of which are recognized by ILAE (Nordli, 2005), even if other are described in the literature (Panayiotopoulos, 2005). The difficulties stem from the practical view that IGE are clinically considered as one disease, leading to an easy clinical approach, but in reality IGE comprise several syndromes. This would imply to consider IGE as a complex ensemble of different syndromes which would be more demanding diagnostically. Therefore, in view of a better differential diagnosis, a well organized classification of epilepsy syndromes would be a useful tool for the physicians.

Table 5: Epilepsy syndromes and related conditions

Benign familial neonatal seizures		
Early myoclonic encephalopathy		
Ohtahara syndrome		
<sup>a</sup> Migrating partial seizures of infancy		
West syndrome		
Benign myoclonic epilepsy in infancy		
Benign familial infantile seizures		
Benign infantile seizures (non-familial)		
Dravet's syndrome		
HHE syndrome		
<sup>a</sup> Myoclonic status in non-progressive encephalopathies		
Benign childhood epilepsy with centrotemporal spikes		
Early onset benign childhood occipital epilepsy (Panayiotopoulos type)		
Late onset childhood occipital epilepsy (Gastaut type)		
Epilepsy with myoclonic absences		
Lennox–Gastaut syndrome		
Landau–Kleffner syndrome		
Epilepsy with continuous spike-and-waves during slow-wave sleep (other than LKS)		

Progressive myoclonus epilepsies		
Idiopathic generalized epilepsies with variable phenotypes	Juvenile absence epilepsy	
	Juvenile myoclonic epilepsy	
	Epilepsy with generalized tonic-clonic seizures only	
Reflex epilepsies	Idiopathic photosensitive occipital lobe epilepsy	
	Other visual sensitive epilepsies	
	Primary reading epilepsy	
	Startle epilepsy	
Autosomal dominant nocturnal frontal lobe epilepsy		
Familial temporal lobe epilepsies		
<sup>a</sup> Generalized epilepsies with febrile seizures plus		
<sup>a</sup> Familial focal epilepsy with variable foci		
Symptomatic (or probably symptomatic) focal epilepsies	Limbic epilepsies	Mesial temporal lobe epilepsy with hippocampal sclerosis
		Mesial temporal lobe epilepsy defined by specific etiologies
		Other types defined by location and etiology
	Neocortical epilepsies	Rasmussen syndrome
		Other types defined by location and etiology
Conditions with epileptic seizures that do not require a diagnosis of epilepsy	Benign neonatal seizures	
	Febrile seizures	
	Reflex seizures	
	Alcohol-withdrawal seizures	
	Drug or other chemically induced seizures	
	Immediate and early post cerebral insult seizures	
	Single seizures or isolated clusters of seizures	
	Rarely repeated seizures (oligoepilepsy)	

Adapted from Engel (2001) with permission. (<sup>a</sup> Syndromes in development)

## *Models of epilepsy*

(For an extensive review of epilepsy modelling see “Models of seizures and epilepsy” Pitkanen A., Schwartzkroin P.A. and Moshé S.L. (2006) Elsevier Academic Press)

The study of epilepsy can not be performed only in humans because of several different reasons, from ethical issues to practical inapplicability, from unavailability of controls to high costs of human research. For all these reasons, modelling epilepsy is of a central importance. Epilepsy models for studying epilepsy are principally used for three reasons:

1. to understand basic mechanisms underlying the pathology;
2. to devise new approaches for diagnosis;
3. to test new drugs or new therapies.

Basic mechanisms of epilepsy are still under strong investigation and epileptic models are a very precious tool to deeply understand the molecular and physiological causes of this complex pathology. Of course, uncovering the mechanisms underpinning the disease will help develop new diagnostic, therapeutic and preventive approaches. Models of epilepsy are the best instruments on

which innovative experimental approaches for diagnosis and therapies can be tested. This will speed up the application of new diagnostic and therapeutic tools, allowing rapid intervention on the disease. In each of these cases, human studies can give a big support, but preliminary data are normally retrieved from in vitro and in vivo experiments.

Epilepsy models should be created or prepared in order to faithfully reproduce the human epileptic condition. Therefore several different models have been developed trying to model all the complex aspects of this pathology. On the other hand, some experimental approaches model only some of the manifestation of epilepsy (epilepsy equivalent) allowing only the investigation on that symptom and not on the whole complex picture of a complete model. In any case, this gives the chance to study an aspect of the disease.

Therefore, the already reported epidemiology that describes 1% of the population as affected from some kind of epilepsy, which in turn explains the economic, social and personal cost of the disease, makes it clear how important is to study epilepsy and how important are the tools that model it.

#### *Induced models of epilepsy*

Induced models of epilepsy are developed by application of chemical, electrical or damaging insults on a healthy brain, in order to transform that brain in an ill one, capable to show features of the chosen kind of epilepsy in study.

Table 6: Induced model of epilepsy (classification based on the tools used to develop the models).

In Vitro	Cell culture Models		
	Acute and organotypic slices		
	Single nerve cells acutely dissociated from animal and human brain		
	In vitro isolated guinea pig brain		
In Vivo	chemical model of epilepsy	GABA	Pentylentetrazole
			Bicuculline
			Picrotoxin
			Glutamic Acid Decarboxylase (GAD) inhibitors
			Beta carbolines and convulsant benzodiazepine Ro 5-3663
			GHB (gamma-hydroxy-butyrate)
		Excitatory Amino-Acid	Kainic acid
			Quisqualic acid/alfa-amino-3-Hydroxy-5-Methyl-4-Isoxazole Propionic acid
			N-Methyl-D-Aspartic acid (NMDA)
			Homocysteine, homocysteic acid
		Acetylcholine related substances	Pilocarpine, and lithium-pilocarpine
			Organophosphorus compounds
		Other drugs	Strychnine
			Aminophylline
			Insulin induced hypoglycemia
Ay-9944			
THIP (4,5,6,7 tetrahydroxyisoxazolo (4,5,c) pyridine 3-ol)			

		Inhalants	Fluoroethyl
		Topical application	Metals (cobalt, zinc, antimony, alumina cream, iron)
			Antibiotic (penicillins and cephalosporins)
			Tetanus toxin
	electrical model of epilepsy	electroshock seizures	
		local electrical stimulation	
		electrical kindling	
		self sustaining status epilepticus by Perforant Path stimulation	
		self sustaining status epilepticus by Amygdala stimulation	
		focal status epilepticus by perforant path stimulation in anesthetized rats	
		continuous hippocampal stimulation	
	lesion model of epilepsy	cortical freeze lesion model	
		antiproliferative agents (5-azacytidine, methyl-mercury, nitrosureas and carmustine)	
		Methylazoxymethanol acetate (MAM) model	
		In-Utero irradiation as a model of cortical dysplasia	
		Hypoxia-induced seizures and hypoxic encephalopathy in neonatal period	
		Lateral fluid percussion brain injury	
		Chronic Partial Cortical Isolation model	
		Head Trauma: haemorrhage-Iron Deposit	
		Stroke	
Others	Complex febrile seizures – experimental model in immature rodents		
	Infection induced seizures	Model of neurocysticercosis	
		Model of herpes virus infection	
	Rasmussen's encephalitis model		

### *Genetic models of epilepsy*

The above reported epilepsy models are related to chemically, electrically or mechanically induced alterations in an otherwise healthy brain. Genetic models of epilepsy, instead, refer to a molecularly and/or anatomically altered brain due to genetic alterations. These models can be naturally selected in animals spontaneously showing epilepsy phenomena or can be created by introducing gene mutations. Either ways, animals show and model a particular kind of epilepsy. On one hand, a naturally occurring seizure behaviour in a spontaneously epileptic animal (i.e. GAERS and WAG/Rij for rats and tottering, lethargic, ducky, stargazer etc for mice); on the other hand, specific seizures and syndromes induced by targeted alteration of genes already known to cause or to be involved in a particular type of epilepsy. Table 7 and 8 reports spontaneous and induced genetic models.



Table 7: Spontaneous models of epilepsy

rats	Absence model	GAERS rats WAG/Rij rats
mice	Spike wave	Tottering
		lethargic
		ducky
		stargazer
		SWE
		Mocha2j
		coloboma
		Dilute lethal
	convulsion	jimpy
		jittery
		megencephaly
		quaking
		staggerer
		torpid
		veritint waddler
wabblers-lethal		
weaver		
writher		
convulsions evoked by sensory stimuli	frings	
	lurcher	

Table 8: Induced model of seizures

Gene involved	Protein modified
NPY	Neuropeptide Y
Syn1	Synapsin 1
Syn2	Synapsin 2
Slc1a (Glt-1)	Glutamate transporter
Akp2	Tissue nonspecific alkaline phosphatase (GABA synthesis)
Gad2	Glutamic acid decarboxylase (GABA synthesis)
Gabrβ3	GABA <sub>A</sub> receptor β3 subunit
Gabrδ	GABA <sub>A</sub> receptor δ subunit
Gria2	Glutamate receptor subunit 2
Htr2c	Hydroxytryptamine receptor 2c
Kcna1	Voltage-gated K <sup>+</sup> channel
Scn2a	Sodium channel type 2a (brain specific)
Kcnj6 (Girk2)	Inwardly rectifying K <sup>+</sup> channel
Camka	Calcium calmodulin kinase α subunit
Itpr1	Inositol 1,4,5 trisphosphate receptor
Gap43	Growth-associated protein
Cdk5r	Neuronal-specific activator of cyclin kinase
Otx1	Orthodenticle homolog (homeobox family)
Jrk	Jerky (DNA-binding protein)
Pcmt1	Protein L-isoaspartate (D-aspartate) O-methyltransferase (protein repair enzyme)
Hex-a	α subunit of β-hexosaminidase (lysosomal enzyme)
Hex-b	β subunit of β-hexosaminidase (lysosomal enzyme)
Stfb (Cstb)	Cystatin-B (cysteine protease inhibitor)
Psap	Sphingolipid activator protein
Il6	Cytokine, interleukin 6
App	Amyloid precursor protein
Hdh	Huntingtin's amino-terminal polyglutamine sequence
Polyglutamine repeat	146 unit CAG repeat inserted in hprt gene
Bdnf (homozygous)	BDNF
Fyn	Tyrosine kinase receptor
Plat	Tissue plasminogen activator
Fos	Protooncogene
Mt-3	Metallothionein-3
...	...
Others	Many others are already available and several others will be modelled as soon as new genes will be found responsible for an epileptic syndrome.

Modified From Puranam and McNamara, 1999

# GENES AND EPILEPSY

Of the about 1% of the population affected by epilepsy, 30-40% are estimated to have a genetic contribution to the aetiology (Gardiner, 2000; Berkovic et al, 2006). Indeed, among all the classifications for epilepsy, one consistent group is represented by the idiopathic epilepsies which are usually described as epilepsies with no known causes and, therefore, probably genetic. Epidemiologic studies describe also the risk of hereditary transmission of epilepsy from parents experiencing epilepsy or having familiarity for this pathology (Winawer and Shinnar, 2005). Beside a general risk of 5% of children born with epilepsy in the general population, there is an increased risk of seizures in siblings and offspring of pro-bands with the epileptic pathology. This is mainly related to parent gender with a maternal higher effect (Ottman et al, 1988), early age at onset (Anderson and Hauser, 1997; Ottman et al, 1996a), number and closeness of affected relatives (Winawer and Shinnar, 2005) and EEG abnormalities including generalized spike waves and photoparoxysmal response (Anderson and Hauser, 1997). Parents affected by idiopathic epilepsies have higher risk to transmit epilepsy than parents affected by symptomatic ones. Hereditary risks depend also from the different seizure types affecting the pro-bands: parents with myoclonic and absence seizures as well as with generalized seizures have higher risk to transmit epilepsy to offspring than people experiencing partial seizures (Eisner et al, 1959; Tsuboi and Christian, 1973; Tsuboi, 1980; Annegers et al, 1982; Beck-Mannagetta and Janz, 1991; Ottman et al, 1998; Ottman et al, 1989). In any event, the data are reassuring; more than 90% of individuals with epilepsy have no affected relatives, and most parents with epilepsy will have no children with epilepsy. In conclusion, the general genetic risk of epilepsy transmission is about 5% for parents having a direct or indirect history of epilepsy (Winawer and Shinnar, 2005).

Genetic epilepsies can be classified based on the mechanism of inheritance in (Gardiner, 2000):

- Mendelian disorders;
- Non-Mendelian disorders or complex epilepsies;
- Chromosomal disorders.

*Mendelian disorders* are those in which one single major locus represents the main genetic modification which is responsible for the epileptic phenotype. Modes of inheritance are the common autosomal dominant, autosomal recessive, X-linked dominant and X-linked recessive. Primary Mendelian epilepsies are considered rare and account only for 1% of the genetic epilepsies.

However, they form an important group because recognition of the characteristic features and presence of a family history enables the correct diagnosis to be made. In these disorders single gene mutation leads to a deleterious change in gene sequence, presumably bringing to either loss of the encoded protein function or gain of function mutation. Either way, a disruption of the pathway including the modified protein is affected, exacerbating the epileptic phenotype. Examples of these diseases are: Benign Familial Neonatal Convulsion (EBN1, EBN2); Autosomal Dominant Nocturnal Frontal Lobe Epilepsy (ADNFLE); Generalized Epilepsy with Febrile Seizures Plus (GEFS+). Several times, epilepsy is one of the major symptoms of Mendelian transmitted disorders such as Unverricht-Lundborg disease, Lafora Disease or Fragile X syndrome.

*Non Mendelian disorders* (complex epilepsies) are characterized by a complex inheritance and represent about 70% of the idiopathic epilepsies (Anneger, 1996). They are usually polygenic, as suggested by rapidly diminishing risks beyond first-degree relatives (Ottman et al, 1996b) and high concordance between monozygotic twins (Berkovic et al, 1998). Mechanisms of inheritance for these diseases showed a complex pattern and not only one gene is presumed to be involved. Complex epilepsy arises when, by chance, meiotic reshuffling creates a combination of susceptibility alleles with sufficient effect in the same individual to push neuronal hyperexcitability over the seizure threshold. Each susceptibility allele alone is insufficient to cause seizures, but requires the additive or epistatic interaction of other susceptibility alleles. Complex epilepsy is most often expressed sporadically in an affected individual, and the phenotype does not follow Mendelian inheritance. Nor do the close relatives, who may or may not be affected, necessarily share the same set of epilepsy susceptibility alleles. During intergeneration transmission, each susceptibility allele is segregated away from other unlinked susceptibility alleles (Mulley et al, 2005). This group of genetic epilepsies include Juvenile Myoclonic Epilepsy (JME), Childhood Absence Epilepsy (CAE) and Benign Childhood Epilepsy with Centrotemporal Spikes (BCECTS) (Mulley et al, 2005).

*Chromosomal disorders* associate to epilepsy many times, as happens in Down Syndrome (trisomy 21p), in trisomy 12p, in trisomy 1q42.3-qter (Tuschl K et al, 2007), monosomy 21q22.3-qter (Tuschl K et al, 2007), etc. They are usually related to a gross cytogenetic abnormality which ordinarily determine a syndrome in which epilepsy may be an important component.

Genetic epilepsy can be also classified based on functional classification as follows (Noebels, 2003):

- those linked to primary defects of membrane and synaptic signalling,
- those linked to neuronal plasticity and metabolism,
- those linked to network development.

Epilepsies of the first group are characterized by genetic alterations that modify general neurotransmission leading to an imbalance between excitatory and inhibitory stimuli that exacerbate epileptic phenomena. Among those classified in this group, a large number is represented by mutations in neuronal ion channels, the so called *channelopathies*. Channelopathies have been among the first disorders associated to genetic modifications in epilepsy and gave a boost for a deeper investigation of the genetics of epilepsy.

Impaired neuronal ion channel function has been associated to several pathologies like neuromuscular disorders (Bernard and shevell, 2008), chronic pain (Catterall et al, 2008), migraine (Catterall et al, 2008) and epilepsy (Noebels, 2003; Heron et al, 2007; Bernard and Shevell, 2008, Catterell et al, 2008). Symptoms of a channelopathy may present either as an abnormal gain of function or as a loss of function of the channel affected by the mutation. Channelopathies usually demonstrate both phenotypic and genetic heterogeneity. Phenotypic heterogeneity means that different mutations in the same gene can cause different diseases. Genetic heterogeneity means that mutations in different genes can result in the same apparent disease phenotype (Bernard and Shevell, 2008).

Numerous are the genetic epilepsies caused by channelopathies, including Autosomal Dominant Nocturnal Frontal Lobe Epilepsy (ADNFLE), caused by mutation in the acetylcholine receptor subunit alfa-4 or subunit alfa-2, Generalized Epilepsy with Febrile Seizures plus (GEFS+) caused by mutation on sodium channel alfa-1 and beta-1 subunit or on GABA-A receptor gamma-2 subunit, Benign Familial Neonatal Seizures (BFNS) due to mutations on potassium channel subunits. Epileptic channelopaties present normally as monogenic inherited alterations (Heron et al, 2007) even though they may associate to complex patterns of inheritance involving more than one mutation to exacerbate the epileptic phenotype (Mulley et al, 2005; Heron et al, 2007).

Synaptic signalling may be disrupted and cause epilepsy also by mutations involving the large protein families that mediate vesicle trafficking and exocytosis (Bock et al, 2001). Seizures appear in mice deleted for the vesicle-anchoring phosphoproteins synapsins 1 and 2; loss of these proteins diminishes the size of the presynaptic vesicle pool and disrupts synaptic depression (Rosahl et al. 1995).

Another synaptic vesicle protein, Sv2A, also regulates synaptic strength by altering the mobility of the releasable pool. Sv2A-deficient mice, alone or combined with Sv2B nulls, show a severe seizure phenotype (Crowder et al. 1999, Janz et al. 1999). In contrast to synapsin mutants, neurons showed sustained release of transmitter in response to brief activation.

At an earlier stage in vesicle biogenesis, incomplete vesicle assembly can also lead to an epileptic phenotype. The *mocha* mouse encodes the AP3-delta subunit of an adaptor complex that facilitates

incorporation of the Znt3 zinc transporter into synaptic vesicles. Lack of the delta subunit leads to loss of vesicular zinc sequestration, severe EEG hypersynchronization, and seizures (Kantheti et al. 1998).

In conclusion, all the mutations affecting neurotransmission may lead to an imbalance of excitatory/inhibitory signalling leading to hyperexcitability, which in turn may manifest as epileptic seizures.

Epilepsy can be observed during the course of many inborn errors of metabolism (IEMs), usually as part of a large clinical spectrum, and several IEMs may manifest with inaugural epileptic seizures (Sedel et al, 2007). These diseases may be subdivided according to the type of clinical presentations into progressive myoclonic epilepsies, epileptic seizures without mental retardation, epilepsies with mental retardation. In the first group are worth to be mentioned ceroid lipofuscinosis, sialidosis, and lafora disease. The second group includes Wilson disease and acute neuropsychiatric porphyries. The last group comprises De Vivo disease, creatine synthesis or transport defects and succinic semialdehyde dehydrogenase. All these disorders are characterized by altered metabolic pathways in which a known protein has been found as the putative responsible for the disease. Unfortunately, the molecular functions of the different proteins remain to be elucidated in most of the diseases and, in many cases, studies are required to better understand the link between protein mutation and epileptic manifestations.

The third group of the functional classification reported above is represented by epilepsies in which the genetic mutation leads to altered cyto-architectonic formation of brain structures. Cortical dysplasias resulting from aberrant patterns of brain development are a frequent substrate for inherited seizure syndromes. The malformations may be microscopic or visible by magnetic resonance imaging and originate from diverse signalling defects affecting migration, proliferation, differentiation, and segmentation. For example, defective proliferation and cell dimension have been associated to deletion of NeuroD/Beta2, a neuronal transcription factor that selectively blocks postnatal proliferation of granule cells in the dentate gyrus. These mice show a hippocampal formation entirely devoid of a granule cell layer, but with a preserved pyramidal cell layer and theta rhythm generation, and exhibit frequent partial motor seizures in adulthood (Liu et al. 2000). The same hippocampal phenotype is associated to a deletion of citron-kinase (citron-K), a Rho effector regulating cytokinesis (Di Cunto et al. 2000). Citron-K-deficient mice lack granule cells and develop fatal seizures before adulthood. Disturbance of cell migration has been linked to mutation of DCX (Kizhatil et al, 2002), the gene encoding for doublecortin, which leads to subcortical band heterotopia syndrome (Feng and Walsh, 2001) comprehensive of seizure phenomena. Periventricular nodular heterotopia has been suggested as consequence of a mutation in the filamin

gene (FLN1) that interacts with F-actin during motility-related cytoskeletal reorganization, and mutation results in a subset of neurons clustered at the ventricular zone. Analysis of mutant human FLN1 neurons and chemically induced heterotopias confirms that key intrinsic excitability changes occur when cells develop outside of their natural lamination pattern; such molecular rearrangements may provoke specific patterns of aberrant receptor expression and loss of repolarizing K<sup>+</sup> currents that favor epilepsy (Castro et al. 2001, Battaglia et al. 2002). Mutations in the human reelin gene (RELN) are linked to a lissencephalic cortical neuronal migration defect with seizures (Hong et al. 2000). Finally, genetic disruption of homeobox genes related to specification, regionalization, and terminal differentiation of the neocortex results in epileptic phenotypes. Example for this are deletion for OTX-1 and mutation of ARX.

