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Mutant heteromeric nicotinic receptors in brain development and sleep-related epilepsy

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Alla mia famiglia e al mio amore

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Chapter 1: Introduction

Autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) is a focal epilepsy with attacks typically arising in the frontal lobe during non-rapid eye movement (NREM) sleep. The penetrance is about 70–80% and includes frequent, usually brief, seizures with onset in early adolescence with hyperkinetic or tonic manifestations, typically in clusters at night during slow-wave sleep (SWS). Paroxysmal arousals, dystonia-like attacks, and epileptic nocturnal wanderings are also frequently observed (Steinlein et al, 2010). To date, hundreds of ADNFLE families have been identified. Nonetheless, because the genetic analysis is incomplete and because misdiagnosis is still frequent (Nobili et al., 2014), the exact incidence of the disease is unknown. Ten to fifteen percent of the ADNFLE families bear mutations on genes coding for subunits of the neuronal nicotinic acetylcholine receptor (nAChR) (Ferini-Strambi et al., 2012). Recently, ADNFLE mutations have been also found in *KCNT1*, which codes for a Na^+-gated K^+ channel (Heron et al., 2012), and *DEPD5*, coding for the Disheveled, Egl-10 and Pleckstrin Domain-containing protein 5 (Ishida et al., 2013). However, little functional evidence is currently available for these genes.

1.1 nAChR and ADNFLE

The neuronal nAChR is a pentameric ion channel permeable to cations, including Ca^{2+}. When opened by Acetylcholine (Ach), it leads to membrane depolarization, which can produce post-synaptic

excitation or, in presynaptic terminals, stimulation of neurotransmitter release. In the mammalian brain, nine subunits concur in forming functional nAChRs: α2-α7 and β2-β4, encoded respectively by the *CHRNA2-CHRNA7* and *CHRNB2-CHRNB4* genes (Becchetti et al, 2015). The two most common subtypes in the brain are the heteropentamer α4β2 and the homopentamer $(\alpha7)_5$ (Dani and Bertrand, 2007). The first ADNFLE mutation was identified in *CHRNA4* (Steinlein et al., 1995). To date, four mutations are known in *CHRNA4* and six in *CHRNB2* consistent with the major role of α4β2 nAChRs in regulating neocortical excitability (Wallace and Bertrand, 2013). Some mutations are more frequently associated with psychiatric symptoms, but a specific relation between these symptoms and the functional alterations produced by the mutant subunits is not apparent (Steinlein et al., 2012). Finally, *CHRNA2* has also been causally associated with ADNFLE (Aridon et al., 2006). The α2 subunit can form heteromeric receptors by associating with both β2 and β4 (Hoda et al., 2009; Di Resta et al., 2010).

1.2 Animal ADNFLE models

Since 2006, several murine models of ADNFLE have become available. Klaassen used C57BL/6J mice to generate knock-in strains expressing either α4-S252F or α4-+L264, respectively homologous to the human α4-S248F and α4-(776ins3). Heterozygous mice present recurrent seizures accompanied by increased nicotine-dependent GABA release in layer II/III of the PFC (Klaassen et al., 2006) and layer

V of the motor cortex (Mann and Mody, 2008). On the other hand, in a different genetic background (CD1-129/Sv), expression of α4-S248F was found to confer a nicotine-induced dystonic arousal complex similar to the motor features of human ADNFLE, but no spontaneous seizures (Teper et al, 2007).

Another mutant subunit that has been widely studied in mice is β2-V287L. A knock-in strain expressing β2-V287L in C57BL/6 background displays a disturbed sleep pattern, abnormal excitability in response to nicotine, but no overt seizure phenotype (Xu et al., 2011). Conditional strains were generated expressing β2-V287L in FVB background, under control of the tetracycline promoter (TET-off system; Manfredi et al., 2009). Expression of β2-V287L causes spontaneous seizures, generally during periods of increased *delta* wave activity, presumably associated with SWS. The epileptic phenotype is not reversed when β2-V287L is silenced by administering doxycycline in adult mice. Conversely, when the transgene is silenced up to postnatal day 15, no seizures are observed, even if the transgene is expressed at later stages.

Prefrontal circuit alterations that lead to ADNFLE are likely to be produced during synaptogenesis: the role of nicotinic receptors in neuronal development is demonstrated by *in vitro* and *in vivo* evidence (Berg et al, 1996).

1.3. nAchRs contribute to synaptogenesis and epilepsy during development

As is also indicated by conditional expression of β2-V287L (Manfredi et al., 2009), the subtle prefrontal circuit alterations that cause ADNFLE seizures are likely to be produced during the developmental phases of network stabilization. In mammals, a "brain growth spurt" occurs around birth, characterized by neurite outgrowth, synaptogenesis, myelinisation and circuit pruning (Eriksson et al., 2000). In rodents, this phase spans the first 2-3 postnatal weeks and is accompanied by maturation of the cholinergic system and a transient increase of nAChR subunit expression.

1.3.1 Nicotinic receptors developmental expression.

The early presence nAChRs and of the machinery devoted both to synthesizing ACh during early embryogenesis suggests that important roles are exerted by nicotinic signaling in early neural development. Choline acetyltransferase, the synthetic enzyme for ACh, has been observed as early as the neural plate stage in presumptive crest (Smith et al. 1979). Specific neuronal nAChR subunit transcripts are detected as early as embryonic day 2 throughout the mouse CNS (Zoli et al. 1995), and both subunit mRNA and functional nAChRs are expressed in premigratory crest cells in vitro (Howard et al. 1995) and in autonomic and sensory neurons early in development (Role et al, 1996). Moreover, neuronal nAChR subunits are also expressed in presumptive myoblasts and related cellular types at early stages (Corriveau et al. 1995). The high relative Ca^{2+} permeability of nAChR

subtypes expressed during these early developmental stages (prior to neuronal differentiation) suggest a role for these receptors in regulating gene expression. In human (Hellstrom-Lindahl et al., 1998) and rodent brain (Zhang et al., 1998; Dwyer et al., 2008; Abreu-Villaca et al., 2011), most nAChR subunits reach their highest expression levels at late prenatal and early postnatal developmental stages. In the murine cortex, the majority of nAChR subunits show a transient peak of expression at early postnatal life (P7–P14). This is associated with dendrite outgrowth and synapses formation that leads to the formation of a proper neuronal network (Lin and Koleske, 2010). Shortly afterwards (P14–P21), a drop in some nAChR genes expression is observed. This temporal window is focal for the shaping of excitatory glutamatergic system by affecting dendrites and synapses maturation and pruning (Lai and Ip, 2013). These findings suggest that the down-regulation of some nAChR genes may be required for brain circuits maturation. As the brain matures (P21–P60), the expression of most nAChR genes is relatively low as compared to developmental stages. In the adult brain, nAChRs have been involved in the maintenance and plasticity of hippocampal and cortical excitatory systems (Picciotto et al., 2012; Poorthuis and Mansvelder, 2013; Proulx et al., 2013; Yakel, 2013).

1.3.2 Cholinergic and glutamatergic signalling interplay: nicotinic receptor contribute to dendritic spine formation and consolidation.

Dendritic spines provide critical postsynaptic compartments for excitatory glutamatergic transmission in the mammalian brain. Spines facilitate the transmission of the excitatory signal to the dendrites and they are focal for intracellular signalling compartmentalization. For example, they limit calcium influx to specific cellular compartments, therefore mediating synapse-specific effects (Lozada et al, 2012). Heteromeric nAChRs alterations have been found to deeply affect dendritic spines formation and glutamatergic synapses localization in CNS: β2 KOs mice ($\beta2^{-/-}$,) or mice pharmacologically treated in order to reduce β2- nAChRs expression (with RNA interference) does not show any reduction in glutamatergic synapses number; synapses functionality seems not to be affected (Lozada et al., 2012). Adopting the same genetic and pharmacological manipulations other studies have revealed a reduction in the number of dendritic spines. The spine loss in β2KOs is observed in CA1 hippocampal and cortical pyramidal neurons at early postnatal stages (P4) and persists until adulthood (P40–P60). When the β2 subunit expression is silenced, glutamatergic synapses appear to shift their localization to dendritic shafts (Lozada et al., 2012). Synapses localization plays an extremely important role in signal integration and transmission (Yuste and Bonhoeffer, 2001): therefore the redistribution of synapses in the absence of β2 subunit induces significant alterations on network connectivity and plasticity.

Size and complexity of the pyramidal dendritic trees appear to be reduced in the adult β2KOs mice cortex (P60). Not all areas are equally affected, probably due to different nAChR subunit expression in different cortical areas (Ballesteros-Yanez et al., 2010). β2-nAChRs may thus represent a central component that controls spine-based synapses and, subsequently, circuitry refinement.

1.3.3 The cholinergic system and cytoskeletal dynamics.

During development, the cytoskeletal dynamics in nascent dendrite branches and axon growth cones promotes the initial contact between presynaptic and postsynaptic sites (Yoshihara et al, 2009). nAChRs activation, by allowing the influx of Na^+ and Ca^{2+} into the cell, can regulate several intracellular signalling cascades affecting cytoskeletal dynamics (MacNicol and Schulman, 1992; Yamashita and Isa, 2003; Steiner et al., 2007; Gubbins et al., 2010). nAChR opening has been found to affect Ca^{2+}/CaMKII signalling, which is involved in activity-dependent dendrite stabilization (Wu and Cline, 1998; Vaillant et al., 2002) and dendritic spines induction (Jourdain et al., 2003; Pi et al., 2010). In addition, nAChRs can lead to the activation of other signalling cascades which affect the cytoskeleton dynamics in dendritic structures (Vaillant et al., 2002; Goldin and Segal, 2003; Middei et al., 2012; Zhang et al., 2013). However how nAChRs contribute to excitatory system shaping is not still clear. Intriguingly, nAChRs localization and function is under the control of the same proteins that regulate cytoskeletal dynamics, suggesting a crosstalk

between cholinergic and cytosketal dinamics. RhoA and ROCK proteins, central mediators of cytoskeletal processes in neurons (Koleske, 2013), have been recently found to influence nAChR assembly and clustering in vitro. RhoA and ROCK pharmacological inhibition favors the accumulation of α3β4-nAChRs on the membrane (Bruses, 2013). The function of nAChRs can be regulated by the non-receptor protein kinases of Abelson family kinases (AFKs, Abl1 or Abl2) (Jayakar and Margiotta, 2011). Abl2 is involved in dendritic spines localization and selectively regulate spine and dendrite stability (Lin et al., 2013). Abl2 can therefore specifically stabilize either spines or dendrites through a nAChR-dependent mechanism. (Molas et al, 2014)

Synapses and dendritic structures formation and long-term stability appear to be affected by the cytoskeleton control machinery (Koleske, 2013), and cell adhesion molecules at synaptic sites play important roles in the induction of synapse formation and in the stabilization of the contact between presynaptic and postsynaptic compartments (Molas et al, 2014). Ample evidence highlights that nAChRs directly interact with cell adhesion molecules: α4β2-nAChRs, when expressed in heterologous cells, have been found to co-immunoprecipitates with neurexin-1 (Cheng et al., 2009) while α7-nAChRs seems to interact with ephrinB2 (Liu et al., 2008). The interaction probably account for nAChRs specific membrane sites localization: however nAChRs could affect the glutamatergic system influencing these proteins function (Molas et al, 2014).

1.3.4 nAChRs contribute to glutamatergic system transmission and plasticity.

Heteromeric nAChRs can affect the glutamatergic system development by directly affecting neurotransmitter release: presynaptic nAChRs have been found to promote both glutamate and GABA release on layer V prefrontal cortex pyramidal neurons (Aracri et al, 2010). Maintenance of synaptic input is critical for dendritic stabilization (Trachtenberg et al., 2002; Cline and Haas, 2008). nAChRs located on presynaptic glutamatergic terminals are known to facilitate the release of glutamate that help to convert "silent" synapses to functional ones in developing hippocampal and cortical neurons (Molas et al, 2014). Conversion of silent synapses into functional ones constitutes an efficient mechanism for enhancing synaptic efficacy in the immature brain; it is commonly accepted that this conversion occurs mainly at a postsynaptic site with the insertion of new α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors on the subsynaptic membrane. Nicotine administration has been found to elicit persistent changes in synaptic efficacy in immature glutamatergic synapses. (Maggi et al, 2003). Activation of nAChRs localized on pre-synaptic glutamatergic nerve endings seems to be involved in the process: nicotine has been found to be responsible for EPSCs appearance in silent neurons and their frequency increase the frequency in those exhibiting spontaneous events. Both nicotine and endogenously released acetylcholine (ACh) increase the probability of neurotransmitter release. Several mechanisms may account for the nicotine-promoted glutamatergic

synapse activation: calcium influx through nAChRs and voltage gated Ca^{2+} channels may directly promote neurotransmitter vesicular release or affect synaptic efficiency through the activation of several intracellular signalling cascade. The activity of nAChRs seems also to enhance postsynaptic responses mediated by NMDARs thus participating in the induction of synaptic plasticity both in the hippocampus and cortex. A recent hypothesis is that nAChRs and NMDA receptors have complementary roles in synaptic plasticity: both nAChRs and NMDA receptors show relatively high Ca^{2+} permeability, and activation of these receptors modulates intracellular Ca^{2+}concentrations. However, nAChRs can conduct Ca^{2+} at resting membrane potentials, whereas NMDA receptors cannot, due to the Mg^{2+} blockade. Mansvelder and McGehee proposed that nAChRs and NMDA receptors can fulfill complementary excitatory system long-term enhancement (Mansvelder and McGehee,2000; Feduccia et al., 2013). Evidence also suggests that nAChRs control excitatory transmission and plasticity indirectly, through GABAergic interneurons: nAChRs location on inhibitory populations provide these receptors with a broad range of possible modulation of glutamatergic system being able to inhibit or stimulate pyramidal neurons (Griguoli and Cherubini, 2012; Gu and Yakel, 2011; Poorthuis and Mansvelder, 2013). In addition, nAChRs can also modulate the excitatory system via non-neuronal cells: astrocytic nAChRs enhance synaptic plasticity in the hippocampus (Lopez-Hidalgo et al., 2012) and can trigger the recruitment of AMPA receptors on postsynaptic sites (Wang et al., 2013).

1.3.5 Molecular mechanisms of cholinergic driven glutamatergic synapses consolidation

nAChRs are found both presynaptically and postsynaptically at most glutamatergic synapses in cortex and hippocampus (Bertrand et al., 1993; Fabian-Fine et al., 2001). These receptors' activation facilitates glutamate release and can promote LTP in a synapse specific manner requiring coincident glutamatergic transmission at the synapse (Yakel, 2012). An elegant work from Halff and colleagues highlighted a novel mechanism by which nicotinic activity can drive synaptic potentiation independent of fast excitatory transmission. The enhancement can be induced in the absence of coincident action potentials (APs) at the synapse, depending instead on α7 nAChRs acting in a cell autonomous manner on the postsynaptic cell. The enhancement results from a stabilization and accumulation of GluA1-containing AMPARs (GluA1s) at synaptic sites on dendritic spines. The nicotine-induced entrapment of GluA1s depends on intracellular free calcium, the availability of PDZ [postsynaptic density-95(PSD-95/Discs large (Dlg)/zona occludens-1 (ZO-1)]-binding scaffold proteins, and the lateral mobility of surface GluA1s (Halff et al, 2014). Cell-autonomous actions have been proposed for nAChRs in regulating network integration and synapse formation of developing neurons (Campbell et al., 2010; Lozada et al., 2012b). Both acute and chronic nicotine administration leaded to stabilization of GluA1s on spines; this is consistent with persistent ambient levels of ACh thought to occur in the CNS as a result of volume transmission, (Umbriaco et al., 1995; Pepeu and Giovannini, 2004). This mechanism could be

relevant not only for nicotine exposure, but also for endogenous nicotinic cholinergic signalling through ACh and can therefore play a focal role in neuronal network development and stabilization.

1.3.6 Cholinergic influence over GABAergic excitatory/inhibitory switch: how cholinergic alteration can affect early network activity.

Recent findings suggest a possible interplay even between cholinergic and GABAergic signalling in regulating the formation and remodelling of synaptic connections (Liu et al, 2006). Releasing γ-aminobutyric acid (GABA) onto different neuronal population, GABAergic interneurons regulate cells excitability giving rise to networks oscillations which are supposed to allow distinct brain states transition and high cognitive functions (Klausberger and Somogyi, 2008). GABA is the main inhibitory neurotransmitter in the adult brain, but during early stages of development GABAergic play an excitatory role due to a reversed chloride gradient (Ben Ari, 2014). Neuronal $[Cl^-]_I$ is under the control of cation-chloride co-transporters (CCCs). The two main CCCs which control chloride concentration inside the cell are the Na–K–2Cl importer NKCC1 and the K–Cl extruder KCC2. The low expression of KCC2 at birth leads to high $[Cl^-]$ inside the cell (~20 mM) and set the equilibrium potential for chloride (E_{Cl^-}) to more positive values compared to the resting membrane potential (V_m). The progressive reduction in $[Cl^-]_I$ with age, due to the developmentally up-regulated expression of KCC2 and the

concomitant down-regulated expression of NKCC1, leads to relatively low $[Cl^-]_I$ (~10 mM) (E_{Cl} close to V_m)(Cherubini et al, 2013). The GABAergic excitatory period plays a key role in neuronal maturation and integration into circuits during embryonic development and after adult neurogenesis. Immature neurons generate a singular network-driven pattern called *Giant Depolarizing Potentials* (GDPs) that plays a focal role in remodelling of synaptic connections in both neocortex and hippocampus; GDPs, which are mediated by $GABA_A$ and glutamate receptors, disappear as GABA shifts towards its inhibitory role (Ben Ari, 2002). GDPs are network-driven, synaptic events that engage a large number of neurons in a synchronous discharge. GABA-driven depolarization can remove the voltage-dependent Mg^{2+} block of *N*-methyl-D-aspartate receptor (NMDA channels) thereby producing large intracellular calcium influx (Leinekugel et al., 1997). The combination of this depolarization and the large and long-lasting NMDA-mediated EPSCs generates GDPs: this kind of processes can modulate synaptic plasticity and affect the generation of oscillations that modulate activity dependently the formation of functional units. Pair recordings of interneurons and pyramidal cells show that interneurons and pyramidal neurons are synchronized during GDPs. Further evidences suggest that GABA-mediated membrane depolarization provides the first excitatory drive necessary for promoting neurite outgrowth and synapse formation (Cherubini et al., 2013). Therefore, the excitatory/inhibitory GABA shift is associated with a complex timing of events that lead the formation of functional neuronal units. (Ben Ari, 2014). A recent work from Liu

and colleagues reveals that that endogenous nicotinic cholinergic activity drives maturation of GABAergic signalling (Liu et al, 2006). Pharmacological nAChRs blockade in vivo revealed the effects of spontaneous nicotinic cholinergic activity on GABAergic maturation: when nAChR activation is prevented by antagonist administration (both MLA or DHβE) GABA administration remains excitatory: GABA application on acute dissociated chick ciliary ganglion neurons induced a depolarization (leading to a calcium influx through low threshold voltage-gated Ca^{2+} channels VGCCs) even in advanced developmental stages (E14 when this kind of response is usually loss). Also α7-nAChRs knock-out prevents the excitatory inhibitory (I/E) transition in GABAergic signalling. The depolarizing chloride gradient, resulting from an immature expression pattern of chloride transporters (NKCC1/KCC2) in pyramidal cells, appears to be responsible for the extended period of GABAergic excitation Morphological analysis revealed reduced spines density and dendritic arborization in treated neurons. Also nAChRs genetic knock-out exerts a similar effect (Liu et al, 2006). Cholinergic signaling was thus proposed to cooperate with GABAergic ones in regulating neurogenesis probably affecting gene expression. nAChR activation directly promote calcium influx besides inducing a depolarization which can lead to calcium influx through VGCCs. Concomitant GABA release hyperpolarize the membrane preventing VGCCs activation in mature neurons, while may exerts an opposite effect in immature neurons. Reduced VGCCs activation is required for nicotinic CREB (cAMP response element-binding protein) activation which leads to

alteration in gene expression: In contrast, VGCCs-induced calcium influx, activates phosphatases which prevent CREB activation. Concomitant GABAergic receptor and nAChRs activation may have different outcome in term of synaptic reinforcement or loss (Chang and Berg, 2001). Besides affecting the timing of GABAergic I/E shift ACh release promotes GABA and glutamate dependent GDP-like activity in spinal cord (GICs, spontaneous giant inward currents) (Czarnecki et al., 2014). Blocking nAChR clearly inhibits spontaneous GIC occurrence and prevents the burst propagation along the cord (Hanson and Landmesser, 2003). ACh release is unlikely to control GIC rhythmicity because these events are partially restored in the presence of nAChR antagonists when external K^+ concentration is increased to depolarize the cells. The main function of ACh release could probably be the activity control of interneurons which drives GDPs. ACh seems to exert complex effects on interneurons promoting these neurons activity by acting on DHβE-insensitive nAChRs and regulating GABA and glutamate synaptic release by acting on α-containing heteromeric presynaptic nAChRs (Czarnecki et al., 2014). Mutation in nAChRs are therefore very likely to alter the synchronized neuronal firing which drives the formation of coherent neuronal network.

