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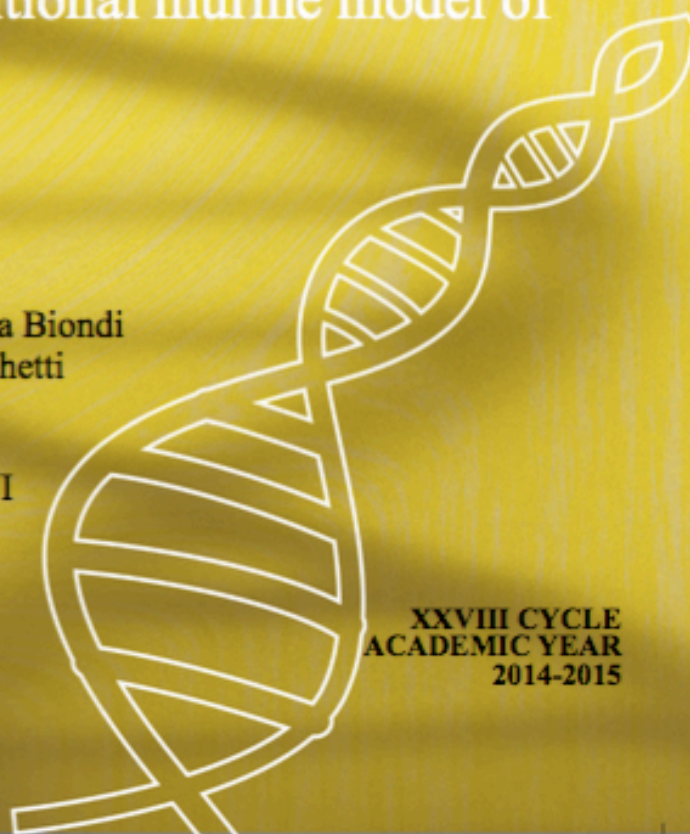
Cholinergic transmission in the cerebral cortex of a conditional murine model of ADNFLE

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To my family, friends and colleagues

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The role of nicotinic acetylcholine receptors in autosomal dominant nocturnal frontal lobe epilepsy

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Abstract

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. It is characterized by clusters of complex and stereotyped hypermotor seizures, frequently accompanied by sudden arousals. Cognitive and psychiatric symptoms may be also observed. Approximately 12% of the ADNFLE families carry mutations on genes coding for subunits of the heteromeric neuronal nicotinic receptors (nAChRs). This is consistent with the widespread expression of these receptors, particularly the $\alpha 4\beta 2^*$ subtype, in the neocortex and thalamus. However, understanding how mutant nAChRs lead to partial frontal epilepsy is far from being straightforward because of the complexity of the cholinergic regulation in both developing and mature brains. The relation with the sleep-waking cycle must be also explained. We discuss some possible pathogenetic mechanisms in the light of recent advances about the nAChR role in prefrontal regions as well as the studies carried out in

murine models of ADNFLE. Functional evidence points to alterations in prefrontal GABA release, and the synaptic unbalance probably arises during the cortical circuit maturation. Although most of the available functional evidence concerns mutations on nAChR subunit genes, other genes have been recently implicated in the disease, such as *KCNT1* (coding for a Na⁺-dependent K⁺ channel), *DEPD5* (Disheveled, Egl-10 and Pleckstrin Domain-containing protein 5), and *CRH* (Corticotropin-Releasing Hormone). Overall, the uncertainties about both the etiology and the pathogenesis of ADNFLE point to the current gaps in our knowledge the regulation of neuronal networks in the cerebral cortex.

Introduction

Sleep has been long known to facilitate the occurrence of epileptic seizures, particularly during non-rapid eye movement (NREM) sleep (Boursouliau et al., 2013). Among the most common epileptic syndromes associated with NREM sleep, we recall Idiopathic Generalized Epilepsy (IGE, comprising a heterogeneous groups of epilepsies), Idiopathic Focal Childhood Epilepsies (IFCE), the Lennox-Gastaut Syndrome (LGS) and Temporolimbic Network Epilepsy (TLNE). Another major epileptic syndrome related to NREM sleep is nocturnal frontal lobe epilepsy (NFLE), whose features were originally defined during the detailed characterization of cohorts of patients affected by paroxysmal nocturnal dystonia (Provini et al., 1999; Tinuper and Lugaresi, 2002). The familial form of NFLE has autosomal dominant inheritance and was named ADNFLE (Scheffer et al., 1995). Because similar clinical and electroencephalographic (EEG) features are displayed by ADNFLE and the sporadic non-lesional cases of NFLE, ADNFLE is a good model of NFLE in general (Picard and Brodtkorb, 2008). ADNFLE is a partial epilepsy often arising in childhood or early adolescence. Attacks arise in the frontal lobe, usually during stage 2 of sleep, and are characterized by clusters of complex and stereotyped hyperkinetic seizures. This suggests that motor patterns controlled by subcortical structures are released during the attacks. Sudden arousals are also frequent in ADNFLE, which is an indication of increased sleep fragmentation (Scheffer et al., 1995; Picard and Brodtkorb, 2008). Cognitive deficit and psychiatric comorbidities can also be present

and are probably more common than initially assumed (Bertrand et al., 2005; Picard et al., 2009). As in many other epilepsies, about one third of the patients is refractory to pharmacological treatment. 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 receptor (Di Corcia et al., 2005; Picard and Brodtkorb, 2008; Ferini-Strambi et al., 2012; nAChR). The identification of other genes linked to ADNFLE has proceeded slowly. Recently, ADNFLE mutations have been detected 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). Evidence is also available about the implication of the corticotropin-releasing hormone gene (*CRH*; Combi et al., 2005). Because little functional evidence is yet available for these genes, we here mostly focus on how mutant nAChRs may cause ADNFLE. The nAChR is a pentameric ion channel permeable to cations, including Ca²⁺ (Fucile, 2004). When opened by 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: α 2- α 7 and β 2- β 4, encoded respectively by the *CHRNA2-CHRNA7* and *CHRNA2-CHRNA4* genes. The two most common isoforms in thalamus and isocortex are the heteropentamer α 4 β 2* and the homopentamer (α 7)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 *CHRNA2* (**Table 1**), consistent with the major role of $\alpha 4\beta 2^*$ nAChRs in regulating neocortical excitability (Wallace and Bertrand, 2013). The penetrance ranges from 60 to 80%. 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* (**Table 1**) has also been causally associated with ADNFLE (Aridon et al., 2006). The $\alpha 2$ subunit can form heteromeric receptors by associating with both $\beta 2$ and $\beta 4$ (e.g., Hoda et al., 2009; Di Resta et al., 2010). Besides the pathological relevance, the study of ADNFLE may offer important insight into the function of nAChRs in the cerebral cortex. In the present review, after summarizing some relevant morphofunctional features of the frontal lobe and its cholinergic innervation, we briefly discuss the sleep stages and their relation with ADNFLE and nAChRs. Next, we review the functional studies carried out with mutant nAChRs expressed in cellular systems or animal models. These studies are interpreted in the light of recent evidence about the physiological role of heteromeric nAChRs in different neocortical regions, in mature as well as developing brains. We next discuss the possibility of targeting the cholinergic system to treat ADNFLE. Finally, we briefly analyze the other genes that have been implicated in ADNFLE, as a perspective for future studies.

Table 1 | Mutations in ion channel-coding genes linked to ADNFLE.

Gene	Protein/subunit	Mutation	Reference
<i>CHRNA4</i>	nAChR ($\alpha 4$)	S248F	Steinlein et al., 1995
		S252L	Hirose et al., 1999
		776ins3	Steinlein et al., 1997
		T265I	Leniger et al., 2003
<i>CHRNA2</i>	nAChR ($\beta 2$)	V287L	De Fusco et al., 2000
		V287M	Phillips et al., 2001
		I312M	Bertrand et al., 2005
		L301V	Hoda et al., 2008
<i>CHRNA2</i>	nAChR ($\alpha 2$)	V308A	
<i>KCNT1</i>	Kca4.1	I279N	Aridon et al., 2006
		M896I	Heron et al., 2012
		R398Q	
		Y79 6H	
		R928C	

The frontal lobes and the cholinergic system

In primates, the frontal lobes can be broadly divided into three functional regions: a caudal motor/premotor, a paralimbic (comprising the anterior cingulate complex, the parolfactory gyrus and the posterior orbitofrontal regions), and an extensive rostral heteromodal association area. The expression prefrontal cortex (PFC) is generally reserved to the latter two sections, which are extensively interconnected (Mesulam, 2000). The heteromodal PFC is also connected with the unimodal and heteromodal association cortices, while the paralimbic areas are directly linked to the limbic system. In this way, the emotional and visceral states are integrated with the sensory perception of external environment. The PFC is crucially implicated in cognitive tasks, and particularly in working memory, attention and goal-directed behavior. Extensive connections also exist between PFC and subcortical structures such as the basal nuclei (particularly the caudate nucleus) and the mediodorsal thalamus. PFC damage generally decreases the flexibility of behavior (Shallice and Burgess, 1991; Aron et al., 2004) and the capability of controlling impulse-driven responses (Mesulam, 2000). Importantly for the present perspective, prefrontal lesions in rodents produce similar alterations of executive functions (Dembrow and Johnston, 2014). Therefore, rodents seem to constitute a reasonable model to study several aspects of the frontal lobe physiology. For consistency with literature, we retain the expression PFC in rodents, although we are aware of the difficulties of carrying out comparisons with the much larger and complex dorsolateral PFC of primates (Uylings et al., 2003).

The frontal functions are regulated by ascending projections from hypothalamic and brain stem nuclei, which control the behavioral state and the sleep-waking cycle (Steriade and McCarley, 2005). In particular, the cerebral cholinergic system is composed of distinct groups of cholinergic neurons located in the pons and basal forebrain. These project diffusely to the brain, but especially to the cerebral cortex and thalamus (Mesulam, 2004). ACh cooperates with noradrenaline, histamine and serotonin to stimulate the neocortical tone, thus regulating arousal, attention and goal-directed behavior (Lucas-Meunier et al., 2003; Sarter et al., 2006; Wallace and Bertrand, 2013), as well as the switch between sleep stages (Saper et al., 2010). Work carried out in rodents indicates that the ascending cholinergic fibers densely innervate the somatosensory, visual, motor and prefrontal cortices, with a broadly homogeneous laminar distribution, especially in PFC (Eckstein et al., 1988; Avendaño et al., 1996; Mechawar et al., 2000; Henny and Jones, 2008; Aracri et al., 2010). The mechanisms by which ACh exerts its physiological functions are very complex. First, ACh can activate both ionotropic nAChRs and metabotropic (muscarinic) mAChRs. Early work tended to highlight the contribution of mAChRs, and the observed depolarizing effect of ACh on thalamic and neocortical neurons during wakefulness and REM sleep was initially attributed to a mAChR-dependent K^+ channel inhibition (Krnjevic et al., 1971; McCormick, 1992). More recently, the prominent role of nAChRs in regulating brain excitability and cognitive functions has been recognized (Picciotto et al., 1995, 1998; Hahn et al., 2003; Bailey et al., 2010; Howe et al., 2010; Guillem et al., 2011). However, the distinct roles of the numerous nAChR subunits

expressed in the neocortex is poorly understood (Gotti et al., 2007; Pistillo et al., 2015). Second, although the overall effect of ACh in the neocortex is stimulatory, recent results indicate that the cholinergic terminals are often adjacent to the soma and dendrites of GABAergic interneurons, suggesting a strict relationship between the cholinergic and the GABAergic systems (Henny and Jones, 2008; Aracri et al., 2010). Therefore, alterations in cholinergic transmission may have subtle effects on the balance between excitation and inhibition in PFC circuits. Finally, several lines of evidence suggest that non-synaptic (paracrine) ACh release is widespread in the mammalian frontal cortex, but the balance of synaptic and non-synaptic effects of ACh is still matter of debate (Umbriaco et al., 1994; Mrzijek et al., 1995; Turrini et al., 2001; Descarries et al., 2004; Lendvai and Vizi, 2008; Aracri et al., 2010; Sarter et al., 2014).