Transcription factors and protein silencers act together to control the activation and repression of the genetic differentiation program (Robertson and Wolffe 2000). Mutation of genes acting in this pathway are now linked to epilepsy. In this group, two mutations are worth to be mentioned: mutation of the MECP2 gene associated to Rett Syndrome, an X-linked disorder including mental retardation and seizures (Shabazaian et al, 2002; Johnston et al, 2001; Tudor et al, 2002); and deletion of *jerky* gene, which leads to a limbic seizure phenotype in mice, through a putative defect in translational processing (Liu et al. 2002). A mutation in the human homologue, JH8, has been identified in a case of childhood absence epilepsy (Moore et al. 2001).

Epilepsy is therefore well known as a complex neurological disorder and it is now quite clear that a number of human epilepsy syndromes can result from gene mutations (Crino, 2007). Particular gene mutations may account also for many epilepsy phenotypes that show little if any evidence for inheritance patterns. This may be due to a de-novo mutation. Indeed, one fascinating possibility is that sporadic epilepsies result from somatic mutations occurring in brain progenitor cells, which are not present in the germline DNA (Crino, 2005; Weiss, 2005). Thus, while sporadic epilepsies might not follow Mendelian inheritance patterns, they are nonetheless the result of a genetic or mutational event. In this scenario, a deleterious gene mutation might occur during the many rounds of cellular mitosis occurring throughout the course of brain development. Moreover, a corollary hypothesis is that an accumulation of mutations in a variety of distinct genes, insufficient alone to cause seizures, could lead to neuronal hyperexcitability. Thus, somatic mutations in multiple genes during brain development might lead to seizure onset.

This mechanism may provide a further explanation for complex epilepsies (Crino, 2007). Considering these possibilities, the genomics approach is important for the possible explanation of idiopathic epilepsies. In this respect, single nucleotide polymorphism (SNPs) may play an important

role in the onset of several epileptic manifestations. SNPs represent single basepair changes that are present in more than 1% of the general population and may be synonymous, with no changes in amino-acid in the primary structure of the protein or non-synonymous with amino-acid substitution. In non-synonymous SNPs an altered protein function may be expected and may have functional consequences in terms of excitability or seizure susceptibility. Following this reasoning, clearly results that SNPs relevant to epilepsy occur throughout the genome and thus that subtle alterations in basepair sequence in a set number of candidate genes can culminate in an epilepsy phenotype. This so-called “common variant-common allele” phenomenon suggests that certain types of epilepsy result from an inherent predisposition or predilection based on the unique or additive effects of SNPs within a single or multiple genes (Ottman, 2001; Ottman, 2005). Thus, epilepsy may reflect a “SNP dose effect” in which seizure susceptibility is directly related to the number and functional significance of relevant SNPs throughout the individual genome. Genomic influences may account for the majority of patients with no obvious structural or inherited etiology for their seizures, so-called idiopathic epilepsy.

SNP variations may also account for some forms of symptomatic epilepsy. This notion has practical importance since it may account for the incidence of seizures in some, but not all, patients with tumors, stroke, trauma and so on. For example, even in the setting of brain injury, cortical malformation, or tumor, only a proportion of patients will develop epilepsy. It is conceivable that these individuals develop seizures not just because of injury to the cortex but rather because of combinatorial effects of a brain lesion plus a particular set of predispositional SNPs (Crino, 2007). This point of view opens a discussion on how the genetic background may influence epilepsy onset. Therefore, the so called modifier genes may account for synergistic or antagonistic interactions with known epilepsy susceptibility loci (Durner et al., 2001; for review, see Mulley et al., 2003). These sites in the genome, while not directly responsible for seizures, can affect the impact of gene mutations or sequence alterations that culminate in seizures. Modifier genes may affect the epilepsy phenotype in idiopathic epilepsies and this is well documented by the fact that, in mice, strain backgrounds can determine seizure onset in the setting of an engineered gene defect. Moreover, in humans, variable expressivity among family members is a common feature of inherited epilepsy syndrome of most types, suggesting that genetic modifiers may influence the clinical manifestation of epilepsy.

In the same frame, the genetic background may be responsible for epilepsy onset in symptomatic epilepsies. The idea that genes and acquired factors (traumas, injuries, etc.) interact in human epilepsies is an old one, dating back to Lennox, in the mid-20th century (Lennox, 1951), and is generally accepted for any complex disorder. In this respect, what is thought plausible is that after a

brain damaging event, reparation of the injury may activate mechanisms that enhance hyper-excitability of the damaged areas, which in turn may allow exacerbation of seizures. This relates to the influence of the background by the fact that, in the presence of the same damaging event, not all patients develops seizures over time. This points out how susceptibility loci created by the injury associate to the genetic predisposition to seizures in triggering epileptic phenomena.

Genes and their protein products are also related to epilepsy as a consequence of the seizures. Gene expression is perhaps one of the first studied aspects of gene involvement in epilepsy. Indeed several studies report altered gene expression in the brain experiencing seizures (Aronica and Gorter, 2007; Elliot and Lowenstein, 2004; Likasiuk and Pitkanen, 2004; Lukasiuk et al, 2006; Majores et al, 2004; Morgan et al., 1987; Gall et al., 1991; Newton et al., 2003). The modified gene expression correlates to pathophysiology in all the possible ways, either in a protective attempt to prevent seizures to happen again or, in an aberrant acting fashion, leading to a worsening of the disease. From a mechanistic perspective, it is tacitly assumed that altered mRNA levels predict similar changes in functional protein expression that disrupt normal cell function, although this relationship is not universal. Seizure-induced changes in mRNA expression are usually associated with long-lasting changes in protein expression that have functional relevance in terms of cellular architecture, network reorganization, cell proliferation, cell death, and perhaps most interesting, fostering recurrent seizures. Under a certain point of view, the “holy grail” of gene expression analysis studies in epilepsy has been to define either a single gene or a panel of genes that are the first steps in the epileptogenesis cascade. This mainly in relation to the fact that symptomatic epilepsies are usually a consequence of a plastic modification occurring after a damaging event that may lead to the manifestation of recurrent seizures. Deep investigation in gene expression put in motion during epileptic events may shed light on the putative starters of the neuronal plasticity which at a certain point may exacerbate in epileptic phenomena. The therapeutic implications of these essential genes are numerous.

Finally, it is important to stress how difficult is to associate genetic defects to an epileptic syndrome. Very few genetic associations for idiopathic epilepsy have been replicated and this has tempered enthusiasm for the results of genetic studies in epilepsy. Failure to replicate is most often attributed to multiple testing, type I error considerations (i.e., the belief that the original finding was a false positive) (Tan et al., 2004), population stratification, sample size (Tan et al., 2004; Durner et al., 2006), and genetic heterogeneity (Pal et al, 2008). The main concepts to keep in mind while trying to evaluate a putative gene modification attributable to a idiopathic epilepsy must be independent



replication of the gene association and coherence of evidence. Indeed, independent replication of association is an important criterion in judging evidence, yet the absence of replication may not necessarily invalidate an original finding. Therefore, it has been suggested to adopt a perspective integrating results from different experimental methods, rather than place one over another in importance or insisting only on replication. Coherence of experimental methods is a more informative approach than simple replication, because it forces one to evaluate different kinds of experimental data in the context of findings from other approaches.

Concluding, the last few decades have seen an increasing number of reports on the genetic basis of idiopathic epilepsies and on the involvement of genetics and gene expression in epileptic phenomena, confirming that genes and their protein products play a pivotal role in defining the outcome of the disease and the subtle differences in epileptic manifestations. Therefore, comprehension of the complex genetic mechanisms underlying epileptogenesis may allow a better understanding of the pathology and may help define new therapeutic targets.

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# GENERAL AIMS OF THE STUDY AND THESIS STRUCTURE

The overall aim of this thesis has been to characterize three different genetic modifications that are directly or indirectly related to epilepsy, in an attempt to shed light on diverse genetic mechanisms leading to diverse epilepsy types or to susceptibility to seizures. These three different genetic mutations have been investigated under different points of view, with the common goal of increasing knowledge of the mechanisms by which they may eventually lead to the pathology.

Data have been organized in separate chapters:

1. the first chapter deals with a primary genetic epileptic syndrome, the Unverricht-Lundborg disease, and leads to the proposal of a pathogenetic mechanism for its onset and progression;
2. the second chapter deals with Fragile X mental retardation, a genetic disease that associates with epileptic seizures in 20-25% of the cases, and focuses on the study of the subcellular targeting of the gene product whose silencing causes Fragile X (FMRP);
3. in the last chapter, we investigated a putative susceptibility gene, the bradykinin B1 receptor gene, by studying the susceptibility to seizures of B1 knockout mice in two different models of epilepsy.



Chapter II

# **Unverricht-Lundborg disease**

a mouse model for CSTB deficiency

(this chapter is based on the paper Franceschetti et al, 2007)

(work have been conducted in collaboration with the Institute C. Besta in Milan with Prof Franceschetti Silvana and collaborators)





# INTRODUCTION

## *Myoclonus*

Myoclonus is a brief, sudden and involuntary movement of a muscle or a group of muscles. It is defined as positive myoclonus when active muscle contraction is involved, and negative when an inhibitory muscle stimulus underpins the phenomenon. It can present as a single myoclonus or a group of subsequent muscle movements and may develop casually or following a precise scheme. Myoclonus usually arises from brain alterations involving cortical, sub-cortical and spinal cord areas. It can be benign, as it is hiccups (a myoclonus of the diaphragm), or represent a symptom in different pathologies: multiple sclerosis, Parkinson disease, Alzheimer disease, Creutzfeldt-Jakob disease, epilepsy.

ILAE (International League Against Epilepsy) defined 4 different main kinds of myoclonus:

1. Cortical myoclonus (CM): CM is due to hyperexcitability of the sensorimotor cortex, and each muscle jerk results from a neuronal discharge in the sensorimotor cortex.
2. Thalamocortical myoclonus: in thalamocortical myoclonus a thalamocortical loop is involved. In both instances, CM and thalamocortical myoclonus conduction velocity from cortex to muscles is fast: -60 m/s. Timing of muscle innervation after a cortical discharge shows a rostrocaudal lag, with muscles innervated by first cranial nerves contracting initially and those innervated by last cranial nerves contracting later. CM combines a brief (20-75 ms) contraction of both agonist and antagonist muscles.
3. Reticular reflex myoclonus (RRM): RRM results from hyperexcitability of the caudal reticular formation. In contrast to those in CM, electromyographic (EMG) events reflect sequential innervation, with the ninth, seventh and then fifth cranial nerves innervated in that order. Muscle jerks, which last 80-330 ms, precede cortical events. RRM may affect a single muscle or both agonist and antagonist muscles, with proximal muscles often affected, usually bilaterally.
4. Negative myoclonus (NM): NM consists of the inhibition of muscular activity and can be demonstrated as lapses interrupting previously continuous muscular activity when the patient is maintaining a posture.

In terms of bodily involvement, myoclonus and myoclonic seizures may be focal (i.e. confined to one region), regional (i.e. affecting two or more contiguous regions) or generalized involving the whole body. They differ from tonic-clonic seizures in that are of much shorter duration and involve much less movement. In terms of timing, myoclonic seizures can consist of single jerk or repetitive

jerks. Myoclonic seizures may also be rhythmic or arrhythmic. In terms of amplitude, they can be small (e.g. no joint movement) or massive contractions (e.g. movement of extremities, trunk and/or head). To further complicate their classification, myoclonic seizures can occur unilaterally or bilaterally and, if bilaterally, symmetrically or asymmetrically (Leppik, 2003).

A myoclonus is termed epileptic when it occurs in combination with cortical epileptiform discharges, and as illustrated by ILAE epileptic myoclonus should be distinguished from the following conditions:

- Non-myoclonic epileptic seizures, including spasms, which are more prolonged, occur in clusters, and are combined with a high-amplitude EEG slow wave. They should also be distinguished from tonic seizures, which are associated with EEG low amplitude fast activity;
- Non-epileptic myoclonus, including opsoclonus myoclonus syndrome, sleep myoclonus and progressive distonia;
- Non-epileptic, non-myoclonic phenomena, particular tremor in which the contraction affects agonist and antagonist muscles alternatively and is more rhythmic than myoclonus. Examples may be TICs and Chorea.

Electrophysiological criteria for differentiating epileptic from non-epileptic myoclonus have been summarized by Hallett (Hallett, 1985). In general, epileptic myoclonus has an EEG correlate of spikes, multispikes and spike-wave or multispikes-wave complexes. The EGM pattern for epileptic myoclonus usually consists of a short burst, <59 ms in duration, whereas non epileptic myoclonic events are associated with long bursts of 50-300 ms in duration (Hallett, 1985)

### *Myoclonus and epilepsy*

Myoclonus and myoclonic seizures may associate to epilepsy in various combination. Positive myoclonus of cortical type associates to progressive encephalopathies such as Unverricht-Lundborg disease or Rasmussen pathology, and positive myoclonus of thalamo-cortical origin is usually associated to non-progressive generalized epilepsy and mainly involves idiopathic generalized epilepsies [i.e. Benign Myoclonic Epilepsy of Infancy (BMEI), Juvenile Myoclonic Epilepsy (JME), Severe Myoclonic Epilepsy of Infancy (SMEI), etc]. Myoclonus also associates to malformations and chromosomal aberrations such as Angelman syndrome. Therefore, myoclonus associates to

several kinds of epilepsies and its classification is sometimes very difficult. On the other hand, its classification is of high importance because counselling and treatment depend on accurate diagnosis and confusion between the different kinds of myoclonus may be devastating.

Myoclonus can be classified in *epileptic* and *non-epileptic* myoclonus. Epileptic myoclonus is then divided in *inherited* and *acquired* type.

Acquired myoclonic epilepsies are secondary to brain altering events which predispose the brain to manifest myoclonic epileptic symptoms. Such precipitating traumatic events may be represented by anoxia, head trauma, stroke, tumors, metabolic encephalopathies, degenerative central nervous system disease and/or viral infections (Leppik, 2003).

Inherited myoclonic epilepsies are related to genetic alterations and can be divided in progressive and non progressive diseases. Non progressive myoclonic epilepsies are clinically stable and their symptoms do not progress in time. The most representative ones are: the Benign Myoclonic Epilepsy of Infancy (BMEI) and the Juvenile Myoclonic Epilepsy (JME). Both show generalized myoclonic epilepsy, tonic-clonic seizures and absence seizures. Other non progressive myoclonic disorders are: severe myoclonic epilepsy of infancy (SMEI) and myoclonic atstatic epilepsy (MAE). Progressive myoclonic epilepsies (PME), on the other hand, show progression in symptoms with worsening of the clinical picture. Progressive myoclonic epilepsies are characterised by myoclonic seizures, tonic-clonic seizures, and progressive neurological deterioration. Myoclonus, in PME, is typically fragmentary and multifocal, and is often precipitated by posture, action, or external stimuli such as light, sound, or touch. It is particularly apparent in musculature of the face and distal extremities. Bilateral massive myoclonic jerks that tend to involve muscles of proximal limbs may also occur (Shahwan et al, 2005). The most representative PMEs are reported in tab 1. Among these, Unverricht-lundborg disease is the most common in the world population. Moreover, recent studies have been able to uncover the genetic alterations which underpin several PME (see table 1 for a schematic description and references).

Table 1: genetic alterations of PME

<i>disease</i>	<i>locus/chromosome</i>	<i>protein</i>	<i>references</i>
Unverricht-Lundborg disease	EPM1/21q22.3	Cystatin B (CSTB)	Pennacchio et al. (1996)
Lafora disease	EPM2/6q24	laforin	Minassian et al. (1998), Ganesh et al. (2000)
Neuronal ceroid lipofuscinosis (NCL)	TTP1/11p15 CLN3/16p12, CLN6/15q21, CLN5/13q22,	tryptidylpeptidase 1 (NCL2) membrane protein CLN	Sleat et al. (1997), Rawlings and Barret (1999), Vines and Warburton (1999) International Batten Disease Consortium (1995) ; Gao et al. (2002), Wheeler et al. (2002) ; Savukoski et al. (1998)

<i>disease</i>	<i>locus/chromosome</i>	<i>protein</i>	<i>references</i>
Myoclonic epilepsy with ragged red fibres (MERRF)	MTTK/mtRNA	tRNA for lysine	Shoffner et al. (1990), Yoneda et al. (1990)
Sialidoses	NEU1/6p21 NEU1/6p21 e cr.20	$\alpha$ neuraminidase. (S type1) N-acetyl neuraminidase and $\beta$ -galactosialidase, (S type 2)	Bonten et al. (1996), Pshezhetsky et al. (1997)
Dentatorubral-pallidolusian atrophy (DRPLA)	DRPLA/12p13	atrophin 1	Shahwan et al. (2005), Leppik (2003)

## *Clinical features of Unverricht-Lundborg disease*

### *Unverricht-Lundborg disease (ULD)*

The most common form of PME is the Unverricht–Lundborg disease (EPM1; OMIM #254800), an autosomal recessively inherited disorder. EPM1 was first described by Unverricht in 1891 and subsequently by Lundborg in 1903 (Unverricht, 1891; Lundborg, 1903). Initial epidemiology studies reported high incidence in defined world regions such as the Baltic peninsula and the mediterranean basin, giving it its first names of Baltic myoclonus (or Baltic myoclonic epilepsy) and Mediterranean myoclonus. In the last few decades, though, several have been the descriptions of this disease all over the world, making it more widely spread, and maybe underdiagnosed, then expected (Janssen, 1954; Shakir et al, 1992; Acharya et al, 1995; Vistorte et al, 1999; Mazarib et al, 2001; Kalviainen et al 2008).

Onset of this disorder, which occurs between 6 and 16 years of age, is characterized by stimulus-sensitive myoclonic jerks in half of the patients, and generalized tonic clonic seizures in almost the other half, as first recognized symptoms. Myoclonus is usually precipitated by stimuli such as light, physical exertion, noise and stress. Either myoclonus and tonic-clonic seizures worsen in frequency and severity as the pathology progress, with their main evolution 5-10 and 3-7 years from disease onset respectively (Kalviainen et al 2008, Lehesjoki and Koskiniemi, 1999). During the progression of the disease, worsening of the myoclonus, which leads sometimes to status myoclonicus, makes one third of the patients severely incapacitated, like wheelchair bounded and unable to eat or drink by themselves (Kalviainen et al 2008). Neurological findings are initially absent or very mild, but, as the disease progresses, signs of cerebellar ataxia, incoordination, intentional tremor, and

dysarthria start to become evident (Kalviainen et al 2008; Lehesjoki and Koskiniemi, 1999; Shahwan et al, 2005). Mild intellectual performance impairment is also observed in patients with Unverricht-Lundborg disease which, however, maintain a relatively good mental alertness. In a few cases though, dementia may progress, leading to severe cognitive involvement (Shahwan et al, 2005). Moreover, patients show emotional lability and depression (Kalviainen et al 2008).

Debate on EEG characteristics is raging, questioning the initially reported slow background activity in EPM1 patients (Koskiniemi et al, 1974b). In fact, a retrospective evaluation of the EEG from 25 ULD patients, reports no signs of slow background, or a very mild one (Ferlazzo et al, 2007). These discrepancies may be justified by the use, in the old studies, of therapies (such as phenytoin) that can worsen the clinical features of the pathology (Iivanainen and Himberg, 1982; Ferlazzo et al, 2007). What seems to be true is that EEG abnormalities, such as spike-wave discharges, photosensitivity, and polyspike discharges during REM sleep (Tassinari et al., 1974), are more pronounced during the initial stages of the disease, usually associated to onset of myoclonus, tonic-clonic seizures and their worsening, which in turn tend to diminish while, in years, the disease stabilizes. On average, in 15 years from disease onset a reduction of epileptic seizures and physiological sleep patterns are evident (Lehesjoki and Koskiniemi, 1999) reflecting a stabilization of the EEG (Kalviainen et al 2008).