1.4 Aim of the work

The aim of my work is to analyze the role of cholinergic signalling in the shaping of glutamatergic synapses, in order to understand how mutant heteromeric nAChRs mutation can contribute to an ADNFLE phenotype in an established neuronal network, and how they can affect neuronal development leading to the formation of an aberrant neuronal network.

Chapter 2 describes the cholinergic system and $GABA_A$ receptor expression during development and particularly the effect of a gain-of-function mutant β2 subunit (β2-V287L) in the establishment of ADNFLE. Taking advantage of the murine model developed by Manfredi and colleagues (Manfredi et al, 2009), I considered the developmental aspects of this pathology, and particularly the effect of β2-V287L over GABAergic excitatory/inhibitory switch, which is known to be deeply affected by cholinergic signalling.

Chapter 3 shows the effect β2-V287L subunit over the development of glutamatergic transmission, again taking advantage of the previously mentioned murine model (Manfredi et al, 2009).

Chapter 4 shows how loss of function CHRNA2 (cholinergic receptor nicotinic alpha 2 subunit) mutations can be related to ADNFLE. In particular, it considers the pathogenic effect of an α2 subunit mutation (Ile297Phe) identified in a cohort including ADNFLE and NFLE patients.

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Chapter 2: The expression of mutant β2-V2878L nAChR subunit does not affect the timing of GABAergic excitatory/inhibitory switch.

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Unpublished data

2.1 Introduction

Early postnatal life is a critical time for CNS development. During this period, a strong synaptic remodeling that allows the development of a well-balanced and functional neuronal network, takes place. Coordinated neuronal activity requires structural and functional coupling of neurons. Synchronized neuronal firing plays a key role in determining which synaptic connections will be maintained and which will be lost. GABAergic signalling has gained interest in recent years, due to the role of GABAergic interneurons in orchestrating dynamic pattern formation in neuronal networks (Egurov and Draguhn, 2013). GABAergic polarity largely depends on the intracellular chloride concentration: GABAergic current reversal potential (E_{GABA}) is primarily determined by chloride equilibrium potential (E_{Cl}) because the receptor permeability for Cl^- is five times that of HCO^{3-} (Bormann et al, 1987). The balance between intracellular and extracellular Cl^- concentration is determined by the activity of the two chloride cotransporter NKCC1 and KCC2 (which respectively absorbs and extrudes chloride). During early postnatal stages GABA acts as excitatory neurotransmitter (Ben Ari, 2015) as the low expression of KCC2 at birth leads to high $[Cl^-]$ inside the cell (~20 mM) and sets the equilibrium potential for chloride (E_{Cl^-}) to more positive values compared to the resting membrane potential (V_{rest}). The developmental KCC2 up-regulation and the concomitant NKCC1 down-regulation, leads to relatively low $[Cl^-]_I$ (~10 mM), and E_{Cl^-} close to V_{rest} (Cellot and Cherubini et al, 2013). This switch takes place in mice between the first and the second week of postnatal life.

The GABAergic depolarizing action and the extensive electrical coupling may (due to the high presence of gap-junction, in immature neurons, which are subjected to downregulation with development) promote unspecific recruitment of excitatory and inhibitory neurons into propagating waves of activity in immature CNS. The GABA-driven network activity, called GDPs, is likely to deeply influence the establishment of proper connections between different neuronal populations (Picardo et al, 2011). Alterations in the timing of GABAergic switch can therefore lead to an imbalance between excitatory and inhibitory connections, thus promoting the formation of an aberrant neuronal network highly susceptible to seizures. Recent findings highlighted an interplay between GABAergic and cholinergic signalling. Pharmacological inhibition or KO of nAChRs have been found to affect the timing of GABAergic E/I transition (Liu et al, 2006). Treated neurons retained an excitatory response to GABA administration at later developmental stages compared to the controls. Besides influencing the timing of this process, the cholinergic system plays an important modulatory effect on GABAergic system: heteromeric nAChRs activation has been found to promote GABA-driven network oscillation in spinal cord (Czarnecki et al, 2014). Since pyramidal neurons and interneurons may differ in terms of ion channels' expression, nAChRs' mutation can also affect the strength of synaptic connections between different neuronal subpopulations.

A murine ADNFLE model, conditionally expressing a mutant β2 subunit (V287L), under the control of a Tet-off promoter, suggested that mutant heteromeric nAChR can account for an epileptic phenotype affecting the neuronal network development (Manfredi et al, 2009). In this murine strain, transgene expression from E15 to P15 is necessary for the establishment of an epileptic phenotype; transgene expression at later stages does not lead to ADNFLE onset.

We thus considered if, in this murine ADNFLE model, GABAergic switch timing is affected: we first carried out our characterization in acute dissociated neurons from PFC and how nAChR and $GABA_A$ receptor expression change during the first two postnatal week through a combined electrophysiological and immunohistochemical approach. We then proceeded to consider the timing of GABAergic E/I switch taking advantage of the perforated patch-clamp method (to determine E_{Cl} without perturbing intracellular chloride homeostasis). Due to high acute dissociated neurons' sensitivity to perforated patch (which often leaded to cell death before allowing to record GABAergic currents) we replicated our characterization in primary neuronal culture.

2.2 Material and Methods

Drugs and solutions. Stock solutions were daily prepared from dry powders. Unless otherwise indicated, chemicals and drugs were purchased by Sigma-Aldrich.

Brain slices preparation and acute neuronal dissociation. FVB wild type and mutant mice, aged 4–12 days, were deeply anesthetized with isoflurane and decapitated. Brains were removed and placed in ice-cold solution containing (mM): 87 NaCl, 21 $NaHCO_3$, 1.25 NaH_2PO_4, 7 $MgCl_2$, 0.5 $CaCl_2$, 2.5 KCl, 25 D-glucose, and 75 sucrose, equilibrated with 95% O_2 and 5% CO_2 (pH 7.4). Coronal slices (300 μm thick) were cut with a VT1000S vibratome (Leica Microsystems) from the medial PFC and in particular from the Fr2 region (between +2.68 and +2.10 mm from Bregma; Paxinos and Franklin, 2004).1 hour after excision, we proceeded to enzymatic digestion with Protease XIV: brain slices have been incubated at 37°C in Dissociation Medium(DM) (in mM: 134 Sodium Isethionate, 23 Glucose, 15 Hepes, 2 KCl, 4 $MgCl_2$, 100 $CaCl_2$ and 0.75mg/mL Protease XIV) for different times accord to mice age (from 5 minute for P5 to 15 minute for P15). After wash in DM, we then proceeded to mechanical dissociation using Pasteur pipettes whose diameter was progressively reduced. The cell suspension was then transferred to Concanavalin A coated Petri dishes for patch clamp analysis.

Cell cultures. Cerebral cortices were removed from decapitated postnatal mice (P1–P3) cut into 1 mm^3 pieces, and digested by trypsin (0.15%) and DNAse (10 μg/ml) at 37°C for 20 min. After enzyme digestion, the cells were mechanically dissociated by means of trituration, and plated on 35 mm polyethyleneimine pre-coated Petri dishes 0.1% (w/v). After 3 h incubation, the plating medium was replaced by Neurobasal medium (NB) with B27 (Invitrogen, Italy), 1

mM glutamine and 10 ng/ml bFGF, and the culture was maintained at 37° C in 5% CO_2. One-half of the medium volume was replaced every 3 days.

Cortical tissue preparation for immunocytochemistry. Young mice (aged 3–28 days) were anesthetized with intraperitoneal 4% chloral hydrate (2 mg/100 g) and sacrificed by intracardiac perfusion with 4% paraformaldehyde, in phosphate buffer (PB) 0.1*M*(pH 7.2–7.4). Perfusion of paraformaldehyde was preceded by heparin followed by 1% paraformaldehyde in PB. Such a procedure allows to maintain adequate tissue antigenicity. Brains were removed from skulls and immersed in 4% paraformaldehyde in PB, at 4°C, overnight. Coronal sections (50 µm thick) were cut with a VT1000S vibratome (Leica Microsystems) from Fr2 and SS (between +0.50 and +0.02 mm from Bregma; Paxinos and Franklin, 2004) cortex. For each cortical region, three to four sections were selected for immunocytochemistry.

Immunofluorescence assay. Fluorescent labeling was studied with confocal microscopy. After aldehyde quenching with NH_4Cl for 30 min, sections were rinsed with PB. They were next treated with BSA (1%) and Triton X-100 (0.2%) in PBS for 30 min. Finally, sections were washed with PBS and incubated for two nights with polyclonal anti-α4 nAChR antiserum (made in guinea pig, diluted at 1/1000 ;Millipore, Milan, Italy). For primary antibody detection we used biotinylated anti-guinea pig IgG, made in donkey (bDAGp; 1/200; Vector Laboratories, CA), and Alexa-488-labeled streptavidin (1/200; Molecular Probes). For α4 nAChR subunit densitometric analysis

neuronal bodies have been identified through Neurotrace (1:50, Molecular Probe). After rinsing, samples were mounted on coverslips with PBS/glycerol and inspected with a TCS-NT (Leica Laserteknik GmbH) laser scanning confocal microscope, to visualize double fluorescent labeling. Immunoreaction specificity was tested by carrying out negative controls as in previous works (Aracri et al, 2013). In this case, no specific signal was ever observed. Fluorescence intensity change has been quantified using Image J (Wayne Rasband, National Institute of Health, USA)

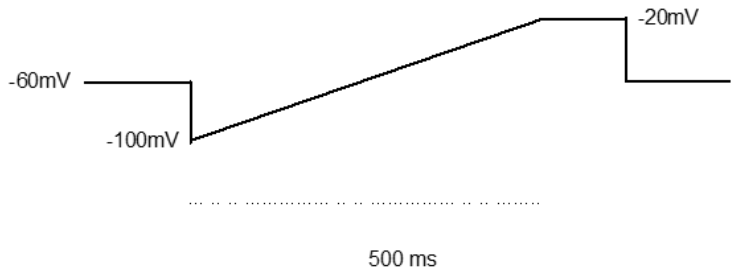
Densitometric analysis. Sections were inspected with a Leica TCS NT confocal microscope maintaining the same acquisition parameters. For each animal we compared three different PFC areas. Mean fluorescent intensity for has been evaluated with ImageJ (Version 1.37, National Institutes of Health, USA). For each tested age, four sections from three different animals have been considered for both PFC and SS cortex. Mean fluorescence intensity has been normalized for the number of cells (identified with Neurotrace) in the considered area. Data are showed as mean ± s.e.m.. Statistical comparisons between data were carried out with unpaired Student's *t*-test, at the indicated level of significance (*p*).

Patch-clamp recordings. Patch-clamp recordings were obtained from freshly PFC dissociated neurons. To assess how $GABA_A$ receptor expression changes during development, we adopted voltage clamp in whole-cell configuration. We recorded GABA-evoked currents at -60 mV adopting an internal solution with high chloride concentration

(in mM: 70 K-Gluconate, 70 KCl, 1 $MgCl_2$, 10 HEPES, 0.5 BAPTA , 2 Mg-ATP, 0.3 Na-GTP buffered to pH 7.25 with KOH). The standard external solution contained (mM): 150 NaCl, 5 KCl; 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 10 Hepes (buffered to pH 7,4 with NaOH). In these experimental conditions E_{Cl} is more positive than recording potential (- 17mV as determined from Nernst equation) therefore GABAergic currents are depolarizing. The $GABA_A$ mediated currents were distinguished based on their fast kinetics and by administration of 10 µM Gabazine, a specific antagonist for these receptors. Pyramidal neurons were distinguished by their morphology.

To analyze the developmental shift of E_{GABA} we used the perforated-patch technique with gramicidin (5/7 mg/mL) to preserve the intracellular chloride concentration. We adopted the same external solution used for whole-cell recordings. Patch pipettes contained (mM): 135 K-Gluconate, 5 KCl, 1 $MgCl_2$, 10 HEPES, 2.5 BAPTA buffered to pH 7,25 with KOH).The pipette tip was initially filled with a gramicidin-free pipette solution by a brief immersion. The remainder of the pipette was then backfilled with the same solution also containing gramicidin diluted to a final concentration of 5 µg/mL. Gramicidine D stock solution was daily prepared by dissolving the drug in DMSO (100 mg/ml). After each dilution, the solution was sonicated for about 5 seconds. The extracellular solution and drugs were applied with an RSC-160 Rapid Solution Changer (BioLogic Science Instruments).

Currents were recorded with an Axopatch 200B amplifier (Molecular Devices), at room temperature (20–22°C). Micropipettes (3–4 MΩ) were pulled from borosilicate capillaries (GMBH, Science Product) with a P-97 Flaming/Brown Micropipette Puller (Sutter Instrument Co.). The cell capacitance and series resistance (up to about 75%) were always compensated. Currents were low-pass filtered at 2 kHz and acquired on-line at 5–10 kHz with Molecular Devices hardware and software (pClamp 8 and Axoscope 8). Cell were recorded after the access resistance reach a value lower than 30 MOhm (usually taking between 20 to 60 minutes). Cells were discarded if the resistance dropped too quickly (suggesting membrane rupture). No corrections for junction potentials have been applied. E_{GABA} was estimated applying voltage ramp from -100 mV to -20 mV. To isolate GABA evoked currents, background currents have been removed by subtracting from currents recorded in presence of the agonist those recorded before GABA application and after drug washout. Five ramps were applied for each condition and data expressed as averages to decrease fluctuation in measurements.



Patch clamp statistical analysis. For evaluating the $GABA_A$ receptor expression we considered peak current amplitude evoked by 100 µM GABA. The GABAergic current density was obtained by normalizing peak current amplitude for the cell capacitance (expressed as pA/pF).

To estimate E_{GABA} we generated for each cells I/V curve. From each of the resulting I/V relations, we estimated E_{GABA} visually after fitting the I/V curve with a polynomial function.Data are given as mean ± s.e.m. with "*n*" indicating the number of determinations. Statistical comparisons between two populations of data were carried out with unpaired Student's *t*-test, at the indicated level of significance (*p*), after testing that the data were normally distributed (with KS test), with equal variances (with *F*-test). Intracellular chloride concentration has been determined through Nernst Equation:

$$V_{eq} == \frac{RT}{zF} \ln\left(\frac{[X]_{out}}{[X]_{in}}\right)$$

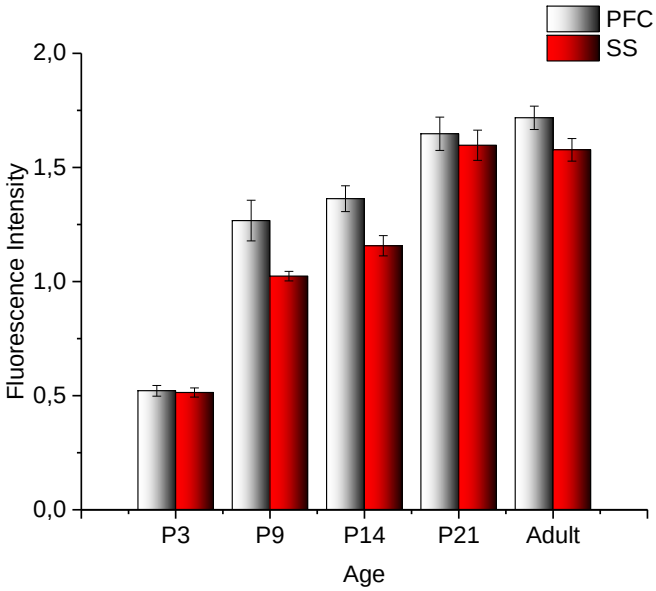
Where *R* is the universal gas constant, *T* is the temperature in Kelvin, *z* is the valence of the ionic species, *F* is the Faraday's constant and $[X]_{in}$ and $[X]_{out}$ ion concentration in the intracellular and extracellular environments .

All statistical analysis have been performed using Origin 9.1.