The stages of sleep and their relation with cholinergic transmission and epilepsy

Human sleep can be divided into several stages (Siegel, 2002; Steriade and McCarley, 2005). During attentive wakefulness, EEG mostly comprises low voltage (5-10 μ V), high-frequency (20-40 Hz) *beta* waves. Ampler *alpha* oscillations at approximately 10 Hz appear on relaxed waking. On falling asleep, these are substituted by mixed EEG rhythms, with frequency band similar to the *beta*'s but higher amplitude (*stage 1* sleep). This brief phase is followed by *stage 2* sleep, in which the EEG displays *K-complexes* and *sleep spindles*, superimposed to the *beta* waves. The former are sharp, high voltage transient waves that may occur spontaneously or be triggered by

sensory stimuli. The sleep spindles are wave discharges of 7-14 Hz, lasting 1-2 s, and recurring at 0.1-0.3 Hz with a waxing and waning pattern (Lüthi, 2014). During *stage 3* and *stage 4*, the EEG becomes progressively dominated by *delta* waves, with amplitudes up to 300 μ V and a frequency of 0.5-3 Hz. These stages are collectively named *slow wave sleep* (SWS). Stage 1 to stage 4 constitute the NREM sleep, as indicated by electro-oculogram. From stage 4, the sleeper reverts to stage 3 and stage 2, from which the *rapid eye movement* (REM) state can be reached. In REM sleep, the EEG resembles the one observed during wakefulness, but the subject is unconscious and the skeletal muscle tone is minimum. The most vivid oneiric activity takes place in this stage. Sleep in non-human mammals is often simply described in terms of SWS and REM sleep, with durations shorter than in humans. ACh release is high in wakefulness, strongly decreases during NREM sleep, and rises again in REM sleep (Jones, 2008). Many evidences point to the nicotinic receptors as important mediators of these effects. More specifically, studies with mice in which specific nAChR subunits were deleted show that the $\beta 2^*$ nAChRs mediate most of the arousing effects of nicotine and regulate the NREM sleep stability. The transient activity of $\beta 2^*$ nAChRs, caused by the low-level ACh release during NREM sleep, can stimulate “micro-arousal” events, at least in mice (Léna et al., 2004). In ADNFLE, this phenomenon could be potentiated by hyperfunctional $\alpha 4\beta 2$ nAChRs, thus causing the typical hyperexcitability with sudden arousals during NREM sleep. Such interpretation would also point to a strong implication of local neocortical cholinergic mechanisms in ADNFLE, which could explain

an interesting neurophysiological difference between NFLE and most of the other sleep-related epilepsies (i.e., IGE, IFCE, LGS, and TLNE). In fact, the latter syndromes are characterized by EEG spike-wave discharges (Halász, 2013). These are thought to be caused by the interplay of cortical and thalamic cells. The “spike” reflects the strong activation of pyramidal cells, which is followed by the slower “wave” caused by the inhibition produced on these cells by the activation of local GABAergic interneurons as well as reticulothalamic (RT) cells. Spike-wave activity is facilitated during NREM sleep by the lower excitatory drive from the brain stem, which induces neuronal synchrony in the thalamocortical network (Amzica and Steriade, 2002; Halász, 2013). In contrast, in NFLE patients the spike-wave seizures are much less frequent (Halász, 2013), which points to an epileptogenic mechanisms characterized by a stronger implication of local neocortical circuits. This would be also in agreement with recent studies showing that cortical arousal is particularly sensitive to the excitatory input provided by the afferent fibers relayed through the basal forebrain, as compared to the thalamic afferents (Fuller et al., 2011 and references therein).

The ADNFLE mutant nAChRs studied in expression systems

Most of the functional studies carried out to date concern the $\alpha 4\beta 2$ subtype, to which the mutant ADNFLE subunits confer a variety of functional alterations, whose physiological interpretation has given rise to controversy (Becchetti, 2012). Part of this diversity probably derives from the usage of different expression systems (*Xenopus* oocytes or mammalian cell lines), and of human or rodent nAChR subunit clones. Nonetheless, in the simulated heterozygous condition, which is the most relevant for rare and dominant mutations, a gain of receptor function is commonly observed. This is often caused by increased sensitivity to the agonists, accompanied or not by altered current kinetics (De Fusco et al., 2000; Phillips et al., 2001; Itier and Bertrand, 2002; Hoda et al., 2008). A similar increase was observed in the first mutation identified in the $\alpha 2$ subunit (Aridon et al., 2006; Hoda et al., 2009). One possible explanation of such an effect is that ADNFLE subunits modify the nAChR subunit ratio (Son et al., 2009). The $\alpha 4\beta 2$ receptor can exist in at least two stoichiometric forms: $(\alpha 4)_3(\beta 2)_2$ and $(\alpha 4)_2(\beta 2)_3$. In heterologous expression systems, the former prevails and presents an EC_{50} for ACh of approximately 70 μM , whereas the latter subtype accounts for about 20% of the expressed receptors and presents an EC_{50} of around 1 μM (Nelson et al., 2003). In fact, five ADNFLE subunits ($\alpha 4$ -S248F, $\alpha 4$ -S252L, $\alpha 4$ -776ins3, $\beta 2$ -V287L, and $\beta 2$ -V287M) were found to increase by 10-20% the proportion of the high-affinity subtype, when expressed in mouse neuroblastoma cells (Son et al., 2009). Another possible pathogenetic mechanism was suggested based on the nAChR response to

extracellular Ca^{2+} ($[\text{Ca}^{2+}]_o$). The nAChRs are normally potentiated by increasing $[\text{Ca}^{2+}]_o$ up to the physiological concentration (Mulle et al., 1992; Vernino et al., 1992). Higher $[\text{Ca}^{2+}]_o$ produces channel inhibition (Buisson et al., 1996). The above five subunits, expressed in *Xenopus* oocytes, were observed to decrease the allosteric potentiation caused by $[\text{Ca}^{2+}]_o$ (Rodrigues-Pinguet et al., 2003). This could affect presynaptic $\alpha 4\beta 2$ receptors, leading to enhance glutamate release during synchronous discharges of pyramidal neurons (Rodrigues-Pinguet et al., 2003). Regardless of the specific properties of individual mutations, it remains to be explained why mutant subunits widely expressed in the mammalian brain facilitate seizures in the frontal regions during light NREM sleep and why the attacks are accompanied by hyperkinetic motor events. Functional imaging studies show that complex alterations of heteromeric nAChR expression take place in human patients (Picard et al., 2006), which are difficult to explain on the basis of the functional alterations observed in cellular expression systems. Therefore, to better understand the effects of ADNFLE mutations in the complex cerebral context, it is necessary to proceed with broader studies of the cholinergic transmission in animal models of ADNFLE.

Murine models of ADNFLE

Since 2006, several murine models of ADNFLE have become available. Klaassen et al. (2006) used C57BL/6J mice to generate knock-in strains expressing either $\alpha 4$ -S252F or $\alpha 4$ -+L264, respectively homologous to the human $\alpha 4$ -S248F and $\alpha 4$ -(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 $\alpha 4$ -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).

The other mutant subunit that has been widely studied in mice is $\beta 2$ -V287L. A knock-in strain expressing $\beta 2$ -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; O'Neill et al., 2013). Moreover, conditional strains were generated expressing $\beta 2$ -V287L in FVB background, under control of the tetracycline promoter (TET-off system; Manfredi et al., 2009). Expression of $\beta 2$ -V287L does not alter the surface expression level of heteromeric nAChRs, but causes spontaneous seizures, generally during periods of increased *delta* wave activity. The epileptic phenotype is not reversed when $\beta 2$ -V287L is silenced by administering doxycycline in adult mice. Conversely, when the transgene is silenced between the embryonic day 1 and the postnatal day 15, no seizures are observed, even if the transgene is expressed at

a later stage. Finally, in transgenic rats expressing $\alpha 4$ -S284L, epileptic seizures are observed during SWS. These are accompanied by attenuation of synaptic and extrasynaptic GABAergic transmission before the onset of the epileptic phenotype and abnormal glutamate release at the onset of seizures (Zhu et al., 2008). In summary, these murine models of ADNFLE do not display gross morphological alteration in their brains, but tend to display abnormal excitability, generally accompanied by disturbed sleep. The presence of spontaneous seizures is strain-dependent. The physiological analysis is incomplete, but points to altered GABAergic transmission in PFC. Furthermore, the only conditional model presently available suggests that critical stages of synaptic stabilization are implicated in the pathogenesis of ADNFLE.

Nicotinic transmission in the cerebral cortex nAChRs in the PFC

As was mentioned earlier, the heteropentamer $\alpha 4\beta 2^*$ and the homopentamer $\alpha 7_5$ are the main nAChR subtypes in thalamus and isocortex (Gotti et al., 2007). Although the proportion of the different stoichiometries of $\alpha 4\beta 2^*$ receptors *in vivo* are unknown, overall these receptors largely account for the highly sensitive and slowly desensitizing component of the response to ACh and nicotine. Therefore, the heteromeric nAChRs are thought to give a major contribution to the tonic control of neocortical excitability even in the presence of low ACh concentrations. In contrast, $(\alpha 7)_5$ receptors have an EC₅₀ for ACh of 100-200 μ M, much faster desensitization and

higher permeability to Ca^{2+} (Dani and Bertrand, 2007), and seem thus to be better suited to regulate the phasic responses to high ACh concentrations. This may explain why no ADNFLE mutation has ever been observed in *CHRNA7*, despite the widespread expression of $\alpha 7$ subunits in the brain. The best characterized of the other subunits is $\alpha 5$, which can associate with $\alpha 4\beta 2^*$ receptors (Kuryatov et al., 2008) and regulate its function in both mature and developing PFC (Ramirez-Latorre et al., 1996; Bailey et al., 2012). Unfortunately, nothing is known about the interaction of $\alpha 5$ with the known ADNFLE subunits. Very little is also known about the physiology of $\alpha 2$, which is directly implicated in ADNFLE (Aridon et al., 2006). The difficulty of interpreting the mutations on $\alpha 2$ resides in the fact that its pattern of expression is different in primates as compared to rodents. In particular, $\alpha 2$ is much more widely expressed in primates' brain (Han et al., 2000; Aridon et al., 2006), where there seems to be much more overlap with $\alpha 4$ than in rodents' brain. The other complication is that $\alpha 2$, as $\alpha 4$, can associate with both $\beta 2$ and $\beta 4$ to yield functional nAChRs. The cerebral expression of $\beta 4$ is also relatively widespread in mouse (Gahring et al., 2004), squirrel monkeys (Quik et al., 2000) and human *feti* as well as aged post-mortem samples (Hellström-Lindh et al., 1998). However, once again, scarce attention has been devoted to the physiology of $\beta 4$. Nevertheless, the above evidences suggest that heteromeric receptors different from $\alpha 4\beta 2$ could have important physiological roles in the mammalian brain, and particularly in primates.

nAChRs regulate both excitatory and inhibitory transmission

In general, $\beta 2^*$ nAChRs regulate glutamate release from thalamo-cortical fibers (Vidal and Changeux, 1993; Gioanni et al., 1999; Lambe et al., 2003). The expression and roles of nAChRs on pyramidal cell somata and terminals are more variable, depending on species, layer and cerebral region (Chu et al., 2000; Couey et al., 2007; Dickinson et al., 2008; Kassam et al., 2008; Zolles et al., 2009; Marchi and Grilli, 2010; Poorthuis et al., 2012; Aracri et al., 2013a). Nonetheless, the global effect of ACh release in deep layers is thought to be excitatory and dominated by $\beta 2^*$ receptors (Poorthuis et al., 2012). This is relevant in the present context, as layer V is particularly prone to develop seizures (Richardson et al., 2008). During NREM sleep, the cholinergic tone is low, but hyperfunctional nAChRs could maintain abnormal glutamate release, even in the face of low ACh levels, thus increasing sleep fragmentation. In fact, the rat strain expressing $\alpha 4$ -S284L shows higher nicotine-dependent glutamate release during SWS (Zhu et al., 2008). It seems however unlikely that hyperfunctional nAChRs can produce paroxysmic hyperexcitability by a moderate stimulation of glutamate release in the neocortex. Because the pyramidal neuron activity is potently regulated by the feed-back control exerted by GABAergic neurons, some degree of circuit disinhibition is generally required to lead to seizure-like activity (Richardson et al., 2008). This is confirmed by theoretical modeling showing that altering the “weight” of inhibitory connections is the most effective way of modulating the excitability of recurrent

networks (Tsodyks et al., 1997; Murphy and Miller, 2009; Ozeki et al., 2009). Therefore, to understand the nAChR-dependent hyperexcitability it is necessary to also consider the nicotinic regulation of neocortical GABAergic transmission. In fact, expression of heteromeric nAChRs on the soma of different interneuronal populations is established in rats (Xiang et al., 1998; Porter et al., 1999; Christophe et al., 2002), humans (Alkondon et al., 2000) and mice (Couey et al., 2007; Aracri et al., 2010). Heteromeric nAChRs also exert presynaptic control of GABA release onto pyramidal cells (Klaassen et al., 2006; Aracri et al., 2010).