One MRI study reports very mild anatomical changes in patients in late stages of ULD, with loss of neuronal volume in pons, medulla and cerebellar hemispheres (Mascalchi et al, 2002). At disease onset MRI is usually normal.

Outcome of the disease was very dramatic in the past, with patients dying before their thirties (Koskiniemi et al., 1974a, Lehesjoki and Koskiniemi, 1999). With the new therapeutic approaches life expectancy of EPM1 affected individuals has increased, spanning till their sixties or seventies (Kalviainen et al, 2008; Shahwan et al, 2005; Lehesjoki and Koskiniemi, 1999). Indeed, even though pharmacological therapy for ULD is, unfortunately, only symptomatic, this leads to a much ameliorated condition. Valproic acid is the drug of choice for the management of myoclonus and can also reduce generalized seizure frequency (Shahwan et al, 2005; Kalviainen et al, 2008). Clonazepam and other benzodiazepines are used as add-on therapy for the treatment of myoclonic seizures or as intravenous emergency treatments when myoclonus is exacerbated in series or into status myoclonicus (Shahwan et al, 2005; Kalviainen et al, 2008). For emergency intervention valproate and levetiracetam prove the same efficacy (Kalviainen et al, 2008). Piracetam has been found useful only in myoclonus treatment and topiramate and zonisamide can be used as add-on therapies (Shahwan et al, 2005; Kalviainen et al, 2008). Phenytoin, and sodium channel blockers in general, must be avoided and GABAergic drugs (tiagabine and vigabatrin) as well as gabapentin

and pregabalin must not be used because they may aggravate myoclonus and myoclonic seizures (Shahwan et al, 2005; Kalviainen et al, 2008). Brivacetam, a SV2A ligand, which is under study and appear to provide a good control of seizures and myoclonus in epileptic experimental models, is being currently investigated as add-on therapy for ULD (Truong & Tai, 2005; von Rosenstiel, 2007). Management of Unverricht-Lundborg disease comprehend also psychosocial support, which is of key importance for a comprehensive rehabilitation of patients. Alleviating psychological liability and depression, usually associated to the disease, allows patients to be better accepted socially. A constant clinical follow-up is mandatory for EPM1 affected individuals (Kalviainen et al, 2008).

Other therapeutic approaches are: vagus nerve stimulation, which reduces seizures and may significantly improve cerebellar function on neurological examination (Smith et al, 2000; Kalviainen et al, 2008), and apomorphine, due to the involvement of the dopaminergic system shown to underlie the pathogenesis of EPM1 (Mervaala et al, 1990; Korja et al, 2007). Indeed, apomorphine has been proven to be effective in a case of ULD (Mervaala et al, 1990).

The molecular basis of Unverricht-Lundborg Disease has been identified using positional cloning techniques (Lehesjoki et al, 1991; Lehesjoki et al 1993; Pennacchio et al, 1996; Stone et al, 1996; Virtaneva et al, 1996). Alterations of the gene for Cystatin B are responsible for the phenotype. To date, 10 mutations have been reported (fig. 1) and the one related to a dodecamer expansion accounts for about 90% of affected individuals. The other gene alterations are point mutations responsible for miss-sense transduction, altered splicing, frame shifts and introduction of stop codons. An extensive description of molecular basis of the disease is reported below.

Diagnosis for ULD has specific parameters to evaluate and occurrence of more than one of the following particular symptoms must be considered predictive of the disease:

1. age of onset between 6 and 16 years
2. evident or mild myoclonic jerks, especially if stimulus-precipitated
3. experience of generalized tonic-clonic seizures
4. mild neurological signs in motor function (i.e. clumsiness, mild dysmetria, mild ataxia)
5. photosensitive induced EEG abnormalities (i.e. generalized spike-and-wave and polyspike-and-wave paroxysms)
6. MRI uncovered brain anatomic changes such as cortical and central atrophy in old patients (MRI is normal in the beginning of the disease)
7. progression of initially reported symptoms, mostly myoclonus and neurological signs.

Therefore, clinical diagnostic examination should include evaluation of walking, coordination, handwriting, school performance and emotional features. In addition, essential evaluation of myoclonus must be carried out in conditions of rest, action and in response to precipitating stimuli.

Confirmation of a diagnosis of ULD consists in genetic detection of the CSTB gene alteration (Kalviainen et al, 2008).

## *Molecular basis of Unverricht-Lundborg disease*

### *Cystatin B (CSTB)*

EPM1 is associated with a defect in the cystatin B (CSTB) gene. The CSTB gene, positioned on chromosome 21 in the 21q22.3 locus, has been identified by positional cloning in the nineties (Lehesjoki et al, 1991; Lehesjoki et al 1993; Pennacchio et al, 1996; Stone et al, 1996; Virtaneva et al, 1996) it encodes Cystatin B, a cysteine protease inhibitor (Jarvinen and Rinne, 1982; Ritonja et al, 1985; Abrahamson et al, 2003). CSTB is a member of the cystatin superfamily, which includes three distinct types: type-1 (also termed stefins), -2 and -3. Based on molecular properties and evolutionary relationship, CSTB is classified as a type-1 cystatin (Abrahamson et al, 2003). Firstly described as a cytoplasmic protein with apparent concentration at membranes of vesicular structures (Calkins et al, 1998), CSTB was then reported to be mainly nuclear in proliferating cells and present in both cytoplasm and nucleus in differentiated cells (Riccio et al, 2001).

Principal substrates for CSTB are a group of proteins called cathepsins. Based on in vitro experiments, CSTB seems to mainly interact with cathepsins B, H, L and S (Jarvinen and Rinne, 1982; Ritonja et al, 1985; Turk and Bode, 1991; Lenarcic et al, 1996; Saha and Usdin, 2001). In vivo, in lymphoblastoid cells from EPM1 patients, reduced expression of CSTB results in an increased B, L, and S cathepsin activity (Rinne et al, 2002) proving the fact that CSTB has a fundamental interaction with cathepsins. On the other hand, CSTB and cathepsins tissue distribution do not always overlap (Calkins et al, 1998) leading to think that CSTB may also act via other interactions. Indeed, Di Giaimo and coworkers reported a CSTB interaction with non-cathepsin proteins in cerebellar cells. With GST-pull down and co-immunoprecipitation experiments Di Giaimo demonstrated CSTB involved in a multi-protein complex which function is still unknown (Di Giaimo et al, 2002).

Structurally, CSTB is a low weight protein of about 100 amino-acid in length with no disulphide bonds; its secondary structure consists of a five-stranded beta sheet wrapped around a five-turn alpha helix (the human one contains an additional C-terminal strand that runs along the convex side of the beta sheet) (Bode et al, 1988; Stubbs et al, 1990). Three have been demonstrated to be the sites of major interaction for cathepsins on CSTB: 1) a Glycine at N-terminal of the protein, 2) a highly conserved amino acid sequence, QVVAG, in the middle of the peptide and 3) LP at the C-

terminus (Stubbs et al, 1990). The extended C-terminal region in human CSTB introduces an additional hydrophobic interacting site (Joensuu et al, 2007).

The main function of CSTB, as of other cystatins of the family, is to ensure protection of cells against the proteolytic activity of lysosomal peptidases (cathepsins in the CSTB case) that are released occasionally during normal cell death or activated by proliferating cancer cells. Indeed, the main function of cathepsins is the nonselective degradation of intracellular proteins to peptides and amino acids, but they also participate in antigen processing (Turk et al., 2000; Rinne et al., 2002). Cathepsins have been also involved in apoptosis and caspase-independent cell-death (such as autophagy, paraptosis and slow cell death) leading to the idea that CSTB can indirectly regulate, in physiological conditions, cathepsins action on these two phenomena (Broker et al, 2005; Chwieralski et al, 2006).

*Molecular alterations of CSTB leading to the Unverricht-Lundborg phenotype.*

To date, 10 genetic alterations have been related to ULD phenotype (fig. 1)



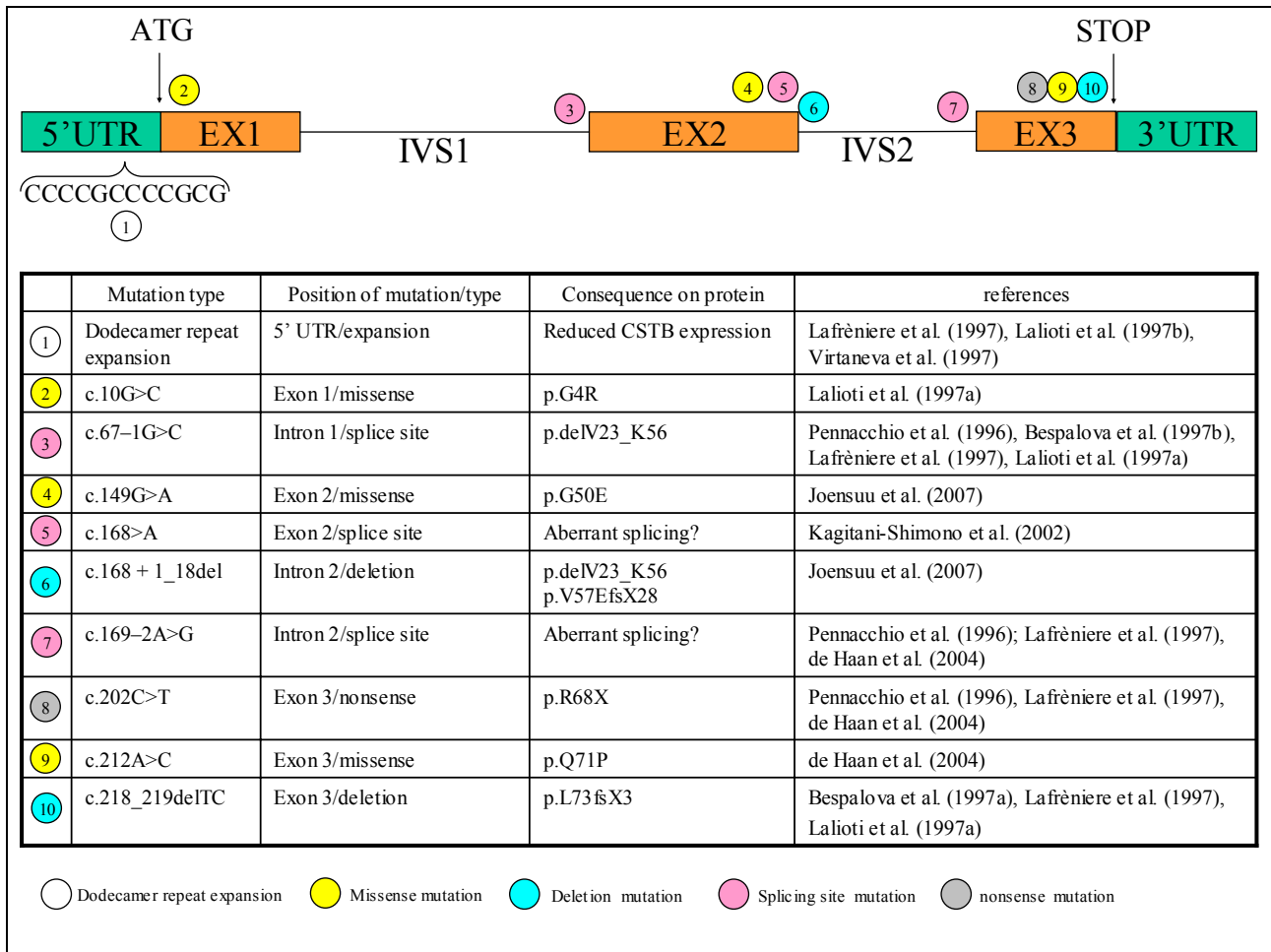


fig. 1: Genetic modifications associated to ULD phenotype. (adapted from Joensuu et al 2008).

Among these, the 12-nucleotide repeat unstable expansion accounts for about 90% of EPM1 affected individuals (Pennacchio et al., 1996; Bernalova et al., 1997; Lafrèniere et al., 1997; Lalioti et al., 1997a, 1997b; Virtaneva et al., 1997; Kagitani-Shimono et al., 2002; Joensuu et al., 2007). The 5'-CCCCGCCCCGCG-3' dodecamer is found at 175 bp from the promoter region of the normal CSTB gene in 2-3 copies and results in a functional expressing gene. The expansion of at least 30 copies of this dodecamer alters the CSTB gene reducing its mRNA expression to less than 10% (Joensuu et al, 2007) and, consequently, to a very low expression of its own protein (Rinne et al, 2002). The worst case reported has 125 repetitions of the dodecamer in its promoter region (Virtaneva et al, 1997). Human pre-mutation, found to date only in the form of 12 to 17 dodecamer copies (Lalioti et al, 1997b; Alakurtti et al, 2000), caused a reduced mRNA expression with no evident symptoms compatible with EPM1. To date, no other kind of pre-mutation has been found in the human population. Correlations between the length of dodecamer expansion and phenotype, mainly age of onset and severity, have never been found. The lowest expression level of CSTB protein compatible with a normal phenotype is yet to be determined (Joensuu et al, 2008).

The reduction of gene expression due to the dodecamer expansion can be explained in different ways:

- dodecamer repetition introduces more space between promoter elements, leading to the impossibility for the transcription factor complex to properly interact with the promoter region (Lalioi et al, 1999)
- dodecamer repetition allow DNA to form tetraplex secondary structures which, again, result in disrupt spacing of promoter elements and in turn to reduced transcription of the gene. Even mRNA could be affected by reduced transduction decreasing the quantity of protein available. (Pataskar et al, 2001; Saha and Usdin, 2001)
- iper-methylation may be another mechanism for the reduced expression of the CSTB gene altered by dodecamer repetition, but experimental data do not support this evidence (Lalioi et al, 1997b).

The nine remaining CSTB mutations underlying EPM1 affect splice sites (c.67-1G>C, c.168G>A, c.168 + 1 18del, c.169-2A>G), result in amino acid changes (c.10G>C, p.G4R; c.149G>A, p.G50E; c.212A>C, p.Q71P) or predict truncated proteins (c.202C>T, c.218 219delTC) by producing a premature stop codon (p.R68X) or a frameshift (p.L73fsX3) (fig 1). These mutations usually appear in compound heterozygous form with the dodecamer expansion.

Only p.G4R has been reported to be capable to induce the EPM1 phenotype alone (Lalioi et al, 1997a). This mutation results in substitution of highly conserved Glycine amino acids in position 4 of the protein, with disruption of important interaction sites of CSTB with cathepsins. This latter mechanism seems also to be the base for the other miss-sense mutations.

Because of the high prevalence of the dodecamer expansion mutation in individuals affected by ULD, we can conclude that a general loss of function for the CSTB protein, mainly due to its decreased expression, seems to be the primary pathological mechanism in the majority of EPM1 patients.

Another, still not fully identified, mutation has been associated to an inbred Arab family by Berkovic and collaborators which found, through homozygosity mapping in a genomewide scan, another underlying locus for EPM1 (called EPM1B) linked to a 15-megabase region on chromosome 12 (Berkovic et al., 2005). The corresponding mutated gene remains to be identified. This new finding complicates the genetic picture for Unverricht-Lundborg disease, implying that other, still unknown, genes may be involved in establishing the EPM1 phenotype.

### *CSTB Knock-out mice: an animal model of Unverricht-Lundborg disease*

A mouse model for the human disease has been developed by targeted disruption of the CSTB gene (Pennacchio et al., 1998). Mice have been created on the base of two genetic background: isogenic 129Svj and mixed background of C57Bl6/129Svj. Several studies support the notion that, in general, CSTB knock-out (KO) mice are a good model of EPM1. Indeed, CSTB knock-out mice display a behavioural phenotype that replicates main symptoms reported in human patients, including progressive ataxia and myoclonic jerks, although convulsive seizures appear to be rare (Pennacchio et al., 1998). In mice, myoclonus is not stimulus sensitive and action activated as it is in humans, but develops already at 1 month of age during sleep and only in mice with an isogenic 129Svj background (Pennacchio et al., 1998). Moreover, no tonic-clonic seizures, photosensitivity, or spike-wave complexes in EEG have been observed in KO mice (Joensuu et al., 2008). Progressive ataxia starts in mice around 6 months of age as shown by still and rotating rotarod tests (Pennacchio et al., 1998). Ataxia is independent from genetic background. Analysis of the CSTB-deficient brain, in keeping with human studies (Haltia et al., 1969, Koskiniemi et al., 1974a, Eldridge et al., 1983, Mascalchi et al., 2002, Lehesjoki, 2003), reveals the presence of apoptosis, mainly involving cerebellar granule cells (Pennacchio et al., 1998), and cerebellar atrophy (Shannon et al., 2002). It has also been reported that neuronal apoptosis associated with gliosis involve other brain structures, including the cortical and subcortical grey matter (Shannon et al., 2002). These findings are in line with the notion that CSTB has a role in preventing cell damage and, thus, EPM1 may be classified as a primary neurodegenerative disorder.

Other features of CSTB KO mice, even though not in line with human phenotype, are development of progressive corneal lesions that start around three months of age (Pennacchio et al., 1998) and a significant reduction of body and brain weight (Shannon et al., 2002).

Since their creation, CSTB KO mice helped to shed light on the molecular and structural alterations associated to CSTB deficiency.

1. Lieuallen and collaborators demonstrated, in micro-array experiments, that Cystatin B deficiency leads to over-expression of cathepsins S, C1q B-chain of complement, beta2 microglobulin, glial fibrillary acid protein, apolipoprotein D, fibronectin one and metallothionein II in the brain of CSTB KO mice, which are expected to be involved in increased proteolysis, apoptosis and glial activation (Lieuallen et al., 2001).
2. In a study involving double mutant CSTB KO/Bid KO mice, Houseweart and collaborator found that cathepsins are still able to promote apoptosis even in the absence of Bid, indicating that these proteases mediate apoptosis via a different pathway, or that some other

molecule can functionally substitute for Bid in this system (Houseweart et al, 2003b).

3. Again, Houseweart and colleagues demonstrated that removal of cathepsins L or S from cystatin B-deficient mice did not ameliorate any aspect of the EPM1 phenotype, but removal of cathepsin B resulted in a 36–89% reduction in the amount of cerebellar granule cell apoptosis depending on the mouse age. These findings establish cathepsin B as a contributor to the apoptotic phenotype of cystatin B-deficient mice and humans with EPM1. They also suggest that the identification of cathepsin B substrates may further reveal the molecular basis for EPM1 (Houseweart et al, 2003a).
4. Arbatova and colleagues demonstrated that the tryptophan metabolism along 5-HT and KYN pathways is disrupted in EPM1 (Arbatova et al, 2005).
5. Kopitar-Jerala et al. found that sensitization to apoptosis induced by STS in thymocytes of CSTB-deficient and wild-type mice is not dependent on cathepsin inhibition by stefin B (Kopitar-Jerala et al, 2005).
6. Kopitar-Jerala and Turk observed increased cleavage of MARCKS in brain and macrophages of CSTB-deficient mice compared to wild-type mice. They also showed that processing of cathepsin B was unaltered in the brain of CSTB-deficient mice, leading to the conclusion that increased cleavage of MARCKS could be attributed to the lack of inhibitor (Kopitar-Jerala and Turk, 2007).
7. Kaasik et al, showed a partial decrease in cystatin B expression in heterozygous mice for CSTB, which in turn leads to development of a mild EPM1 phenotype (Kaasik et al, 2007).
8. Cipollini et al showed that, *in vivo*, in the CSTB KO model, cystatin B has a highly resistant, polymeric structure which is sensitive to the redox environment and when over-expressed generates aggregates (Cipollini et al, 2008).
9. O. H. Manninen and collaborators detected, by the means of MRI, a significant decrease in volume of the whole brain, hippocampus and striatum in the CSTB KO mice. Moreover, the MRS revealed significant differences in metabolic profiles between the two groups, which showed significant decreases in glutamate, N-acetyl aspartate, taurine, and other metabolites (Manninen et al, 2008).

In conclusion, CSTB KO mice mimic fairly well the human disease and represent a very good tool to study the pathophysiology of Unverricht-Lundborg disease and to uncover the still hidden functions of Cystatin B in the brain and in other tissues. Extensive investigation on this animal model may shed light on the mechanisms underpinning EPM1 leading to a possible, more targeted, therapy for ULD affected individuals.