2.3 Results

Heteromeric nAChRs are expressed during early developmental stages. Before proceding to analyze the role of heteromeric nAChRs in the development of GABAergic signalling and their contribute to ADNFLE establishment we considered how α4β2 nAChRs are expressed in wild type mice cortex. In collaboration con prof. Alida Amadeo's laboratory we performed a series of immunofluorescence assays to evaluate α4 subunit expression (which associates with β2 subunit to form the most common isoform of heteromeric nAChRs in brain). Densitometric analysis highlighted that α4 subunit expression is higher in PFC cortex (layer V) than in somatosensory cortex (SS) at any tested ages; fluorescence intensity progressively increase over the first fifteen days of postnatal life, reaching stable values around P21. The fluorescence increase is more pronounced between P3 and P9. Between P9 and P14 a the fluorescence signal is significantly higher in PFC than in SS region (Fig 1)

a


PFC
SS
2,0
1,5
1,0
0,5
0,0
Fluorescence Intensity
P3
P9
P14
P21
Adult
Age

b

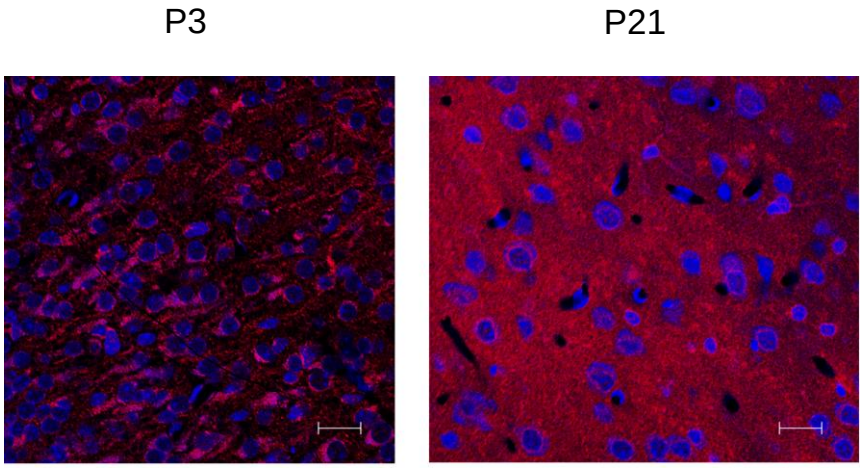
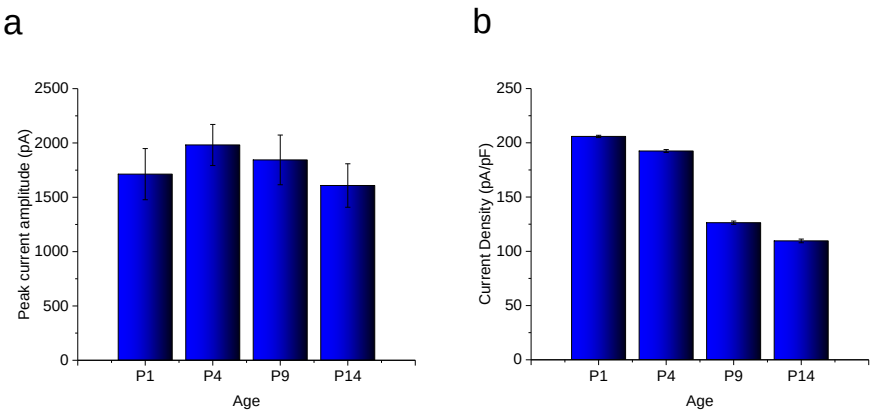
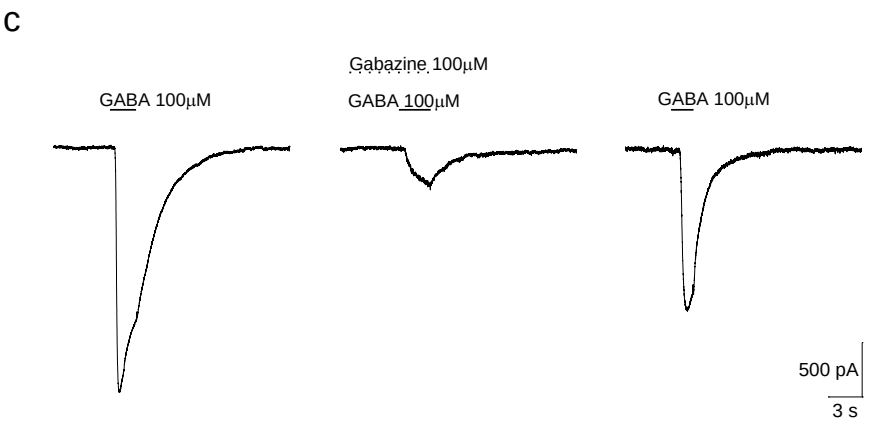

P3
P21

Figure 1: Cholinergic system development. A) Histogram showing developmental changes in α4 nAChR's subunit expression in PFC and SS cortex. For each age tested, four sections from three different animals have been considered for both PFC and SS cortex; between P9 and P14 a difference in fluorescence intensity between the two areas was detected ($p<0.05$). B) High magnification confocal images from P3 and P21 mice prefrontal cortex (α4 nAChR's subunit: red signal; Neurotrace: blue signal)(scale bar= 20µm)

$GABA_A$ receptor expression during development in wild type mice PFC. Since GABAergic transmission play a focal role in the development of a coherent neuronal network, we considered how $GABA_A$ receptor ($GABA_AR$) expression changes during the first two postnatal weeks. Whole-cell patch-clamp recordings from acute dissociated pyramidal neurons show a high $GABA_AR$ expression around birth, which remains rather stable over the considered temporal window (Figure 2a) (P1: 1713.12 ± 235.47 pA, n=7 ;P4: 1981.41 ± 189.46 pA, n=12; P9: 1844.18 ± 228.97 pA, n=17; P14: 1608.36 ± 200.33 pA, n=11). Because during this time the neuronal capacitance progressively increase, while the channel expression remains stable, a reduction in GABAergic current density is observed (Figure 2b) (P1: 205.86 ± 1.06 pA/pF, n=7 ;P4: 192.45± 1.35 pA/pF, n=12; P9: 126.34 ± 1,52 pA/pF, n=17; P14: 109,59 ± 1.7 pA/pF, n=11). Most pronounced reduction is observed between P4 and P9 ($p<0.05$) $GABA_A$ receptors mediated currents have been distinguished by using 10 µM Gabazine. In presence of this specific antagonist, the phasic

GABAergic current disappears. Only a reduced tonic current persists, probably reflecting the existence of a particular $GABA_AR$ subfamily, differing in terms of subunit composition and pharmacological sensitivity.


a
b
Peak current amplitude (pA)
2500
2000
1500
1000
500
0
P1
P4
P9
P14
Age
Current Density (pA/pF)
250
200
150
100
50
0
P1
P4
P9
P14
Age


c
Gabazine 100µM
GABA 100µM
GABA 100µM
GABA 100µM
500 pA
3 s

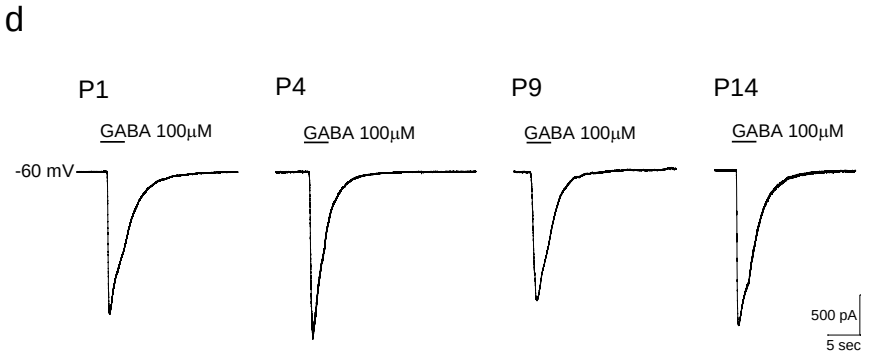

d
P1
P4
P9
P14
GABA 100µM
GABA 100µM
GABA 100µM
GABA 100µM
-60 mV
500 pA
5 sec

Figure 2: Developmental changes in GABAergic transmission. A) Peak GABAergic current amplitude is high during the first two weeks of postnatal life (P1: 1713.12 ± 235.47 pA, n=7 ;P4: 1981.41 ± 189.46 pA, n=12; P9: 1844.18 ± 228.97 pA, n=17; P14: 1608.36 ± 200.33 pA, n=11). B) The GABAergic current density slightly decreases during this temporal window, due to an increase in neurons' capacitance (P1: 205.86 ± 1.06 pA/pF, n=7 ;P4: 192.45± 1.35 pA/pF, n=12; P9: 126.34 ± 1,52 pA/pF, n=17; P14: 109,59 ± 1.7 pA/pF, n=11). C) 10 µM Gabazine was used to assess the nature of $GABA_AR$ driven currents: 100 µM GABA administration evoked little residual current, showing a different kinetics from those observed without antagonist (suggesting the involvement of Gabazine-insensitive $GABA_A$ receptor subpopulation). D) Whole-cell patch-clamp recordings showing acute dissociated neurons' response to 100 µM GABA at different developmental stages.

The expression of β2 V287L nAChRs subunit does not influence GABAergic E/I switch. Since cholinergic transmission has been recently demonstrated to influence the GABAergic E/I transition (Liu et al, 2006), we studied E_{GABA} during the developmental as in wild type and mutant mice. We carried out our gramicidine perforated patch-clamp experiments in acute dissociated neuron from the third to the fourteenth day of post-natal life. A progressive hyperpolarization in E_{GABA} is observed in both experimental group. The value observed in the wild type group are consistent with literature (Ben Ari, 2014), and reflects the progressive reduction in the intracellular chloride concentration. Our data show that this process is unchanged in mice expressing β2 V287L subunit.

	Wild type	**β2 V287L**
P3/4	-48.97 ± 2.95 mV (n=6)	
P5/6	-51,95 ± 2.13 mV (n=6)	-51,98±3.06 mV (n=5)
P7/8	-55,8 ± 1,01 mV (n=7)	-54,93 ± 5,97 mV (n=7)
P9/10	-56 ± 1,43805 mV (n=5)	-57,29 ± 0,93 mV (n=7)
P13/14	-62,22±1,35 mV (n=8)	-60,66 ±1,85 mV (n=7)

Table 1: Developmental shift in E_{GABA}.

Developmental E_{Cl} hyperpolarization follows a similar time course ex vivo and in vitro. Acute dissociated neurons constitute a very perturbed system which does not allow long-term stable patch-clamp recordings. We therefore opted to analyse GABAergic E/I transition in primary neuronal cultures. We replicated our gramicidine perforated patch-clamp in cultured cortical neurons: interestingly, E_{Cl} time course strongly resembled our findings in acute dissociated neurons (Figure 3c and f).(4 DIV:-52.79 ± 1.70 mV, n=13; 6 DIV: -58.1 ± 1.9, n=10; 7 DIV: -59.15 ± 1.48 mV, n=12; 8DIV: -63.74 ± 1.98 mV, n=5; 14 DIV: -64.1 ± 0.5 mV, n=4). These results indicate that primary neuronal cultures are a good model to study the GABAergic switch in vitro.

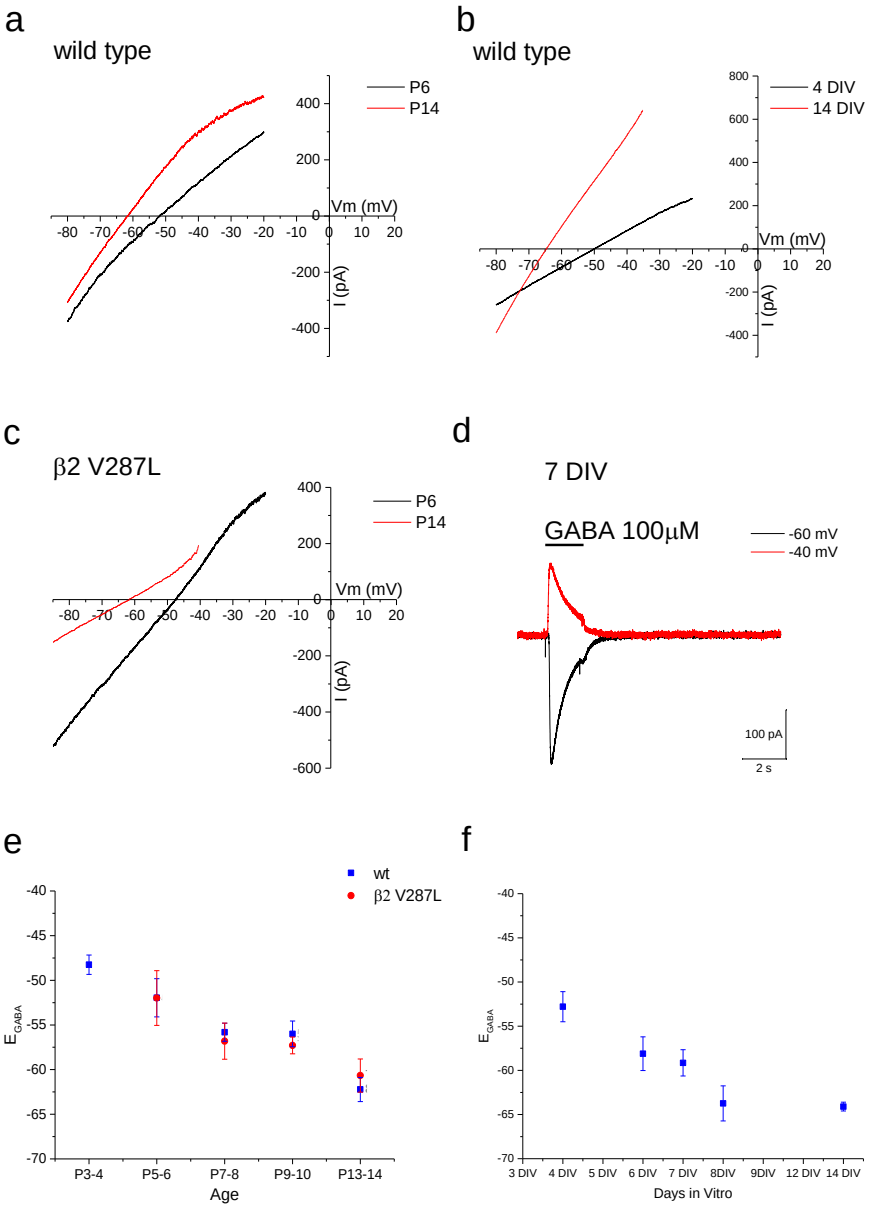

a
wild type
P6
P14
Vm (mV)
I (pA)
b
wild type
4 DIV
14 DIV
Vm (mV)
I (pA)
c
β2 V287L
P6
P14
Vm (mV)
I (pA)
d
7 DIV
GABA 100μM
-60 mV
-40 mV
100 pA
2 s
e
wt
β2 V287L
E_GABA
P3-4
P5-6
P7-8
P9-10
P13-14
Age
f
E_GABA
3 DIV
4 DIV
5 DIV
6 DIV
7 DIV
8DIV
9DIV
12 DIV
14 DIV
Days in Vitro

Figure 3: The GABAergic E/I developmental switch is not affected in mice expressing the mutant β2-V287L subunit. A) I/V curves from P6 (in black) and P13 (in red) wild type mice (in acute dissociated neurons). E_{GABA} shows a progressive hyperpolarization, which reflects a concomitant reduction in intracellular chloride concentration. B) I/V curves recorded from primary neuronal cultures (prepared from P0-1 wild type mice) after 4 and 14 days in vitro. C) I/V curves from P6 and P14 (respectively in black and red) transgenic mice reveal E_{Cl} values very similar to those observed in wild type mice (in acute dissociated neurons). D) Voltage-clamp traces from primary neuronal cultures after 7 days: we can observe the different polarity of current evoked by 100 μM GABA at -40 mV and -60 mV. E) Analysis of mutant and wild type mice shows a close correspondence in E_{GABA} values at any tested ages. F) E_{GABA} developmental hyperpolarizing shift follows a similar time course in acute dissociated neurons and cultured cortical neurons.

2.4 Discussion

Our work shows that heteromeric nAChRs are extensively expressed in mice cortex during postnatal development: the α4 subunit, which we considered in our immunofluorescence assays, is likely to associate with β2 subunit to form α4β2 nAChRs, which exert an important neuromodulatory role, and are implied in ADNFLE pathogenesis (Becchetti et al, 2015). Since several ADNFLE animal models are characterized by disregulation of GABAergic transmission (Klaassen et al., 2006; Mann and Mody, 2008) we considered if mutant our murine ADNFLE model (Manfredi et al, 2009) presented alteration in GABAergic system development. In particular, we focused on developmental aspects. In mammals, a "brain growth spurt" occurs around birth, characterized by neurite outgrowth, synaptogenesis, myelinization and circuit pruning (Eriksson et al., 2000). In rodents, this phase spans the first 3, 4 postnatal weeks and is accompanied by maturation of the cholinergic system and an upsurge of nAChRs expression. In developing brain GABA release from interneurons drives synchronized neuronal firing which plays a focal role in synaptic remodelling during embryonic and early postnatal phases (Ben Ari, 2014). Our findings highlighted that $GABA_A$ receptors expression is high during the first two postnatal week, providing strong evidence about their involvement in the establishment of a proper neuronal network. Liu and colleagues (2006) showed a role for cholinergic transmission in the modulation of GABAergic E/I inhibitory switch. This process is known to play a fundamental role in determining the shape of cortical circuitry;

excitatory GABA action provides the first synaptic input to developing neurons (Tyzio et al, 2009), driving the early GDPs. With progressive hyperpolarization of E_{GABA} GDPs disappear (around P9-10). Our perforated patch-clamp recordings from acute dissociated neurons did not highlight any difference between wild type and mutant mice in E_{GABA} time course. We can therefore exclude that mutant heteromeric nAChR influence chloride homeostasis. In our work we considered only pyramidal neurons: we cannot exclude that the same characterization carried out in interneurons could have highlighted significant differences. Moreover our mutation could affect GABAergic transmission in a different way, maybe affecting GDP frequency: cholinergic activity has been observed to regulate GDP-like activity in developing spinal cord neurons (Czarnecki et al, 2014). Hyperfunctional nAChRs such as those containing β2 V287L subunit could lead to a reinforcement of particular synapses at expense of other, maybe promoting the establishment of a neuronal network highly susceptible to epilepsy.

Acute dissociated neurons constitute a very unstable experimental system: our E_{GABA} characterization in cultured cortical neurons highlighted that the GABAergic system maturation in vitro follows the same kinetics as the one observed in vivo development, at least as for as the GABAergic excitatory/inhibitory switch is concerned.

2.5 Conclusions and future perspectives

Our observation highlighted that both cholinergic and GABAergic tone are high during the transition between the first and the second

postnatal weeks and are therefore likely to affect synaptic connections development and remodelling. Our characterization of mice expressing a mutant β2 V287L subunit led us to exclude that the mutation exerts its developmental effect affecting GABAergic E/I switch. However in future will be interesting extend our analysis to interneurons to understand if E_{GABA} hyperpolarizing shift follows the same time course observed in pyramidal neurons and if our mutation affect the process in different interneurons subpopulations, which show different nAChRs' expression levels (Verhoog et al, 2016). Interneurons activity regulates pyramidal neurons sinchronized firing and are the real conductors of coherent cortical neuronal activity. In particular fast spiking interneurons are thought to be the real regulators of fast network activity(Kann et al, 2016); FS interneurons are stimulated through presynaptic nAChRs on glutamatergic inputs (Poorthuis and Mansvelder, 2013) therefore nAChRs' mutations are likely to affect their activity. Understand the role of these neurons in neuronal network development will be useful to shed light on ADNFLE and other brain disease pathogenesis.