The origin of seizures in ADNFLE

In the light of the above discussion, ADNFLE seizures could be triggered by several mechanisms. (1) During SWS, thalamocortical neurons tend to be inhibited (Steriade and McCarley, 2005). In these conditions, an upsurge of ACh in the presence of hyper- functional nAChRs could cause excessive GABA release in the PFC and thus abnormal hyperpolarization of pyramidal neurons. This would deinactivate low-threshold, voltage-gated Ca^{2+} channels and activate pacemaker H-type currents, thus making pyramidal cells more sensitive to post-inhibitory rebound (Klaassen et al., 2006). (2) Alternatively, nAChR activation could stimulate reciprocal inhibition between GABAergic interneurons, thus producing pyramidal cell disinhibition (**Figure 1**). This latter mechanism has been excluded in Klaassen's work (2006), but may explain Zhu's results (2008). (3) Another possible mechanism considers thalamocortical interplay. In stage 2 of sleep, spindle waves are generated in the thalamus by the

regulatory action of RT cells onto thalamocortical cells (Lüthi, 2014), but cortical neurons are essential in synchronizing their appearance in wide thalamocortical regions. Sleep spindles can turn into epileptiform activity and could be promoted by nAChR-dependent stimulation of glutamate release onto RT cells and the release of GABA from RT cells onto thalamocortical cells (Sutor and Zolles, 2001). A more detailed interpretation is made difficult because of the current uncertainties about the GABAergic cell populations and their physiological roles in the neocortex. The classification of GABAergic interneurons must take into account a number of factors, such as morphology, axonal and dendritic connectivity, efficacy and dynamics of input and output synapses, intrinsic electrophysiological properties, combinations of molecular markers, such as Ca^{2+} -binding proteins and neuropeptides, and developmental origin (Markram et al., 2004; DeFelipe et al., 2013; Cauli et al., 2014). The role of different GABAergic populations in the cortical microcircuit is unclear, and differences occur between layers, brain areas and species. A simplified classification (**Figure 1**), which covers more than 85% of cortical interneurons, is based on expression of parvalbumin (PV), somatostatin (SOM) and vasoactive intestinal peptide (VIP). PV neurons are generally fast-spiking (FS) cells (Hu et al., 2014), which are thought to be the main responsible of surround inhibition, particularly in layer V. Models of interactions between the GABAergic populations were proposed based on studies on hippocampus and sensory, but not associative, cortices (Pfeffer et al., 2013). While PV-cells mainly inhibit the perisomatic compartments of pyramidal cells, SOM-cells form

synapses onto dendrites of pyramidal neurons and inhibit PV interneurons (but not *vice versa*). SOM-cells would thus not only increase inhibition in the dendrites but also decrease the perisomatic inhibition mediated by PV-cells (Pfeffer et al., 2013). In contrast, VIP-cells produce scarce inhibition of pyramidal cells, but seem to specifically target SOM-cells. In this way, VIP-cells may indirectly stimulate PV-cells. This simplified picture is complicated by the presence of chandelier cells, a subtype of PV-cells that mainly targets the axon hillock of pyramidal cells. Their function seems critical in PFC (Hardwick et al., 2005) and shows species-specific characteristics possibly related to neurologic disorders (Woodruff and Yuste, 2008). The study of how nAChRs regulate GABAergic populations is in its infancy (**Figure 1**). Nonetheless, the non-FS cells (mainly SOM, probably) that form GABAergic synapses onto FS-PV cells (Harris and Mrsic-Flogel, 2013; Pfeffer et al., 2013) express $\alpha 7$, $\alpha 4$, and $\beta 2$ nAChR subunits (Freund, 2003; Couey et al., 2007; Armstrong and Soltesz, 2012). Therefore, mechanism (2) above could be facilitated in SWS, when glutamatergic transmission weakens. In these conditions, inhibition between interneurons may prevail because of nicotinic excitation of the cell bodies of the non-FS cells that innervate the FS-PV-cells. Moreover, FS-cells also present reciprocal inhibitory connections (Gibson et al., 1999; Galarreta and Hestrin, 2002). Because FS-cell terminals express $\alpha 4\beta 2$ nAChRs, it is possible that when the overall glutamatergic input decreases, the action of hyperfunctional nAChRs on presynaptic terminals shifts the synaptic balance toward interneuronal inhibition.

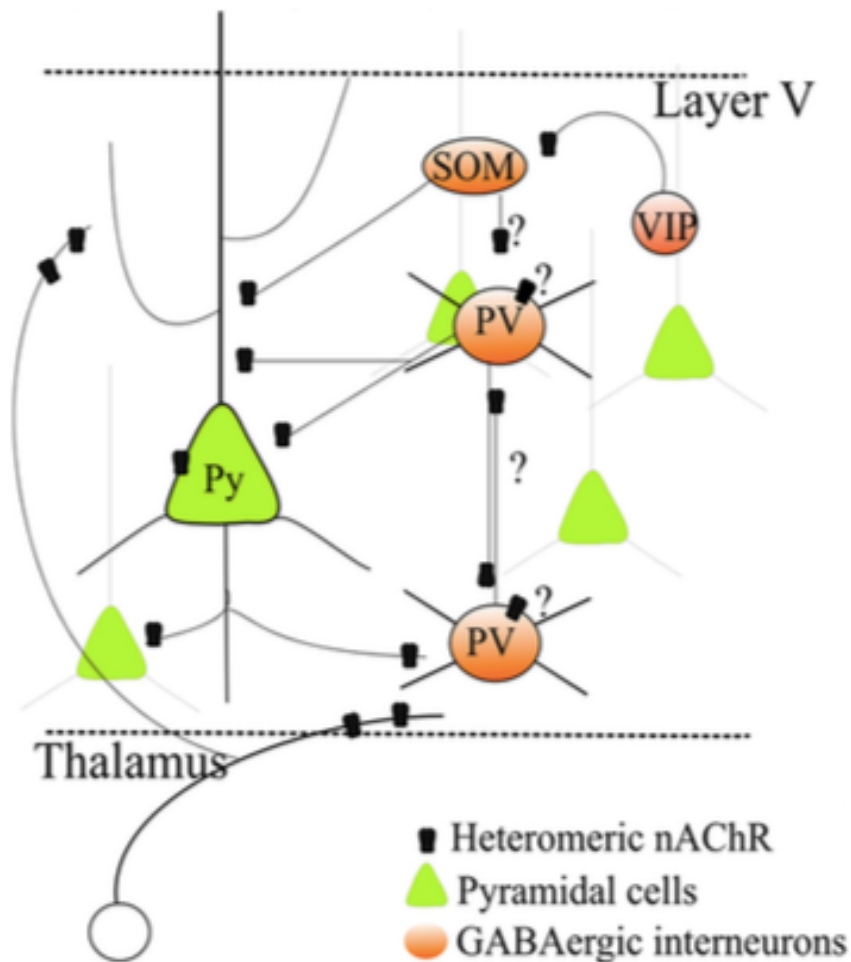


FIGURE 1 | Regulation by heteromeric nAChRs of a simplified layer V neocortical microcircuit. Heteromeric $\beta 2^*$ nAChRs regulate excitatory transmission by stimulating glutamate release from thalamocortical as well as intrinsic glutamatergic terminals. Expression of nAChRs on pyramidal cell somata has also been observed, although it is more variable. Heteromeric nAChRs also control GABA release onto pyramidal neurons. Moreover, growing evidence indicates that nAChRs are also expressed in several distinct types of GABAergic cells. However, because their precise physiological roles in the different cell types is unclear, a comprehensive picture cannot be given yet. This uncertainty is indicated by the question marks in the graph. For full discussion and references, see the main text.

Why do seizures arise in the frontal regions?

Besides the local mechanism by which ADNFLE mutations can alter the excitability of neocortical circuits, one must also explain why seizures arise in frontal regions. Although the literature on cholinergic transmission is immense, the analysis of the differences in cholinergic and, in particular, nicotinic regulation of different areas of the isocortex is rather neglected. Interestingly, recent results suggest that important differences exist in cholinergic innervation and response to nicotine between distinct regions of the cerebral cortex. In mouse, for instance, the response of layer VI pyramidal neurons to nicotine in the medial PFC is more sensitive than the one observed in primary somatosensory and motor cortices (Tian et al., 2014). This appears to be consistent with the distribution of cholinergic fibers in infragranular layers, which is denser in PFC, as compared to primary sensory and motor regions (Aracri et al., 2010). Differences in cytoarchitectonics and physiology are also observed between different prefrontal regions (for a brief discussion, see Aracri et al., 2013a). Moreover, a typical feature of ADNFLE is the presence of stereotyped motor events accompanying the seizures, suggesting that motor patterns are released during the attacks. Therefore, we hypothesize that the implication of premotor areas such as Fr2 (also known as M2; discussed in Aracri et al., 2013a) is particularly relevant for ADNFLE. This area projects to the motor cortex and dorsolateral striatum (Berendse et al., 1992; Condé et al., 1995). Moreover, it is very sensitive to nicotinic stimulation in layer V (Aracri et al., 2010, 2013a), and its well-developed layer V is seemingly accompanied by

lesser inhibitory weight than is typical in regions such as the somatosensory cortex (Aracri et al., 2013a).

Developmental aspects

The subtle prefrontal circuit alterations that cause ADNFLE seizures are likely to be produced during the developmental phases of network stabilization, as is also indicated by conditional expression of $\beta 2$ -V287L (Manfredi et al., 2009). In mammals, a “brain growth spurt” occurs around birth, characterized by neurite outgrowth, synaptogenesis, myelination 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 nAChR expression. In rat forebrain, $\beta 2$ appears in mid-gestation and peaks in the second postnatal week, along with $\alpha 4$ and $\alpha 7$ (Mansvelder and Role, 2006). A similar pattern is observed in mouse (Kassam et al., 2008; Bailey et al., 2012), although evidence is less extensive. At this stage, the density of extrinsic cholinergic innervation (Mechawar et al., 2002) and cortical cholinergic cells (Consonni et al., 2009) increases dramatically. Treatment with nicotinic ligands in the second postnatal week produces persistent behavioral and morphological alterations (Eriksson et al., 2000) and mice lacking $\beta 2$ show region-specific changes in cortical structure (Cordero-Erausquin et al., 2000). Nonetheless, the specific nAChR roles during neural circuit wiring are still largely unknown. The spontaneous nAChR activity was reported to regulate the developmental switch between the excitatory and inhibitory roles of GABA (Liu et al., 2006). The latter transition depends on the

progressive substitution of the transporter NKCC1, which absorbs Cl⁻ and is mainly expressed in early stages, with KCC2, which extrudes Cl⁻ and is expressed at later stages (Rivera et al., 1999; Ben-Ari et al., 2012). Both homo- and heteromeric nAChR activity seem to regulate expression of these transporters (Liu et al., 2006). In particular, KCC2 appears after postnatal day 3 in layer V pyramidal neurons. Its expression accompanies the formation of GABAergic synapses and KCC2 variants have been associated to epilepsy (Kaila et al., 2014; Puskarjov et al., 2014). Precise timing of early GABAergic excitation is important for early neuronal development and integration into circuits (Ge et al., 2006; Ben-Ari et al., 2012). Recent work in transgenic rats expressing $\alpha 4$ -S284L (Zhu et al., 2008) suggest that this mechanism may also be active at a later stage. In these mice, the onset of the epileptic seizures (around 8 weeks after birth) is accompanied by a decrease of the expression ratio of KCC2 and NKCC1, with a consequent depolarizing shift of the GABA_A reversal potential, which could explain the observed alterations in GABAergic transmission (Yamada et al., 2013).

Pharmacological treatment of ADNFLE

A first-line choice for treating ADNFLE is carbamazepine (CBZ). As many other antiepileptics (AEDs), CBZ has inhibitory effects on voltage-gated Na⁺ channels (McLean and Macdonald, 1986). Nonetheless, CBZ has been proposed to be particularly effective on ADNFLE because it also blocks heteromeric nAChRs at therapeutic doses, and ADNFLE mutations alter the channel sensitivity to the drug (Picard et al., 1999; Hoda et al., 2009). However, CBZ can cause

serious toxic side-effects produced by its metabolites. A related less toxic second generation compound is oxcarbazepine (Beydoun et al., 2008), which has recently found to give good results in ADNFLE patients, including some refractory to other drugs (Raju et al., 2007; Romigi et al., 2008). The steady-state plasma concentration of oxcarbazepine is negligible and the clinically relevant compound is thought to be the monohydroxy derivative MHD, which can reach effective plasma levels between 30 and 150 μ M (Johannessen et al., 2003). In agreement with the notion that good efficacy in treating ADNFLE may depend on modulation of nAChRs, oxcarbazepine and MHD can also inhibit heteromeric nAChRs, especially $\alpha 4\beta 2$ (Di Resta et al., 2010). Lamotrigine, another AED commonly used in partial epilepsy (Labiner et al., 2009), has also been found to block $\alpha 4\beta 2$ nAChRs (Zheng et al., 2010). Unfortunately, the effects of common AEDs on ligand-gated ion channels have begun to be studied only recently (Di Resta and Becchetti, 2013). Deeper analyses on the action of AEDs on different ion channels and the search of more specific compounds are clearly needed to advance the pharmacology of ADNFLE and epilepsy in general. Considering that many ADNFLE mutations produce an over- all increase of receptor's function and that several AEDs block nAChRs, it may seem paradoxical that treatment with nicotine has also been suggested to be beneficial in ADNFLE. After the first report in a patient refractory to standard antiepileptic therapy (Willoughby et al., 2003), the effect of tobacco habit was studied in a wider cohort of patients carrying either $\alpha 4$ -776ins3 or $\alpha 4$ -S248F (Brodtkorb and Picard, 2006). Seizure freedom was associated with smoking habits and transdermal nicotine application had

beneficial effects in one patient (Brodtkorb and Picard, 2006). The possible explanations of these observations are as follows. First, tonic nicotine administration tends to produce partial nAChR desensitization, which may counteract the effects of receptors' hyperfunctionality. Second, nicotine can act as a molecular chaperon that regulates the nAChR subunit expression (Kuryatov et al., 2005). In several ADNFLE mutations studied *in vitro*, tonic nicotine application was observed to decrease the overexpression of the high-affinity $(\alpha 4)_2(\beta 2)_3$ subtype (Son et al., 2009). Therefore, in ADNFLE patients, nicotine may normalize an altered subunit stoichiometry.