## AIM OF THIS STUDY

This study has been designed to begin exploring the mechanism of EPM1 onset and progression. Our working hypothesis stems from the observation that treatments capable of counteracting seizures are also capable of lessening the rate of disease progression. This suggests that seizure-linked excitotoxic events (see, for a review, Sutula et al., 2003) might play a role in determining the progression of the degenerative processes sustaining the severity of the disease. Thus, we hypothesized that the onset of the disease may be related to a latent hyperexcitability of the EPM1 brain, and that the progression may depend on higher susceptibility to seizure-induced damage. To challenge this hypothesis, we performed a series of experiments in the CSTB-deficient mouse model of EPM1. First, by means of electrophysiological recordings, we investigated the presence of changes in excitability and in susceptibility to seizures in the hippocampus of CSTB-deficient mice *in vitro*. Second, we explored the susceptibility of CSTB-deficient mice to kainate induced generalized seizures and to seizure-induced damage *in vivo*, by analysing markers of degeneration.



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Chapter III

# **Fragile X**

Dendritic targeting of Fmr1 and FMRP in a model of status epilepticus.

(based on submitted paper Ferrari et al.)

(work have been conducted in collaboration with the University of Rome, Torvergata, with Prof. Claudia Bagni and collaborators)



# INTRODUCTION

## *Fragile X*

Fragile X syndrome (FXS) is the most common inherited cause of mental retardation. Being an X-linked pathology, prevalence results worse in male than females with values of 1 out of 4000 for males and 1 out of 8000 for females (Crawford et al, 2001). FXS is an X-linked dominant disorder which inheritance transmission follows what is called Sherman Paradox. Genetic penetrance for the disease increases in succeeding generations of the pedigree (O'Donnell and Warren, 2002), with non penetrant male carrier of the Fragile X gene defect which transmits its modified allele to a non penetrant daughter which, in turn, may give birth to full mutant off-springs (Sherman et al, 1985; Fu et al, 1991; Bassell and Warren, 2008).

This particular inheritance is due to the genetic characteristics of the defect associated to the pathology: a trinucleotide (CGG) expansion in the 5'untranslated (UTR) region of the *fmr1* gene positioned on the X chromosome.

*Fmr1* has been identified as the main cause for FXS by Verkerk and collaborators (Verkerk et al, 1991) using the positional cloning technique; this has been the first example of a trinucleotide repeat mutation. The principal mutation on this gene is the reported expansion of the CGG triplet in its 5'UTR. Normal alleles contain less than 40 repetitions for CGG trinucleotide, with an average 30 trinucleotides expansion (Snow et al, 1993). The full mutation, instead, is more than 200 trinucleotide repetitions (Snow et al, 1993; Fu et al, 1991). Average repetitions in full mutation usually span in the order of 800 triplets. Individuals with that many triplets show the full phenotype of Fragile X. In between there are other two situations: one called *intermediate allele* with 41-54 repetitions, which does not associate with gene instability, and phenotypical features, and *premutation allele*, which spans from 55 to 200 repetitions (Garber et al, 2008). Premutations are meiotically unstable (Snow et al, 1993; Fu et al, 1991) and are not clinically phenotype free. Indeed, the premutation allele associates with two different adult syndromes: FXTAS, Fragile X Tremor and Ataxia Syndrome, and POF, Premature Ovarian Failure. FXTAS appears in a subgroup of men presenting premutation which develop neurological symptoms beyond age 50: intentional tremors, balance problems, frequent falls, neuropathy, autonomic dysfunction, cognitive decline and progressive dementia (Jacquemont et al, 2004; Willemsen et al, 2005). FXTAS has a very low prevalence in females. POF occurs in about 20% of women carriers for the premutation and is

represented by premature cessation of menses before age 40 (Allingham-Hawkins et al, 1999). Both clinical manifestations seem paradoxically related to an over expression of *fmr1* mRNA, which occurs only in premutation carriers (Cornish et al, 2008).

Since when repeat expansion has been discovered and thought to be the main cause for FXS (98-99% of FXS individuals has this kind of mutation), other conventional mutations, such as non-sense and missense mutations, for *fmr1* went underestimated (Bassell and Warren, 2008), even though, 1% of FXS displaying patients have recognised missense mutations (Sherman et al, 2005; Feng et al, 1997a).

The full mutation associates with hypermethylation, which is responsible for silencing *fmr1* gene expression (Sutcliffe et al, 1992; Coffee et al, 1999). The phenotype for Fragile X is therefore due to absence of FMRP, the protein product of the *fmr1* gene.

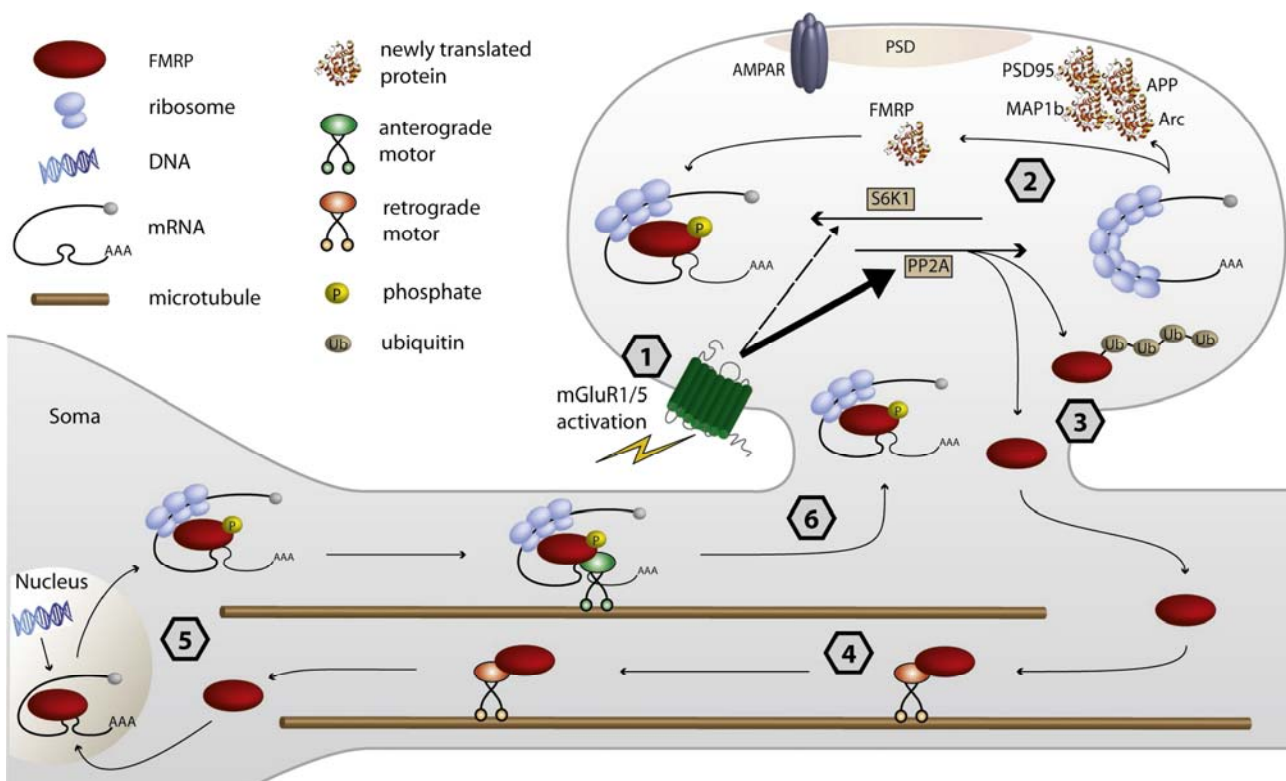
FMRP is a protein of about 71 KDa, depending on its different isoforms deriving from alternative splicing of the *fmr1* gene (Bassell and Warren, 2008). FMRP, an mRNA binding protein (Ashley et al, 1993), is widely expressed in several tissues included the brain, where is thought to play an important role in regulating mRNA expression (Bassell and Warren, 2008). Structurally, it is a protein expressing both signals for nuclear localization and for nuclear export, and it seems to shuttles between nucleus and cytoplasm of the cells (Eberhart et al, 1996; Sittler et al, 1996). The best characterized motifs on FMRP are two hnRNP-K-homology (KH) domains and an Arginine-Glycine-Glycine (RGG) box. In in-vitro experiments the, RGG box recognize a stem-G-quartet loop structure in RNA and most of the RNAs that have been associated to FMRP (Brown et al, 2001; Darnell et al, 2001) seem to contain this structure. Several studies confirm this interaction and enlarge this concept to a more general interaction of the RGG box to G-rich regions, including G-quartet regions (Menon et al, 2008; Menon and Mihailescu, 2007; Schaeffer et al, 2001; Lu et al, 2004; Zalfa et al, 2003; Zhang et al, 2001; Zalfa et al, 2007; Todd et al, 2003; Darnell et al, 2001; Westmark and Malter, 2007). The putative role of KH-domains is to bind FMRP to actively translating polyribosomes (Feng et al, 1997b; Khandjian et al, 2004; Stefani et al, 2004). Their possible interactions with mRNAs are still unclear: current hypothesis include the formation of the so called “kissing complex”, represented by more than one mRNA that, combined, create a KH recognized structure (Darnell et al, 2005) or by mRNA complexed with microRNAs that allow indirect interaction between KH FMRP domains and mRNAs (Jin et al, 2004). Another possible interaction is the controversial binding of FMRP to mRNA through BC1 RNA (Zalfa et al, 2003; Iacoangeli et al, 2008a; Bagni, 2008; Iacoangeli et al, 2008b).

Due to the above described interactions, FMRP is thought to regulate local protein synthesis (Antar and Bassell, 2003; Bagni and Greenough, 2005; Grossman et al, 2006) and several works support

the hypothesis that FMRP binds and regulates expression of dendritic mRNAs (Antar et al, 2005; Kindler et al, 2004; Miyashiro et al, 2003; Zalfa et al, 2003; Bramham and Wells, 2007). FMRP has not been demonstrated to be important for steady-state maintenance or constitutive localization of mRNAs at the dendrites (Steward et al, 1998; Muddashetty et al, 2007; Miyashiro 2003) and its role in mRNA stabilization is debated (Zalfa et al, 2007; Muddashetty et al, 2007). More convincing evidence suggests that FMRP may be implicated in shuttling a subset of dendritic mRNAs, accordingly to a model whereby the dynamic association of FMRP with translationally repressed, microtubule-associated mRNP complexes and polyribosomes might be a mechanism linking mRNA transport in dendrites with translational regulation at synapses (Wang et al., 2008).

Regulation of mRNAs expression by FMRP is mainly repressive, leading to an excessive protein synthesis when FMRP is absent, as happens in FXS. In FXS excess protein expression is found even under steady-state conditions, leading to reduced plasticity response (i.e. increased protein synthesis) to synaptic activation. The prevalent view in fragile X syndrome is that the synaptic dysfunction and cognitive impairment are the result of excess protein synthesis at the synapse (Ronesi and Huber, 2008) and, while the excess translation is likely to be a major cause, it may be of equal concern that there is a loss of stimulus-induced translation. The inability of synapses in the FXS brain to control where and when translation precisely occurs and the inability to augment translation in response to synaptic activity are likely to have consequences on long-term plasticity, which influences learning and memory. Indeed, a loss of protein synthesis-dependent plasticity has been observed, at least with regard to Gp1 mGluR LTD (Hou et al., 2006; Huber et al., 2002) which introduce to the so called mGluR theory of fragile X syndrome. This theory states that FMRP normally acts as a negative regulator of translation downstream of Gp1 mGluRs and, in the absence of FMRP, there is an increase in protein synthesis that leads to excessive AMPAR internalization and exaggerated LTD (Bear et al., 2004). In addition, the mGluR theory suggests that many of the synaptic phenotypes in FXS may be directly attributed to exaggerated mGluR signaling, and therefore mGluR antagonists could be a useful therapy for FXS. There is solid evidence to support this theory (McBride et al, 2005; Tucker et al, 2006; Yan et al, 2005).

A model for FMRP functions and dynamic activation/repression in neurons is reported in figure 1 .



(from Bassel and Warren, 2008).

Figure 1: **The Stimulated Travels and Function of FMRP throughout the Neuron.**

FMRP is in a complex with several translationally arrested mRNAs at the synapse. Following mGluR stimulation, FMRP-target mRNAs are rapidly derepressed, allowing for local translation. A second phase of FMRP-dependent plasticity is shown that involves the subsequent transport of new mRNAs from the cell body into dendrites. The model shown here illustrates translational repression at the level of elongation, as suggested by Ceman et al. (2003). The translational activation and repression of mRNA is regulated by a PP2A/S6K1 signaling module (Narayanan et al., 2007; Narayanan et al., 2008). (1) Upon mGluR1/5 activation, PP2A is rapidly activated and dephosphorylates FMRP, thereby allowing for (2) local translation of proteins that affect AMPAR trafficking, i.e., PSD-95, Arc, Map1b, and App (Westmark and Malter, 2007; Todd et al., 2003; Muddashetty et al., 2007; Hou et al., 2006; Davidkova and Carroll, 2007; Waung et al., 2008; Park et al., 2008). Following mGluR activation, FMRP is rephosphorylated by S6K1 with slower kinetics, leading to translational repression. (3) FMRP can also be ubiquitinated following mGluR stimulation, and its proteasome-dependent degradation is necessary for mGluR-LTD (Hou et al., 2006). The local degradation of FMRP may contribute to local protein synthesis underlying mGluR-LTD. A mechanism of local FMRP degradation may be balanced by its synthesis. FMRP is synthesized in synaptoneurosomes upon mGluR activation (Weiler et al., 1997), which may provide a feedback mechanism to restore translational repression. Upon mGluR stimulation, (4) there may be a retrograde signal that leads to the transport of new FMRP-associated mRNAs from the soma. The active bidirectional transport of FMRP granules in dendrites has recently been described (Dichtenberg et al., 2008). This model speculates that FMRP itself may traffic from the synapse to the cell body and/or nucleus, where it may complex with new target mRNAs, and return to the activated synapse. (5) In that FMRP can shuttle into the nucleus (Eberhart et al., 1996), it will be interesting to assess whether nucleocytoplasmic trafficking is regulated by mGluR signaling. (6) FMRP has recently been shown to be necessary for the transport of several mRNAs into dendrites, whereas neurons cultured from *Fmr1* KO mice show impaired mRNA transport dynamics (Dichtenberg et al., 2008). This model speculates that the trafficking population of FMRP is phosphorylated; however, future work is needed to assess whether FMRP phosphorylation may influence mRNA trafficking.

FXS affects the individual from birth, even though it becomes evident only when retardation in the developmental milestones (such as motor and/or language delays) becomes evident. Individuals affected by FXS display very subtle physical characteristics all involving connective tissue dysplasia that brings to long and narrow face, prominent ears, joint hypermobility and flat feet (Hagerman et al, 1984). Macro-orchidism is also present and become apparent just prior puberty (Lachiewicz and Dawson, 1994).

Behavioural aspects of this disease comprehend autistic-like features including hand flapping, hand biting, gaze avoidance, tactile defensiveness and hyperarousal to sensory stimuli (Hagerman et al,



1986; Brown et al, 1986). All these features are expressed in varying degrees in children with FXS. Other features may be anxiety, mood disorders, hyperactivity, impulsivity and aggressive behaviour. Females with FXS display an higher emotional instability being more prone to social anxiety, shyness, social avoidance, withdrawal, language deficits, mood lability, and depression (Freund et al, 1993). Average IQ for full mutation affected patients may vary depending on degree of methylation of the triplet expansion. Fully methylated trinucleotide expansions lead to a very low IQ score of about 40 (Merenstein et al, 1996) but, when CGG repeats are not fully methylated, the IQ score is in the borderline or low normal range. Cognitive deficiency for FXS patients consists of working and short-term memory problems, impairment of executive function and mathematic and visuospatial abilities (Kemper et al, 1988). Females are much less impaired in cognitive function but, as already stated before, show a higher risk for emotional problems (Freund et al, 1993).

Neuroanatomical analysis of the FXS brain shows no gross abnormalities, but neurons have immature and dense dendritic spines: this is a neuroanatomical key feature in FXS (Irwing et al, 2001). It is believed that these differences represent a defect in dendritic spine development and maturation.

Finally, about 20% of FXS affected individuals present with seizures, correlating FXS to epileptic phenomena.

Diagnosis for FXS is based on clinical observation of developmental delay which eventually leads to a FXS molecular testing. Genetic testing for the CGG expansion consists of PCR analysis, with primers designed for the flanking regions of the expansion mutation, and southern Blot, that measures the entity of the expansion. Sequencing of the *fmr1* gene should be considered in the 1% of patients that display the clinical features of FXS and no expansion mutation is revealed by PCR and Southern Blot (Garber et al, 2008). Prenatal diagnosis has been performed and proved to be highly reliable (Brown et al, 1993).

Treatment for FXS is mainly symptomatic. Psychopharmacological therapies are usually combined with supportive strategies including speech therapy, sensory integration occupational therapy, individualized educational plans and tailored behavioural interventions to maximize functioning (Garber et al, 2008). General pharmacological approach consists of *stimulants* for symptoms of hyperactivity, impulsivity and distractability (Berry-Kravis and Potanos, 2004); of *alfa-2-adrenergic agonists*, which may control response to sensory input and provide good containment of hyperarousal behaviour (Berry-Kravis and Potanos, 2004); of *selective serotonin reuptake inhibitors*, quite often used to treat mood disorder, anxiety and obsessive-compulsive behaviours; of *atypical antipsychotics*, (drug of choice: aripiprazole), used to prevent self injury, aggressive behaviour and autism. Based on the mGluRs theory, antagonists for this system may become a good

tool to treat several aspect of FXS (Bear et al, 2004); indeed, mGluR5 antagonists are under investigation and preclinical reports showed good results (Slassi et al, 2005; Berry-Kravis et al, 2006). Of course, the surrounding environment is essential in the outcome of Fragile X affected individuals and environmental variables may influence the development of adaptative behaviour, cognitive abilities and behavioural symptoms.

Due to the very complex molecular picture drawn for the function of FMRP and the highly differentiated clinical outcome associated to FXS, it is of high importance to better understand how a single gene silencing mutation may lead to all the features described, in order to better address the therapeutic efforts designed to ameliorate the condition of Fragile X patients.

### *Fragile X and Epilepsy*

Epilepsy associates to the Fragile X phenotype in almost 20-25% of the cases (Berry-Kravis, 2002; Incorpora et al, 2002; Sabaratnam et al, 2001; Musumeci et al, 1999). Although high variability is associated in human studies for the onset and recurrence of seizures, FragileX-related-seizures generally appear around early childhood and usually disappear in a period that spans from adolescence through early adulthood (Kluger et al, 1996; Musumeci et al, 1999; Sabaratnam et al, 2001). Semiology of Fragile X–linked-epilepsy shows a variable phenotype and, although generalized seizures have been reported as the prominent ones (Wisniewski et al, 1991), some other authors report that complex partial seizures also appear with high frequency (Musumeci et al, 1999; Sabaratnam et al, 2001). According to the literature (Musumeci et al, 1991), types of seizures in Fra-X present similarities with some epilepsy syndromes such as benign childhood epilepsy with centrotemporal spikes (BCECTS (\*)) (Aicardi, 1986), childhood epilepsy with occipital paroxysms (Gastaut, 1982), partial motor seizures with adersion in patients with extreme somatosensory evoked potential (DeMarco and Tassinari, 1981; Plasmati et al, 1992), Landau– Kleffner syndrome (Landau and Kleffner, 1957), partial frontal epilepsy with favourable evolution (Beaumanoir and Nahory, 1983), and status epilepticus during sleep (Tassinari et al, 1982). Main correlation reported in literature is with BCECTS, with which FXS-linked epilepsy share, in most of the cases, benign outcome and EEG pattern (Incorpora et al, 2002; Berry-Kravis et al, 2002; Sabaratnam et al, 2001; Musumeci et al, 1999; Kluger et al, 1996). Some works have also been directed to find a genetic correlation between BCECTS and FXS but no molecular association has been demonstrated to date (Kluger et al, 1996; Rees et al, 1993).

**(\*)Benign Childhood Epilepsy with Centrotemporal Spikes (BCECTS)**

Benign epilepsy of childhood with centrotemporal spikes (BCECTS) is the most common partial epilepsy syndrome in the pediatric age group, with an onset between age 3 and 13 years. The typical presentation is a partial seizure with parasthesias and tonic or clonic activity of the lower face associated with drooling and dysarthria. Seizures commonly occur at night and may become secondarily generalized. They are usually infrequent and may not require antiepileptic drugs but, if treated, they tend to be easily controlled. Children with BECT are neurologically and cognitively normal. The EEG shows characteristic high-voltage sharp waves in the centrotemporal regions, which are activated with drowsiness and sleep. In this typical form, BCECTS is easily recognized.