Our findings that E_{GABA} follows the same time course both in vitro and in vivo provided us some proofs that the processes that regulate neuronal network development are, at least partially, conserved in neuronal culture. In future we will take advantage of this system to better characterize this process (maybe also monitoring nAChRs expression and function in relation to GABAergic developmental switch) and to attempt some pharmacological manipulation. We will

characterize our cultures not only through patch clamp but also with MEA (Multi-Electrode Array), which allows to monitor whole neuronal network activity. We hope our findings will one day lead to a better comprehension of ADNFLE pathogenetic mechanism and of CNS development in general.

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Chapter 3: The role of heteromeric nAChRs in the development of glutamatergic signaling in mice prefrontal cortex.

Simone Brusco and Andrea Becchetti

Unpublished data

3.1 Introduction

The cholinergic system is one of the most important modulators of neuronal activity in CNS. Besides directly promoting neurotransmitter release in mature brain (Aracri et al, 2006), nAChRs play a fundamental role in shaping cortical circuitry during early developmental stages (Role et al, 1996; Zhang et al, 2008). Distinct nAChRs present different permeability to Ca^{2+} and can activate distinct signalling pathways, so that the channel ability to modulate brain development is also dependent on their subunit composition. In particular, recent findings suggest an important role of heteromeric nAChRs in regulating the morphogenesis and mantainance of dendritic spines (Molas and Dierssen, 2014); nAChRs activation have been found to deeply affect glutamatergic synapses localization and composition in term of subunits (Lozada et al, 2012). Mutation in genes coding for nAChRs subunits can deeply glutamatergic signalling and therefore promote alteration in excitatory/inhibitory balance in cortex that can ultimately lead to epilepsy onset (Molas and Dierssen, 2014). Our murine ADNFLE model, in which the expression of a β2-V287L subunit can be silenced through doxycycline administration, provides strong evidence for a nAChRs implication in the formation of a neuronal network highly susceptible to epilepsy (Manfredi et al, 2009).

Here we consider how heteromeric nAChRs regulate glutamate release on pyramidal neurons in the PFC layer V, in wild type and mutant mice (expressing the β2-V287L) from the 4^{th} to the 12^{th} day of

postnatal life. We also consider if direct nAChR agonist administration can evoke somatic currents in pyramidal neurons of wild type mice, to better understand the localization and role of these receptors in mice cortex.

Our findings reveal that heteromeric nAChRs start to exert their effect on glutamatergic transmission at the end of the first postnatal week in mice. No somatic nicotinic currents have been detected at this as well as at later developmental stages in pyramidal neurons, suggesting that heteromeric nAChRs are mainly located at synaptic level where they stimulate neurotransmitter release. Analysis of transgenic mice highlighted an increase in EPSC frequency both in control condition and following nicotine exposure, compared to wild type. Cumulative distribution of the EPSC amplitudes showed a larger increase in EPSC amplitude between P7-9 mice and P10-12 in transgenic mice

3.3 Material and Methods

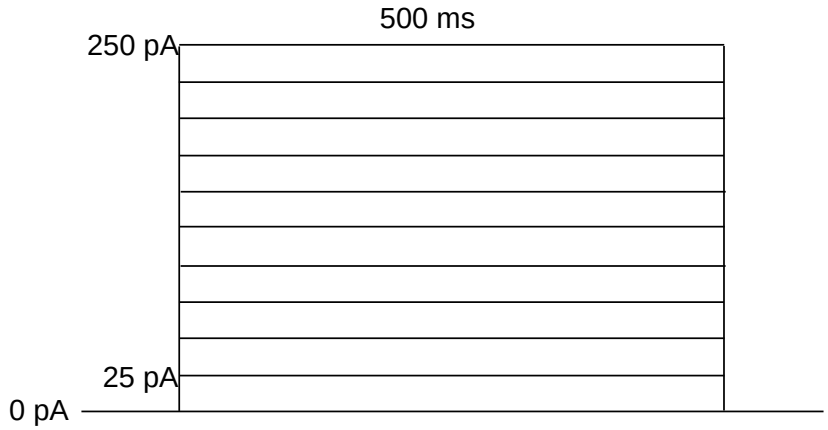
Brain slices preparation. FVB wild type and mutant mice, aged 4–12 days, were deeply anesthetized with isoflurane and decapitated. Brains were removed and placed in ice-cold solution containing (mM): 87 NaCl, 21 $NaHCO_3$, 1.25 NaH_2PO_4, 7 $MgCl_2$, 0.5 $CaCl_2$, 2.5 KCl, 25 D-glucose, and 75 sucrose, equilibrated with 95% O_2 and 5% CO_2 (pH 7.4). Coronal slices (300 µm thick) were cut with a VT1000S vibratome (Leica Microsystems) from the Fr2 prefrontal region

(between +2.68 and +2.10 mm from Bregma; Paxinos and Franklin, 2004). Slices were incubated at room temperature for at least 1 hour, before being transferred to the recording chamber. During the experiments, slices were superfused at 1.8 mL/min with artificial cerebrospinal fluid (ACSF) at 33°-34°C, containing (mM): 135 NaCl, 21 $NaHCO_3$, 0.6 $CaCl_2$, 3 KCl, 1.25 NaH_2PO_4, 1.8 $MgSO_4$, and 10 -glucose, aerated with 95% O_2 and 5% CO_2 (pH 7.4). Cells were examined with an Eclipse E600FN microscope (Nikon Instruments) equipped with a water immersion differential interference contrast (DIC) objective and an infrared (IR) C8484-05G01 camera (Hamamatsu Photonics).

Cell cultures. The primary cultures of cortical neurons were prepared as described by others (Gullo et al, 2012). Briefly, all of the cerebral cortices (excluding the hippocampus) were removed from P1–P3 mice, cut into 1 mm^3 pieces, and digested by trypsin (0.15%) and DNAse (10 µg/ml) at 37°C for 20 min. After enzyme digestion, the cells were dissociated by means of trituration, and plated on 35 mm petri dishes pre-coated with polyethyleneimine 0.1% (w/v). After 3 h incubation, the plating medium was replaced by neurobasal medium, supplemented with B27 (Invitrogen, Italy), 1 mM glutamine and 10 ng/ml bFGF. The culture was maintained at 37° C in 5% CO_2. One-half of the medium volume was replaced every 3 days.

Patch-Clamp recordings. For EPSCs characterization in PFC slices, pyramidal neurons were voltage or current-clamped with a Multiclamp 700A patch-clamp amplifier (Molecular Devices) at room temperature. Low-resistance micropipettes (3–4 MΩ) were pulled

from borosilicate capillaries (Science Product GmbH) with a P-97 Flaming/Brown Micropipette Puller (Sutter Instrument Company). The cell capacitance and series resistance were always compensated. Synaptic currents were low-pass filtered at 2 kHz and digitized at 5 kHz with pClamp/Digidata 1322A (Molecular Devices). Pipettes contained (mM): 135 K-Gluconate, 5 KCl, 1 $MgCl_2$, 10 HEPES, 2.5 BAPTA. Pyramidal neurons were identified considering their morphology and their firing profile in response to 500 ms - 25 pA current steps.



Excitatory post synaptic currents (EPSCs) were recorded -68 mV and inspected one by one: those not presenting the typical shape of synaptic currents were rejected. The baseline noise (peak to peak) was generally lower than 5 pA. The threshold was generally set at 7–8 pA.

To record somatic nicotinic currents in neuronal cultures we used an Axopatch 200B amplifier (Molecular Devices), at room temperature (20–22°C). Micropipettes' resitance was 2–3 MΩ. The cell capacitance and series resistance (up to about 75%) were always compensated. Currents were low-pass filtered at 2 kHz and acquired on-line at 5–10 kHz with Molecular Devices hardware and software (pClamp 8 and Axoscope 8). Pyramidal neurons were distinguished according to their firing profile and shape. Extracellular solution and drugs were applied with an RSC-160 Rapid Solution Changer (BioLogic Science Instruments).

Drugs and solutions. Drugs and chemicals were purchased from Sigma-Aldrich Srl except CNQX, AP-5 and 5-Iodo-A-85380 dihydrochloride (5-IA; Tocris, UK). Drugs were kept as frozen stock solutions in distilled water at −20°C until diluted to their appropriate concentrations in external solution for each experiment. CNQX was diluted in DMSO and stored at room temperature. Nicotine and DHβE - an antagonist specific for α4-containing heteromeric nAchRs - were perfused in the bath and their effect calculated at the steady state, which was usually reached within 2 min. Nicotinic somatic currents were elicited by applying 100 nM 5-IA (a selective agonist for α4-containing nAChRs) in presence of glutamatergic channel blockers (CNQX 10 μM and AP-5 40 μM).

Data analysis. EPSCs were analyzed off-line by using Clampfit 9.2 (Molecular Devices), and OriginPro 9.1 (OriginLab Corporation, Northampton, MA, USA). Data are given as mean values ± standard

error of the mean. The distributions of EPSC amplitudes and inter-event intervals were analyzed in each cell with the Kolmogorov–Smirnov (KS) test, on at least 2-min continuous recording for each experimental condition. Statistical significance of averages from several cells was determined with paired Student *t*-test (with significance level set to 0.05 after testing that the data were normally distributed (with Kolmogorov-Smirnoff test), with equal variances (with *F*-test).

3.4 Results

Pyramidal neurons identification. We first analysed the development of glutamatergic signalling in prefrontal cortex of wild type mice, from the 4th to the 12th day of postnatal life. Pyramidal neurons were distinguished by their laminar location, morphology and firing profile. The firing properties were tested in current-clamp mode: in response to 500 ms depolarizing current steps they exhibited a low-frequency action potential firing (20 Hz in response to 200 pA current injection).

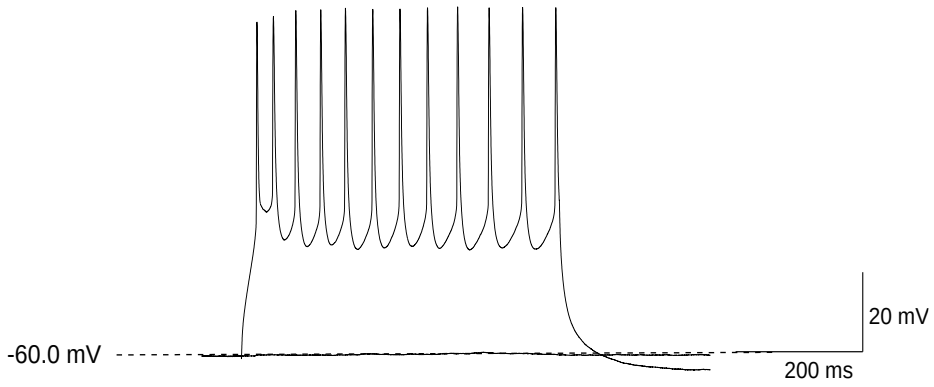
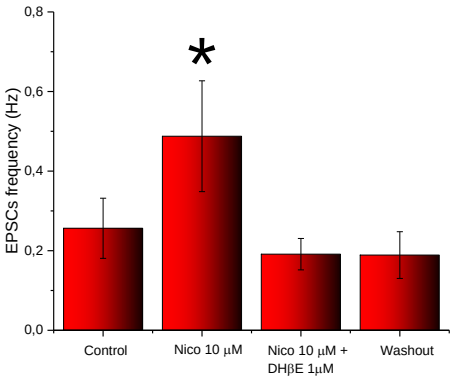


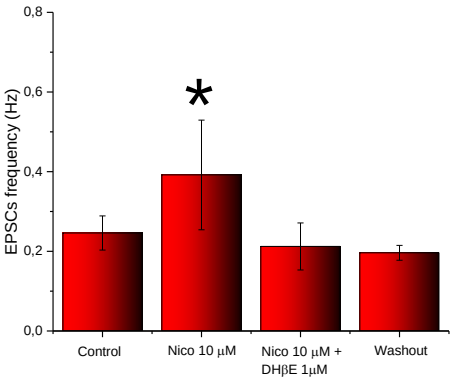
Figure 1: Firing profile of a layer V pyramidal neuron recorded from P9 PFC slices: firing pattern was determined through the application of consecutive 0.5-s depolarizing currents (50 pA steps). In response to a 200 pA injection these neuron showed a relatively low AP firing frequency (~20Hz).

Cholinergic regulation of glutamatergic system development. EPSCs were characterized in wild type mice from the 4th to the 12th day of postnatal life. We carried out our patch-clamp experiments in whole configuration: cells were clamped at -68 mV (near the E_{Cl} in our experimental conditions) to minimize the contribution of post synaptic inhibitory currents. We found that EPSCs frequency was low immediately after birth and remained stable over the first two weeks (Figure 2) (P4-6: 0.25 ± 0.07 Hz, n=6; P7-9: 0.24 ± 0.04 Hz, n=9; P10-12: 0.24 ± 0.03 Hz, n=5). To analyze if nAChRs activation promoted glutamate release, like in adult mice, we used nicotine instead of acetylcholine to avoid muscarinic receptor activation (whose effect on nAchRs are not fully defined). Exposure to 10 μM nicotine increased EPSC frequency at all tested ages ($p<0.05$ with paired *t*-Test), with a more pronounced effect in older mice (Figure 2) (P4-6: 0.48 ± 0.13 Hz, n=6; P7-9: 0.39 ± 0.13 Hz, n=9; P10-12: 0.58±0.10 Hz, n=5). Administration of 1 μM DHβE, a selective blocker of heteromeric nAChRs strongly reduced frequency, providing strong evidence about the heteromeric nature of the nAChRs involved (Figure 2) (P4-6: 0.19 ± 0.03 Hz, n=6; P7-9: 0.21 ± 0.0.5 Hz, n=9; P10-12: 0.24±0.11 Hz, n=5). The block was promptly reversible after washout confirming that the reduction in EPSCs frequency was not caused by rundown (Figure 2) (P4-6: 0.19 ± 0.05Hz, n=6; P7-9: 0.19 ± 0.0.1 Hz, n=9; P10-12: 0.32±0.01 Hz, n=5).

P4-6



P7-9



P10-12

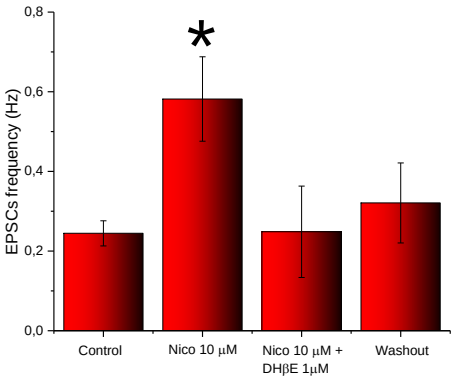


Figure 2: developmental changes in EPSCs frequency and the effect of nicotine: during early post natal stages EPSCs show a reduced frequency compared to adults (5.06 ± 1.03 Hz, Aracri et al, 2013). An increase in EPSCs frequency was observed in both control conditions (P4-6: 0.25 ± 0.07 Hz, n=6; P7-9: 0.24 ± 0.04 Hz, n=9; P10-12: 0.24 ± 0.03 Hz, n=5) and following 10 µM nicotine administration (P4-6: 0.48 ± 0.13 Hz, n=6; P7-9: 0.39 ± 0.13 Hz, n=9; P10-12: 0.58 ± 0.10 Hz, n=5). 1 µM DHβE promptly abolished the effect of nicotine administration over EPSCs frequency confirming that the effect is mediated by α4-containing nAChRs (P4-6: 0.19 ± 0.03 Hz, n=6; P7-9: 0.21 ± 0.0.5 Hz, n=9; P10-12: 0.24 ± 0.11 Hz, n=5). Washout restored EPSCs frequency to value observed in control conditions (P4-6: 0.19 ± 0.05 Hz, n=6; P7-9: 0.19 ± 0.0.1 Hz, n=9; P10-12: 0.32 ± 0.01Hz, n=5).

The EPSC amplitude distribution was not affected by heteromeric nAChR activation at any tested age, as judged by administration of 10 µM Nicotine. 1 µM DhβE coadministration also showed no effect. (Figure 3a and b). Comparing the events' distribution at P7-9 and P10-12, a clear increase in amplitude was observed both in control condition and following nicotine administration, probably reflecting the maturation of glutamatergic signalling ($p<0.05$ with Kolmogorov-Smirnoff test) (Figure 3c and d).