Investigating the pathogenesis and pharmacology of epilepsy is complicated by the scarcity of good *in vitro* models, as it is difficult to obtain spontaneous epileptiform activity in cultured neuronal networks or brain slices. In the case of ADNFLE, long-term neuronal cultures dissociated from mice expressing $\beta 2$ -V287L display spontaneous hyperexcitability features (i.e., not requiring the application of pro-convulsants), as measured with multi-electrode arrays (Gullo et al., 2014). The network excitability can be modulated by both GABAergic drugs and CBZ. Besides facilitating the study of the role of $\beta 2$ -V287L on synaptic formation, this experimental model allows to determine the effects of tonic pharmacological treatment on network excitability. In general, *in vitro* models such as this one should considerably facilitate the screening of antiepileptic drugs (AEDs).

Other genes implicated in ADNFLE

KCNT1

Four missense mutations in the *KCNT1* gene (**Table 1**), coding for a Na⁺-gated K⁺ channel (KCNT1 or K_{Ca}4.1, also known as Slo2.2 or Slack), were associated to severe ADNFLE with psychiatric symptoms, and a penetrance of 100% (Heron et al., 2012). Consistently, KCNT1 is expressed in the frontal region (Bhattacharjee et al., 2002). The ADNFLE mutations tend to cluster around the cytoplasmic NAD⁺ binding domain (Heron et al., 2012), which regulates the channel sensitivity to [Na⁺]_i (Tamsett et al., 2009). When expressed in *Xenopus laevis* oocytes, these mutations produce higher currents than the WT counterparts. In analogy with what was discussed for the mutant nAChRs, the balance of the possible physiological effects on excitatory and inhibitory transmission is uncertain. It is possible that hyperfunctional KCNT1 channels accelerate the action potential repolarization, thus increases the firing frequency of pyramidal neurons (Milligan et al., 2014). Because KCNT1 is also expressed in interneurons (Bhattacharjee et al., 2002) it has been also suggested that the slow accumulation of Na⁺ in FS interneurons could stimulate KCNT1 to dampen excitability. The effect would be stronger in the case of mutant channels, with ensuing network disinhibition (Milligan et al., 2014).

DEPD5

Loss-of-function mutations in *DEPDC5* are linked to different types of focal epilepsies, including ADNFLE (Dibbens et al., 2013; Ishida et al., 2013; Lal et al., 2014; Picard et al., 2014; Scheffer et al., 2014). Differently from the mutations on ion channel genes, the epileptogenic mutations in *DEPDC5* are unrelated to the specific area of seizure initiation. Therefore, *DEPDC5* appears to be implicated in overall excitability, rather than being specifically associated with ADNFLE. The DEPDC protein participates in a molecular complex implicated in repressing the activity of mTORC1 (Target Of Rapamycin Complex 1; Bar-Peled et al., 2013). However, how the mTORC1 pathway may be linked to epileptogenesis is unknown (Baulac, 2014).

The possible involvement of neuropeptides

Putative disease-causing mutations have been identified in the promoter (Combi et al., 2005) or in the pro-sequence region of *CRH* (Combi et al., 2005; Sansoni et al., 2013). *In vitro*, the P30R substitution in the pro-sequence of CRH decreases peptide secretion (Sansoni et al., 2013). How suppressed CRH signaling may lead to ADNFLE seizures is unclear. Among neuropeptides, CRH is known to exert the most potent pro-epileptogenic effects during development (Baram and Hatalski, 1998). The hypothalamic neurons secreting CRH also innervate the neocortex, and CRH receptors are densely distributed in rodents' prefrontal regions (Radulovic et al., 1998; Van Pett et al., 2000), thus cooperating with the ascending arousal systems,

including the cholinergic, particularly during the stress response. The other hypothalamic neuropeptide implicated in neocortex arousal and sleep-wake cycle is orexin (also known as hypocretin). Orexin peptides directly regulate synaptic transmission in the frontal cortex (Li et al., 2010; Aracri et al., 2013b), and cooperate with ACh in modulating glutamate release from thalamocortical fibers (Lambe et al., 2005). Until now no ADNFLE-linked mutations have been observed on orexin-related genes (Bouchardy et al., 2011). Nonetheless, we believe that the interplay between ACh, orexin and CRH in regulation neurotransmission in the cerebral cortex may be another fruitful line of research with implications for sleep-related epilepsy in general and ADNFLE in particular.

Conclusions

Several genes have been associated with ADNFLE, but the available functional studies mostly concern heteromeric nAChRs. Murine models suggest that alteration in the nicotinic control of GABAergic transmission may be a major pathogenetic mechanism. In general, the prefrontal regions may be particularly sensitive to nicotinic stimulation in the deep layers. However, a full comprehension of epileptogenesis in ADNFLE will require a better understanding of how the different nAChR subtypes regulate both excitatory and inhibitory neurons, at pre- and postsynaptic level, in the frontal regions. In particular, how nAChRs control different interneuronal populations is unclear. Moreover, assessing how the altered excitability is generated during the maturation of neocortical connections will need deeper studies of the nAChR function in the

early postnatal weeks. From a pharmacological standpoint, mounting evidence indicates that several classic AEDs can target neurotransmitter-gated ion channels, and particularly nAChRs. Modulating nAChRs is a possible therapeutic strategy in ADNFLE, which merits further investigation.

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Regulation of GABA release onto fast-spiking cells by $\alpha 4\beta 2^*$ nicotinic receptors in layer V of prefrontal (Fr2) cortex

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Abstract

Heteromeric nicotinic acetylcholine receptors (nAChRs) regulate the prefrontal cognitive functions and are implicated in sleep-related epilepsy. In mouse prefrontal cortex (PFC), we studied the tonic modulation produced by $\alpha 4\beta 2^*$ nAChRs on inhibition of fast spiking (FS) interneurons in layer V of the frontal area 2 (Fr2). When the ionotropic glutamate receptors were blocked, 10 μ M nicotine stimulated the spontaneous inhibitory postsynaptic currents (IPSCs) recorded on FS cells. A similar effect was produced by 10 nM 5-iodo-3-[2(S)-azetidinylmethoxy]pyridine (5-IA), a selective agonist of $\beta 2^*$ nAChRs. Stimulation was antagonized by 1 μ M dihydro- β -erythroidine (DH β E), which blocks heteromeric $\alpha 4^*$ nAChRs, but not 10 nM methyllicaconitine (MLA), which blocks $\alpha 7^*$ nAChRs. Similar results were obtained on miniature IPSCs (mIPSCs), in the presence

of tetrodotoxin (TTX). Moreover, approximately 40% of FS cells displayed whole-cell nicotinic currents that were blocked by DH β E. By confocal microscopy, the α 4 nAChR subunit was immunocytochemically identified on interneurons expressing either parvalbumin (PV), which mainly labels FS cells, or somatostatin (SOM), which labels the other major interneuron population in layer V. GABAergic terminals expressing α 4 and PV were observed to be juxtaposed to PV-positive (PV+) cells. A fraction of these terminals displayed PV immunoreactivity. These results suggest that α 4 β 2* nAChRs can produce sustained regulation of FS cells inhibition in layer V, and that the effect presents a presynaptic component mainly dependent on PV+ cells. This mechanism may provide a powerful way to regulate PFC excitability during cognitive tasks as well as contributing to cause hyperexcitability in epileptic patients carrying hyperfunctional mutant heteromeric nAChRs.

Significance Statements

By modulating neocortex excitability, heteromeric nAChRs regulate arousal and cognition. When mutated, they can cause sleep-related frontal lobe epilepsy. We found that nAChRs modulate inhibition of fast-spiking interneurons in the Fr2 region of the murine prefrontal cortex. Fr2 elaborates the network activity preceding the motivation-dependent goal-oriented tasks, and may be implicated in facilitating the uncontrolled motor pattern observed in frontal epilepsy. Experimental and theoretical work indicate that some degree of disinhibition is necessary to generate hyperexcitability in neuronal networks. Moreover, inhibitory network deficit is thought to be

implicated in both epilepsy and psychiatric disorders such as schizophrenia. Overall, our results reveal a powerful mechanism by which ACh release can unleash prefrontal excitability in physiological as well as pathological conditions.

Introduction

In the neocortex, ACh regulates arousal and executive functions (Sarter et al., 2006; Jones, 2008), and the implication of nAChRs is increasingly recognized (Wallace and Bertrand, 2013). The main nAChR subtypes are the low-affinity and quickly desensitizing homopentamer $(\alpha 7)_5$ and the high-affinity and slowly desensitizing heteropentamer $\alpha 4\beta 2^*$. Both regulate cognitive processes, although their specific roles and the contribution of other subunits are debated (Dani and Bertrand, 2007; Parikh et al., 2010; Guillem et al., 2011; Picciotto et al., 2012; Bloem et al., 2014; Zoli et al., 2015). In general, the kinetic and pharmacological features of heteromeric nAChRs make them particularly fit to regulate overall excitability (Fonck et al., 2005), particularly on a relative slow timescale, as is likely to occur during non synaptic, diffuse transmission (e.g., Descarries, 1997; Lendvai and Vizi, 2008). This concept agrees with the observation that mutant $\alpha 4$ (Steinlein et al., 1995) and $\beta 2$ (De Fusco et al., 2000) subunits are frequently linked to autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE). In rodents, heteromeric nAChRs regulate both excitatory and inhibitory neurons in medial (Lambe et al., 2003; Couey et al., 2007; Kassam et al., 2008; Zolles et al., 2009) and dorsomedial (Aracri et al., 2010, 2013) PFC. Functional heteromeric nAChRs are also observed in GABAergic interneurons dissociated

from surgical samples from human neocortex (Alkondon et al., 2000). However, how nAChRs expressed in different compartments interplay in prefrontal circuits is poorly understood. In particular, little is known about the nicotinic regulation of interneuron classes, despite the well-known sensitivity of neocortical excitability to the GABAergic tone (Avoli et al., 2005; Isaacson and Scanziani, 2011; Paz and Huguenard, 2015). Another source of interpretive uncertainty is the increasing recognition that different PFC regions present heterogeneous structure and connectivity (Franklin and Chudasama, 2012), which suggests that nAChRs may exert different roles in different areas. We studied the dorsomedial shoulder region of murine PFC, or Fr2, which is related to the human dorsolateral PFC (Uylings et al. 2003). This region projects to the motor cortex and dorsolateral striatum and receives afferents from sensory and parietal cortices as well as ventral tegmental dopamine neurons (Berendse et al., 1992; Condé et al., 1995). Hence, Fr2 appears to be a pivotal target of cholinergic transmission, as in rodents it mediates the decision-execution steps that precede the motivation-dependent goal-oriented tasks, particularly those requiring immediate attention (Kargo et al. 2007). Moreover, because Fr2 is connected with both motor cortex and striatum, the nicotinic regulation of excitability in this region may be particularly relevant in the development of ADNFLE seizures. These are in fact typically accompanied by hypermotor seizures and the release of stereotyped motor patterns (Picard and Brodtkorb, 2008). We here focus on the regulation of fast-spiking (FS) cells by heteromeric nAChRs in Fr2 layer V. This latter constitutes the main output channel to subcortical structures and is crucial for spread of

synchronized activity (Richardson et al., 2008). Pyramidal neurons in layer V are tightly controlled by a dense network of GABAergic cells (Shu et al., 2003; Ozeki et al., 2009). In particular, FS cells are the main responsible of feed-forward inhibition in the neocortex (Cammarota et al., 2013). They are major regulators of neocortical output during cognitive operations and drive network oscillations at different frequencies (Cardin et al., 2009; Sohal et al., 2009). In agreement with these notions, several lines of evidence suggest that abnormal prefrontal disinhibition may cause the cognitive deficits observed in schizophrenia (Marin, 2012; Murray et al., 2014). Inhibitory interneurons are known to be connected by reciprocal as well as autaptic GABAergic synapses, and such evidence is particularly ample in sensory cortices (Tamas et al., 1997, 1998; Galarreta and Hestrin, 2002; Bacci et al., 2003; Jiang et al. 2013). In fact, disinhibition of local interneurons is implicated in regulating visual signal elaboration (Pfeffer et al., 2013), auditory discrimination (Pi et al., 2013), learning in auditory cortex (Letzkus et al., 2011), and may be a widespread mechanism contributing to learning (Letzkus et al., 2015). In contrast, scarce information is available about how neurotransmitters shape reciprocal inhibition between interneurons in prefrontal regions, and generally in associative areas. We here show that $\alpha 4\beta 2^*$ nAChRs regulate GABA release onto FS cells in layer V, which reveals a potentially potent mechanism to stimulate physiological excitability as well as cause pathological hyperexcitability in prefrontal areas.

Materials and Methods

Brain slices. We used FVB mice of both sexes (Harlan, Italy), as previously described (Aracri et al., 2013). Our procedures followed the Principles of Laboratory Animal Care (86/609/EEC Directive of 1986), and were approved by the Italian Ministry of Health. In brief, Fr2 coronal sections were cut between + 2.68 mm and +2.10 mm from bregma (**Fig. 1A**). For patch-clamp experiments, brains from P17-P35 mice were placed in ice-cold solution containing (mM): 87 NaCl, 21 NaHCO₃, 1.25 NaH₂PO₄, 7 MgCl₂, 0.5 CaCl₂, 2.5 KCl, 25 D-glucose, 75 sucrose, 400 μ M ascorbic acid, and aerated with 95% O₂ and 5% CO₂ (pH 7.4). Slices (300 μ m thick) were cut with a VT1000S vibratome (Leica Microsystems), incubated at 32-33°C for 30 min in the above solution, and maintained at room temperature before being transferred to the recording chamber. For immunocytochemistry, five mice (2 to 3 months old) were anesthetized with intraperitoneal 4% chloral hydrate (2 mg/100 g) and intracardially perfused with 4% PFA, in 0.1 M phosphate buffer (pH 7.2-7.4). Brains were removed and incubated in the same solution, at 4°C for 16 h. Coronal vibratome sections (50 μ m thick) were maintained in phosphate buffer.