However, atypical cases are common and the definition of BCECTS can become blurred. Although further investigations are not required in cases with typical clinical and EEG findings and normal neurologic examinations, neuroimaging studies may be required in atypical cases to rule out other pathology. The long-term medical and psychosocial prognosis of BCECTS is excellent, with essentially all children entering long-term remission by mid-adolescence (Wirrel, 1998).

Most of the cases of epilepsy associated to FXS have, as in the BCECTS, a benign course responding well to anticonvulsant therapy, mainly carbamazepine, and with a high incidence of total remission of the symptoms within childhood or adolescence (Sabaratnam et al, 2001). Even though this is the general outcome of the epileptic disease linked to FXS, some patients still experience seizures during adulthood and sometimes are unable to control the pathology by AEDs therapy (Musumeci et al, 1988; Incorpora et al, 2002).

EEG analysis of FXS patient, aside finding analogies with BCECTS, reveals a particular EEG pattern that associates to about 50% of Fragile X affected individuals, even in those that do not show seizures. Common EEG features are slow background and abnormal intermittent rhythmic theta or delta activity (Sabaratnam et al, 2001; Wisniewski et al, 1991; Musumeci et al, 1991; Vieregge and Froster-Iskenius, 1989). EEG activity, either in epileptic patients or in patients not showing any seizure, has been reported as age-dependent because of its relation to a particular period of time that spans from childhood, when the EEG pattern usually appears, and late adolescence-early adulthood, when the EEG pattern slowly disappears (Musumeci et al, 1999; Kluger et al, 1996). EEG activity is usually stronger and dramatically activated by sleep (Musumeci et al, 1999; Kluger et al, 1996). The significance for this EEG alterations in FXS patients is not clear and different hypothesis have been proposed. Vieregge and Froster-Iskenius suggested the dendritic spine abnormalities observed in fragile X syndrome as the cause of excessive neuronal excitation and spiking (Vieregge and Froster-Iskenius, 1989). This would also be in line with the idea that impairment in brain maturation is at the base of EEG abnormalities in FXS and in BCECTS. Another possible cause could be a deficit of GABA neurotransmission; Binstock has proposed that the absence of the FMRP leads to a dysfunction in the GABAergic system (Binstock, 1995). Indeed, recent studies demonstrate that GABAergic inhibition is impaired in different ways, at cellular (Selby et al, 2007), physiological (Curia et al, 2008b; Centonze et al, 2008) and molecular level (El Idrissi et al, 2005; Curia et al, 2008; D'Hulst et al, 2006). Therefore, decrease of interneurons number, altered GABAergic transmission and/or altered GABA-A subunit expression

may be the cause for the epileptic seizures and/or the EEG abnormalities associated to FXS. Finally, Kluger, who performed EEGs in children with fragile X and genetic analysis on children with BCECTS, has suggested that the final common pathway of impairment in the two conditions is the FMR-1 gene (Kluger et al, 1996). The latter hypothesis still awaits experimental confirmation.

Brain excitability and susceptibility to seizures has been shown also in mice deficient for Fmr1, the genetic model reproducing FXS in mice (Bakker et al, 1994; Kooy et al, 1996). Even if the mouse model for FXS does not display spontaneous seizures, it is susceptible to audiogenic induced seizures (Musumeci et al, 2000; Chen and Toth, 2001; Kooy, 2003). Although seizures age dependency is still on debate, a trend has been reported with older mice more prone to acoustic seizures (Chen and Toth, 2001; Kooy, 2003). Chemically induced seizures, by the means of kainic acid, bicuculline and pentylentetrazole injection, do not show difference between null Fmr1 mice and their controls, suggesting that Fmr1 KO mice have specific susceptibility to audiogenic stimuli (Todd and Mack, 2000; Chen and Toth, 2001). Possible explanation for this specific susceptibility in Fmr1 Knock out mice may be related to an increased cortical excitability or connected to a deficiency in long-term plasticity (Kooy, 2003). Indeed, as proposed by Chen and Toth, a developmental impairment of the auditory system may occur in fragile X mice (Chen and Toth, 2001).

In conclusion, there is still a huge amount of work to do to uncover the implication of Fmr1 mutation in epilepsy. Human studies are very important to deeply describe the follow up of the pathology and to widely report the subtle differences among the patients. Preclinical investigation, on the other side, may shed light on the intrinsic mechanisms that may apply to the still unclear functions of Fmr1-FMRP. Therefore, it is of vital importance to deepen our knowledge on every aspect of the manifestations of FXS, in order to better target the efforts in dealing with the pathology and making amelioration possible.

## *Pilocarpine*

The pilocarpine model is a model of Temporal Lobe Epilepsy (for an extensive review see Curia et al, 2008a). It is an isomorphic model capable to reproduce the main features of the epileptic disorder. Implications of the cholinergic system in epileptic phenomena have been demonstrated since the beginning of last century, when it resulted clear that cholinergic agonists induce seizures (Sjortrand, 1937; Miller et al, 1938; Brenner and Merritt, 1942; Chatfield and Dempsey, 1943; Turski et al 1989). First description of the pilocarpine model has been done by Tursky and

collaborator in 1983 (Tursky et al, 1983a,b) and, from then on, it has been widely used to mimic and model human TLE.

The pilocarpine model is characterized by three different chronological stages: an *acute period*, of status epilepticus instauration, a *latent* (referred also as silent or quiescent) *period*, of seizures free behaviour, and a *chronic period*, when spontaneous recurrent seizures occur.

The *acute period* is represented, in animals, by rapid changes in behaviour after pilocarpine administration. Five minutes after injection, animals begin to be motionless displaying oro-facial movements, salivation, eye blinking, twitching of vibrissae and yawning. At 30 minutes, discontinuous seizures are observed, which will last until limbic motor seizures take over. The latter are accompanied by intense salivation, rearing, upper extremity clonus and falling. Animal is, therefore, entered in status epilepticus (Turski et al, 1983a). Usually, 60% of injected animals enter status epilepticus (Cavalheiro et al, 1991) which last for a few hours (5 to 6 h) before entering the post-ictal coma which lasts 1-2 days. Loss of weight is appreciated in animals undergoing this treatment which is recovered after 1 week from pilocarpine injection (Turski et al, 1989) . High mortality is associated to this model and increases in relation to the length of status epilepticus (Turski et al, 1983a, Turski et al, 1989; Cavalheiro et al, 1991; Liu et al, 1994). Reduction of mortality is achieved by stopping status epilepticus with anticonvulsant drugs such as diazepam or others.

EEG-associated-activity perfectly follows ictal and interictal epileptic events in the pilocarpine model. Pilocarpine doses capable to develop status epilepticus range between 300 and 400 mg/Kg (Turski et al, 1983a, Turski et al, 1989; Cavalheiro et al, 1991; Liu et al, 1994).

The *latent period* starts after status epilepticus recovery and is characterized by a seizures free period in which a normal behaviour and EEG activity is recorded in the animal. The latent period have a variable duration of 1-6 week(s) (Cavalheiro et al, 1991) depending on different factors: length of status epilepticus (Lemos and Cavalheiro, 1995; Fujikawa, 1996; Biagini et al, 2006; Goffin et al, 2007), pilocarpine dose (Liu et al, 1994), background strain of the animal and age (Biagini et al, 2006; Goffin et al, 2007). In this period several pathophysiological rearrangements occur in the brain, that might lead to the third phase of the model (spontaneous seizures). Tissue and cell alterations in the latent period include: mossy fiber sprouting, interneuron and neuron loss, rewiring of synaptic circuits, glial cell activation and ectopic cell proliferation (Dalby and Mody, 2001; Pitkanen and Sutula, 2002).

The *Chronic period* is characterized by spontaneous recurrent seizures (SRSs) with different behaviour, all aspects summarized and reclassified by Veliskova (Veliskova, 2006) according to the following criteria: 1, staring and mouth clonus; 2, automatisms; 3, monolateral forelimb clonus; 4,

bilateral forelimb clonus; 5, bilateral forelimb clonus with rearing and falling; 6, tonic-clonic seizure. Usually, SRSs begin as partial seizures (classes 1, 2 or 3) and develop in to secondary generalized seizures (classes 4, 5 or 6) (Goffin et al, 2007). Once started, SRSs recur relatively regularly for the whole life of the animal and their recurrence has been described as cyclic, occurring in definite clusters each 5-8 days or more on average (Goffin et al, 2007; Arida et al, 1999). SRSs occur more often during the diurnal period (Arida et al, 1999), are associated with EEG activity starting from the hippocampus and spreading to the neocortex in 90% of the cases (Cavalheiro et al, 1991) and usually last less than 60 seconds (Cavalheiro et al, 1991).

The main mechanism by which pilocarpine acts in producing this model is its action on the M1 muscarinic receptor subtype, which causes imbalance between excitatory and inhibitory transmission resulting in status epilepticus (Priel and Albuquerque, 2002). This imbalance may be due to increased glutamate release (Smolders et al, 1997). This may explain why pilocarpine starts its action by stimulation of M1 muscarinic receptors and then, once seizures are activated, is sustained by NMDA receptor activation (Nagao et al, 1996; Smolders et al, 1997). A plausible molecular explanation may be related to a reduction of voltage and  $Ca^{2+}$  dependent  $K^{+}$  conductance (Benardo and Prince 1982) which may favour  $Ca^{2+}$  and  $Na^{+}$  cellular influx, that in turn may be responsible for the depolarizing excitatory events exacerbating the seizures (Pumain et al, 1983).

For the aims of our experiments we focused our attention only on the first stage of the model (acute period).

## **AIM OF THIS STUDY**

The aims for this study lay on the intention to deeply investigate whether Fmr1 mRNA is localized in the dendrites of mature neurons, whether the distribution of the mRNA is altered by synaptic activation of epileptogenic type, and whether the mRNA is locally translated in response to signals that induce synaptic plasticity and epilepsy. This investigation is a part of a wider project in which Fmr1 mRNA localization, distribution and translation is studied also in another experimental setting, perforant path stimulation. The reported results come from the submitted paper Ferrari et al, based on the work performed in collaboration with Claudia Bagni and colleagues, at the University of Rome-Tor Vergata, and Oswald Steward and colleagues, at the University of California at Irvine, CA.

# MATERIALS AND METHODS

## *Animals*

Adult male Sprague-Dawley rats (Harlan) were used for the experiments. Animal care was conducted according to the institutional guidelines that are in compliance with national (DL N116, GU, suppl 40, 18- 2-1992) and international laws and policies (European Community Council Directive 86/609, OJa L 358, 1, December 12, 1987; National Institutes of Health Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996).

## *Pilocarpine treatment*

To induce seizures, rats (250-300g) received pilocarpine (300 mg/Kg). Within 30 min from pilocarpine administration, animals entered a convulsive status epilepticus, which was arrested 2 hrs after onset by administration of 10 mg/Kg Diazepam. Animals were killed by anaesthetic overdose and perfused (as described below) after 2, 4 or 24 hours from the onset of status epilepticus. Control animals received saline and were perfused at matching time points.

## *Preparation of tissue for in situ hybridization and immunostaining*

Animals were deeply anesthetized and then perfused with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS pH 7.4). Brains were removed and postfixed in ice-cold 4% paraformaldehyde in 0.1 M PBS. For cryoprotection, brains were placed in 25% sucrose/4% paraformaldehyde/0.1 M PBS until the brains sank. Brains were then frozen in cold (-80°C) isopentane or dry ice and stored at -80°C until use. Twenty µm sections were cut using a cryostat and either thaw-mounted on polylysine-coated slides and stored at -80°C (for in situ hybridization) or left floating in 1X PBS and stored at 4°C (for immunocytochemistry experiments).



## *In situ Hybridization*

Non-isotopic *in situ* hybridization was carried out as previously described (Steward et al., 1998). Slides that had been stored at  $-80^{\circ}\text{C}$  were thawed at room temperature for 5-10 min, and were then dried in a  $55^{\circ}\text{C}$  oven for 10-15 min. Sections were post-fixed with 4% paraformaldehyde in 0.1 M PBS for 30 min, then rinsed with 0.5X SSC (0.1% DEPC treated) for 5 min. Sections were treated with Proteinase K (1.25  $\mu\text{g}/\text{ml}$ ) for 30 min, rinsed again with 0.5X SSC (0.1% DEPC treated) for 10 min and air-dried. The sections were covered with 75 $\mu\text{l}$  of pre-hybridization buffer (2X SSC, 25% formamide, 1% Denhardt's solution, 10% dextran sulfate, 0.5 mg/ml heparin, 0.5 mg/ml *E. coli* tRNA and 0.25 mg/ml of denatured salmon sperm DNA) and incubated at  $42^{\circ}\text{C}$  for 2-3 hrs. After the pre-hybridization, 0.5  $\mu\text{g}$  of digoxigenin-labeled cRNA probe, in 75  $\mu\text{l}$  of hybridization buffer, was added to each section. Sections were covered with baked coverslips and incubated overnight at  $55^{\circ}\text{C}$  in a humidified box with 25% formamide and 2X SSC. The next day, coverslips were removed and sections were washed with 2X SSC/1 mM EDTA twice (10 min each). Sections were treated with RNaseA for 30 min and then washed twice with 2X SSC/1 mM EDTA. The stringency wash was performed at  $55^{\circ}\text{C}$  for 2 hrs in 0.5X SSC/1 mM EDTA. After that, sections were washed with 0.5X SSC twice (10 min each at room temperature). Sections were dried and incubated with blocking solution (1X TBS, 0.2% Triton-X100, 10% BSA) for 30 min at room temperature. To detect the hybridized probes, we used an alkaline phosphatase conjugated anti-digoxigenin Fab fragment (1:1000 in blocking solution, 1 hr at  $37^{\circ}\text{C}$ ). Sections were rinsed twice with 1X TBS and then NBT/BCIP solution was applied overnight ( $4^{\circ}\text{C}$ ) to detect the alkaline phosphatase. The next day, sections were washed with 0.1 M Tris pH 8.5/1 mM EDTA 3 times (10 min each). Then slides were briefly rinsed with nanopure water and covered with Kaiser mounting medium (nanopure water, gelatin, glycerol).

## *Immunohistochemistry*

Free-floating vibratome sections were heat-treated ( $95^{\circ}\text{C}$  for 5 min) to recover antigenicity. After antigen retrieval, sections were blocked for 2 hrs, at RT, in Mix solution (0.1 M Tris pH 7.4, 0.5% Triton-X100, 0.25% Carrageenan lambda)/10% normal goat serum (NGS) and then incubated overnight with anti-FMRP-rAM2 antibodies (1:100) at  $4^{\circ}\text{C}$ . The FMRP (rAM2) antibodies were produced by immunizing rabbits against the human C-Terminus of FMRP (Ferrari et al., 2007).

Sections were washed with 1X TBS 3 times and then incubated in biotin conjugated secondary antibody (goat anti-rabbit IgG, 1: 500) for 2 hrs at room temperature.

After washing, sections were incubated in Vector ABC kit (Vector, Burlingame, CA) for 1 hr and then reacted with DAB and H<sub>2</sub>O<sub>2</sub> for the colorization reaction. Sections were mounted on poly-L-lysine slides, dehydrated through alcohols to xylene, and coverslipped.

### *Preparation of cRNA probes*

For in situ hybridization the following probes were used (Fmr1-3' UTR, Fmr1 coding) in both sense and antisense orientation. The primers used to clone the cDNAs fragments from mouse brain total RNA were as follows:

Fmr1-3' UTR forward: 5'-GGT AAA GAT CGT AAC CAG AAG-3'

Fmr1-3' UTR reverse: 5'-CAA GTA CAT CAG AGG CAG AAC-3'

Fmr1 coding forward: 5'-CCC GGG CGA TGG AGG AGC TGG TGG TGG-3'

Fmr1 coding reverse: 5'-GGA ATT CCT GGG GTA CTC CAT TCA CGA GT-3'

The cDNA for Fmr1-3' UTR, was cloned into pGemTeasy vector and linearized with SacII or SacI to transcribe, in vitro with SP6 or T7 polymerases, antisense or sense digoxigenin-labeled cRNA probes. The cDNA for the Fmr1 coding region was cloned into the EcoRI-XhoI sites of the pBluescript-SK II plasmid and linearized with EcoRI for antisense cRNA probe synthesis. Specificity of the probe for Fmr1 mRNA was assessed performing in situ hybridization and Northern blot on Fmr1-KO and WT mice.

### *Optical Density Measurement*

Optical density measurements were taken across the granule cell layer/molecular layer, pyramidal cell layer/stratum radiatum and pyramidal cell layer/stratum lucidum/stratum radiatum using Image J software (version 1.36b). Images were acquired through a Nikon Coolpix digital camera mounted on a Zeiss Axioscop microscope (plan-neofluar 20X objective). Densitometric analysis was performed on a 350 x 250 µm frame. For each animal and hippocampal subfield, we generated curves in which each data point represents the average gray level over a line of 700 pixels (corresponding to 350 µm) at a given distance from the cell layer. To compare the different animals, each section was positioned to align the cell layer parallel to one border of the field visualized by

the camera. For statistical analysis, we used the average grey level measured in 20  $\mu\text{m}$  intervals of the different layers in the different hippocampal subfields. The data obtained were analyzed using one-way ANOVA and the post hoc LSD test. Values in the graphs illustrate the mean and standard error of four to five animals per group.

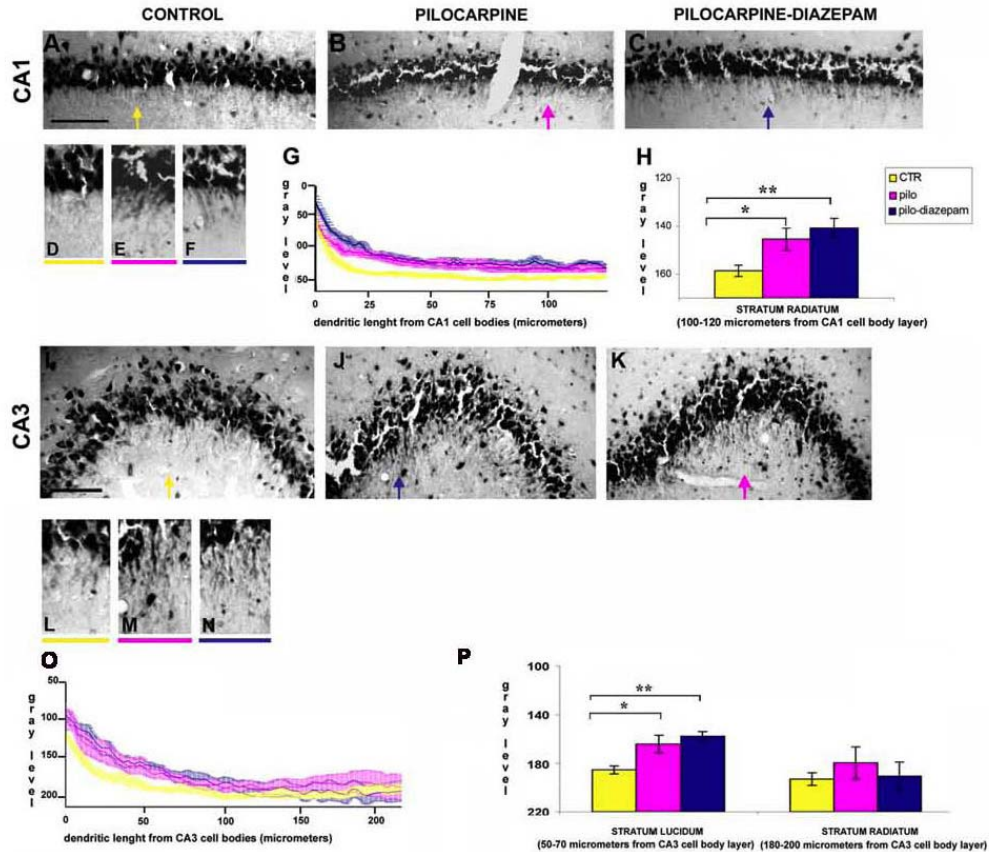
## RESULTS

### *Pilocarpine seizures cause dendritic targeting of Fmr1 mRNA and FMRP in hippocampus*

Because of evidence that loss of FMRP may be associated with epilepsy (Musumeci et al., 2000; Chen and Toth, 2001; Berry-Kravis 2002), we assessed whether epileptogenic events (acute episodes of status epilepticus) altered the distribution of Fmr1 mRNA or FMRP. To address this question, a discrete episode of status epilepticus was induced by treating rats with the muscarinic acetylcholine receptor agonist pilocarpine, terminating seizures after 2hrs with diazepam, and killing animals 2, 4, and 24 hr later (Turski et al., 1989). The hippocampus is strongly activated during pilocarpine-induced status epilepticus, so that dendritic laminae contacted by synapses of intrinsic hippocampal pathways are strongly activated (for example, stratum radiatum, which is the site of termination of commissural/associational synapses from the CA3 subfield and stratum lucidum, which is the site of termination of mossy fibers from dentate granule cells). In situ hybridization revealed that level of Fmr1 mRNA was elevated in the stratum radiatum of the CA1 region (figure 2A-H) and in the stratum lucidum of the CA3 subfield (figure 2I-P). This effect was detected 2 and 4, but not 24 hr after the onset of status epilepticus (data not shown for the 24 h timepoint). The change of distribution in pilocarpine treated animals compared to control is statistically significant as shown in figures 2H and 2P.