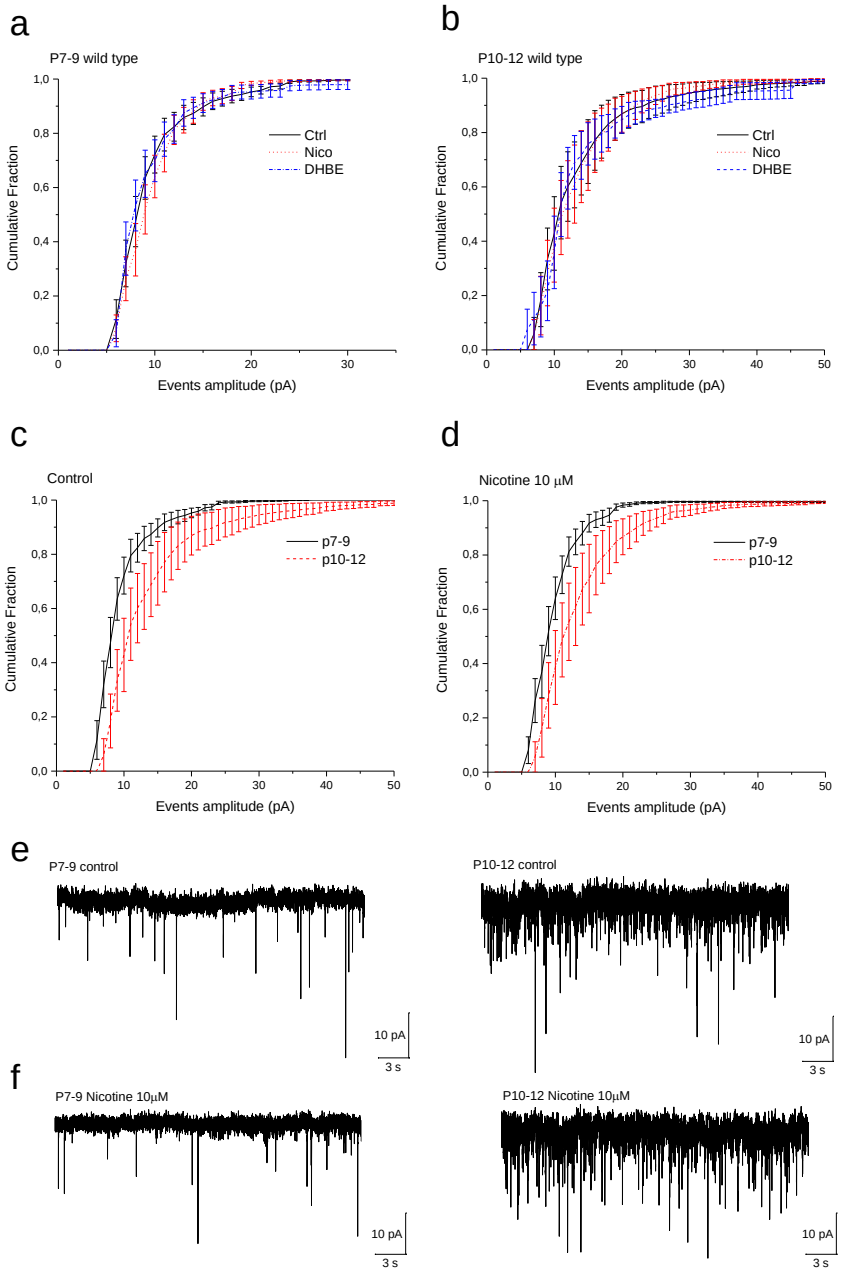
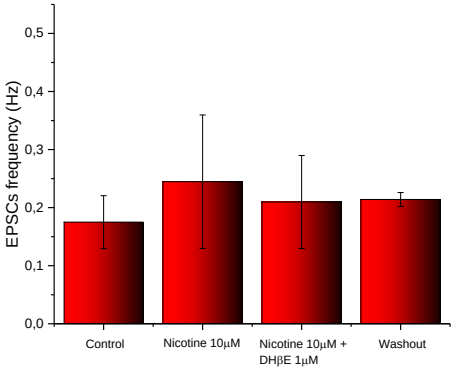

a
P7-9 wild type
Cumulative Fraction
Events amplitude (pA)
Ctrl
Nico
DHBE
b
P10-12 wild type
Cumulative Fraction
Events amplitude (pA)
Ctrl
Nico
DHBE
c
Control
Cumulative Fraction
Events amplitude (pA)
p7-9
p10-12
d
Nicotine 10 µM
Cumulative Fraction
Events amplitude (pA)
p7-9
p10-12
e
P7-9 control
10 pA
3 s
P10-12 control
10 pA
3 s
f
P7-9 Nicotine 10µM
10 pA
3 s
P10-12 Nicotine 10µM
10 pA
3 s

Figure 3: Developmental of glutamatergic signaling is associated to change in EPSCs amplitude distribution. A and B) Heteromeric nAChR activation did not affect EPSCs amplitude distribution in wild type mice at any tested age. C and D) Traces showing how EPSCs amplitude progressively increased with age.

β2 V287L nAChR subunit's expression affected both spontaneous and nicotine evoked glutamate release. In order to understand the role of our mutant β2 subunit in the establishment of an epileptic phenotype we replicated our EPSCs characterization in transgenic mice expressing the β2 V287L (Figure 4). Once again EPSC frequency progressively increased between P7 and P10 in both control conditions (P7-9: 0.17 ± 0.03 Hz, n=3; P10-12: 0.68 ± 0.11Hz n=7), and was stimulated by 10 μM nicotine, although the effect was smaller than in control (P7-9: 0.24 ± 0.11 Hz, n=3; P10-12: 0.89 ± 0.26 Hz, n=7). DhβE reverted the effect of nicotine (P7-9: 0.20 ± 0.08 Hz, n=3; P10-12: 0.73 ± 0.32 Hz, n=7). All the effects of acute drug administration were promptly reversible on washout (P7-9: 0.21 ± 0.01 Hz, n=3; P10-12: 0.46 ± 0.07 Hz, n=7).

P7-9


EPSCs frequency (Hz)
0,0
0,1
0,2
0,3
0,4
0,5
Control
Nicotine 10μM
Nicotine 10μM + DHβE 1μM
Washout

P10-12

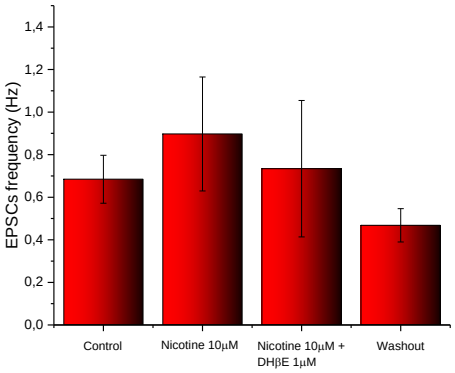

EPSCs frequency (Hz)
0,0
0,2
0,4
0,6
0,8
1,0
1,2
1,4
Control
Nicotine 10μM
Nicotine 10μM + DHβE 1μM
Washout

Figure 4: EPSCs characterization of mice harboring the β2 V287L nAChR' subunit: similar to what was observed in wild type mice we found a progressive increase in EPSCs frequency between P7 and P10 in both control conditions (P7-9: 0.17 ± 0.03 Hz, n=3; P10-12: 0.68 ± 0.11Hz n=7), and following 10 μM nicotine administration (P7-9: 0.24 ± 0.11 Hz, n=3; P10-12: 0.89 ± 0.26 Hz, n=7). 1 μM DhβE administration showed a reduced effect on EPSCs frequency increase induced by nicotine administration (P7-9: 0.20 ± 0.08 Hz, n=3; P10-12: 0.73 ± 0.32 Hz, n=7). Drugs washout restored EPSC frequency to the values observed in control conditions (P7-9: 0.21 ± 0.01 Hz, n=3; P10-12: 0.46 ± 0.07 Hz, n=7).

A comparison between wild type and transgenic mice highlighted several differences. In P7-9 mice the EPSCs' frequency was similar in wild type and mutant mice (respectively 0.19 ± 0.03 Hz, n=6 for wild type and 0.17 ± 0.04 Hz, n=3 for transgenic mice). On the other hand 10 μM nicotine promoted an increase in EPSCs frequency in wild type mice (0.59 ± 0.16 Hz, n=6) but not in mice expressing β2 V287L (0.24 ± 0.11 Hz, n=3). Therefore, mutant nAChRs seem unable to modulate glutamate release onto pyramidal neurons in P7-9 mice ($P<0.05$ with unpaired *t*-test) (Figure 5 a)

In older mice (P10-12) we instead observed differences in EPSCs frequency between the two experimental groups in control

conditions (respectively 0.24 ± 0.03 Hz, n=5, for wild type, and 0.68 ± 0.11 Hz, n=7, for transgenic mice). Hence mice expressing β2 V287L showed a statistically significant higher EPSCs frequency in absence of any pharmacological stimulation (*P*=0.03 with unpaired *t*-test). In these experimental conditions nicotine administration increased EPSCs frequency in both wild type and mutant mice (respectively 0.58 ± 0.10 Hz, n=5, for wild type, and 0.89 ± 0.26 Hz, n=7, for transgenic mice). The observed increase was less pronounced in mutant mice, so that the difference observed between the two experimental groups is reduced in presence of nicotine compared to control conditions (Figure 5 b).

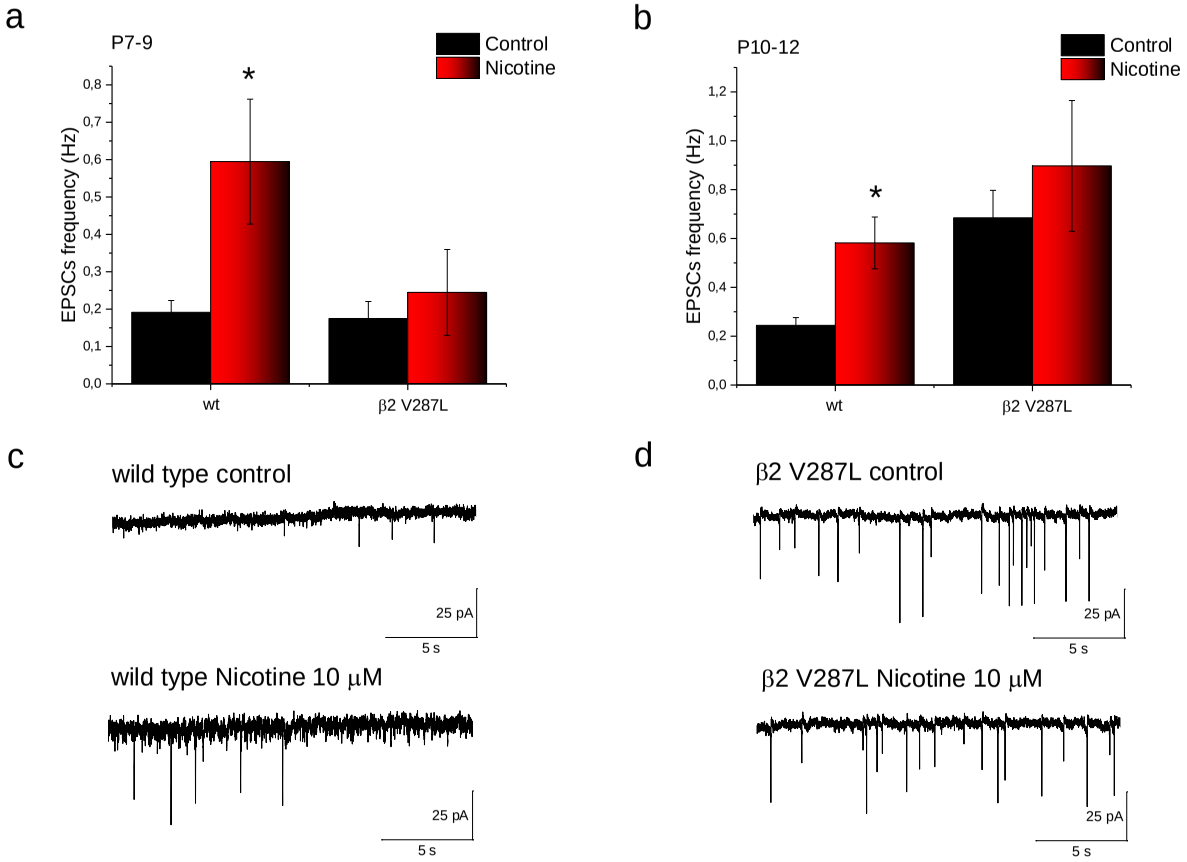

a
P7-9
Control
Nicotine
EPSCs frequency (Hz)
0,0
0,1
0,2
0,3
0,4
0,5
0,6
0,7
0,8
*
wt
β2 V287L
b
P10-12
Control
Nicotine
EPSCs frequency (Hz)
0,0
0,2
0,4
0,6
0,8
1,0
1,2
*
wt
β2 V287L
c
wild type control
25 pA
5 s
wild type Nicotine 10 μM
25 pA
5 s
d
β2 V287L control
25 pA
5 s
β2 V287L Nicotine 10 μM
25 pA
5 s

Figure 5: the expression of β2-V287L affects the EPSCs frequency at different stages in a complex way. a) wild type and mutant mice showed no difference in EPSCs' frequency in absence of any pharmacological stimulation at P7-9; 10 μM nicotine increased EPSCs frequency in wild type mice (0.59 ± 0.16 Hz, n=6) while did not exert any significant effect on mice expressing mutant heteromeric nAChRs (0.24 ± 0.11 Hz, n=3). b) Recordings from older mice (P10-12) highlighted that EPSCs' frequency was increased in transgenic mice (0.68 ± 0.11 Hz, n=7) compared to the control group (0.24 ± 0.03 Hz, n=5) (*P*=0.03 with *t*-test); the difference observed between the mutant and wild type mice is lowered following 10 μM nicotine administration (0.58 ± 0.10 Hz, n=5 for wild type and 0.89 ± 0.26 Hz, n=7 for transgenic mice). C and D) EPSCs traces from wild type and mutant mice in control conditions and after 10 μM nicotine administration.

As in wild type mice, the distribution of EPSCs amplitudes analysis in mutant mice was not affected by acute nicotine administration (Figure 6a and b). Similarly an increase in EPSCs amplitude as mice grew older was also observed in mutant mice ($p<0.05$ with Kolmogorov-Smirnoff test). This increase was more pronounced in transgenic mice than in wild type ones.

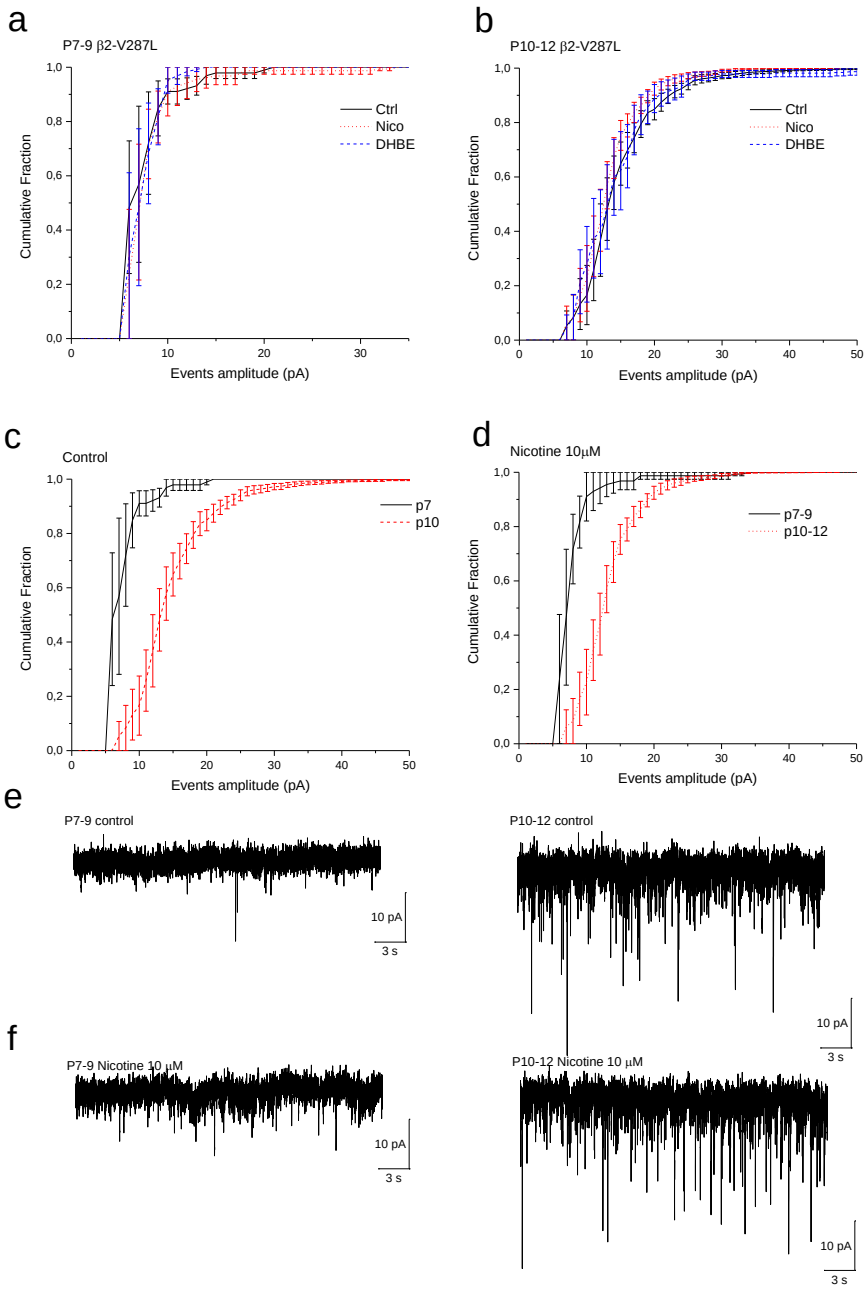
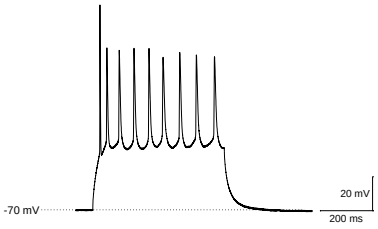

a
P7-9 β2-V287L
Cumulative Fraction
Events amplitude (pA)
Ctrl
Nico
DHBE
b
P10-12 β2-V287L
Cumulative Fraction
Events amplitude (pA)
Ctrl
Nico
DHBE
c
Control
Cumulative Fraction
Events amplitude (pA)
p7
p10
d
Nicotine 10μM
Cumulative Fraction
Events amplitude (pA)
p7-9
p10-12
e
P7-9 control
10 pA
3 s
P10-12 control
10 pA
3 s
f
P7-9 Nicotine 10 μM
10 pA
3 s
P10-12 Nicotine 10 μM
10 pA
3 s

Figure 6: EPSCs amplitude distribution in mice expressing the β2 V287L subunit. A and B) Like in wild type mice nAChRs activation did not influence EPSCs amplitude distribution. C and D) The increase in EPSCs amplitude associated with glutamatergic system development seems more pronounced in mutant mice than in wild type. E and F) Traces showing the progressive increase in EPSCs amplitude.

Somatic nicotinic currents characterization in cortical neuronal culture. To better understand the role of nAChR in the modulation of neuronal development we analysed if nAChR activation evokes somatic currents. We carried out these experiments in primary neuronal culture: In these conditions direct drugs application on the cell is easier than in brain slices. Again we considered pyramidal neurons, which have been distinguished by shape and firing profile (Figure 7a). Cells were clamped at -68 mV; we blocked EPSCs with 10 µM CNQX and 40 µM AP-5 (which respectively blocks AMPA and NMDA glutamate receptors). 100nM 5-IA (an agonist selective for α4β2 nAChRs) failed to evoke somatic currents in pyramidal neurons cultured for 8, 12 or 15 days (Figure 7b). These findings suggest that heteromeric nAChRs are located at presynaptic level and exert their effect mainly by modulating other neurotransmitter release.

a


-70 mV
20 mV
200 ms

b

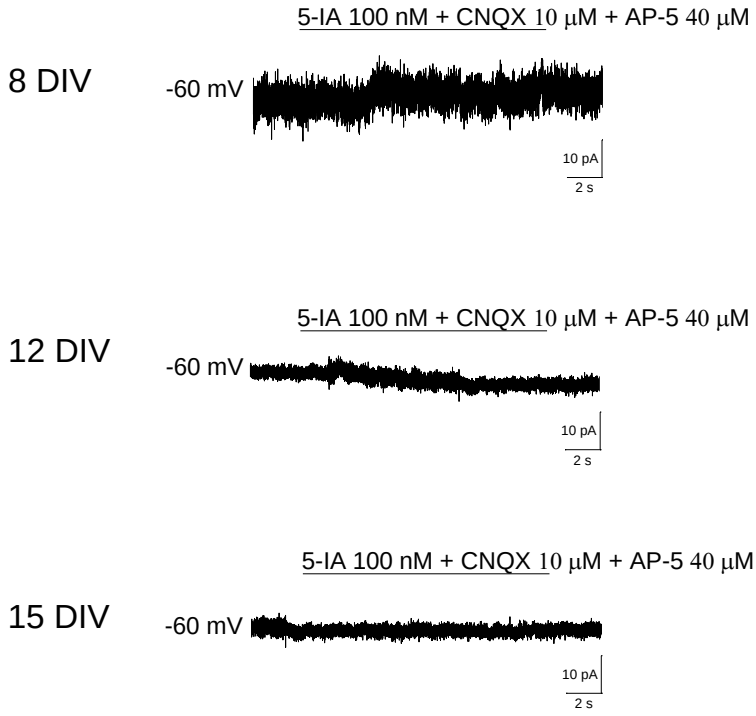

5-IA 100 nM + CNQX 10 μM + AP-5 40 μM
8 DIV
-60 mV
10 pA
2 s
5-IA 100 nM + CNQX 10 μM + AP-5 40 μM
12 DIV
-60 mV
10 pA
2 s
5-IA 100 nM + CNQX 10 μM + AP-5 40 μM
15 DIV
-60 mV
10 pA
2 s

Figure 7: characterization of somatic nicotinic currents characterization in vitro. A) firing profile of a cultured pyramidal neuron, deeply resembling what is generally observed in brain slices. B) direct application of 100 nM 5-IA on pyramidal neurons failed to induce somatic currents in presence of GluRs blocker, after 8, 12 or 15 days in vitro (DIV).