Whole-cell recordings. Cells were examined with an Eclipse E600FN microscope, equipped with a water immersion differential interference contrast objective (Nikon Instruments), and an infrared digital CCD (C8484-05G01) equipped with HCLImage Live acquisition software (Hamamatsu). Neurons were voltage- or current-clamped with a

Multiclamp 700A (Molecular Devices), at 33-34°C. Micropipettes (2-3 M Ω) were pulled from borosilicate capillaries (Corning) with a P-97 Flaming/Brown Micropipette Puller (Sutter Instruments). The cell capacitance and series resistance (up to 75%) were always compensated. Series resistance was generally below 10 M Ω . Input resistance was usually close to 100 M Ω . IPSCs were low-pass filtered at 2 kHz and digitized at 5 kHz, with pClamp9/Digidata 1322A (Molecular Devices). Slices were perfused at 1.8 ml/min with ACSF, containing (mM): 135 NaCl, 21 NaHCO₃, 0.6 CaCl₂, 3 KCl, 1.25 NaH₂PO₄, 1.8 MgSO₄, 10 D-glucose, aerated with 95% O₂ and 5% CO₂ (pH 7.4). Pipette contained (mM): 70 K-gluconate, 70 KCl, 2 MgCl₂, 0.5 BAPTA, 1 MgATP, 10 HEPES (pH 7.2). Drugs were applied in the bath and their effects calculated at the steady-state (usually reached within 2-3 min). The resting membrane potential (V_{rest}) was measured in open circuit mode, soon after obtaining the whole-cell configuration. No correction was applied for liquid junction potentials.

Drugs. Stock solutions of (-)-nicotine hydrogen tartrate salt, DH β E hydrobromide, MLA citrate hydrate, (-)-bicuculline methiodide, AP5 [D(-)-2-amino-5-phosphono-pentanoic acid] and TTX were prepared in distilled water. Stock solutions of CNQX [6-cyano-7-nitroquinoxaline-2,3-dione] were prepared in dimethylsulfoxide (20 mM). 5-IA85380 (5-IA) was dissolved in our standard extracellular solution, and used at the indicated final concentration. Chemicals and drugs were purchased from Sigma-Aldrich Italy, except AP5, CNQX, TTX and 5-IA (Tocris Bioscience).

Analysis of patch-clamp data. We used Clampfit 9.2 (Molecular Devices), and OriginPro 9 (OriginLab). IPSCs included both smoothly shaped isolated signals with a peak amplitude larger than 40 pA and composite signals. IPSCs were inspected one by one to reject spurious events. The peak-to-peak baseline noise was approximately 5 pA. The detection threshold was set at 7-8 pA. The statistical comparison of cumulative distributions of IPSC amplitudes and interevent intervals was conducted with the Kolmogorov-Smirnov (KS) test, on at least 2 min continuous recording (never containing less than about 250 events). The final events frequency after washout was generally higher than 70% of the initial value. Cells in which this value was lesser than 50% were discarded. The results from populations of experiments are given as mean values $\pm$ SEM. The number of experiments (n) refers to the number of tested neurons (in different brain slices). Unless otherwise indicated, statistical comparison between two groups were carried out with two-tailed paired Student's t-test, at the indicated level of significance (p), by preliminarily assessing normality by a KS test. Non-parametric tests gave analogous results.

Confocal microscopy. Sections were incubated in NH_4Cl 0.05 M for 30 min to block the aldehyde groups residual from fixation. After washing with phosphate buffered saline (PBS), they were pre-treated with 1% bovine serum albumin (BSA) / 0.2% Triton X-100 in PBS for 30 min, and incubated for 2 nights at 4°C, with different primary antibodies diluted in 0.1% BSA. Finally, after washing with PBS, sections were incubated with the appropriate secondary antibodies

diluted in BSA 0.1%, for 2h. For subsequent detection of the biotinylated secondary antibody, a further incubation with Alexa 488-labeled streptavidin (Molecular Probes Inc.) was carried out. Images were analyzed with a TCS SP2 AOBS laser scanning confocal microscope (Leica Microsystems). The specificity of immunoreaction was tested by carrying out negative controls omitting primary antibodies. In this case, no specific signal was ever observed.

Primary antibodies. Anti- $\alpha 4$ nAChR subunit: polyclonal, made in guinea pig, diluted at 1/1000 (Millipore). Anti-vesicular GABA transporter (VGAT): polyclonal, made in rabbit, diluted at 1/1500 (Synaptic Systems). Anti-parvalbumin (PV): polyclonal, made in rabbit, 1/2000 (SWant). Anti-PV: monoclonal, made in mouse, 1/1000 (Sigma Aldrich). Anti-somatostatin (SOM): monoclonal, made in rat, 1/300 (Millipore).

Secondary antibodies. For a $\alpha 4$ nAChR subunit: biotinylated donkey anti-guinea pig IgG (bDAGp; Vector, Inc.), diluted at 1/200, and Alexa-488-labeled streptavidin (1/200; Molecular Probes). For VGAT and PV (polyclonal): indocarbocyanine Cy5-conjugated donkey anti-rabbit antibody (DAR-Cy5, 1/200; Jackson ImmunoResearch Laboratories). For PV (monoclonal): indocarbocyanine Cy3-conjugated donkey anti-mouse IgG (DAM-Cy3, 1/200; Jackson ImmunoResearch Laboratories). For SOM: Alexa-568 labelled donkey anti-rat IgG (DARat-Alexa 568, 1/200; Molecular Probes).

Colocalization analysis. Confocal micrographs were collected and digitized as TIF files with the Leica confocal scanning microscope (Leica Microsystems) and software package (Leica Confocal Software). Digital micrographs were optimized for resolution (final resolution 300 dpi), brightness, and contrast using Adobe Photoshop CS2 9.0 (Adobe Systems, San Jose, CA). Images were not altered in any way, e.g., by removing or adding image details. Nonoverlapping pictures (40X objective) were acquired in layer V to sample the double/triple immunolabeling in at least four cortical fields from different sections containing Fr2, obtained from each animal. For two-color analysis, tissues were excited at 488, 568 and 650 nm and fluorescence of Alexa 488 (green) and Cy5 (blue) or Cy3(red) were detected sequentially to avoid crosstalk. Colocalization of $\alpha 4$ nAChR subunit with either VGAT, PV or SOM was also determined by Leica Confocal software, by generating the 2D cytofluorograms (Bolte and Cordelières, 2006). These allow to select in a region of interest (ROI) certain intensity value pairs and to identify them as white signal in the original images, representing merged antigen localization (**Fig. 6A, B, D**). To estimate the overlapping area between red signal (PV or SOM, with the latter changed to blue only in **Fig. 6D, D'**, for reader's convenience) and green signal ($\alpha 4$ nAChR), we analysed single-plane raw images and Manders' coefficients were calculated applying the JACoP plug-in (Bolte and Cordelières, 2006) for ImageJ software. Statistical significance was determined with paired Student's t-test, at the indicated level of significance (p).

Results

In rodents' layer V, FS cells constitute the majority of PV+ neurons (Kawaguchi and Kondo, 2002; Rudy et al., 2011), and are well recognized by action potential (AP) firing pattern and spike width (Connors and Gutnick, 1990; Bean, 2007). After localizing the putative FS cell by shape and laminar location, we applied a series of 0.5 s depolarizing current steps. Below threshold, cells exhibited a quasi-linear response, whereas above threshold they showed the typical high-frequency firing with little adaptation, and APs with short spike width followed by deep after-hyperpolarization (Connors and Gutnick 1990; Bacci et al., 2003b; Okaty et al., 2009; Tai et al., 2014). In particular, the cells studied in the present work had AP frequencies of 80-100 Hz (with 200 pA stimulus), first spike width at half-maximal amplitude of 0.73 ± 0.04 s ($n = 46$), and after-hyperpolarization of -14.4 ± 0.75 mV ($n = 46$). **Fig. 1B** shows typical recordings and the relation between injected current and firing frequency in a representative sample of our cells ($V_{\text{rest}} = -66.4 \pm 0.4$ mV; $n = 10$). The other major population of interneurons we routinely observed was constituted by regular spiking non-pyramidal cells (Kawaguchi, 1993; Couey et al., 2007; McGarry et al., 2010), showing AP frequencies of 30-40 Hz (with 200 pA stimulus), more pronounced adaptation compared to FS cells, spike widths larger than 1 ms and shallow AHPs. These cells are not considered in the present paper.

Nicotine stimulates GABA release onto FS cells

Because the Nernst potential for Cl^- was -16 mV, we could record spontaneous IPSCs at -70 mV, after blocking ionotropic glutamate receptors with 40 μM AP5 and 10 μM CNQX (**Fig. 1C**). Consistently, the IPSCs isolated in this way were fully blocked by 10 μM bicuculline (data not shown). These experimental conditions allowed us to both conduct our experiments around V_{rest} and exclude the powerful glutamatergic drive onto FS cells. To study the nAChR-dependent modulation of synaptic events, nicotine was preferred to ACh, to avoid applying muscarinic receptors' inhibitors, which can affect nAChRs at relatively low concentrations (Zwart and Vijverberg, 1997). Nicotine was always applied in the bath at a concentration of 10 μM . This approximatively corresponds to the peak of the steady state ('window') current for both $\alpha 4\beta 2$ and $\alpha 7$ receptors (e.g. Fenster et al., 1997; Brusco et al., 2015), thus offering the best experimental condition to test the tonic contribution of both nAChR subtypes in conditions that mimic diffuse ACh transmission (e.g., Descarries et al., 1997). Accordingly, we previously showed that 5 to 10 μM nicotine produce the maximal steady-state effect on neurotransmitter release (see Fig.4 in Aracri et al., 2010). The time course of a representative experiment is shown in **Fig. 1D**, which plots the number of IPSCs in consecutive 10 s intervals, in the indicated conditions. Nicotine was applied after allowing the spontaneous IPSCs frequency to stabilize for about 5 minutes (Control). The drug generally increased the spontaneous IPSCs frequency (**Fig. 1C** and **1D**). In a series of similar experiments, nicotine approximately

doubled the steady-state IPSC frequency (**Fig. 1F**). The effect was reversible and sometimes (30% of the cells) accompanied by a decrease of the average IPSC amplitude (**Fig. 1E**). For all experiments, full statistics are given in the figure legends. The stimulatory effect of nicotine on IPSCs measured on FS cells was also observed in mice of older ages (up to P60). However, because obtaining “giga-seals” in brain slices becomes increasingly difficult with mice age, the full set of cell treatments was carried out in younger mice. In particular, 80% of the displayed experiments were executed in slices cut between P21 and P35. We believe this is an acceptable compromise between network maturity and feasibility of patch-clamp measurements. In fact, the expression of GABAergic markers (Le Megueresse and Monyer, 2013) and nicotinic subunits (Molas and Dierssen, 2014) is reasonably stable after P21.

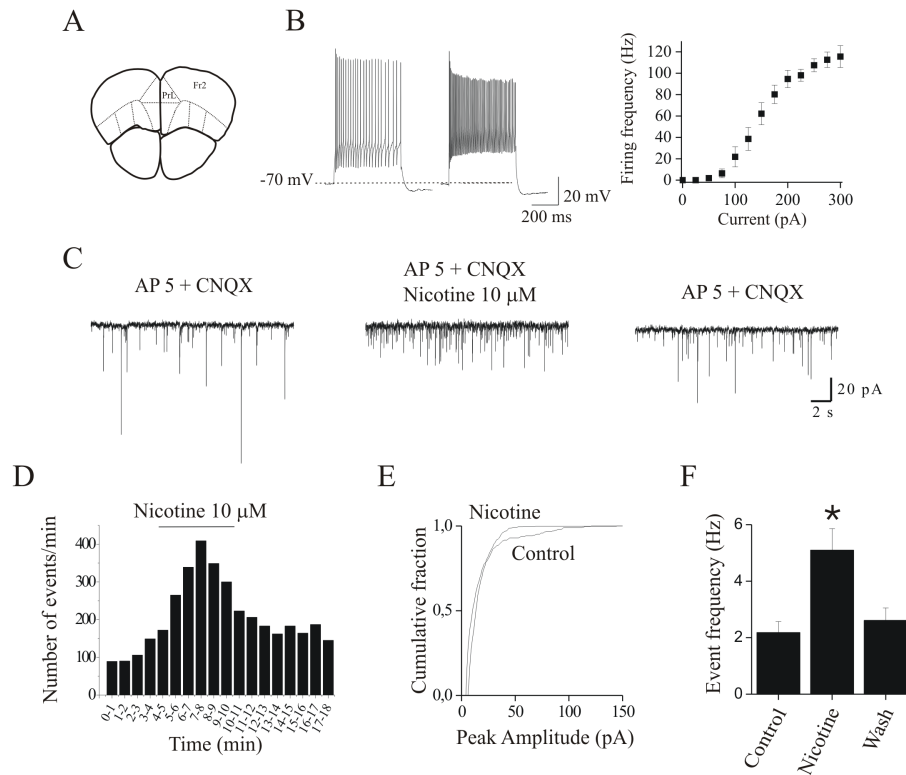


Figure 1. Nicotine stimulates IPSCs in FS cells. **A**, Scheme of a coronal murine PFC section. PrL: prelimbic; Fr2: frontal area 2. **B**, FS cell response to 100 and 300 pA depolarizing currents (500 ms). The right panel plots the average frequency-stimulus relation for ten FS cells. **C**, Typical IPSC traces, in the presence of AP5 and CNQX, at -70 mV. Nicotine (10 μ M) reversibly increased the IPSC frequency. **D**, Time course of the nicotine effect. Bars give the number of IPSCs measured within consecutive 10 s intervals, during approximately 3 min in control condition, 5 min in the presence of nicotine, and 4 min after nicotine was removed (washout). **E**, Amplitude distribution of the IPSCs recorded for 2 min in the indicated conditions, at the steady-state. In 6 out of 9 neurons, nicotine had no effect on the IPSC amplitudes (KS test). **F**, Average effect of nicotine. Bars give the mean IPSC frequency, calculated as illustrated in the previous panels. Nicotine brought the IPSC frequency from 2.2 ± 0.4 Hz to 5.1 ± 0.79 Hz ($0.01 < p < 0.05$; paired t-test; $n = 9$). After washout, the IPSC frequency was 2.6 ± 0.45 Hz.