Immunostaining of sections from the same animals revealed that levels of staining in the dendritic regions of the CA1 subfield were comparable between controls and treated animals (figure 3A-E). In contrast, immunostaining was higher in the stratum lucidum of the CA3 region 2 and 4, but not 24 hr after the onset of status epilepticus (figure 3F-M). This discrepancy between Fmr1 mRNA and FMRP localization in the CA1 region could be due to differences in the timing of accumulation of mRNAs vs. protein in different brain areas and/or at particular subsets of synapses.

Overall, these observations suggest that pilocarpine-induced seizures cause Fmr1 mRNA and FMRP to accumulate selectively at particular dendritic domains.



**Figure 2: Pilocarpine seizures induce dendritic targeting of Fmr1 mRNA in the CA1 and CA3**

(A-C) Part of the coronal brain sections at the level of the dorsal hippocampus labeled with a probe for Fmr1 mRNA in control (A), pilocarpine (B) and pilocarpine- diazepam treated conditions (C) at the level of CA1 region, showing increased dendritic staining for Fmr1 mRNA after onset of status epilepticus (B and C). Specific areas (insets, D-F) marked by yellow, blue and pink lines are enlargements of the areas pointed by the coloured arrows in A, B, C and show the differential distribution in dendrites in control and stimulated conditions. (G and H) represent the densitometric analysis of the dendritic labeling in the CA1 region, expressed as pixel intensity (0-200 gray level; 200=white; 0=black) from the cell soma (in micrometers). Data are the means  $\pm$  standard error of four to five animals per group: control (yellow line; n=5), pilocarpine (pilo, pink line; n=5) and pilocarpine-diazepam (pilotiazepam, blue line; n=4). (I-K) CA3 region labeled with a probe for Fmr1 mRNA in control (I), pilocarpine (J) and pilocarpine-diazepam treated conditions (K)). Specific areas (insets, L-N) marked by yellow, blue and pink lines are enlargements of the areas pointed by coloured arrows in I, J, K. (O and P) represent the densitometric analysis of the dendritic labeling in CA3 region. Quantification has been performed using “Image J” software as described in Materials and Methods. (\*\*p<0,01; \*p<0,05. Bars=100  $\mu$ m)

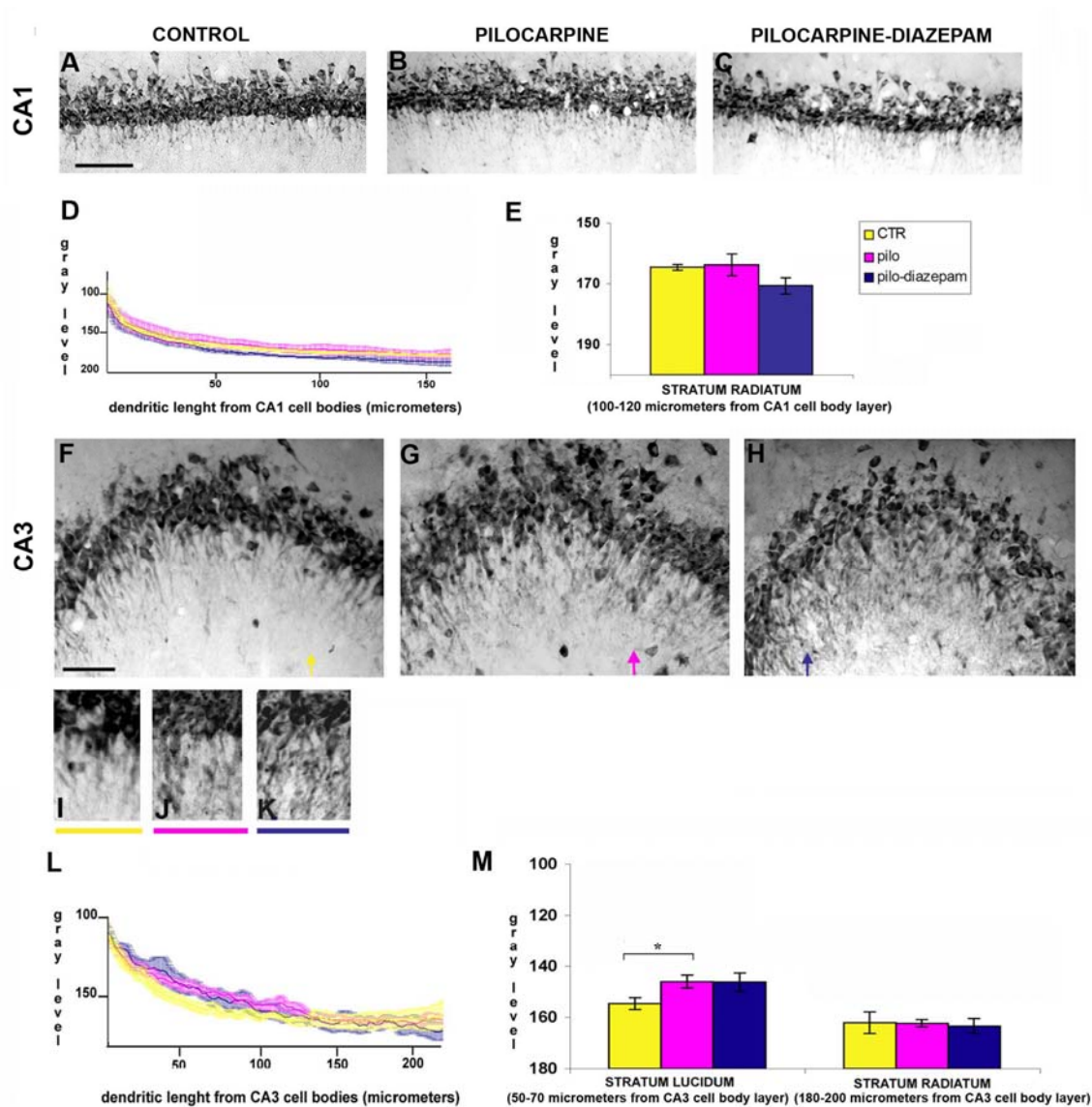


Figure 3: **Pilocarpine seizures increases FMRP levels in dendrites of the CA3 region.**

(A-C) Part of the coronal brain sections at the level of the dorsal hippocampus labeled for FMRP in control (A), pilocarpine (B) and pilocarpine- diazepam treated conditions (C) at the level of CA1 region, showing no differences in FMRP distribution pattern after pharmacological treatments. Graphs (D and E) represent the densitometric analysis of the dendritic immunoreactivity in CA1 region, expressed as pixel intensity (0-200 gray level; 200=white; 0=black) from the cell soma (in micrometers). Data are the means  $\pm$  standard error of four to five animals per group: control (yellow line; n=5), pilocarpine (pilo, pink line; n=5) and pilocarpine-diazepam (pilo-diazepam, blue line; n=4). (F-H) show FMRP level in the CA3 region, with an increased proximal dendritic immunoreactivity after pharmacological treatment. Specific areas (insets, I-K) marked by yellow, blue and pink lines are enlargements of the areas pointed by coloured arrows in F, G, H. (L and M) represent the densitometric analysis of FMRP dendritic staining in CA3 region. Quantification has been performed using “Image J” software as described in Materials and Methods. (\*p<0,05. Bars=100  $\mu$ m)

## DISCUSSION

This study, a part of a wider investigation on Fmr1 and FMRP targeting and dendritic expression (Ferrari et al, submitted) provides several new insights into the mechanisms underlying the expression of FMRP in dendrites. FMRP has been strongly implicated in regulating dendritic mRNA translation, especially during the period in which activity-induced synaptic modifications are “consolidated”. We show here, for the first time, that Fmr1 mRNA is localized in the dendrites of mature neurons *in vivo* at low levels, supporting and extending previous conclusions based on studies of synaptoneurosome fractions from young animals (Weiler et al., 1997; Zalfa et al., 2003) and studies of neurons developing in culture (Antar et al., 2004; Ferrari et al., 2007). Previous studies (Hinds et al., 1993; Valentine et al., 2000), performed using radioactive *in situ* hybridization and probes for the coding region of Fmr1 mRNA, did not report detectable levels of Fmr1 mRNA in dendrites, although the issue of dendritic localization was not explicitly assessed in these studies. The ability to detect labelling in this study may be due to the use of a sensitive non-isotopic *in situ* hybridization procedure, to a slightly different fixation protocol, as well as to a different Fmr1 mRNA probe.

In this discussion we will focus our attention on the fact that we report that Fmr1 mRNA and FMRP dendritic localization pattern in hippocampus can be modified by epileptogenic stimuli *in vivo*. Our findings suggest a direct involvement of Fmr1 mRNA and FMRP in specific synaptic pathways, and indicate that the intra-dendritic distribution of Fragile X mRNA and protein can be modified by synaptic activity related to epileptogenesis. The involvement of FMRP in this kind of activity-dependent modification has been previously suggested based on *in vitro* experiments (Weiler et al., 1997; Huber et al., 2002; Li et al., 2002; Antar et al., 2004; Weiler et al., 2004). Our results here indicate that Fmr1 mRNA accumulates in activated dendritic laminae in response to epileptic stimulation patterns.

Indeed, the alterations in the dendritic localization of Fmr1 mRNA following a 2hr period of status epilepticus supports the conclusion that dendritic localization of Fmr1 mRNA can be regulated by synaptic activation. During pilocarpine-induced status epilepticus, principal neurons in the hippocampus are strongly activated, thus activating synapses that terminate in stratum radiatum (the site of termination of commissural/associational synapses from the CA3 subfield) and stratum lucidum (the site of termination of mossy fibers from dentate granule cells). The episode of status epilepticus initiates epileptogenic processes that eventually, over a period of days or weeks, make the neurons and/or the circuits more prone to seizures. Our results here implicate FMRP in the early

stage of the epileptogenic processes. The delivery of FMRP to dendrites could either contribute to epileptogenic processes in a positive way, enhancing activity-dependent plasticity, or in a negative way by helping to restore synaptic homeostasis after periods of intense, uncontrolled activity. If FMRP does modulate the cellular and molecular events that determine seizure susceptibility, this could explain the increased prevalence of childhood seizures in people with Fragile X syndrome (Berry-Kravis 2002) and increased seizure susceptibility in the mouse model for the syndrome (Musumeci et al., 2000; Chen and Toth, 2001). For example, FMRP might stabilize or translationally repress mRNA molecules involved in the repression of the epileptic seizure. In absence of this/these control(s), susceptibility to seizures would increase. The other possibility is that enhanced seizure susceptibility in Fragile X Syndrome has nothing to do with altered mechanisms of synaptic plasticity in principal neurons, and instead reflects an alteration in the function of GABAergic interneurons. Indeed, there is evidence that GABAergic mechanisms are altered in absence of FMRP and BC1 RNA (Centonze et al., 2008; for a recent review see D'Hulst and Kooy, 2007). Further studies will be required to elucidate the role of FMRP in epileptogenesis.



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Chapter IV

# **Bradykinins and susceptibility to seizures.**

Study on B1 null mouse





# INTRODUCTION

## *Bradykinins*

The biologically active kinins are peptide autacoids. First clues of kinin existence date back to the 1930s (Kraut et al, 1930; Werle and Grunz, 1939) and, since then, several experimental investigations found kinins involved in many biological function. Their main functions are explicated in cardiovascular homeostasis, in contraction and relaxation of smooth muscles, in inflammation and nociception (Marceau et al, 1998;; Calixto et al, 2000; Couture et al, 2001; Rodi et al, 2005). Moreover, Kinins have been recently described as proliferative/anti-proliferative (Patel and Schrey, 1992; Dixon and Dennis, 1997; Tsuchida et al, 1999; Alric et al, 2002; Duchene et al, 2002; Bascands et al, 2003), fibrotic/anti-fibrotic (Wollert et al, 1997; Gallagher et al, 1998; Ricupero et al, 2000; Schanstra et al, 2002), angiogenic/anti-angiogenic (Volpert et al, 1996; Silvestre et al, 2001; Emanuelli et al, 2002) agents, depending on the cellular type taken into consideration. The kinin system is also involved in disease states like asthma, allergies, rheumatoid arthritis, cancer, endotoxic and pancreatic shock (Marceau et al, 1998; Bhoola et al, 2001). Furthermore, kinins are neuromediators of central neuronal pathways associated with the autonomic control of blood pressure and nociception (Couture and Lindsey, 2000; Couture et al, 2001) . The involvement of kinins in neurological diseases has not been intensively investigated thus far. However, recent results suggest their implications in epilepsy, stroke and multiple sclerosis (Leeb-Lundberg et al, 2005; Rodi et al, 2005).

The term “kinins” identifies a group of 9-11 amino-acid peptides, including bradykinin (BK), Lys-bradykinin or kallidin (Lys-BK or KD), T-kinin (Ile-Ser-BK) and their active metabolites (des-Arg9-kinins).

Their activation in-vivo follows two different pathways, a plasmatic one and a tissue one, and both of them are started by an injury event (fig 1)

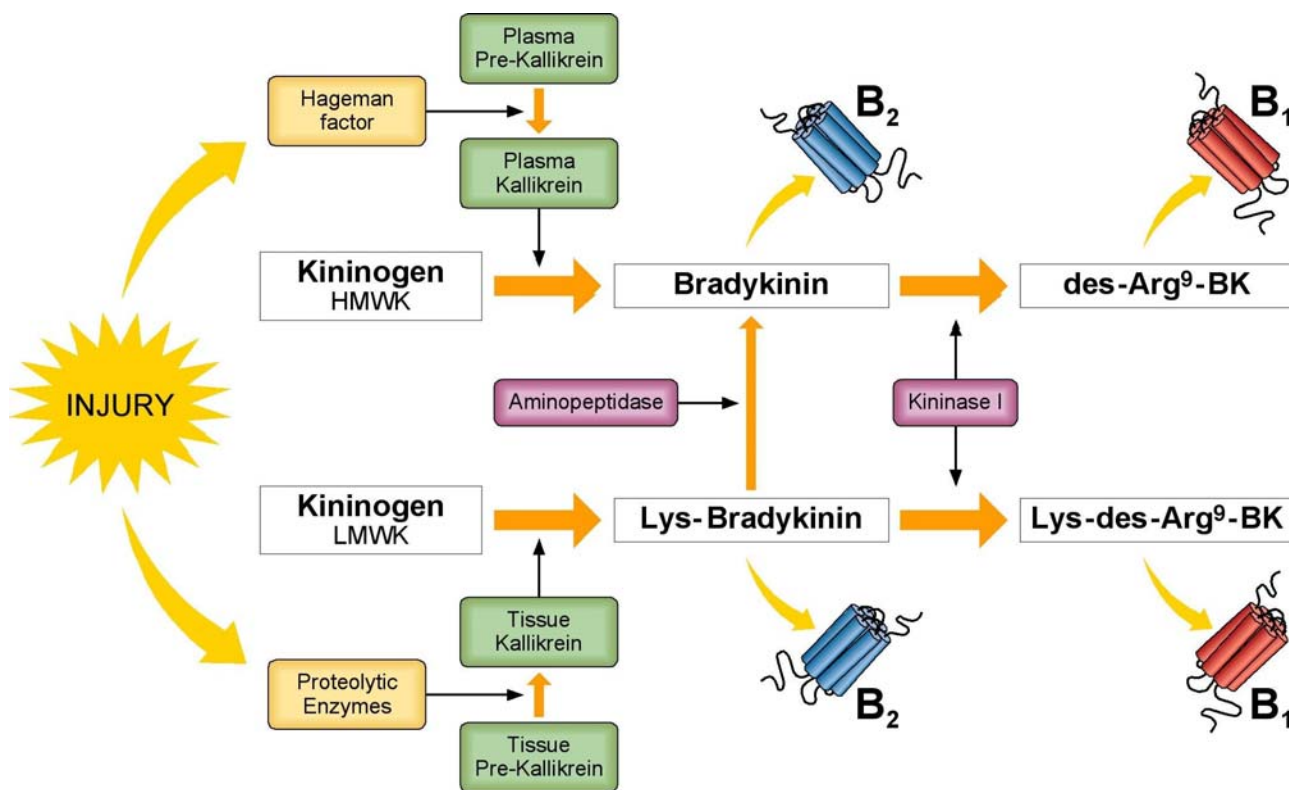


Fig 1: **Biosynthesis of kinins.** See text for details. Abbreviations: HMWK, high molecular weight kininogen; LMWK, low molecular weight kininogen; BK, bradykinin.

In plasma, after tissue injury inducing the activation of the Hageman factor (factor XII of the coagulation cascade), the kallikrein enzyme (kininogenase) is released from plasmatic inactive pre-kallikrein and catalyzes the conversion of the precursor kininogen HMWK (high molecular weight kininogen, 110 kDa) into BK (Fig 1).

In tissues, kallikrein is activated by proteolytic enzymes produced during trauma and induces the release of Lys-BK from the kininogen LMWK (low molecular weight kininogen, 70 kDa), with the exception of the rat where BK, rather than Lys-BK, is released (Bhoola et al, 1992) (fig 1).

HMWK and LMWK derive from alternative splicing of a single gene (Kitamura et al, 1983). Both BK and Lys-BK have a very short life-time, less than 30 seconds in serum or when injected in the cerebral ventricles (Kariya et al, 1982; Decarie et al, 1996). A kallikrein-dependent-mechanism has also been demonstrated, based on mast cell tryptase and elastase action, that are responsible for the production of Met-Lys-BK besides BK and Lys-BK; this alternative pathway of biosynthesis is thought to contribute to the prolonged kinin production at inflammatory sites (Kozik et al, 1998). Lys-BK and T-kinin can be transformed into BK through the action of aminopeptidases (Couture et al, 2000; Kuoppala et al, 2000) (fig. 1). BK and Lys-BK, can be converted in other active metabolites des-Arg<sup>9</sup>-BK and Lys-des-Arg<sup>9</sup>-BK, by removal of the C-terminal Arg. These

biotransformations are catalyzed by a group of enzymes named kininases I: carboxypeptidase N (in plasma) and carboxypeptidase M (associated with cell membranes).

Inactive metabolites are generated by removing the carboxyterminal dipeptides Phe<sup>8</sup>-Arg<sup>9</sup> and Ser<sup>6</sup>-Pro<sup>7</sup> by kininase II, also termed angiotensin-1 converting enzyme (ACE), an enzyme that is mainly located on the luminal membrane of endothelial cells. Another dipeptidyl carboxypeptidase, neutral endopeptidase 24.11 (enkephalinase), removes Phe<sup>8</sup>-Arg<sup>9</sup> from BK in vascular walls. Kininase II cleaves the C-terminal dipeptide Ser<sup>6</sup>-Pro<sup>7</sup> of BK (1-7) to produce BK (1-5), which is the final metabolite of BK and des-Arg<sup>9</sup>-BK in human plasma (Kuoppala et al, 2000). Studies in humans suggest that the BK and the Lys-BK systems are differentially regulated (Duncan et al, 2000).

### *Kinins receptors*

Kinins exert their action through two membrane receptors classified as B1 and B2. Both receptors have been discovered in the 1970s by Regoli and coworkers (Regoli et al, 1977; Regoli et al, 1978; Drouin et al, 1979) and biologically belong to the G-protein-coupled-receptor with seven transmembrane domains (Liao et al, 1993; Marceau et al, 1997; Marceau et al, 1998). B1 and B2 receptors share only part of their primary structure: there is only 36% homology between human B1 (353 amino acids) and B2 receptors (364 amino acids) (Menke et al, 1994). They also show different affinity for BK and its various active derivatives, following this order of agonist potency: for B1 receptors, Lys-des-Arg<sup>9</sup>-BK > Lys-BK  $\approx$  des-Arg<sup>9</sup>-BK  $\gg$  BK and for B2, BK  $\approx$  Lys-BK  $\gg$  des-Arg<sup>9</sup>-BK and Lys-des-Arg<sup>9</sup>-BK (Leeb-Lundberg et al, 2005).