3.5 Discussion

Cholinergic signalling, besides regulating neuronal firing in the adult brain, plays a focal role in cerebral development. In the CNS, nAChRs are predominantly found at presynaptic and pre-terminal sites of different neuronal populations including cholinergic neurons, where they modulate the release of several neurotransmitters. The expression of acetylcholine-synthetizing enzyme starts during early developmental stages (E12-16) and nAChRs subunit transcripts are detectable during embryonic development in mice (Corriveau et al, 1995; Hellström-Lindahl et al, 2008; Abreu-Villaça et al, 2011). During embryonic and early postnatal life, synchronized neuronal firing is crucial for the wiring of a functional neuronal network. The cholinergic system can modulate both GABA and glutamate release (Aracri et al, 2010), thus affecting the balance between excitatory and inhibitory inputs, and influencing the development synaptic connections (Lin and Koleske, 2010; Cherubini et al, 2013;).In facts findings suggest also that nAChR activity is important for the maturation of both the GABAergic system (Liu et al, 2006) and the

glutamatergic system (Halff et al, 2014), and directly regulate dendritic architecture in pyramidal neurons (Cline and Haas, 2008; Lozada et al, 2012). Our findings highlight that heteromeric nAChRs start to exert their modulatory role on glutamatergic system towards the end of the first postnatal weeks. The nicotine-induced increase in EPSC frequency suggests that the Ach promotes glutamate release onto PFC pyramidal neurons, acting mainly on presinaptically located α4β2 nAChRs (as also was confirmed by the inability of 5-IA to evoke somatic current). The effect is greater in older mice (P10-12) suggesting that the expression of heteromeric nAChRs progressively increase after the first postnatal week. Analysis of the EPSCs amplitude distribution shows an increase in events amplitude and relative with time, suggesting that maturation of glutamatergic system is associated with change in receptor subunit composition or receptor recruitment to post-synaptic compartment, as was also suggested by previous studies (Maggi et al, 2003; Halff et al, 2014).

The same experimental approach replicated in mice, expressing the β2-V287L, provided us interesting insight about the role of heteromeric nAChRs in CNS shaping and their possible involvement in epileptic phenotype establishment. Towards the end of the first postnatal week whole-cell patch-clamp recordings from pyramidal neurons show EPSCs frequency values similar to those observed in wild type mice. Nicotine admistration in these conditions had little effect on EPSCs frequency. The mutation probably alters the temporal expression of α4β2 nAChR, because of their important role

in synaptic connections and dendritic structure remodelling (Lozada et al, 2012; Molas and Dierssen 2014). During the second postnatal week EPSC frequency was significantly higher in mutant mice compared to wild type both in absence and in presence of nicotine. These data suggest that the mutation probably alters cortical circuitry development leading to a reinforcement of excitatory synaptic connections or affecting their localization. This is consistent with recent findings which point out that β2 containing nAChR are involved in brain circuits fine-tuning (Ballesteros-Yáñez et al, 2010; Lozada et al, 2012). β2 subunit knockout has been found to alter dendritic branching and to reduce dendritic spine density (Lozada et al, 2012). Since the receptors containing β2 V287L seem to start to exert their effect system later than wild-type ones, glutamatergic synapses in mutant may probably shift their position closer to soma. A recent work from Lozada and colleagues showed that reducing β2-containing nAChRs expression (through KO or RNA-interference) leads to a redistribution of glutamatergic synapses closer to soma (probably due to alteration in dendritic spines formation). Therefore, our mutation, reducing or delaying the expression of heteromeric nAChRs, is likely to exert a similar effect. This synaptic redistribution can lead to the establishment on a neuronal network more susceptible to seizures. Analysis of the EPSCs' amplitude distribution in mutant mice highlights an increase in EPSCs amplitude between the first and second week of life, like previously observed in wild type mice: the amplitude increase was more pronounced in mice expressing mutant heteromeric nAChRs. These findings suggest that

the mutation can act both at presynaptic and postsynaptic level. A greater glutamate release can account for increased EPSCs amplitude, hyperfunctional presynaptic α4β2 nAChRs could produce overactivation of calcium signals that enhance neurotransmitter release in different ways, triggering calcium induced calcium release or promoting its influx through voltage gated calcium channel by membrane depolarization (Role and Berg, 1996). nAChRs often colocalize with glutamatergic receptors and their activation has been found to play a role in their stabilization on spines and to affect their composition in term of subunits (Maggi et al, 2003): hyperfunctional α4β2 nAChRs can lead to the recruitment of GluRs subunits or influence receptor clustering, through an abnormal calcium influx (Yakel, 2012). Calcium influx can also affect GluR subunit composition by influencing gene expression (Berg and Role, 1996; Chang and Berg, 2001; Goldin and Segal, 2003; Pi et al, 2007). Further investigations are needed to clarify the overall effect of the β2-V287L mutation.

3.6 Conclusions and future perspectives

From our findings it appears clear that heteromeric nAChRs are fundamental in regulating synaptic development and that mutations in genes coding for these receptors play a fundamental role in ADNFLE pathogenesis. Besides regulating neurotransmitter release in developed brain, thus directly promoting neuronal firing that can lead to seizure onset, they can alter the fine balance between excitatory an inhibitory inputs in prefrontal cortex (where in ADNFLE

seizures arise). This altered neuronal network probably account for an higher seizure susceptibility which constitute the background for epilepsy onset. We hope our finding will help to identify a temporal window for an early pharmacological intervention able to prevent ADNFLE: the idea is to develop a therapy able to restore the correct balance between excitatory and inhibitory synapses in prefrontal cortex. These approaches combined with traditional anti epileptic drugs in adults one day could hopefully lead to a complete remission from this kind of epilepsy.

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Chapter 4: Nocturnal frontal lobe epilepsy with paroxysmal arousals due to CHRNA2 loss of function

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Neurology 84 (2015): 1520-1528.

4.1 Introduction

Autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) is characterized by clusters of motor seizures arising during non-REM sleep, usually occurring in individuals of normal intellect (Scheffer et al, 1995). Ictal semiology includes a wide spectrum of motor manifestations, ranging from brief motor events to major episodes. Seizures usually appear within the first two decades of life and may disappear in adulthood (Nobili et al, 2014). Carbamazepine, oxcarbazepine, and topiramate have proven effective in both ADNFLE and sporadic nocturnal frontal lobe epilepsy (NFLE) in uncontrolled trials. (Oldani et al, 2006; Romigi et al, 2008)

ADNFLE is the first epilepsy syndrome whose genetic bases have been identified, albeit reported mutations only explain a minority of cases (Combi et al, 2009) Overall, a limited number of mutations affecting neuronal nicotinic acetylcholine receptor (nAChR) subunits have been associated with the syndrome, including 6 in CHRNA4 (α4), 6 in CHRNB2 (β2), and 1 in CHRNA2 (α2). Functional studies indicate gain of function as the main pathogenic mechanism (Ferini-Strambi et al, 2012). Additional mutations leading to a similar phenotype have been identified in the KCNT1 gene, which encodes for a potassium channel subunit (4 mutations), and in the DEPDC5 gene, which is involved in G-protein signaling pathways (5 mutations) (Nobili et al, 2014; Ishida et al, 2013; Picard et al, 2014). A causative role for the corticotrophin-releasing hormone (CRH) gene (1 mutation in the coding sequence, 4 noncoding) has also been suggested (Sansoni et

al, 2014). We identified a missense mutation affecting the first amino acid of the second transmembrane domain of CHRNA2 in a large family in which affected individuals exhibited sleep related seizures with paroxysmal arousals. This is the second point mutation of CHRNA2 to be associated with ADNFLE. Functional studies of the mutant receptor in human embryonic kidney (HEK) cells revealed reduced current density, demonstrating a loss-of-function effect.

4.2 Methods

Subjects. After identifying CHRNA2 as a causative gene for ADNFLE (Aridon et al, 2006), we systematically tested this gene, along with the CHRNA4 and CHRNB2, in patients exhibiting a related phenotype. Overall we tested 150 consecutive probands (78 males, 72 females; 73 sporadic, 77 familial) referred for a clinical and genetic diagnosis between 2006 and 2013. In 74% of patients, diagnosis was validated by video-EEG recording of seizures. We found the p.Ile297Phe mutation in CHRNA2 in the proband of a family in which 8 individuals over 3 generations were affected (figure 1A).

Standard protocol approvals, registrations, and patient consents. All participants and their parents/legal guardians gave informed consent. The study was approved by the human research ethics committees of the Meyer Children's Hospital. A consent to disclose form was obtained for videos of any recognizable patient.

CHRNA2 mutation screening. Genomic DNA was extracted from blood leucocytes using an automated isolation robot (QIA Symphony; Qiagen, Hilden, Germany).

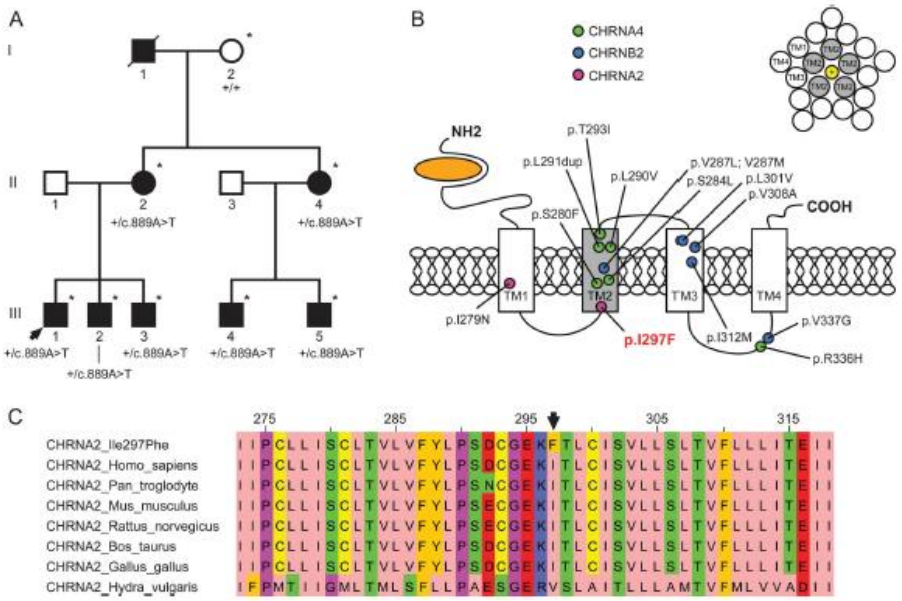

Figure 1: A) Pedigree of the family showing that the mutation segregates in all affected members. Squares = males; circles = females; filled black circles = patients with ADNFLE; black arrow = proband; asterisks = individuals screened for the c.889A.T mutation. B) Basic structure of nAChRs and position in each subunit of all reported mutations. Orange ellipse represents the ligand; circles represent amino acids in which mutations have been found in CHRNA4 (green), CHRNB2 (light blue), and CHRNA2 (pink). p.Ile297Phe (p.I297F) mutation is indicated in red, bold, and with a bigger font size. TM1, TM2, TM3, and TM4 indicate the 4 transmembrane domains of each nAChR subunit, and the second transmembrane domain, which is directly implicated in the gating structure, is colored in light gray. Upper right part of the panel represents the subunits assembly viewed from the top. Yellow circle represents a positively charged ion that moves through the channel. C) In silico multiple sequence alignment, performed using Jalview software, reveals that the mutation affected an amino acid that is conserved among the species. According to the physicochemical properties of the wild-type and mutant amino acids, the mutation resulted in the substitution of an aliphatic, hydrophobic amino acid (isoleucine, I, colored in pink) with an aromatic one (phenylalanine, F, colored in orange). Black arrow indicates the position of the mutation. (ADNFLE = autosomal dominant nocturnal frontal lobe epilepsy; nAChR = nicotinic acetylcholine receptor).

Based on known mutation rates (Ferini-Strambi et al, 2012; Heron et al, 2007), we screened in succession CHRNA4, CHRNB2, and CHRNA2. Exons covering all the coding regions of CHRNA2 (Entrez gene ID: 1135; accession number: NM_000742.3) and their flanking intronic regions were amplified with primers designed using Primer3 Plus software (http://www.bioinformatics.nl/cgibin/primer3plus/primer3plus.cgi/). Genomic DNA template (50 ng) was amplified using FastStart Taq DNA Polymerase (Roche, Mannheim, Germany). PCR products were checked by 1.5% agarose gel electrophoresis, purified using ExoSAP-IT (Affymetrix, Santa Clara, CA) and analyzed by direct sequencing, on both strands, using the BigDye Terminator V1.1 chemistry (Life Technologies, Grand Island, NY), on an ABI Prism 3130XL automated capillary sequencer (Life Technologies). Primers are available on request.

Video-EEG recordings. All affected individuals underwent wholenight video-polysomnographic recordings (EB Neuro, Florence, Italy) with an extended EEG montage, electrooculography, ECG, chin and tibialis anterior muscles EMG, and chest and abdominal respiratory movements. During EEG recordings, none of the patients were receiving antiepileptic medication.

Cell culture and transfection procedure. Either wild-type (WT) or mutant α2 constructs were synthesized in vitro (Biomatik, Cambridge, Canada) and transiently cotransfected with the β2 or β4 subunit in HEK cells (TSA subclone; American Type Culture Collection,

Manassas, VA) using standard procedures. Cells were cultured in DMEM-F12 (Dulbecco's modified Eagle medium F12) (HyClone Laboratories, Logan,UT) supplemented with 10% fetal calf serum (HyClone) and 2 mM L-glutamine, at 37°C and 5% CO2. Cells were seeded in 35-mm culture dishes and transfected with Lipofectamine 2000 (Life Technologies). cDNA ratios were α2:β4 of 1:1, for WT receptors; α 2-Ile297Phe: β4 of 1:1 for homozygous mutant receptor; and α2: α2-Ile297Phe: β4 of 1:1:2, for the simulated heterozygous state. DNA concentration in the transfection mixture was 4 mg of nAChR subunit constructs plus 0.6 mg of expression vector for the enhanced green fluorescent protein E-GFPpcDNA3 (Clontech Laboratories, Mountain View, CA). E-GFP expression allowed easier detection of transfected cells. Cells were incubated with the transfection mixture for 4 to 6 hours. This procedure was slightly modified when testing the α2 β2 form, which yields very low functional expression in mammalian cell lines (see the results section).We increased receptors' expression using the procedure of Cooper et al. In particular, 24 hours before recording, transfected cells were transferred to lower temperature (29°C–30°C, in 5% CO2).

Patch-clamp experiments. We applied the whole-cell configuration of patch-clamp. Currents were registered 36 to 72 hours after transfection, with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), at 22°C to 24°C. Micropipettes (2–3 MV) were pulled from borosilicate capillaries with a P-97 Flaming/Brown Micropipette Puller (Sutter Instrument Co., Novato, CA). Cell capacitance and

series resistance were compensated (up to approximately 75%). Cells were inspected with an Eclipse TE200 microscope (Nikon Corporation, Tokyo, Japan) equipped with a TE-FM epifluorescence attachment for detection of fluorescent cells. Currents were low-pass-filtered at 2 kHz and acquired online at 5 to 10 kHz with pClamp 9 hardware and software (Molecular Devices). Drugs were applied with an RSC-160 Rapid Solution Changer (Bio-Logic Science Instruments, Claix, France).

Solutions and drugs. Unless otherwise specified, chemicals were purchased from Sigma-Aldrich (St. Louis, MO). The extracellular solution contained the following (mM): NaCl 130, KCl 5, CaCl2 2, MgCl2 2, HEPES 10, and D-glucose 5. (pH 7.3). Patch pipettes contained the following (mM): K-aspartate 120, NaCl 10, MgCl2 2, CaCl2 1.3, EGTA-KOH 10, HEPES-KOH 10, and MgATP 1 (pH 7.3). Stock solutions of nicotine (10 mM) were prepared weekly in an extracellular solution and kept refrigerated. Extracellular solutions with the appropriate nicotine concentrations were prepared daily; pH was always rechecked after nicotine addiction.

Analysis of patch-clamp data. Data were analyzed with Clampfit 9.2 (Molecular Devices) and OriginPro 9 (OriginLab, Guangzhou, China). The concentration-response data were fitted to a single-term Hill-Langmuir equation:

$$\frac{I_L}{I_{max}} = \{1 + (EC_{50}/[L]^{nH})\}^{-1}$$

where I_{max} is the maximal current, I_L is the current at a given concentration of agonist L, EC_{50} is the half-effective L concentration, and nH is the Hill coefficient (expressing the degree of apparent cooperativity). To estimate the reversal potential (V_{rev}) of WT and heterozygous currents, current–voltage (I/V) relations were obtained by applying voltage ramps from 260 to 110 mV, with 100 mM nicotine or without it. The background current was subtracted from the current measured in the presence of nicotine. To decrease the fluctuations in V_{rev} measurements, 3 voltage ramps were generally applied before and during nicotine application. Since our aim was to determine V_{rev}, we deemed as irrelevant the small differences (~10%) between current amplitudes obtained with the consecutive voltage ramps caused by slow desensitization of the α2β4 receptor. From each of the resulting I/V relations, we estimated V_{rev} visually after fitting the I/V curve with a polynomial function (e.g., Haghighi and Cooper, 2000). No correction of liquid-junction potential was applied to any of the voltage values provided above. Data are generally given as mean values ± SEM, with "n" representing the number of determinations in different cells. Statistical significance was determined with Student t test for unpaired samples, with the level of significance set at p=0.05.