The effect of nicotine is inhibited by DH β E, but not MLA

The contribution of $\alpha 4^*$ and $\alpha 7^*$ nAChRs can be distinguished by blocking these receptors by 1 μ M DH β E and 10 nM MLA, respectively (Harvey et al., 1996; Murray et al., 2012). After treating the cells with one of the blockers for 3 to 4 min, 10 μ M nicotine was added. The time course of a representative experiment is shown in **Fig. 2A** (left). In the presence of DH β E, the stimulatory effect of nicotine on spontaneous IPSC frequency was abolished (**Fig. 2A**, right). In contrast, MLA did not impair the effect of nicotine on IPSCs (**Fig. 2B**, left). On average, in the presence of MLA, nicotine approximately doubled the IPSCs frequency (**Fig. 2B**, right), consistent with the effect produced by nicotine alone. We conclude that the tonic effect of nicotine on the spontaneous IPSCs recorded on FS interneurons were largely mediated by $\alpha 4^*$ nAChRs.

5-IA stimulates IPSCs in FS cells

To better define the nAChR subtype involved in IPSCs regulation, we applied a submaximal concentration of 5-IA (10 nM), which specifically activates $\beta 2^*$ nAChRs (Mogg et al., 2004). The time course of the IPSC frequency in a representative experiment is shown in **Fig. 2C** (left). 5-IA generally increased the IPSC frequency by approximately 45%. The average results of a series of similar experiments are given in **Fig. 2C** (right). Considering that 10 nM 5-IA activates about 40% of the maximal nicotinic current (Mogg et al., 2004), these data are consistent with those obtained with nicotine. By comparing the results we obtained with DH β E, MLA and 5-IA, we

conclude that GABA release onto FS cells in Fr2 layer V is mainly regulated by $\alpha 4\beta 2^*$ nAChRs, in steady-state conditions.

nAChRs regulate mIPSCs in FS cells

The effect of nicotine on IPSCs could depend on activation of somatic currents on GABAergic cells, or stimulation of presynaptic terminals, or both. Hence, we first tested whether the nicotinic regulation of GABA release had a presynaptic component, by studying the mIPSCs revealed by 0.5 μ M TTX. **Fig. 3A** shows representative current traces, along with the IPSC time course (**Fig. 3B**) and amplitude distribution (**Fig. 3C**), in the indicated conditions. After TTX had revealed the mIPSCs, 10 μ M nicotine was added. On average, nicotine approximately doubled the mIPSC frequency (**Fig. 3D**). After TTX had produced the expected reduction in IPSC amplitude (**Fig. 3E**) and frequency (**Fig. 3F**), subsequent application of 1 μ M DH β E, and DH β E plus nicotine, produced no further effect. These results are summarized in **Fig. 3G**. We conclude that $\alpha 4\beta 2^*$ receptors are expressed in GABAergic synaptic terminals adjacent to FS cells, in Fr2 layer V.

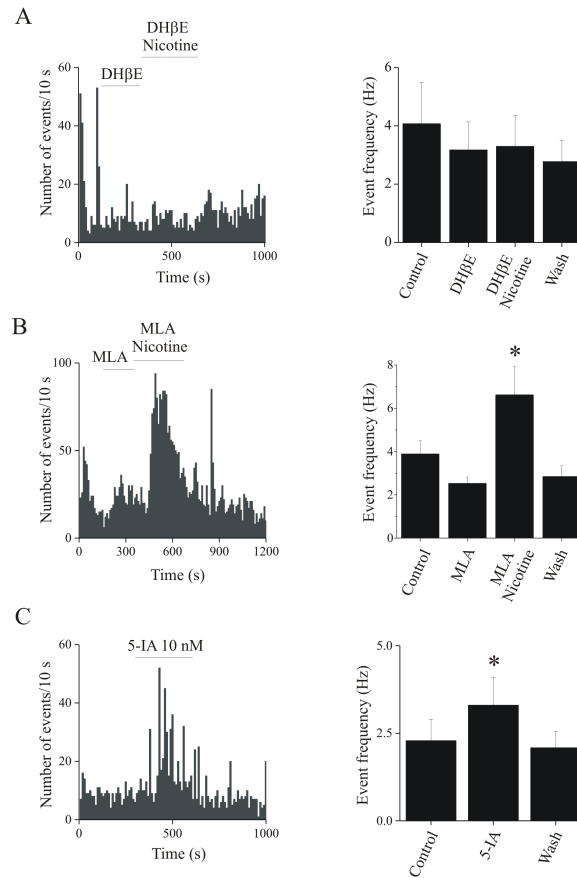


Figure 2. The effects of DHβE, MLA and 5-IA. Data analysis was as illustrated in Fig. 1. **A**, DHβE (1 μM) impeded the effect of nicotine on IPSCs. Left panel: time course of the IPSC frequency in a representative experiment. Right panel: the average IPSC frequencies were 4.07 ± 1.41 Hz (Control), 3.17 ± 0.96 Hz (DHβE), 3.29 ± 1.06 Hz (nicotine + DHβE; NS as compared with DHβE, with paired t-test), 2.77 ± 0.73 Hz (Washout). These values were not statistically different ($n = 9$). **B**, MLA (10 nM) did not block the IPSC increase produced by nicotine. Left panel: representative time course of the IPSC frequency. Right panel: the average IPSC frequencies ($n = 9$) were 3.89 ± 0.6 Hz (Control), 2.53 ± 0.86 Hz (MLA), 6.63 ± 1.32 Hz (nicotine + MLA; $p < 0.05$ compared to MLA; paired t-test), 2.86 ± 0.5 Hz (Washout). **C**, 5-IA (10 nM) stimulated the IPSC frequency. Left panel: representative time course of the 5-IA effect, Right panel: the average IPSC frequencies ($n = 6$) were 2.28 ± 0.61 Hz (Control), 3.3 ± 0.8 Hz (5-IA; $p < 0.05$ compared to Control), 2.08 ± 0.47 Hz (Washout).

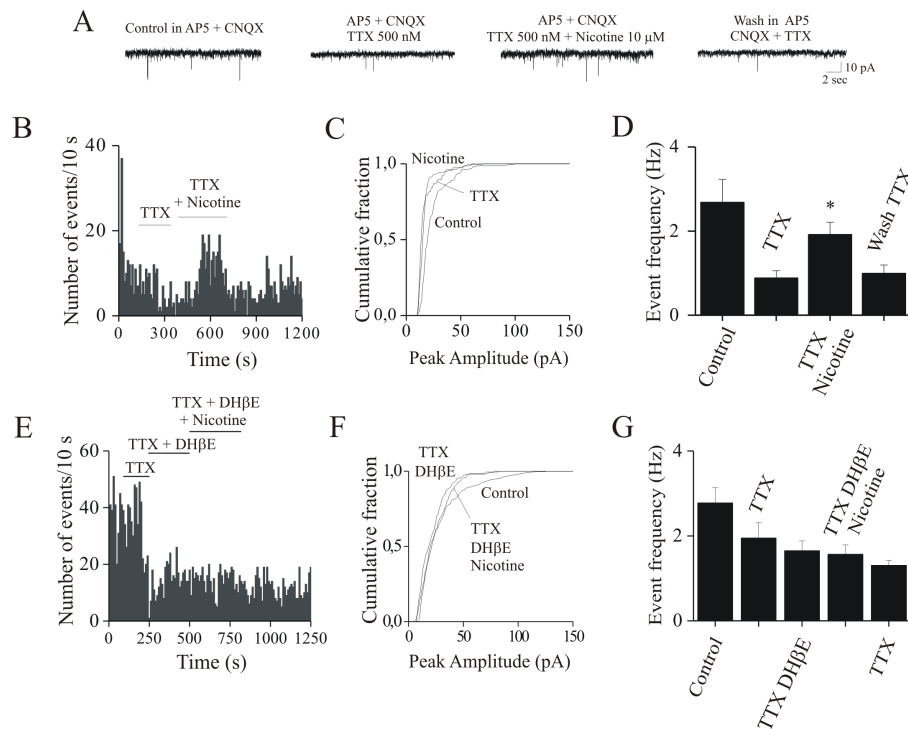


Figure 3. Heteromeric nAChRs regulate the mIPSCs. Data analysis was as in Fig. 1 and Fig. 2. **A**, Representative IPSCs in the indicated conditions. After TTX (0.5 μ M) had revealed mIPSCs, 10 μ M nicotine reversibly stimulated their frequency. **B**, Time course of the IPSC frequency in the same experiment. **C**, Corresponding IPSC amplitude distribution, in the indicated conditions. TTX decreased the IPSC amplitudes. No further effect was produced by nicotine in all of the tested neurons (KS test). **D**, The average IPSC frequencies ($n = 9$) were 2.68 ± 0.55 Hz (Control), 0.88 ± 0.17 Hz (TTX), 1.92 ± 0.29 Hz (nicotine + TTX; $p < 0.05$ compared to TTX), 0.99 ± 0.20 Hz (Washout of nicotine; NS compared to TTX). **E**, Representative time course of the effect of nicotine plus DH β E. The latter blocked the IPSC stimulation produced by nicotine. **F**, Corresponding mIPSC amplitude distribution. TTX decreased the IPSC amplitude. No further effect was produced by either DH β E or DH β E plus nicotine (KS test). **G**, The average IPSC frequencies ($n = 6$) were 2.77 ± 0.36 Hz (Control), 1.94 ± 0.36 Hz (TTX), 1.65 ± 0.23 Hz (TTX + DH β E), 1.56 ± 0.22 Hz (TTX + DH β E + nicotine; NS compared to TTX + DH β E); 1.31 ± 0.12 Hz (final control in TTX).

Whole-cell nicotinic currents on FS cells

Next, we studied if whole-cell nicotinic currents were expressed in FS interneurons and could be activated by slow agonist application. At -70 mV, nicotine activated typical slowly desensitizing inward currents, in a fraction of the tested FS cells (**Fig. 4A**). The same results were obtained irrespective of whether the cells were co-treated (n = 10) or not (n = 8) with TTX, and thus these results were pooled in **Fig. 4**. Whole-cell currents elicited by nicotine were also observed in a fraction of the FS cells pre-treated with 10 nM MLA (**Fig. 4B**), but never in the presence of 1 μ M DH β E (**Fig. 4C**). These results are summarized in **Fig. 5**, showing that 30 to 40% of our FS cells displayed whole-cell currents elicited by nicotine, irrespective of whether MLA was present or not. We conclude that FS cells can also express functional $\alpha 4\beta 2^*$ nAChRs on the somatic compartment, in layer V of murine Fr2 PFC. This suggests that both somatic and presynaptic components can contribute to the tonic stimulation exerted by heteromeric nAChRs in the GABAergic effect recorded in FS cells.