*B1 receptor:* B1 receptor is the protein product of a three exon-gene positioned on chromosome 14 (Leeb-Lundberg et al, 2005). The B1 receptor is mainly activated by des-Arg<sup>9</sup> kinin metabolites. In particular, Lys-des-Arg<sup>9</sup>-BK is thought to be the endogenous ligand for this receptor in humans (Menke et al, 1994; Regoli et al, 1998; Regoli et al, 2001). This receptor is normally scarcely represented and its most prominent biological feature is that it is inducible in consequence of tissue injury, infection and under conditions of massive release of cytokines (IL-1b, TNF-a) and growth factors, i.e. under pathological situations (Ahluwalia and Perretti, 1996; Marceau et al, 1998; Siebeck et al, 1998). In addition, they are induced by the action of the transcription factor NF-kB (Bachvarov et al, 1996; Ni et al, 1998; Schanstra et al, 1998) and by its own agonist (Schanstra et al, 1998; Phagoo et al, 1999; Phagoo et al, 2001). This phenomenon occurs directly at the level of gene transcription and not through the synthesis of intermediate proteins (Phagoo et al, 2001). The

activation of B2 receptors is also thought to induce B1 receptor expression through autocrine production of cytokines and the activation of NF- $\kappa$ B (Pan et al, 1996; Phagoo et al, 1999;).

It is worth noting that B1 receptors with functional haemodynamic functions are induced and overexpressed in B2 knockout mice (Duka et al, 2001; Marin-Castano et al, 2002; Duka et al, 2003). In contrast with the B2, the B1 receptor internalizes its ligand very slowly and it does not undergo desensitization (Austin et al, 1997; Faussner et al, 1998; Marceau et al, 1998; Marceau et al, 2001). The main B1 transduction pathway is the activation of phospholipase C (Marceau et al, 1998; Couture and Lindsey, 2000) with production of DAG and IP<sub>3</sub>, which are responsible for the activation of PKC and for Ca<sup>2+</sup> mobilization, respectively. The B1 receptor can also stimulate the release of arachidonic acid through phospholipase A<sub>2</sub>, which leads to the formation of prostaglandins (Bascands et al, 2003) (Fig. 2).

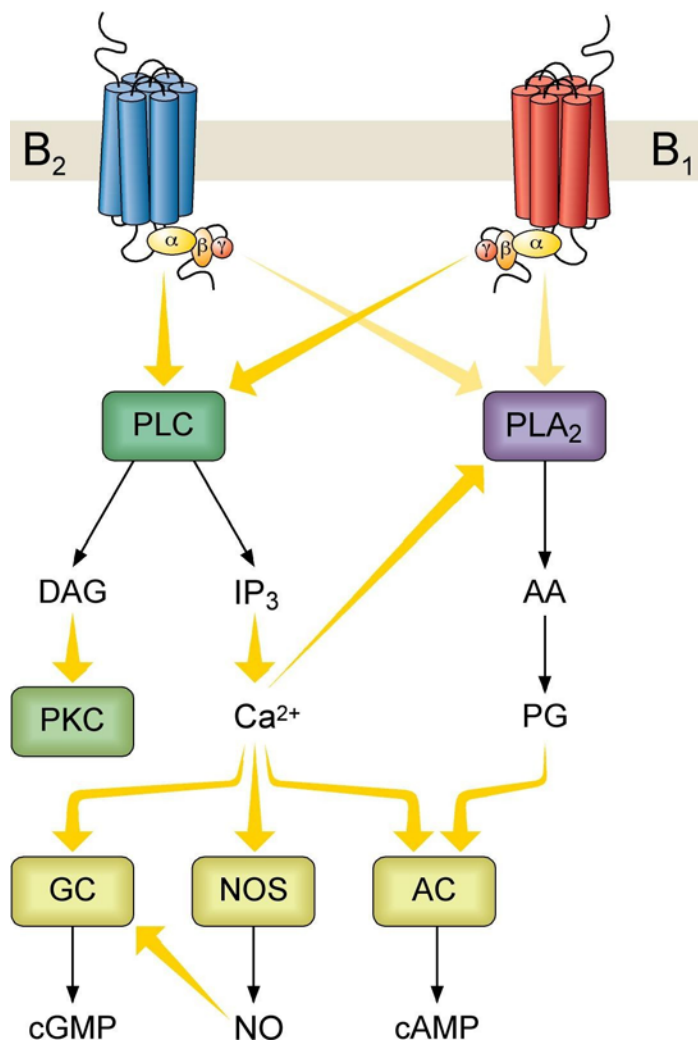


Fig. 2: Kinin receptors. See text for details. Abbreviations: PLC, phospholipase C; DAG: diacylglycerol; IP<sub>3</sub>, inositol triphosphate; PKC, protein-kinase C; GC, guanosine cyclase; cGMP, cyclic guanosine monophosphate; AC, adenylate cyclase; cAMP, cyclic adenosine monophosphate; NOS, nitric oxide synthase; NO, nitric oxide; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; AA, arachidonic acid; PG, prostaglandin.

*B2 receptor*: as reported for B1, B2 receptor is the protein product of a three exon gene on chromosome 14 (Leeb-Lundberg et al, 2005). It is preferentially activated by BK and Lys-BK and

is thought to mediate acute inflammatory reactions (Dray and Perkins, 1993; Couture et al, 2001) by inducing the cascades of both PLC and PLA2. PLA2 releases arachidonic acid (Rang et al, 1991), leading to the production of the prostaglandins PGE2 and PGI2, while PLC induces the activation of PKC and the mobilization of intracellular calcium (Fig. 2). In consequence of these events, neurotransmitters (like excitatory amino-acids and neuropeptides) are released. Furthermore, following depolarization, calcium activates adenylate cyclase and guanylate cyclase (leading to the production of cAMP and cGMP) and NO synthase (with nitric oxide release by endothelial and inflammatory cells).

After the binding of BK to B2 receptors, BK is internalized together with the receptors, interrupting its action (Munoz and Leeb-Lundberg, 1992; Munoz et al, 1993). The B2 receptor can be subsequently recycled.

While B2 receptors are responsible for most of the physiological actions of kinins and are mostly involved in the acute phase of inflammation, B1 receptors seem to participate essentially in the chronic phase (Dray and Perkins, 1993; Dray, 1997; Couture et al, 2001), generating a persistent response due to their up-regulation, to their scarce internalization and to the presence of large amounts of des-Arg<sup>9</sup> kinin metabolites at the inflammatory sites (Decarie et al, 1996) (fig. 3). Furthermore, they seem to be especially involved in pathologies with an autoimmune component, like rheumatoid arthritis and diabetes.

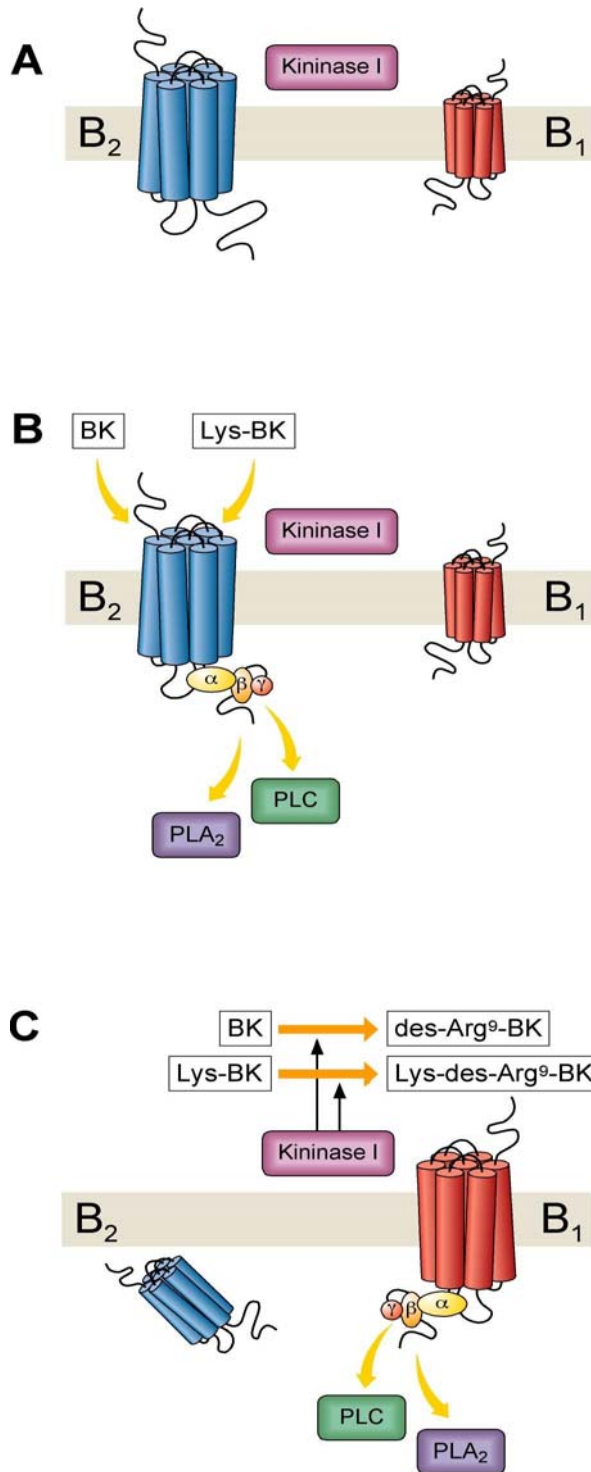


Fig. 3 Coordinated activity of B<sub>2</sub> and B<sub>1</sub> receptors. Under basal, physiological conditions, while B<sub>2</sub> receptors are constitutively expressed, B<sub>1</sub> receptors are little represented in almost all cell types. Acute pathological events (for example acute inflammation, B) cause production of BK and Lys-BK that activate B<sub>2</sub> receptors. Prolonged pathological events (C) lead to internalization of B<sub>2</sub> receptor, biotransformation of BK and Lys-BK into des-Arg<sup>9</sup>-BK and Lys-des-Arg<sup>9</sup>-BK respectively, and up-regulation of B<sub>1</sub> receptors. Since B<sub>1</sub> receptors do not internalize, this mechanism ensures a prolonged cellular response to kinins. See text for further details. Abbreviations: BK, bradykinin, PLC, phospholipase C; PLA<sub>2</sub>, phospholipase A<sub>2</sub>.

The enzymes, the substrates and the receptors of the kallikrein-kinin system have all been identified in different areas of the human (Raidoo et al, 1996a; Raidoo et al, 1996b; de Sousa Buck et al, 2002) and rat (Kizuki et al, 1994; Chen et al, 2000; Ongali et al, 2003; Cloutier et al, 2004;) central nervous system. In particular, tissue kallikrein has been found at high concentrations in the hypothalamus, pituitary and pineal gland, but also in the cortex, brainstem and cerebellum, while kininases are diffuse in all brain areas (Camargo et al, 1972; Camargo et al, 1973). BK-like immunoreactivity has been localized in rat and guinea-pig spinal cord neurons (Lopes and Couture, 1997; Couture and Lindsey, 2000) and in neuronal cells of the rat hypothalamus, periaqueductal gray matter, perirhinal and cingulate cortices, the ventral portion of caudate-putamen, and the lateral septal area (Correa et al, 1979). B2 receptors are widely distributed in mammalian brain (Murone et al, 1997; Chen et al, 2000; de Sousa Buck et al, 2002; Ongali et al, 2003; Cloutier et al, 2004), while in the rat spinal cord they have been identified in the dorsal horn and in particular on the terminals of A $\delta$  and C sensory fibers and of bulbospinal noradrenergic fibers (Couture et al, 2001; Lopes et al, 1995). Thus, it has been suggested that, through B2 receptor activation, kinins exert a role of CNS neuromediator, being involved in the control of nociception and blood pressure (Couture and Lindsey, 2000). B1 receptors are very scarcely represented in the normal rat brain, based on autoradiographic evidence (Ongali et al, 2003). In contrast, their mRNA distribution has been detailed throughout the rostral-caudal extent of the monkey brain and spinal cord, demonstrating a basal level of expression in the nonhuman primate CNS (Shughrue et al, 2003). However, evidence is still lacking for establishing whether this B1 receptor mRNA is translated into functional receptors.

### *Bradykinins and epilepsy*

To date, bradykinins have been scarcely investigated in their implication in pathologies of the CNS. B2 receptors have been implicated in the early phase of stroke-induced inflammation, and the selective blockade of these receptors may prove therapeutically useful. In contrast, B1 antagonists have been found to be detrimental for neuronal recovery and survival, indicating that B1 receptor activation is implicated in the late phase of stroke-induced inflammatory phenomena, in tissue repair. Thus, selective B1 receptor agonists may be of therapeutic value in this phase (Rodi et al, 2005).

B1 receptors may represent a control-point for inflammation in the CNS, usually associated to Multiple Sclerosis, and B1 receptor analogues may be useful in the therapy of this disease. The

critical point for the treatment of this pathology is the dual effect of B1 receptor activation: the use of an antagonist would be appropriate to block the increased permeability of the blood-brain-barrier, while an agonist would be more beneficial for preventing the infiltration of T-lymphocytes in the CNS. Both types of molecules need to be tested in an animal model of multiple sclerosis to address this fundamental issue (Rodi et al, 2005).

In epilepsy, our main field of interest, bradikinin and derivatives have proven to be involved in the pathology, in a series of works. Kinin receptors have been reported to be modified after epileptic stimuli. Ongali and coworkers demonstrated, using autoradiography, that, in fully kindled rats, B2 receptors decrease in many brain areas and B1 receptors increase mainly in the kindling recruited limbic areas (Ongali et al, 2003). At the protein level, in agreement with Ongali, other studies have shown that, in pilocarpine model (Arganaraz et al, 2004b) and in human sclerotic hippocampi from patient suffering of TLE, B1 receptors are increased (Perosa et al, 2007). However, discrepancies were found on the protein expression for B2 receptors (Arganaraz et al, 2004b; Perosa et al, 2007): in the pilocarpine model and in human hippocampi of TLE patients an increase in B2 receptors was observed. At gene expression level, data in the literature are sometimes in agreement with the protein findings (Pereira et al, 2008; Silva et al, 2008) and sometimes they are not a (Silva et al, 2008). Unaltered levels of B1 receptor mRNA in human hippocampi in TLE patients have been shown by Perosa and collaborators (Perosa et al, 2007) and unaltered mRNA levels for B2 receptors have been demonstrated by Silva and colleagues in the pilocarpine model applied to B1 KO mice. Functionally, bradikinin B1 receptors have been involved in epileptic response as favouring glutamate release in fully kindled rats both in slices (Bregola et al, 1999) and using microdialysis (Mazzuferi et al, 2005).

Susceptibility to seizures of B1 and B2 null mouse models have been explored using pilocarpine. B1 KO mice proved to be less prone to seizures, implicating that the B1 receptor contributes to epileptic hyper-excitability, while B2 KO mice were more susceptible to seizures, leading to the speculation that B2 receptors may play a protective role (Arganaraz et al, 2004a; Silva et al, 2008). Taken together, this data suggest a deep implication of the bradykinin system in epilepsy. It is still unclear, however, whether this is detrimental for epileptogenesis or protects brain tissue from damage. B1 receptors seem mainly associated with tissue excitability (Bregola et al, 1999; Mazzuferi et al, 2005; Arganaraz et al, 2004a,b; Silva et al, 2008). In the pilocarpine model, however, B2 receptors mRNA have been shown to be increased in the chronic phase when spontaneous seizures occur (Arganaraz et al, 2004b; Silva et al, 2008). It has been suggested that this phenomenon exerts a protective effect, because B2 KO mice experience a greater number of spontaneous recurrent seizures in the chronic period after pilocarpine (Arganaraz et al, 2004a).



Thus, it is still difficult to draw a complete frame for the involvement of bradykinins in epilepsy and, therefore, more work is needed to complete the picture.

### *B1 KO mice*

At the turn of the century, Pesquero and colleagues created the B1 deficient mouse model by using gene targeting technology (Pesquero et al, 2000). Absence of the target receptor was shown at the mRNA level in several tissues that normally express the B1 receptor. B1 KO mice are grossly normal and fertile and, due to the gene disruption, are insensitive to contractile response of smooth muscle after application of des-Arg<sup>9</sup>-BK. In basal condition they show to be normotensive but, when stimulated with bacterial lipopolysaccharide (LPS), they display a markedly hypotensive response that goes back to normal around 40 minutes after the stimulation. The response to intrathoracic injection of carrageenan or of the specific B1 agonist, des-Arg<sup>9</sup>-BK, which produces pleurisy with plasma extravasation and leukocyte infiltration in normal rodents, is abolished in B1 KO mice. Under the same conditions polymorph nucleate cell invasion is absent in B1 deficient mice, but endothelial permeabilization and mononuclear cell infiltration are preserved.

B1 KO mice response to pain in the tail-flick assay was not different from wild-type mice. However, by using the hot plate assay, B1 receptor-deficient mice showed significant hypoalgesia when mildly painful heat stimuli were used (52.5 and 55.5°C); with more intense stimuli (58.5°C), however, no difference was observed between the genotypes. In addition, capsaicin evoked reduced nocifensive behaviors in B1-deficient compared with wild-type mice. Finally, in a model of acute inflammatory pain, the formalin test, B1-deficient mice showed significantly less nocifensive behaviors in the early and late phase.

Noxious heat has been shown to directly induce inward currents in isolated nociceptors that can be potentiated by kinins (Cesare and McNaughton, 1996; Cesare et al, 1999). To prove that, patch-clamp experiments have been carried out demonstrating that B1 deficient mice are normal in this respect.

The hypoalgesia described in the hot plate, capsaicin, and formalin tests have been studied further by testing the spinal processing of the nociceptive input: B1 KO mice showed a reduced response to the late component of spinal cord afferent transmission due to activation of C-fibbers activated by des-Arg<sup>9</sup>-BK in vivo. The early component of spinal cord afferent transmission, brought about by A-fibbers, is unaltered in B1 KO mice. Indeed, this component is bradykinin-receptor independent. Moreover, repetitive electrical stimulation (500 mA, 500 ms) of the L5 dorsal root at 1 Hz for 20 s

induces an increase in the size of the ventral root potential. This type of plasticity has been termed wind-up. In B1-deficient mice, wind-up was reduced by about 50% compared with wild-type animals, confirming that this receptor modifies nociceptor-induced plasticity of synaptic transmission at the spinal level.

This mouse model has been created to deeply investigate the alterations of the normal homeostasis brought about by disruption of the B1 receptor gene. The data reported above are only a few of the many already published that better define the model and the implication of B1 in several physiological pathways. To date, exploration of the susceptibility to seizures has been reported in the pilocarpine model by Arganaraz and colleagues and Silva and coworkers, who demonstrated a reduced epileptic phenotype in the B1 KO mice. Since Bradykinin has been reported to play a role in epileptic phenomena, this mouse model seems to be a good tool to investigate the functional role of B1 receptor in epileptic experimental condition. For these reasons, we applied the kindling and the kainate model to B1 KO mice.

### *Kindling*

(For an extensive review of the model see “Models of seizures and epilepsy” Pitkanen A., Schwartzkroin P.A. and Moshé S.L. (2006) Elsevier Academic Press)

Discovered by Goddard in 1967, the so called “kindling” refers to the phenomenon by which the repeated administration of an initially sub-convulsive electrical stimulation within a specific brain region results in the progressive intensification of the evoked seizure activity. Indeed, kindling initially present itself as a simple phenomenon, whereby provocation of electrically stimulated focal seizures induces a clear progressive change in response over daily repetitions. The progression begins on the first day with a brief, low frequency electrographic afterdischarge (AD) at the electrode tip, which is associated with little behaviour response. However, the complexity of the phenomenon begins to appear as the response evolves over days, resulting eventually in the triggering of long, high frequency ADs associated with strong convulsive responses (Goddard et al, 1969). In the end, the induced increase in susceptibility to additional seizures is permanent and the brain that underwent kindling may be defined as an “epileptic” brain. This progression is readily apparent from all limbic and most forebrain stimulation sites, but it is dramatic from temporal lobe structures, such as the amygdala and the adjacent cortices, including the piriform, perirhinal, insular and entorhinal cortices (McIntyre et al, 1999). For these reasons, the kindling phenomenon has been employed extensively as a chronic model of temporal lobe epilepsy. It is the daily progressive increase in response severity in both EEG and behaviour that defines kindling.

Practically, kindling consists of the implantation of a bipolar electrode in the chosen brain area using stereotaxic surgery. Beginning one week after surgery, animals will undergo evaluation of AD threshold and, subsequently, daily stimulation with a single train of bipolar rectangular pulses, at the minimal amplitude capable of evoking a focal discharge in the surgically implanted area. This pattern of stimulation does not initially provoke any behavioural response but, in time, a progressively worsening seizure activity will be observed, which can be easily quantified (Racine et al, 1972).