4.3 Results

CHRNA2 mutation screening. Direct sequencing of PCR-amplified products of CHRNA2 demonstrated the c.889A.T (p.Ile297Phe) mutation in 1/150 probands in our cohort (~0.6%of the whole cohort: 95% confidence interval 0.017%–3.658%, 1.2% of familial cases). Segregation analysis demonstrated that all 7 affected individuals available for molecular screening were heterozygous for the mutation, whereas the proband's healthy grandmother was mutation-negative (figure 1A). The p.Ile297Phe mutation affects the first amino acid of the second transmembrane domain of the protein (figure 1B). Bioinformatic analysis using PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.jcvi.org/), and MutationTaster (http://www.mutationtaster.org/) programs predicted a pathogenic role of the Ile297Phe substitution. To exclude that p.Ile297Phe mutation may be a rare, benign polymorphism, we interrogated both the EVS (http://evs.gs.washington.edu) and the ExAC (http://exac.broadinstitute.org) databases, comprising exome data of 6,503 controls and 61,486 individuals sequenced as part of various disease-specific and population genetic studies. Overall, these large datasets list 262 CHRNA2 missense variations, of which 106 are predicted as probably damaging but do not include the p.Ile297Phe mutation, supporting its pathogenic role (Cherepanova et al,). In silico analysis with the Jalview software (Waterhouse et al, 2009) revealed that the p.Ile297Phe mutation, which causes the substitution of an aliphatic, hydrophobic amino acid (isoleucine) with

an aromatic one (phenylalanine), occurred in an evolutionarily conserved residue (figure 1C).

Clinical and neurophysiologic studies in affected individuals carrying the p.Ile297Phe CHRNA2 mutation. Clinical information on affected individuals is summarized in the table. The family pedigree is reported in figure 1A. Clinical findings were homogeneous across affected individuals and characterized by frequent paroxysmal motor events during sleep. Mean age at onset was 6.2 years (median 4.5, range 3–16). In all patients, several seizures per night occurred with no correlation with any specific sleep phase. In undiagnosed, untreated patients (II-2, II-4, and III-2), seizures persisted in adulthood with consequent, frequent traumatic injuries. Personal history was unremarkable except for a diagnosis of attention deficit hyperactivity disorder (patient III-5) and other sleep disorder (somniloquium, patient III-2). Brain MRI was unremarkable in all. Clinical seizures were consistent with epileptic paroxysmal arousals, with sudden motor manifestations, including bilateral tonic or dystonic posturing, with patterned automatic activities. After ictal motor manifestations and EEG activity subsided, the patients remained unresponsive, or uttered unintelligible words, for up to several minutes. No recall of subjective symptoms was possible. Ictal and interictal EEG data are summarized in the table and shown in figures 2. Oxcarbazepine monotherapy was effective in all patients.

Patient	Sex	Age at onset, y	Age at treatment initiation, y	Age at follow-up, y	Seizure frequency and outcome	Interictal EEG	Seizure patterns and semiology	Sleep phase and ictal EEG
II-2	F	10	46	47	Several per night; seizure-free on OXC	Bilateral frontal spikes and slow waves	Paroxysmal arousals with abrupt sitting on the bed, gestural automatisms (right hand swiping nosel, sustained postictal confusion; duration: 1 min; on several occasions, traumatic injuries upon falling from the bed, including leg fracture and subdural hematoma	During N2, in relation to K-complexes, barely recognizable ictal activity covered by movement artifacts
II-4	F	16	36	39	Several per night; seizure-free on OXC	Sporadic bilateral frontal spikes	Paroxysmal arousals with abrupt sitting on the bed, gestural automatisms, sustained postictal confusion; duration: 20-30 s	During N2, in relation to K-complexes, barely recognizable ictal activity covered by movement artifacts
III-1[a]	M	3	8	11	Several per night; seizure-free on OXC	Sporadic bilateral frontal spikes	Paroxysmal arousals with sitting on the bed, asymmetric posturing, patterned leg and pelvic movements, gestural automatisms (right hand swiping nosel, fearful facial expression (inconstant), sustained postictal confusion; duration: 1 min	During N3, bilateral, right predominant, frontotemporal alpha-like activity intermingled with delta and sharp waves, followed by slow waves and awakening EEG
III-2	M	5	14	17	Several per night; seizure-free on OXC	Bilateral frontal spikes and slow waves	Paroxysmal arousals with brisk bilateral posturing of trunk and legs, unintelligible vocalization and postictal confusion; average duration: 1 min; several traumatic facial/head injuries upon falling from bed	During N2, in relationship to K-complexes, diffuse voltage decrement, followed by rhythmic delta waves and awakening EEG
III-3	M	4	5	5	Several clusters per week; seizure-free on OXC	Sporadic right frontal sharp waves	Paroxysmal arousals with sitting on the bed, gestural automatisms (right hand swiping nosel, cycling leg movements, chewing, looking around, screaming, sighing, sustained postictal confusion; duration: 1 min	During N2, abrupt appearance of bilateral frontal high-amplitude rhythmic delta
III-4	M	4	7	8	Several clusters per month; seizure-free on OXC	Sporadic right frontal central spikes	Paroxysmal arousals, sitting on the bed, right hand dystonia, looking around, sometimes fearful expression, trunk and pelvic movements, chewing, postictal confusion; duration: 2 min; several occasions, head/facial injuries upon falling from bed	During N2, in relationship to K-complexes, right frontotemporal rhythmic theta, contralateral slow waves
III-5	M	5	11	11	Several episodes per night; seizure-free on OXC	Sporadic slow waves over right central regions	Paroxysmal arousals, sitting on the bed, gestural automatisms, vocalization, head shaking, prolonged motionless staring; duration: 1 min	During N2, abrupt appearance of bilateral frontal high-amplitude rhythmic delta

Table 1: Clinical information of affected family members. (OXC = oxcarbamazepine; [a] = proband)

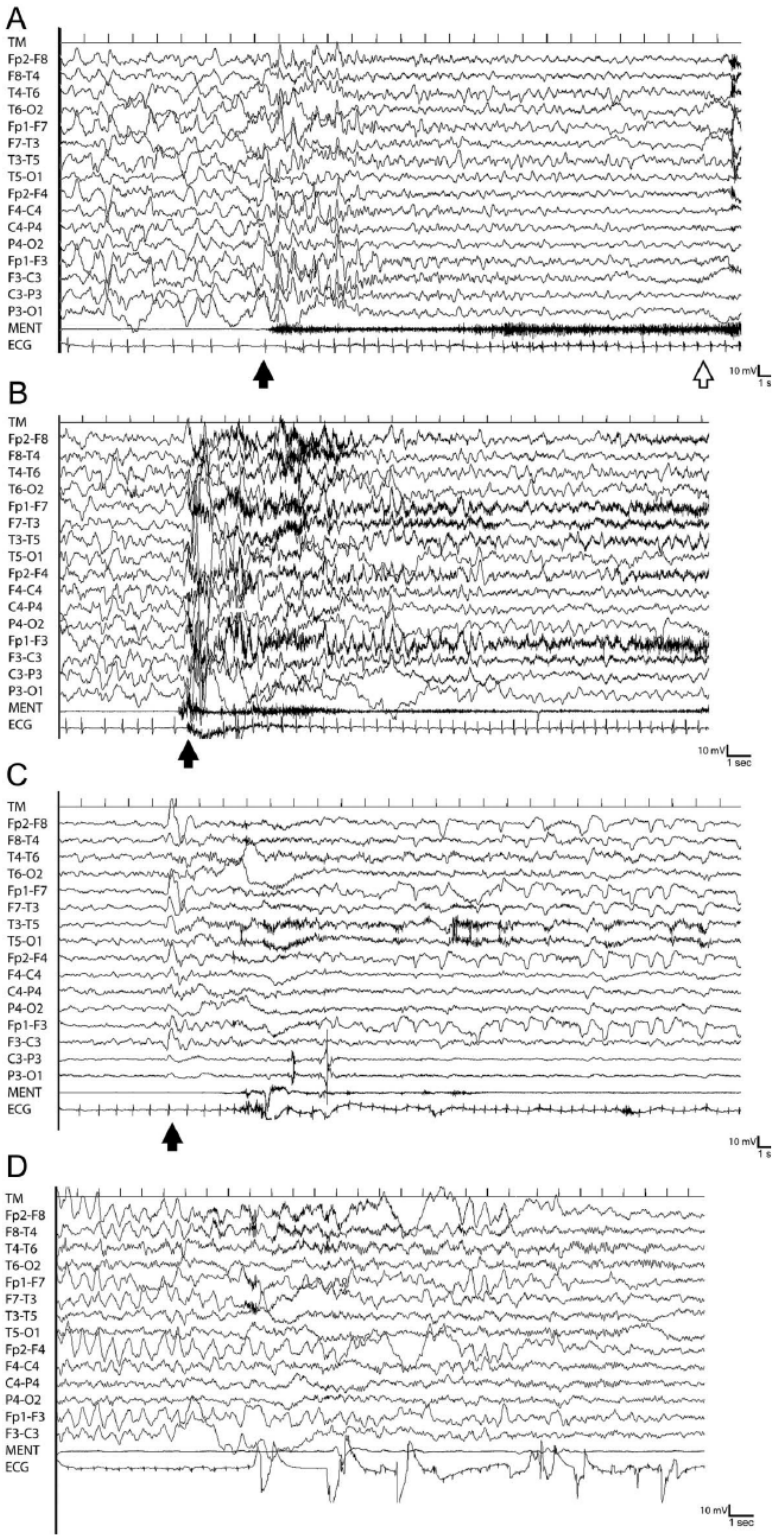
A
TM
Fp2-F8
F8-T4
T4-T6
T6-O2
Fp1-F7
F7-T3
T3-T5
T5-O1
Fp2-F4
F4-C4
C4-P4
P4-O2
Fp1-F3
F3-C3
C3-P3
P3-O1
MENT
ECG
10 mV
1 s
B
TM
Fp2-F8
F8-T4
T4-T6
T6-O2
Fp1-F7
F7-T3
T3-T5
T5-O1
Fp2-F4
F4-C4
C4-P4
P4-O2
Fp1-F3
F3-C3
C3-P3
P3-O1
MENT
ECG
10 mV
1 sec
C
TM
Fp2-F8
F8-T4
T4-T6
T6-O2
Fp1-F7
F7-T3
T3-T5
T5-O1
Fp2-F4
F4-C4
C4-P4
P4-O2
Fp1-F3
F3-C3
C3-P3
P3-O1
MENT
ECG
10 mV
1 s
D
TM
Fp2-F8
F8-T4
T4-T6
T6-O2
Fp1-F7
F7-T3
T3-T5
T5-O1
Fp2-F4
F4-C4
C4-P4
P4-O2
Fp1-F3
F3-C3
MENT
ECG
10 mV
1 sec

Figure 2: Polygraphic video-EEG recordings of nocturnal seizures in 4 patients. See the table for details of clinical semiology and ictal EEG. A) Patient III-1. The black arrow indicates abrupt appearance of bilateral spikes prevalent over the anterior regions during slowwave sleep, followed after a few seconds by diffuse, slow-wave activity. Clinically, the patient awakened and exhibited bilateral dystonic posturing. The white arrow indicates the end of the clinical seizure. B) Patient III-3: paroxysmal arousal. The black arrow indicates the onset of bilateral, frontal predominant, high-amplitude rhythmic delta waves. Clinically, the patient manifested a paroxysmal arousal with sitting on the bed, gestural automatisms, cycling leg movements, chewing, screaming, sighing, and postictal confusion. C) Patient III-4. The black arrow indicates the appearance of right frontotemporal rhythmic theta activity and contralateral slow waves, in relationship to a K-complex. Clinically, the patient exhibited a paroxysmal arousal with right hand dystonia, sitting on the bed, fearful expression, oral automatisms, trunk and pelvic movements, and postictal confusion. D) Patient III-5: paroxysmal arousal. Artifact-free ictal EEG was only available after a few seconds from seizure onset and disclosed a high-amplitude, bilateral, frontal predominant, slow-wave activity. Clinically, the patient manifested a paroxysmal arousal, with gestural automatism, vocalization, head shaking, and motionless staring

Functional studies. In the primate brain, the α2 subunit exhibits a wide distribution that largely overlaps with that of the α4, β2, and β4 subunits (Aridon et al, 2006; Han et al, 2000; Quik et al, 2000). We first tested the effect of coexpressing α2 and α2-Ile297Phe with β4 in HEK cells. Figure 3A shows whole-cell current traces elicited by the indicated concentration of nicotine, applied for 2 to 3 seconds to cells maintained at -70 mV (i.e., close to the neuronal V_{rest}). The maximal current was tested at regular intervals to rule out nAChR rundown, which is sometimes observed in cell lines. Nicotine was preferred to the physiologic agonist acetylcholine (ACh) to avoid applying muscarinic receptor's blockers, whose effect on different nAChR subtypes is only partially defined (Zwart et al, 1997). No current expression was observed in cells transfected with the homozygous α2-Ile297Pheβ4 form. Figure 3B shows the average nAChR current density (i.e., the maximal current divided by cell capacitance) in experiments performed with the indicated subunit combinations. The current density of the heterozygous form was approximately 40% of that observed in WT channels, whereas the homozygous form produced no measurable currents. No significant difference between WT and heterozygous receptors was observed in the kinetics of channel desensitization and deactivation (data not shown). Statistics are given in the figure legend. The functional expression of the α2β2 subtype generally produces lower current amplitudes than other subtypes such as α2β4 and α4β2 (Aridon et al, 2006; Di Resta et al, 2010; Zwart et al, 1997; Groot-Kormelink et al, 2004; Xiao et al, 2004). Therefore, to obtain measurable currents from the α2β2

subtype, before patch-clamp experiments, we incubated the transfected cells at lower temperature for 24 hours (see the methods section). As shown in figure 3D, the results we obtained with α2β2 were qualitatively similar to those obtained with α2β4, in that α2-Ile297Phe strongly decreased nAChR currents. These results show that the p.Ile297Phe substitution decreases the functional expression of the heteromeric nAChR isoforms containing the mutant α2 subunit. We subsequently performed a fuller characterization of the properties conferred to nAChRs by α2-Ile297Phe, conducting a functional study in the α2β4 nAChR subtype. To assess whether the mutant subunit significantly altered ion selectivity, we measured V_{rev} from current/voltage (I/V) relations obtained by applying voltage ramps from -60 to +10 mV. Representative I/V curves from cells expressing WT or heterozygote nAChRs are shown in figure 4A. Both types of receptors showed the inward rectification typical of neuronal nAChRs. We observed no significant difference in the average V_{rev} for WT and heterozygous receptors in a series of similar experiments (figure 4B). These results indicate that α2-Ile297Phe is unlikely to produce major alterations in channel permeability, thus not modifying the depolarizing nature of nicotinic currents in CNS neurons. Through experiments such as those illustrated in figure 3, we generated concentration-response relations to nicotine for WTs and heterozygotes. The peak currents measured at different agonist concentrations were normalized to the maximal value and plotted as a function of ligand concentration (figure 4C). Data points were fitted with a properly scaled Hill-type equation (equation 1). The half-

effective nicotine concentrations (EC_{50}) for the 2 components were approximately 11 μM for WT receptors and 22 μM for the heterozygote, with a Hill coefficient of about 1.5. Statistics are given in the figure legend.

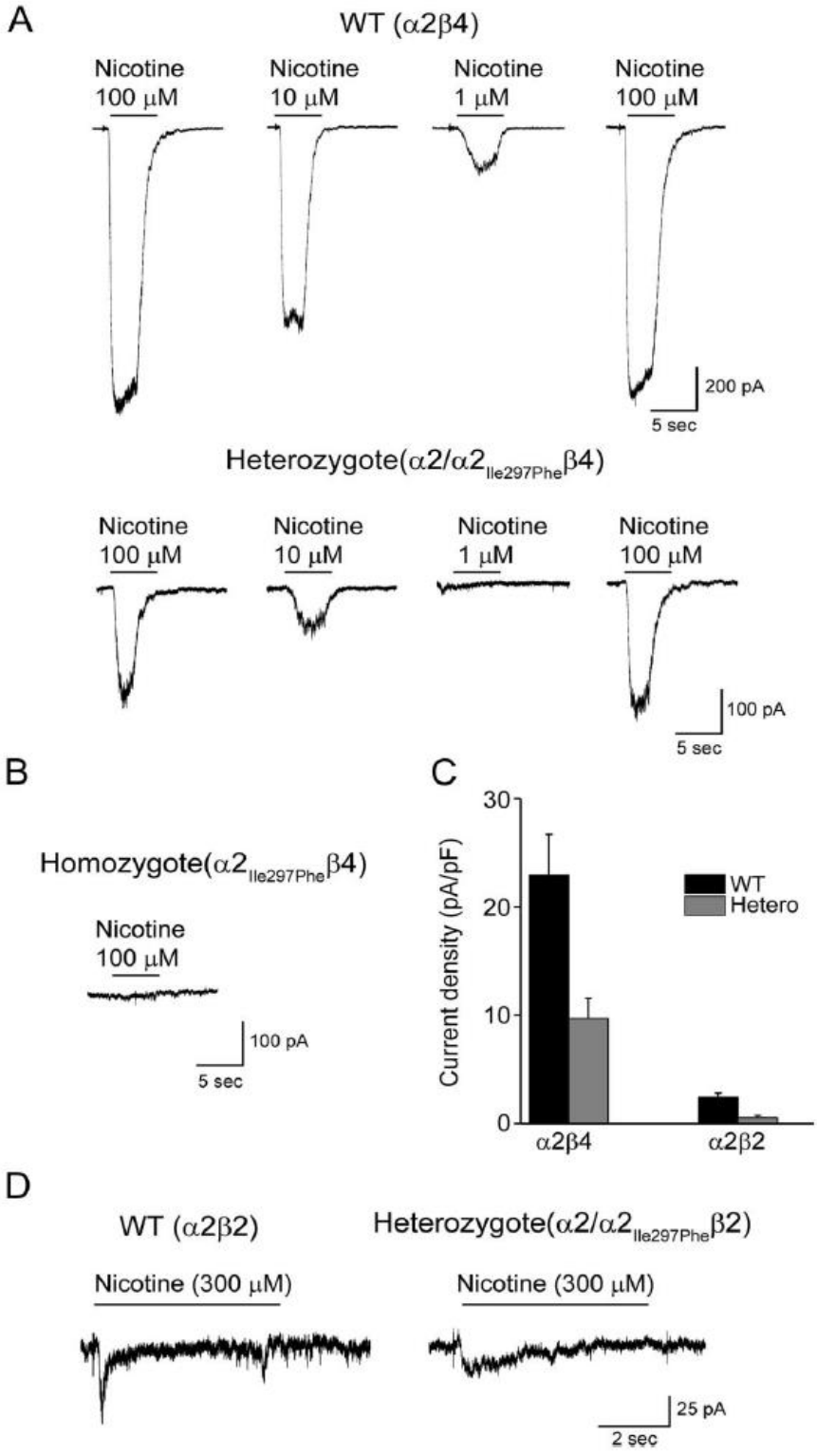
A
WT (α2β4)
Nicotine 100 μM
Nicotine 10 μM
Nicotine 1 μM
Nicotine 100 μM
200 pA
5 sec
Heterozygote(α2/α2Ile297Pheβ4)
Nicotine 100 μM
Nicotine 10 μM
Nicotine 1 μM
Nicotine 100 μM
100 pA
5 sec
B
Homozygote(α2Ile297Pheβ4)
Nicotine 100 μM
100 pA
5 sec
C
Current density (pA/pF)
30
20
10
0
WT
Hetero
α2β4
α2β2
D
WT (α2β2)
Heterozygote(α2/α2Ile297Pheβ2)
Nicotine (300 μM)
Nicotine (300 μM)
25 pA
2 sec

Figure 3: Functional expression of α2β4 and α2β2 nAChR subtypes, containingor not containing α2-Ile297Phe. A) Representative current traces elicited by the indicated concentration of nicotine in HEK cellsexpressing either the α2β4 (WT) or the α2/α2Ile297Pheβ4 (heterozygous) nAChR isoform. V_m was -70 mV. The continuous lines above the current traces mark the time of nicotine application. Panels display typical currents recorded from WT and heterozygous nAChRs, as indicated. B) No measurable current was elicited when cells were transfected with the homozygous (α2Ile297Pheβ4) nAChRs. Experimental conditions were as in panel A. C) Bars indicate the average peak current density for WT and heterozygous receptors in either α2β4 or α2β2 condition, as indicated. Currents were elicited at -70 mV, by 100 μM (for the α2β4 subtype) or 300 μM nicotine (for the α2β2 isoform). In particular, the average current values were (pA/pF): 22.97 ± 3.73 for WT α2β4 (n = 21), and 9.72 ± 1.85 for the corresponding heterozygote (n = 27, p = 0.01). In these experiments, the current densities obtained with 10 μM nicotine were (pA/pF): 11.33 ± 2.47 for WT (n = 21), and 3.49 ± 0.74 for the heterozygote (n = 27). No measurable current was ever detected in the homozygous mutant form (n = 30). Data summarize the results obtained in 4 runs of transfection. For the α2β2 subtype, the peak current density (pA/pF) was 2.45 ± 0.35 (n = 9) for the WT, and 0.55 ± 0.15 for the heterozygote (n = 5, p = 0.05). These data summarize the results obtained in 2 runs of transfection. D) Representative current traces for the α2β2 subtype, in WT and heterozygous condition, as indicated. Currents were elicited by 300 μM nicotine, at -70 mV. Continuous lines mark the time of nicotine application. Once again, no current was ever observed in the homozygous condition (not shown). HEK = human embryonic kidney; nAChR = nicotinic acetylcholine receptor; WT = wild-type.