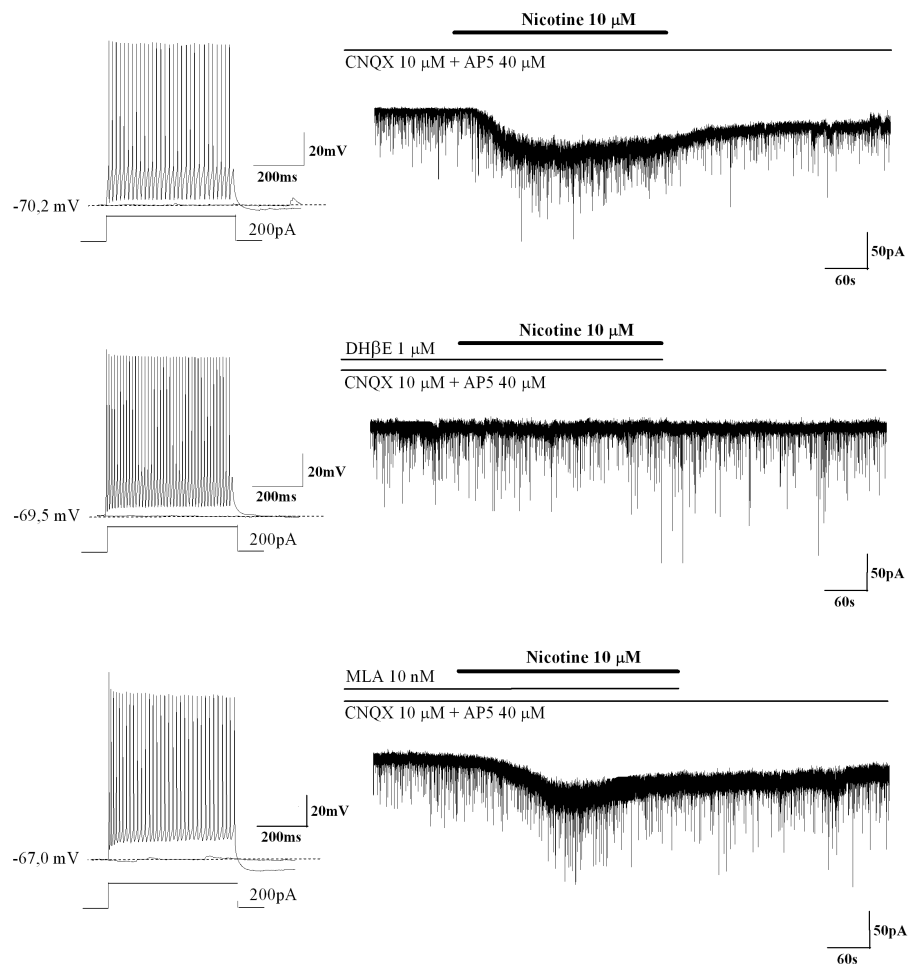


Figure 4. Expression of heteromeric nAChR currents in FS cells. Whole-cell currents elicited by 10 μ M nicotine. The left panels illustrate, for each experiment, the corresponding action potential firing pattern (only the response to a 200 pA current step is displayed). After testing cell firing, each cell was voltage-clamped at -70 mV, and nicotine was added either alone (top panel), or in the presence of 1 μ M DH β E (middle panel), or in the presence of 10 nM MLA, as indicated. In about 50% of the tested cells, nicotine elicited an inward slowly desensitizing current, which was also observed in the presence of MLA, but never in the presence of DH β E.

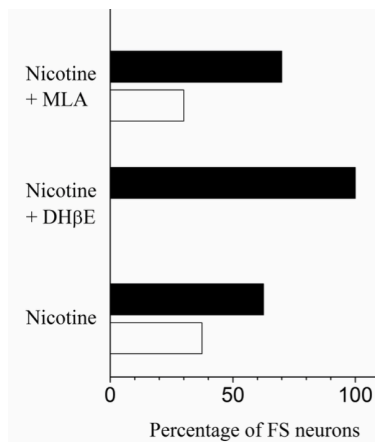


Figure 5. Percentage of FS cells expressing whole-cell nAChR currents.

Experiments were as illustrated in Fig. 4. Bars represent the percentage of FS cells displaying (white) or not (black) whole-cell currents activated by 10 μ M nicotine, at -70 mV. Percentages are given for cells treated with nicotine alone, nicotine plus 1 μ M DH β E, and nicotine plus 10 nM MLA (bottom), as indicated. No nAChR current was ever observed in the presence of DH β E.

Localization of α 4-containing nAChRs on GABAergic terminals adjacent to GABAergic cells

We previously showed that heteromeric nAChRs are expressed in GABAergic terminals, in Fr2 PFC. Most of these terminals surround pyramidal cells and express the calcium binding protein PV, a well-known marker of FS interneurons (Aracri et al., 2010). Here, we have specifically tested the α 4 nAChR subunit expression in GABAergic terminals forming synapses with FS neurons, by immunocytochemically labeling PV (to mark FS cells), α 4 nAChR, and VGAT (to label GABAergic terminals). **Fig. 6A** shows colocalization of α 4 with VGAT (white puncta), demonstrating nAChR expression on GABAergic terminals contacting putative FS cell bodies, as identified in the triple labeling of **Fig. 6A'**, which also shows PV immunoreactivity. A fraction of the VGAT+ synaptic terminals juxtaposed with PV+ interneurons display α 4 nAChR immunoreactivity, while most of them surround PV-negative cells,

mainly pyramidal neurons (**Fig. 6A**). A more detailed examination of the $\alpha 4$ /PV colocalization (**Fig. 6B**) shows that some PV+ cell bodies displaying $\alpha 4$ immunoreactivity are juxtaposed with PV+ puncta (**Fig. 6B'**) that also express $\alpha 4$ (**Fig. 6B''**). These results show a certain degree of interaction between PV+ neurons expressing $\alpha 4$ -containing nAChRs. Next, we labeled SOM to mainly mark Martinotti interneurons. These are thought to largely account for the regular spiking non-pyramidal cells (Kawaguchi and Kondo, 2002; McGarry et al., 2010), which constitute the other major interneuron population we could distinguish on electrophysiological basis. Differently from what was observed with PV+ cells, the $\alpha 4$ nAChR/SOM colocalization was concentrated in SOM+ cell bodies (**Fig. 6D**), clearly distinct from the PV+ somata (**Fig. 6D'**). Few puncta showing $\alpha 4$ nAChR/SOM colocalization (**Fig. 6D**) were adjacent neither to SOM+ or PV+ neuronal bodies (**Fig. 6D, D'**), suggesting that SOM+ interneurons are mainly regulated by $\alpha 4$ -containing nAChRs at the somatic level. Finally, the quantitative analysis of total (i.e. neuronal cell bodies, processes and puncta) colocalization between either SOM and $\alpha 4$, or PV and $\alpha 4$, showed that the amount of PV+/ $\alpha 4$ + structures was significantly higher than the amount of the SOM+/ $\alpha 4$ + ones (**Fig. 6C**). Taken together, our immunocytochemical results suggest that both PV+ and SOM+ cells can be modulated by $\alpha 4^*$ nAChRs, although the presynaptic effect on GABA release appears to be dominated by PV+ cells.

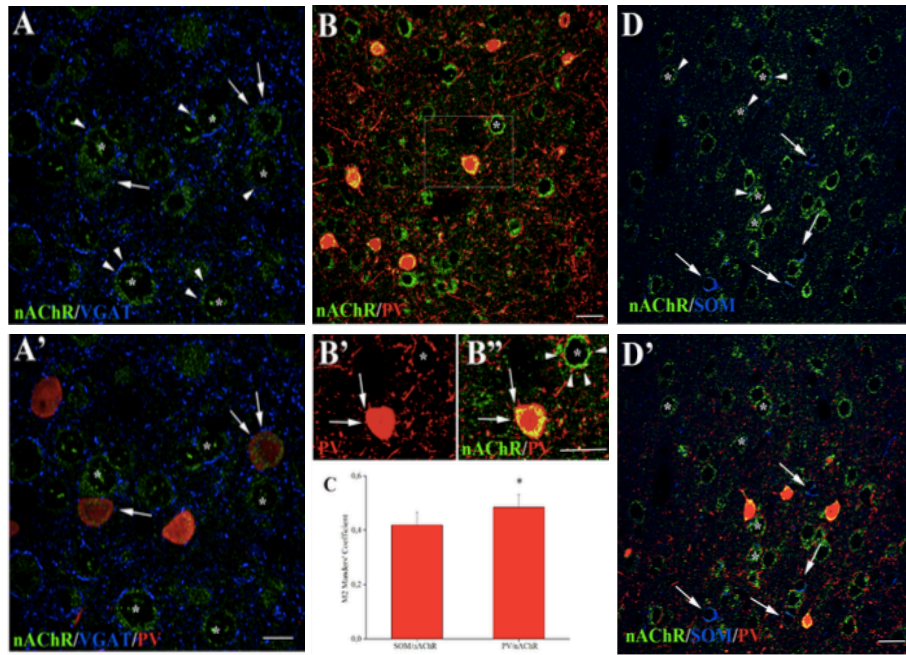


Figure 6. Localization of nAChRs on GABAergic terminals. *A, A'*: Double (*A*) and triple (*A'*) labeling of $\alpha 4$ nAChR (green), VGAT (blue) and PV (red). In *A*, colocalization of $\alpha 4$ and VGAT corresponds to the white signal, which indicates colocalizing puncta selected inside the ROI of a 2D cytofluorogram. Few GABAergic terminals contact PV+ cell bodies (arrows), while they are more frequent (arrowheads in *A*) in proximity of PV negative somata (asterisks), some of which could be pyramidal cells. *B, B', B''*: double labeling of $\alpha 4$ nAChR (green) and PV (red). The rectangle in *B* shows the enlarged area in *B'* (single PV immunolabeling) and *B''*. The colocalization analysis (*B''*) indicates two white puncta (arrows in *B', B''*) on a PV+ cell body, and a larger number (arrowheads in *B''*) on an adjacent neuron (asterisk). *C*: analysis of overlapping colocalization by means of M2 Manders' coefficients, showing the fraction of SOM or PV signal that is also positive for $\alpha 4$ nAChR. Significantly higher colocalization was observed between PV and nAChR than between SOM and nAChR. The M2 Manders' coefficients (in layer V of Fr2 cortical fields) were 0.419 ± 0.047 (for SOM) and 0.48 ± 0.047 (for PV; $p < 0.05$ with Student's t-test; $n = 11$). *D, D'*: Double (*D*) and triple (*D'*) labeling of $\alpha 4$ nAChR (green), SOM (blue) and PV (red). In *D*, the $\alpha 4$ /SOM colocalization (white signal) is concentrated in SOM+ neurons (arrows) and in some puncta (arrowheads in *D*) contacting PV negative neurons (asterisks). Scale bars: 10 mm in *A, A'*; 20 mm in *B, B', B'', D*, and *D'*.

Discussion

Here, we have shown that steady state activation of heteromeric nAChRs can regulate FS cell in layer V of the murine Fr2 prefrontal region. By comparing the results we obtained with DH β E, MLA, and 5-IA, we attribute most of the IPSC stimulation to activation of $\alpha 4\beta 2^*$ receptors. In the murine PFC, these prevail on the other subtypes possibly targeted by 5-IA (such as $\alpha 6\beta 2^*$; Zoli et al., 2015). The concentration of 5-IA we used essentially activates the high-affinity $(\alpha 4)_2(\beta 2)_3$ stoichiometry (Zwart et al., 2006), suggesting that this nAChR subtype is significantly expressed in our preparation. Contrary to the state of knowledge in hippocampus, where expression of heteromeric receptors in several interneuron types, including PV+, is ascertained (e.g., Ji and Dani, 2000; Griguoli and Cherubini, 2012), a comprehensive picture of nAChR expression in different regions, layers and cell populations is not available in the neocortex. In rat, nAChRs are not expressed in FS cells in visual (Xiang et al., 1998) and motor (Porter et al., 1999) cortex, but they are widely expressed in layer I interneurons, which regulate interneuron inhibition in layers II/III (Christophe et al., 2002). Expression of $\alpha 4^*$ nAChRs in layer IV GABAergic neurons was also observed in somatosensory areas, but the cell-specific distribution is unclear (Brown et al., 2012). In deep layers of medial PFC of mouse (Couey et al., 2007) and rat (Gulledge et al., 2007), the expression of $\alpha 4\beta 2$ receptors is mainly restricted to non-FS cells (Couey et al., 2007; Gulledge et al., 2007). Our results with SOM labeling confirms in more dorsal PFC regions that $\alpha 4\beta 2^*$ receptors are expressed by non-FS cells. However, in Fr2 we also

observed that a fraction of FS cells express whole-cell currents carried by heteromeric nAChRs, indicating that these receptors are not necessarily confined to presynaptic structures. At the present time, we cannot distinguish electrophysiologically which proportion of the IPSCs we measure on FS cells depends on GABA release from other FS cells or non-FS cells. Nonetheless, our results suggest that $\alpha 4\beta 2^*$ nAChRs can produce network disinhibition by directly stimulating FS cells in the deep layers of Fr2. If confirmed in different murine strains and species, this result would indicate that differences in the pattern of cholinergic stimulation exist among different prefrontal regions. This notion would be consistent with the morphological observation that the cholinergic fibers innervating the PFC are scarcely collateralized, which is a further indication that there may be area-specific variability in the cholinergic-dependent regulation of neocortical circuits (Price and Stern, 1983; Walker et al., 1985; Koliatsos et al., 1988; Sarter et al., 2009). Altogether, our results suggest that a higher degree of regulatory flexibility is available in the neocortical areas directly implicated in elaborating the decision-execution prefrontal functions. These regions could bear more functional resemblance to the neocortex of Primates, in which nicotinic targeting of FS cells seems more common, also in sensory regions (Disney et al., 2007; Disney et al., 2012; and references therein).