By the description reported above, it is clear that kindling is initially a model of focal partial seizures, that, with daily repetitive stimulation, recruits more brain areas, starting from the implanted focus and spreading to connected circuits, becoming a model of complex partial seizures with secondary generalization. In particular, in kindling there is loss of consciousness, a feature of complex seizures. Even though seldom reported, continuous triggering of seizures over time may lead to spontaneous seizures in the chronic period of the model (Pinel and Van Oot, 1975; Wada and Osawa 1976; Michalakis et al, 1998) making this experimental approach a possible model for studying epileptogenesis.

### *Kainate*

(For an extensive review of the model see “Models of seizures and epilepsy” Pitkanen A., Schwartzkroin P.A. and Moshé S.L. (2006) Elsevier Academic Press)

This model has been developed from the initial observations of Nadler and of Ben-Ari and Lagowska (Ben-Ari and Lagowska, 1978; Nadler et al, 1978) that kainate induces repetitive seizures and neuronal damage in the hippocampus, reminiscent of temporal lobe epilepsy. From then on, kainate administration has been used to produce rodent preparations that display the general characteristics of human acquired or injury-induced epilepsy, including TLE.

The kainate model has at least three major stages: 1) the initial hours-long episode of status epilepticus, 2) the days to weeks-long seizure-free latent period and 3) the gradual development and progressive increase in the frequency of recurrent spontaneous seizures. The final stage is generally permanent and defines chronic epilepsy. During all the three stages anatomical, cellular and molecular changes occur and seem to develop in parallel with the acute and chronic neuropathological alterations reported in humans. These changes and plastic modifications suggest, like in the pilocarpine model, that status epilepticus triggers epileptogenic mechanisms.

Kainate administration may be done in different ways and this may change the outcome of the model. Single dose administration usually allows a rapid onset of status epilepticus, but lethality may be very high or some animals may never enter in status epilepticus. The first problem may be

solved by stopping status epilepticus using a benzodiazepine like diazepam. The second problem may be overcome by giving a second dose of kainate. The kainate model obtained by single high dose seems to reduce the probability in generating animals with robust spontaneous recurrent seizures. Repeated lower (subconvulsant) doses, titrated until the development of status epilepticus, is another protocol found in the preparation of the kainate model. This approach increases the success in inducing status epilepticus and dramatically reduces lethality (Hellier et al, 1998). Spontaneous seizures seem to occur more frequently using this experimental approach.

The kainate model associates to plastic alterations such as neuronal cell loss, reactive gliosis, network rearrangement, metabolic changes and genetic and molecular modifications. These features are in line with those found in human mesial temporal lobe epilepsy, making kainate model a good tool to explore mechanisms underlying epileptogenesis and epileptic induced modifications.

## AIM OF THE STUDY

The aim of this study has been to add new insights on the involvement of bradykinin in epilepsy by exploring the susceptibility of B1 null mice to two different epilepsy models: kindling, an electric model, and kainate, a chemical model.

# MATERIALS AND METHODS

## *B1 null mice*

Adult male B1 null mice and their controls C57Bl/6 were used for all experiments. The animals were housed under standard conditions: constant temperature (22–24°C) and humidity (55–65%), 12 h dark–light cycle, free access to food and water. Procedures involving animals and their care were carried out in accordance with European Community and national laws and policies. All efforts were made to minimize animal suffering, and to reduce the number of animals.

## *Kindling*

For kindling, a twisted bipolar electrode was implanted in the right amygdala (coordinates according to Paxinos and Franklin (Paxino and Franklin, 2001) and experimentally adjusted: 3,2 mm lateral and 1,2 mm posterior to bregma, 4,7 mm deep from dura) under isoflurane anaesthesia. Animals were allowed seven days to recover, and then stimulated to uncover their threshold. Threshold has been found for each animal following a protocol with a starting stimulation of 40 microA and increasing it by 20 microA every 2 minutes until a single after discharge of at least 5 seconds became evident on EEG recording. Animals not producing an AD below 300 microA were not included in the study. Starting from the day after evaluation of threshold, animals were stimulated once daily with a single 1 s train of bipolar pulses (1 ms, 60 Hz, mA 25% above after-discharge threshold). For every animal, behaviour (staging according to Janumpalli et al, 1998) and the duration of afterdischarge measured in the right amygdala were recorded after each stimulation. Kindling criteria were reached after three consecutive stage 4 or higher seizures.

## *Kainate*

Animals were treated with kainic acid (20 mg/kg, i.p.). After administration of this chemoconvulsant, seizure activity was monitored for 2 h by blind investigators, and scored as follows: 1, chewing and drooling; 2, head nodding; 3, unilateral forelimb clonus; 4, bilateral forelimb clonus; 5,

bilateral forelimb and/or hindlimb clonus with falling; 6, running or jumping seizure; 7, tonic hindlimb extension; 8, death (Janumpalli et al, 1998). Control mice were aged-matched litter-mates which were injected with saline.

The *cumulative seizure score* (CS) was rated as the sum of the maximal scores recorded in the 24 5 min intervals of the 2 h observation. The *seizures index* (SI) was rated as follows; 0, no severe seizures in the two hour observation; 1, one seizure classified equal or above to 4 in the two hour observation interval; 2, 2-5 seizures classified equal or above 4 in the two hour observation interval; 3, 6-10 seizures classified above or equal to 4 in the two hour observation interval; 4, more than 10 seizures classified above or equal to 4 in the two hour observation interval; 5, death of the animal within the two hour of observation. *Latency* was calculated as the time between the injection and the first class 4 or higher.

# RESULTS

## *Kindling development*

The kindling procedure has been applied to B1 KO mice in order to compare the response of WT and B1 KO mice. Indeed, B1 KO mice displayed a lower threshold compared to WT mice, indicating an already hyper-excitabile tissue ( $195,38 \pm 17,71$  microA for WT;  $143,08 \pm 14,47$  microA for KO;  $p=0,0313$ ) (Fig 4A) . This reflected in the number of stimulations required to reach the kindled condition that resulted lower in KO compared to WT mice ( $8,6 \pm 0,6$  stimulations in WT;  $5,7 \pm 0,8$  stimulations in KO;  $p=0,0048$ ) (Fig 4B). The average class experienced by mice for each stimulation resulted higher in B1 deficient mice in comparison with their C57Bl control mice. This phenomenon is more evident during the initial phases of kindling development (Fig 4D). The duration of the electrical manifestation (the after-discharge, AD) was slightly longer in B1 null mice than in controls (Fig 4C).

Taken together, the results of this experiment lead to the conclusion that B1 KO mice are more prone to kindling than normal mice.



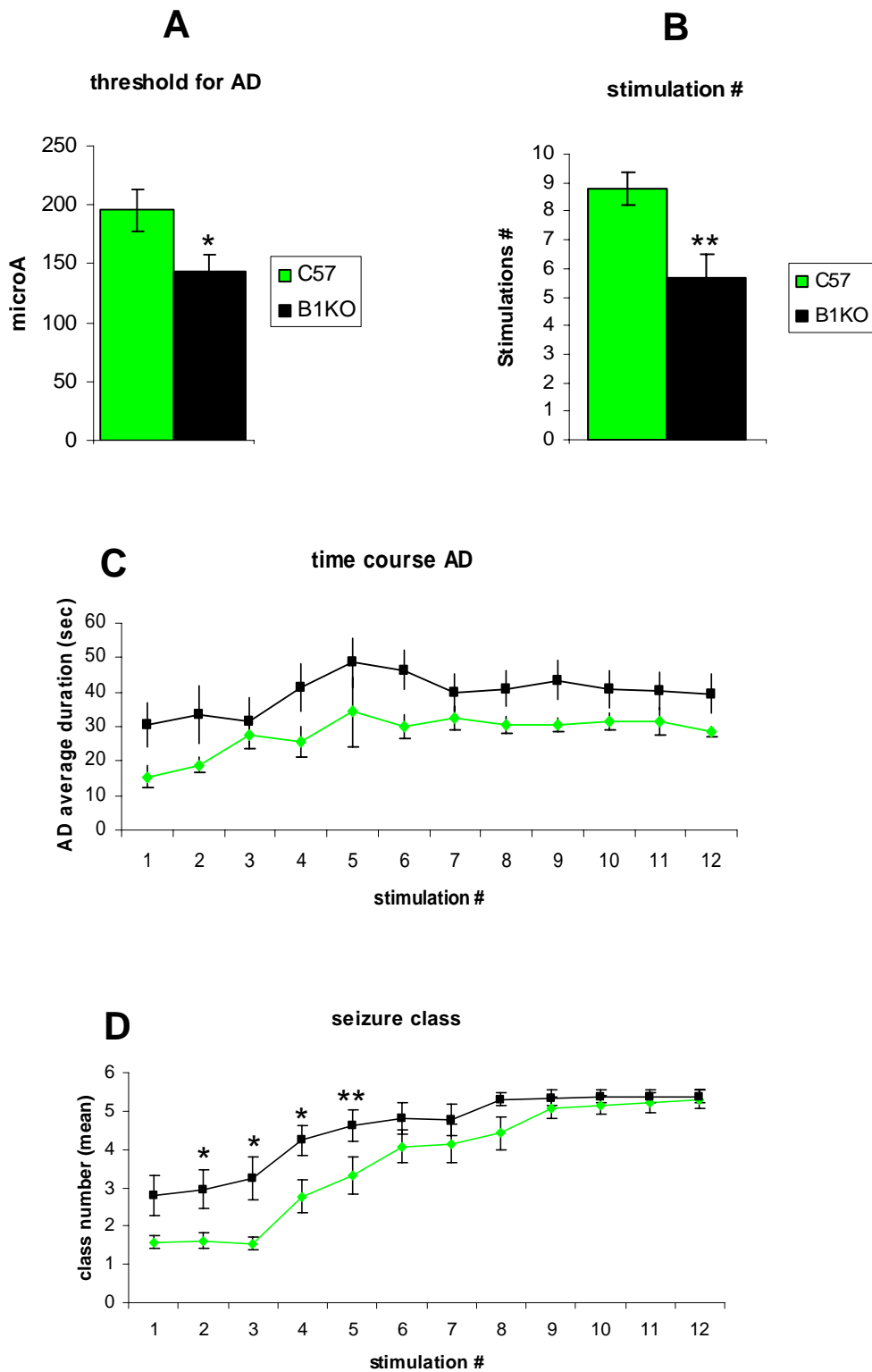


Figure. 4: kindling development in B1 KO mice. (A) Comparison of average threshold values between KO and WT mice: a lower threshold has been found in KO mice compared to WT. (B) number of stimulations required to reach the kindling status. A reduced number of stimulation is required in KO mice compared to controls. (C) Average AD duration for each stimulus in WT and KO mice. (D) Average seizure class (staged as in Janumpalli et al, 1998) during kindling development. KO mice show higher class for each stimulation than WT control mice.

## *Susceptibility to kainate seizures*

To explore the susceptibility to seizures of B1 null mice, WT and B1 KO age matched mice were injected with 20 mg/kg i.p. kainate, and their behavior monitored for 2 h thereafter. This dose of kainate was chosen because it causes relatively mild seizures in WT animals, allowing proper observation of possible alterations in the B1 KO group. The response to kainate was consistent with a latent epileptic phenotype in B1 KO mice. (1) Behavioral seizure scores, based on two distinct scoring systems, were significantly increased in B1 KO mice (Fig. 5 B and C). (2) The latency to generalized seizure onset (in the subset of animals displaying generalized seizures) was significantly shorter in B1 KO mice ( $26\pm 4$  min versus  $76\pm 12$  min in WT;  $p < 0.001$ , Student's t test for nonpaired data) (Fig. 5A). (3) The percentage of animals displaying generalized seizures (stage 4 or higher) was much higher in the B1 KO group: 6 of 13 WT animals (46,15%) versus 13 of 14 kainate-treated B1 KO mice (92,86%). During most of the 2 h after kainate administration, the seizure class was indeed higher for the B1 KO mice compared with the WT group (Fig. 5E). Furthermore, at the end of the observation period, all WT animals were behavioral seizure-free, whereas some B1 KO mice were still seizing (2 of 14, i.e., 14,28%). (4) Lethality was increased: whereas no WT mouse of 13 treated with kainate died, 4 of 14 (28,57%) B1 KO died during observation (i.e., in the first 2 h after kainate administration) (Fig. 5D).

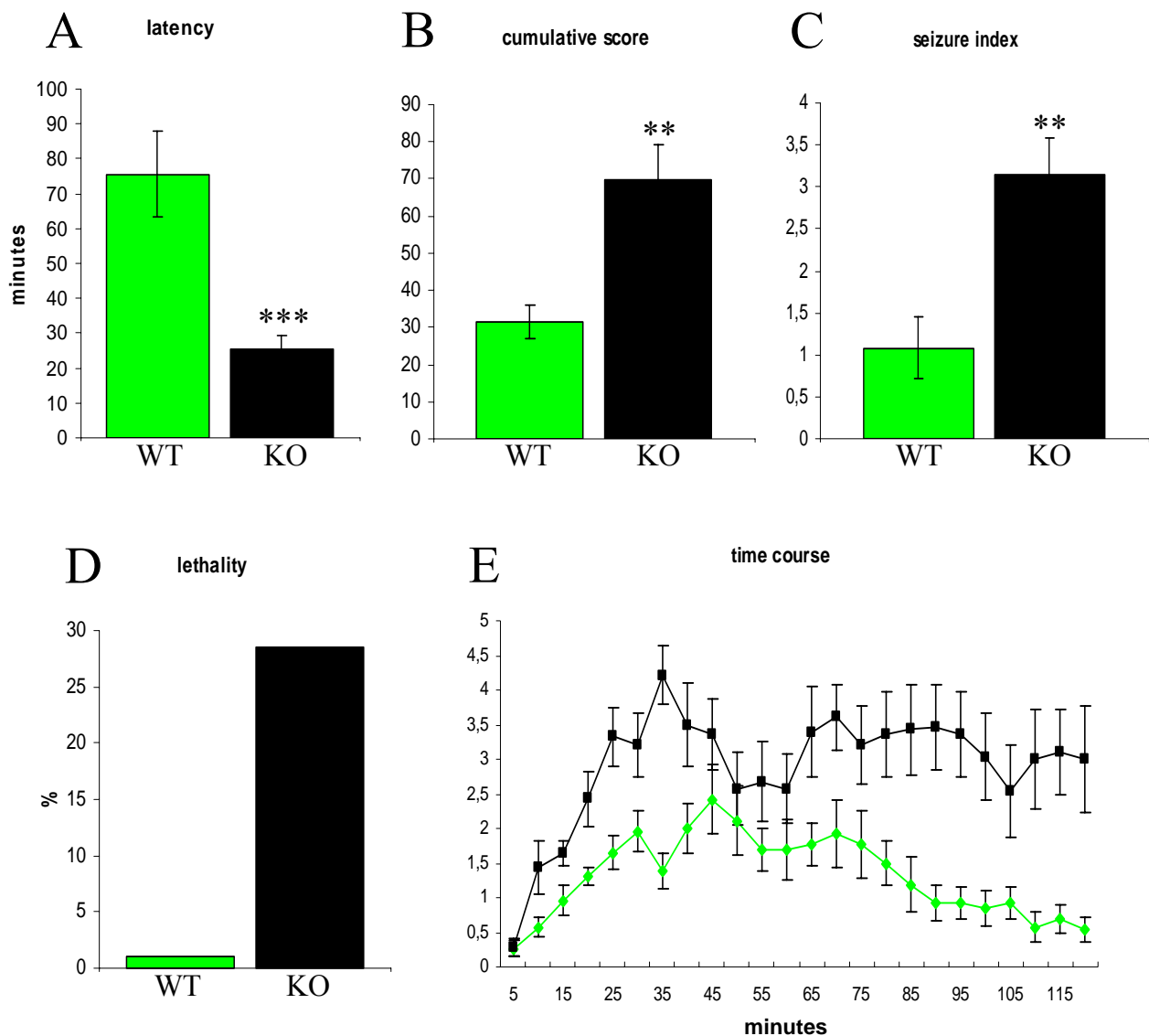


Fig. 5: Kainate seizure susceptibility in B1-deficient mice. (A) Latency to the first generalized (grade 4 or above) seizure after 20 mg/kg i.p. kainate injection in WT (green) and B1-deficient (KO) (black) mice. (B) Cumulative seizure score, rated as the sum of the scores recorded in each 5-min interval of the 2 h following kainate injection. (C) Seizure index: 0, no seizure; 1, 1 severe (class 4–5) seizure; 2, 2–5 severe seizures; 3, 6–10 severe seizures; 4, >10 severe seizures, or very severe seizures (class 6–7); 5, death within 2 h. (D) lethality expressed in %. (E) Time course of seizure grade. Data are the mean±SE of 13 WT and 14 KO mice per group; \*P<0.05, \*\*P<0.01; Mann–Whitney U test for panels B and C; \*\*\*P<0.001 Student's t test for panel A.

## DISCUSSION

In this work we report that B1 KO mice are hyper excitable in two epilepsy models. In kindling, animals displayed a lower AD threshold and this observation was confirmed in all the other parameters we analysed: a higher number of stimulations was required to reach the kindled state, the average class and the average AD duration was higher for each stimulation. In the kainate model, B1 KO mice resulted more prone to seizures than their age-matched controls, displaying higher severity scores.

These results are in contrast with what we were expecting based on our previous results (Bregola et al, 1999; Mazzuferi et al, 2005) and reports from other groups (Arganaraz et al, 2004a; Arganaraz et al, 2004b; Perosa et al, 2007; Pereira et al, 2008; Silva et al, 2008). Indeed, our previous findings demonstrated B1 receptors to mediate a higher excitatory response in the late phase of kindling, by increasing glutamate release (Bregola et al, 1999; Mazzuferi et al, 2005). Following this observation, the rest of the literature reports excitatory implications for B1 receptor in the pilocarpine model (Arganaraz et al, 2004a; Arganaraz et al, 2004b; Pereira et al, 2008; Silva et al, 2008).

What is then the role of the bradykinin system in epilepsy? Our findings may suggest a protecting role for B1 receptors. However, another hypothesis may be proposed: that B2 receptor may be over-expressed in the brain of B1 KO mice as a compensatory response to the absence of the inducible B1 receptors. Based on this hypothesis, the increased acute response to kainate may be explained by a high compensatory expression of B2 receptors. B2 receptors have already been demonstrated to be involved in the acute inflammatory response (Couture et al, 2001; Rodi et al, 2005;) and inflammation has been shown to have a compounding effect on brain tissue excitability (Vezzani et al, 2002; Vezzani and Granata, 2005). The same hypothesis may apply to kindling and its evolution. The lower threshold of B1 KO, when compared to controls, may be due to the putative excitatory effect of B2 receptor over-expression. Accordingly, if one assumes higher density of B2 receptors in many brain areas, this would imply a lower resistance to seizure spread and the faster kindling development that we observed. These speculations obviously need an experimental investigation which will involve a more accurate analysis of the B1 KO brain, in particular an evaluation of B2 receptor density.

In conclusion, it can be stated that, in both epileptic models we used, B1 KO mice displayed the same increased susceptibility to seizures, allowing the speculation that B1 receptors may be somehow protective in epilepsy or that a compensatory over-expression of B2 receptors may be detrimental for epilepsy phenomena. Both hypotheses are amenable of experimental investigation.

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## CONCLUDING REMARKS

The genetics of epilepsy are undoubtedly complicated. Genetic mechanisms are fundamental to explain why seizures appear, progress, worsen or eventually disappear. The data reported in this thesis add contributions to the vast amount of work that has been done to uncover how genes and their expression may be involved or influence the outcome of epilepsy.

In the Unverricht-Lundborg disease, a primary genetic epilepsy, we proposed a putative cellular and physiological mechanism that may underlie the pathophysiology of the onset and development of the disorder. In Fragile X, a major genetic defect that presents as a mental retardation syndrome with epilepsy, we demonstrated that *Fmr1* (the gene whose silencing causes the disease) is activated during epileptic phenomena and may contribute to the plastic response occurring after an ictal event. Finally, we found that brain excitability is exacerbated in B1 null mice after electric and chemical epileptic insults, suggesting that modifications in the bradykinin system create a fertile condition in which other genetic or acquired factors may induce epilepsy.

Increasing our knowledge of the genetic mechanisms and the consequent molecular, cellular, histological and physiological alterations is of key importance for a better treatment of epilepsy: this goal is highly expected by millions of people who deal with seizures and their consequences every day. In a wider perspective, the knowledge retrieved by genetic studies on epilepsy may help to shed light on the physiological functions of the brain.