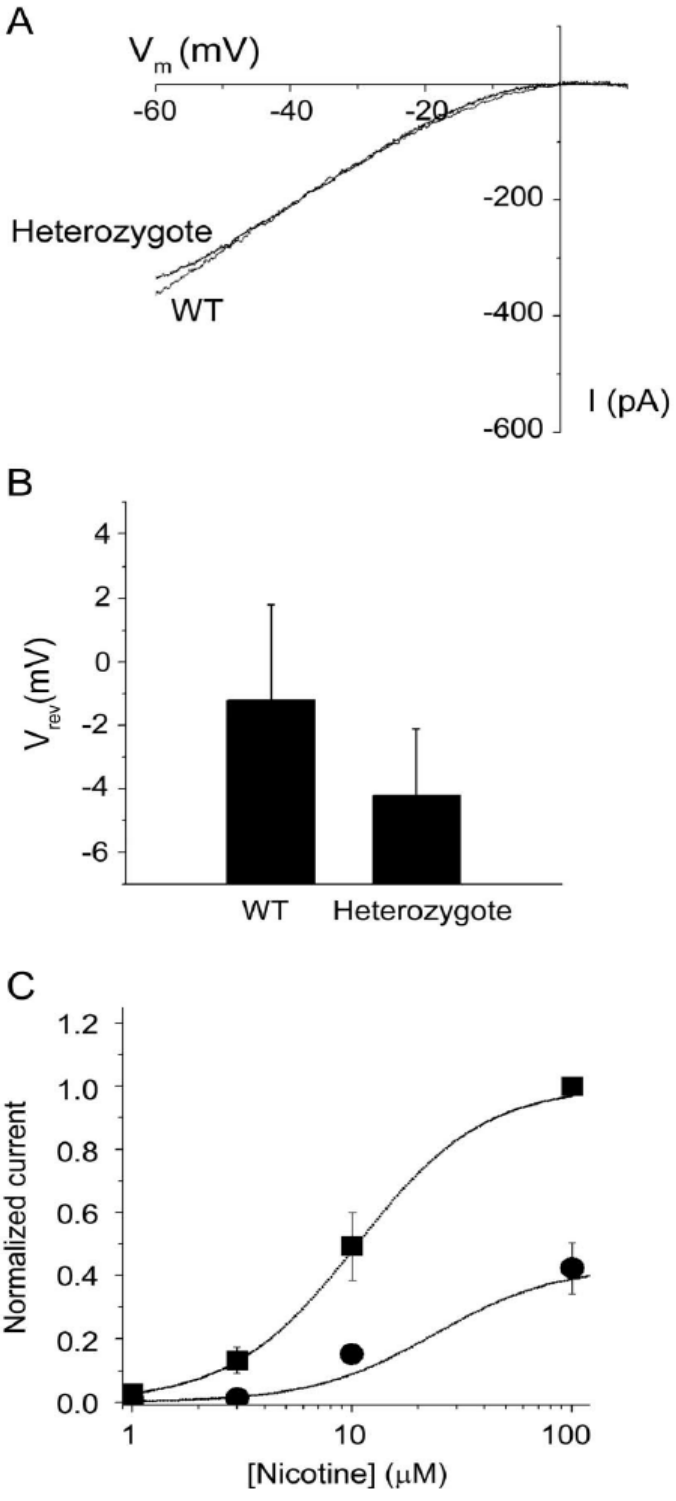
A
V_m (mV)
-60
-40
-20
-200
-400
-600
I (pA)
Heterozygote
WT
B
V_{rev} (mV)
4
2
0
-2
-4
-6
WT
Heterozygote
C
Normalized current
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
10
100
[Nicotine] (μM)

Figure 4: Functional features of α2β4 nAChRs containing or not containing α2-Ile297Phe. A) I/V relationships for WT and heterozygous receptors, from 2 cells expressing whole-cell currents of similar amplitude. Currents were elicited by applying voltage ramps from -60 to +10 mV (duration was 1 second). The current flowing through nAChRs was isolated by subtracting the background current from the current recorded in the presence of 100 µM nicotine. The illustrated currents are averages of 3 trials, applied consecutively (interval between trials was 1 second). Notice the typical inward rectification displayed by neuronal nAChRs, and the V_{rev} between -10 mV and 0 mV. B) Bars give the average V_{rev} calculated from a series of experiments in the same run of transfection, for WT and heterozygous receptors. Experimental procedure was as illustrated in panel A. The average V_{rev} was 21.1 ± 3mV for the WT (n = 9) and 24.2 ± 2.1 for the heterozygote (n = 5, not statistically different from the WT). C) Concentration-response relations obtained by applying different nicotine concentrations to WT (squares) and heterozygous (circles) receptors. The applied nicotine concentrations were 1, 3, 10, and 100 µM. V_m was -70 mV. Data points are average peak currents normalized to the maximal WT value, and plotted as a function of ligand concentration. Each point represents at least 6 determinations in different cells. Continuous lines were obtained by fitting the data with equation 1. The EC_{50} was 10.6 ± 0.03 µM (n_H = 1.52 ± 0.06) for WT, and 23.9 ± 2.4 µM (n_H = 1.56 ± 0.53) for heterozygous nAChRs. Data summarize the results obtained from 48 cells, in 4 runs of transfection. EC_{50} = 50% effective concentration; nAChR = nicotinic acetylcholine receptor; WT = wild-type.

4.4 Discussion

ADNFLE is genetically heterogeneous, with only a few disease-causing mutations having been reported so far in 6 different genes (Nobili et al, 2014). DEPDC5 was recently identified as a major ADNFLE-causing gene, with about 13% of affected families exhibiting mutations of this gene (Picard et al, 2014). DEPDC5-related ADNFLE is often drug-resistant and associated with diurnal seizures. Families carrying DEPDC5 mutations are smaller than those related to other causative genes, possibly as a consequence of some degree of reproductive disadvantage resulting from severe epilepsy. Mutations in the 5 remaining ADNFLE-related genes CHRNA4, CHRNB2, CHRNA2, KCNT1, and CRH) collectively account for about 10% to 15% of familial cases (Nobili et al, 2014; Picard et al, 2014). Among these genes,CHRNA2 has until now revealed the lower mutation rate, with only one reported mutation in one family (Aridon et al, 2006). The second ADNFLE point mutation in CHRNA2 we are reporting here confirms the causative role of this subunit for this syndrome. Although CHRNA2 only accounts for 1.2% of the ADNFLE families we screened after reporting the first mutation (Aridon et al, 2006),neither CHRNA4 nor CHRNB2 were ever mutated in the same cohort. The previously reported CHRNA2 mutation (p.Ile279Asn) resulted in a marked increase of the receptor sensitivity to the agonist, suggesting gain of function (Aridon et al, 2006; Hoda et al, 2009). Conversely, the p.Ile297Phe mutation has scarce effect on the receptor sensitivity to nicotine, but causes complete loss of current expression in homozygosis and a decrease to about 40% in heterozygosis, thus

pointing to loss of receptor function. These functional differences might be due to either the position of the mutated amino acid in the protein or the physicochemical alterations introduced by the substitution, or both. The p.Ile279Asn mutation resulted in the non conservative substitution of an amino acid with a hydrophobic side chain with another having a polar uncharged side chain (Aridon et al, 2006). The affected amino acid is located in the first transmembrane domain and, apparently, does not directly contribute to the structure of the channel pore, but is probably implicated in the conformational transition operated by agonist binding (Hoda et al, 2009). The p.Ile297Phe mutation affects an amino acid located in the second transmembrane domain, which is more directly implicated in the gating structure (Albuquerque et al, 2009). The decreased current expression caused by p.Ile297Phe might depend on either impaired channel expression onto the cell surface or on a drastic decrease in the channel open probability. Although both Ile and Phe have a hydrophobic side chain, the substitution cannot be considered conservative, because of the different sizes and chemical properties of the aliphatic and aromatic benzyl side chain (Liu et al, 2011). As a consequence of these differences, p.Ile297Phe might cause a steric hindrance that disrupts the tight association of amino acids in the ion gate, impairing folding and trafficking of the mutated receptor, or the gating transition, or both. Observing similar phenotypes in relation to nAChR mutations exhibiting opposite cellular effects in vitro is not entirely surprising. This phenomenon has been demonstrated, for example, in congenital myasthenia in which most alterations in the

CHRNE gene result in loss of function, but a small fraction cause gain of function, in either case ultimately resulting in reduced efficiency of the synaptic transmission (Steinlein et al, 2008). Loss of receptor function as a consequence of disease-causing mutations of nAChR has also been reported in ADNFLE. Analysis of the Ser248Phe (S248F) CHRNA4 mutant revealed that these receptors exhibit a marked desensitization (Weiland et al, 1996; Kuryatov et al, 1997; Bertrand et al, 1998). Based on these data, it was hypothesized that seizures were somehow caused by loss of receptor function, although a gain-of-function mechanism seems to be operating in most functionally characterized mutations. Heteromeric nAChRs control both excitatory and inhibitory transmission in the frontal cortex and elsewhere, and the normal firing activity is generated by a delicate balance of excitation and inhibition. For example, a gain-of-function nAChR expressed in GABAergic cells can produce hyperexcitability because abnormally strong bouts of γ-aminobutyric acid (GABA) release can synchronize pyramidal cells (Klaassen et al, 1996). However, a loss-of-function nAChR expressed in the same GABAergic cells can also produce hyperexcitability by decreasing feedback inhibition of pyramidal cells (Aracri et al, 2010). Focusing on the p.Ile297Phe mutation, one might assume that because of its strong effect on channel expression, it would cause an abnormal reorganization of the subunits participating in the heteromeric nAChRs comprising or not α2, resulting in a global alteration of the nicotinic function in vivo. Clinical manifestations occurring in the family we are describing were conclusively diagnosed as ADNFLE only

after video-EEG recordings. ADNFLE is indeed often misdiagnosed as parasomnias and may consequently be underdiagnosed. When ADNFLE is suspected, mutation screening of CHRNA2 should be considered along with CHRNA4, CHRNB2, and KCNT1. Since ADNFLE caused by nAChR mutations carries an overall good long-term seizure outlook and is likely responsive to medication, assigning it to its specific genetic etiology may have implications for management.

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Chapter 5: Summary, conclusions and future perspectives

5.1 Summary and conclusions

This three years' work allowed our laboratory to understand the molecular mechanism which account for a NFLE/ADNFLE phenotype. In particular we dissected the role of heteromeric nAChRs in the establishment of epileptic phenotype. This kind of brain disorder is commonly associated to gain of function mutation, often caused by increased sensitivity to the agonists, accompanied or not by altered current kinetics (De Fusco et al., 2000; Hoda et al., 2008; Nichols et al, 2016). However our work in collaboration with A. Meyer Children's Hospital–University of Florence led to the identification of an α2 subunit loss of function mutation responsible for a NFLE-like phenotype (Conti et al, 2015). At first sight, an explanation for these findings appears to be difficult: α4β2 and α2β2 are the two most common form of heteromeric nAChR in the CNS. One hypothesis is that hypofunctional α2β2 nAChRs can shift the balance towards the α4β2 isoform, which shows an higher affinity for acetylcholine. Moreover, heteromeric nAChRs can modulate both excitatory and inhibitory neurotransmitter release. Therefore, a hypofunctional nAChR could hinder the ability of inhibitory interneurons to contain seizure propagation. Testing this hypothesis would require further studies on the role of α2 in different neuronal population. Altered GABAergic signaling is commonly observed in ADNFLE animal models (Steinlein et al, 2010), but it is not clear if epileptiform activity leads to a disruption of chloride homeostasis (shifting GABA from an inhibitory to an excitatory role) (Zhu et al, 2008) or viceversa (Liu et al, 2006).

Besides analyzing the contribution of heteromeric nAChRs to ADNFLE in a mature brain, we also considered their role in neuronal network development. Besides affecting GABA and glutamate release in adult mice (Aracri et al, 2010), recent finding suggest that nAChRs regulate the development GABAergic and glutamatergic systems (Liu et al, 2006; Molas and Dierssen 2015, Lozada et al, 2012). Adopting the murine ADNFLE model developed by Manfredi and colleagues, we observed that β2-V287L does not interphere with the GABAergic excitatory/inhibitory switch but affect glutamatergic transmission.

The timing of GABAergic excitatory/inhibitory switch plays a central role in CNS development: GABA driven network oscillation underlied by GDP is focal for the establishment of long range hub connections between neurons (Cellot and Cherubini, 2013; Ben Ari, 2015). This process takes place during the first two postnatal weeks and is influenced by cholinergic signaling (Liu et al, 2006); our findings highlighted that the E/I switch timing is not affected in our ADNFLE model. However we cannot exclude that heteromeric nAChRs contribute to GABA driven synaptic development in a different way, maybe affecting GDP's frequency and therefore the strength of synaptic connections.

How cholinergic signaling affects the glutamatergic system development is still unclear. Mutant mice show a strengthening of glutamatergic signaling starting from the end of the first postnatal week, in terms of EPSCs frequency and amplitude. Change in cortical circuitry and synaptic efficiency may account for these changes.

nAChRs can affect synaptic development at different levels (Molas and Dierssen, 2014) and heteromeric receptors seem to mostly affect more synapse maturation, dendritic branching and spine stabilization rather than the initial formation of these structures (Lozada et al, 2012). Heteromeric nAChRs are thus likely to exert their effect on brain development from the end of the first postnatal week. A change in glutamatergic synapses localization may probably account for the higher seizure susceptibility observed in mice harboring the β2-V287L subunit. By slowing receptor desensitization (De Fusco et al, 2000) or stabilizing the high affinity $(\alpha4)_2(\beta2)_3$ stoichiometry (Son et al, 2009; Becchetti et al, 2015; Nichols et al, 2016) β2-V287L can lead to an abnormal calcium influx from extracellular compartment which may activate a series of important intracellular effectors. These effectors can affect cytoskeletal dynamics (Lozada et al, 2012), cell adhesion molecule (Koleske, 2013) or influence synaptic plasticity (Trachtenberg et al., 2002; Cline and Haas, 2008). Interplays between these processes are also possible.

It appears clear that mutation in genes coding for heteromeric nAChRs can contribute to an epileptic phenotype at different levels, promoting excitability in adult neuronal networks (which are still susceptible to remodeling), or affecting the development of a functional cortical circuitry, or both. ADNFLE appears therefore not only to be a channelopaty but a more complex developmental disease.

5.2 Future perspectives

A first issue for future studies is to extend our physiological characterization to interneurons: in these studies we limited our interest to layer V pyramidal neurons which represent the main channel of cortical output to subcortical structures. However inhibitory interneurons represent the computational machinery that integrates the signals from pyramidal neurons. Understand how connections between different interneurons population are regulated to control different types of inhibition (feedback and feedforward inhibition), will be focal to understand how ADNFLE arises.

The use of a conditional murine model will also allow us in future to understand how β2-V287L affects the correctly developed brain. Doxycycline administration during pregnancy and early post natal life to silence mutant subunit expression will lead to the formation of a coherent neuronal network. Stopping pharmacological treatment after P15 will allow us to understand if the differences observed in adult mice are attributable only to developmental changes or if the mutation exerts significant effect in a well-developed brain.

In future we will also consider the exact temporal window during which β2-V287L expression is crucial for epilepsy establishment, by limiting the transgene expression to progressively restricted temporal windows.

We also developed an in vitro ADNFLE model: we used Manfredi's strains not treated with doxycycline, to compare the action potential firing activity of neocortical cultures from mice expressing or not β2-V287L subunit. Cell firing is recorded by using multi-electrode array (MEA) platforms, which permit long-term continuous extracellular recording of the main excitability features (Gullo et al., 2009, 2012). The neuronal networks from ADNFLE mice produce spontaneous epileptiform activity, characterized by prolonged up-states interspersed among the normal up- and down-states. On these neuronal cultures we also carried out patch-clamp recordings to monitor EPSCs/IPSCs amplitude and frequency. On this in vitro ADNFLE model we will test cholinergic agents and anti-epileptic drugs, able to prevent the establishment of an highly epileptogenic neuronal network. The ultimate aim is to identify a temporal window during which a targeted pharmacological intervention will allow to prevent ADNFLE establishment. By combining this approach with early genetic characterization of individuals with high familiarity for this kind of epilepsy we hope to identify an effective and totally remissive treatment of this brain disorder.

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