Nicotinic control of global PFC excitability

In deep layers, nAChR stimulation is thought to produce overall excitatory effects (e.g., Poorthuis et al., 2013). Layer V, in particular, provides a major output to subcortical targets and is particularly susceptible to develop and spread hyperexcitability (Shu et al., 2003; Ozeki et al., 2009; Cammarota et al., 2013). This is consistent with the well-known stimulatory effect produced by $\beta 2^*$ nAChRs on glutamate release throughout the mouse PFC (Lambe et al., 2003; Couey et al., 2007; Aracri et al., 2013). However, to better define the nAChR roles on the dynamics of neocortical output as well as the possible pathological implications, it is essential to understand how nAChRs regulate the balance of excitation and inhibition in different regions. Experimental work in both hippocampus and neocortex (Avoli et al., 2005; Isaacson and Scanziani, 2011; Paz and Huguenard, 2015) shows that, because of the strong local inhibitory restraint on pyramidal neurons, it is generally difficult to generate hyperexcitability in the absence of some degree of circuit disinhibition. These observations are increasingly supported by theoretical work on recurrent network models containing balanced excitatory and inhibitory elements, which show that the network output is very sensitive to the regulation of inhibitory activity (Tsodyks et al., 1997; Murphy and Miller, 2009; Ozeki et al., 2009; Murray et al., 2014). These studies also suggest that engagement of inhibitory neurons can lead to unexpected effects on excitability. Hence, more thorough studies of the regulation of excitatory/inhibitory networks in the neocortex are currently needed to allow in-depth comparisons between experimental and theoretical

results. Most experimental studies have addressed the sensory cortices, in which the relevance of circuit disinhibition for sensory elaboration and learning has been recently revealed (Letzkus et al., 2011; Pfeffer et al., 2013; Pi et al., 2013). However, very little is known about the regulation exerted by the ascending regulatory systems on these processes. In layer II/III of murine sensory cortex, disinhibition of FS cells was found to be indirectly regulated by non- $\alpha 7$ nAChRs, which stimulate non-FS cells that form synapses onto FS neurons (Arroyo et al., 2012). Virtually nothing is known about these mechanisms in associative cortices. Our results suggest that, in Fr2, the excitatory effect produced by nAChRs through stimulation of glutamate release (Aracri et al., 2013) is accompanied and potentially reinforced by direct regulation of network disinhibition.

Possible implications for epileptogenesis

Motor disturbances are frequently observed in sleep disorders and ADNFLE is no exception, as it is characterized by seizures as well as sudden arousal episodes during non-REM sleep, accompanied by hyperkinetic events (Picard and Brodtkorb, 2008). Therefore, fits of uncontrolled firing activity in the frontal cortex can unleash stereotyped motor patterns. Because of its connections, layer V of the Fr2 region may be a particularly sensitive area to produce these effects. Nonetheless, the precise mechanism of hyperexcitability in ADNFLE remains unclear. The mutant $\alpha 4$ and $\beta 2$ nAChR subunits linked to ADNFLE tend to cause gain of receptor function, at least in heterologous expression systems (Dani and Bertrand, 2007; Becchetti et al., 2015). As previously discussed, activation of heteromeric

nAChRs is known to stimulate glutamate release in PFC. However, because of the potent negative feedback exerted by basket cells onto pyramidal neurons, it is unclear whether a moderate increase in the nAChR-dependent glutamate release caused by mutant nAChRs is sufficient to generate epileptiform activity, as is also suggested by the experimental and theoretical evidence mentioned in the previous paragraph. In fact, alterations of GABAergic transmission seem to be common in rodent models of ADNFLE (reviewed in Becchetti et al., 2015). One possibility is that abnormal GABAergic activity leads to synchronization of pyramidal neurons by rebound firing after bouts of inhibition (Klaassen et al., 2006). Another possibility, suggested by the present results, is that hyperfunctional heteromeric nAChRs have a supplementary, and more powerful, way of generating epileptiform activity in layer V, by inhibition of FS cells. This effect could be particularly effective in conditions of low glutamatergic drive, as is generally the case in non-REM sleep.

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Cholinergic transmission in the cerebral cortex of a conditional murine model of ADNFLE

Introduction

In recent years, several mutations on CHNRA2, CHNRA4 and CHRNB2 genes, coding for heteromeric nAChRs subunits, have been linked to a mendelian form of nocturnal frontal lobe epilepsy (ADNFLE). These mutations have been reported to alter the gating properties of the receptor, generally resulting in a gain of function (Aridon et al., 2006; Chen et al., 2009; Cho et al., 2008; Hirose et al., 1999; Phillips et al., 2001; Sutor and Zolles, 2001; Steinlein et al., 1995, 1997; for extensive review see chapter 1). Furthermore, a new mutation has been recently characterized in CHNRA2 gene demonstrating that also loss of function of $\alpha 2$ subunit may have pathogenic effects. These findings tightened the already strong bonds that link heteromeric $\alpha 4\beta 2$ nAChRs disfunction to ADNFLE (Conti et al., 2015). Several mouse and rat models, carrying these human mutated genes, have been generated to investigate the pathologic mechanisms responsible for the onset of the disease. The severity of the phenotype shown by these animals and the presence of spontaneous seizures are variable and, in some of the cases, very different from the human counterparts. Nonetheless, abnormal cortical excitability and a disturbed sleep pattern have been found to be common features shared by these murine models (Klaassen et al., 2006, Mann and Mody, 2008; Zhu et al., 2008). Among them, the conditional $\beta 2$ -V287L mouse model emerges not only for its

similarities to human ADNFLE symptoms, but also for its contribution to the study of the developmental effect of the mutation. In fact, the transgene expression during early stages of neuronal development (from E1 to P14) is necessary to induce the epileptic phenotype (Manfredi et al., 2009). Once expressed in heterologous systems, in this case HEK293 cells, electrophysiological studies report that the overall effect comes down to a widening of the channel window current. This is determined by a combination of higher sensitivity to the agonists and retarded desensitization (De Fusco et al., 2000; Itier and Bertrand, 2002). We conducted electrophysiological investigations on the cholinergic modulation of the excitability in the PFC Fr2 region of mature animals, expressing or not $\beta 2$ -V287L* heteromeric nAChRs. To this purpose, we performed patch-clamp recordings of the spontaneous excitatory and inhibitory post-synaptic currents (EPSCs; IPSCs) in layer V pyramidal neurons. We focused our attention on the prefrontal cortex because this is where seizures originate in ADNFLE patients. In particular, a typical feature of this pathology is the presence of stereotyped motor events accompanying the seizures, suggesting that motor patterns are released during the attacks. Therefore, premotor areas such as Fr2 seem to be particularly relevant for ADNFLE. This area projects to the motor cortex and dorsolateral striatum (Berendse et al., 1992) and it is very sensitive to nicotinic stimulation in layer V (Aracri et al., 2010; 2013). Moreover, layer V pyramidal neurons constitute the main output to subcortical nuclei and they are probably responsible for the onset of the typical hypermotor seizures that characterize ADNFLE. We here show that $\beta 2$ -V287L expression alters the excitatory/inhibitory balance in

response to nicotine exposure and that this effect is mediated by $\beta 2^*$ heteromeric nAChRs. This may represent a mechanism through which hyperexcitability can be promoted in PFC areas. Moreover, we studied the modulation produced by nAChRs stimulation on reciprocal inhibition in the two major components of the inhibitory part of the cortical network, the Fast Spiking interneurons (FS) and the Regular Spiking Non Pyramidal neurons (RSNP). These results showed several differences between control and mutant mice. In particular, in $\beta 2$ -V287L mice RSNP neurons we observed a strong depression in the basal inhibitory tone sensed. Moreover, IPSCs recorded in FS interneurons in control conditions were doubled compared to control animals and showed opposite behaviours in response to 10 μ M nicotine application. Taken together, these data raise the possibility of an altered structure of inhibitory connections among interneurons producing network instability during periods of transient PFC stimulation, as it occurs during stage II of sleep.

Materials and Methods

Murine model. We used the S3 line of double transgenic mice [FVB-Tg(tTA:*Chrnb2*^{V287L}), which express $\beta 2$ -V287L and the tetracycline-controlled transcriptional activator tTA (Manfredi et al., 2009). When the transgene is not silenced by doxycyclin, animals show spontaneous seizures with a mean duration of 25 s. Seizures mostly (75%) occur during the light period, which in mice corresponds to the resting sleeping phase. The vast majority (>90%) of these seizures take place during periods of increased delta (0.5 – 4 Hz) electroencephalographic activity (for details, see Manfredi et al.,

2009). As controls, we used the littermates [FVB-Tg(PrnP-tTA)] expressing the transcriptional activator but lacking the *Chrn2*^{V287L} transgene (reported in the following sections as tTA mice). Animals were housed in SPF conditions on a 12h light-dark cycle, at $21 \pm 1^\circ\text{C}$, $55 \pm 10\%$ humidity and free access to food and water. Mice genotyping was carried out as previously described (Manfredi et al., 2009). Experiments were carried out by following the Principles of Laboratory Animal Care (directive 86/609/EEC), as previously described (Aracri et al., 2013).

Brain slices. In brief, Fr2 coronal sections were cut between +2.68 mm and +2.10 mm from bregma. During this procedure, brains (from P20-P50 mice) were placed in ice-cold solution containing (mM): 87 NaCl, 21 NaHCO₃, 1.25 NaH₂PO₄, 7 MgCl₂, 0.5 CaCl₂, 2.5 KCl, 25 D-glucose, 75 sucrose, 400 μM ascorbic acid, and aerated with 95% O₂ and 5% CO₂ (pH 7.4). Slices (300 μm thick) were cut with a VT1000S vibratome (Leica Microsystems), incubated at 32-33°C for 30 min in the above solution, and maintained at room temperature before being transferred to the recording chamber.

Whole-cell recordings. Cells were examined with an Eclipse E600FN microscope, equipped with a water immersion differential interference contrast objective (Nikon Instruments), and an infrared digital CCD (C8484-05G01) equipped with HCLImage Live acquisition software (Hamamatsu). Neurons were voltage- or current-clamped with a Multiclamp 700A (Molecular Devices), at 33-34°C. Micropipettes (2-3 M Ω) were pulled from borosilicate capillaries (Corning) with a P-97 Flaming/Brown Micropipette Puller (Sutter Instruments). The cell

capacitance and series resistance (up to 75%) were always compensated. Series resistance was generally below 10 M Ω . Input resistance was usually close to 100 M Ω . Post synaptic currents were low-pass filtered at 2 kHz and digitized at 5 kHz, with pClamp9/Digidata 1322A (Molecular Devices). Slices were perfused at 1.8 ml/min with ACSF, containing (mM): 135 NaCl, 21 NaHCO₃, 0.6 CaCl₂, 3 KCl, 1.25 NaH₂PO₄, 1.8 MgSO₄, 10 D-glucose, aerated with 95% O₂ and 5% CO₂ (pH 7.4).

During EPSCs recordings, pipettes contained (mM): 140 K-gluconate, 5 KCl, 1 MgCl₂, 0.5 BAPTA, 2 Mg-ATP, 0.3 Na-GTP, 10 HEPES (pH 7.26).

During IPSCs recordings in layer V pyramidal neurons, pipettes contained (mM): 140 Cs-gluconate, 2 MgCl₂, 0.5 BAPTA, 10 HEPES (pH 7.26). The tip of the pipettes was filled with an internal solution with the same composition of the one used for EPSCs experiments. This procedure allowed us to rapidly record the neuronal firing pattern, injecting steps of depolarizing current, during the first minutes after the whole-cell configuration achievement.

During IPSCs recordings in layer V FS and RSNP neurons, pipettes contained (mM): 70 K-gluconate, 70 KCl, 2 MgCl₂, 0.5 BAPTA, 1 MgATP, 10 HEPES (pH 7.2). This procedure set the chloride reversal potential around -16 mV, thus making IPSCs visible as inward positive currents with the membrane potential clamped at -70 mV. During these experiments, EPSCs were then abolished using saturating concentrations of glutamate receptors inhibitors (AP5 10 μ M and CNQX 40 μ M).

Drugs were applied in the bath and their effects calculated at the steady-state (usually reached within 2-3 min). The resting membrane potential (V_{rest}) was measured in open circuit mode, soon after obtaining the whole-cell configuration. No correction was applied for liquid junction potentials.

Drugs. Stock solutions of (–)-nicotine hydrogen tartrate salt, dihydro beta erythroidine hydrobromide (DHβE) and [D(-)-2-amino-5-phosphono-pentanoic acid] (AP5) were prepared in distilled water. Stock solutions of CNQX [6-cyano-7-nitroquinoxaline-2,3-dione] were prepared in dimethylsulfoxide (20 mM). These solutions were dissolved in our standard extracellular solution, and used at the indicated final concentration. Chemicals and drugs were purchased from Sigma-Aldrich Italy except AP5 and CNQX (Tocris Bioscience).

Analysis of patch-clamp data. We used Clampfit 9.2 (Molecular Devices), and OriginPro 9 (OriginLab). Both EPSCs and IPSCs included smoothly shaped isolated signals with a peak amplitude larger than 40 pA and composite signals. Post-synaptic currents were inspected one by one to reject spurious events. The peak-to-peak baseline noise was approximately 5 pA, therefore, the detection threshold was set at 7-8 pA to avoid errors during event sorting. The statistical comparison of cumulative distribution of both EPSCs and IPSC amplitudes was conducted with the Kolmogorov-Smirnov (KS) test, on at least 2 min continuous recording (never containing less than about 300 events). The results from populations of experiments are given as mean values $\pm$ SEM. The number of experiments (n) refers to the number of tested neurons (in different brain slices). Unless

otherwise indicated, statistical significance was assessed with two-tailed paired Student's t-test, at the indicated level of significance (p), by preliminarily assessing normality by a KS test.

Confocal microscopy. After patch-clamp recordings with internal solution containing 2mg/ml neurobiotin, slices were incubated for 24 hours in 4% paraformaldehyde at 4°C. Then tissue samples were placed in phosphate buffer solution for 48 hours at 4°C. Sections were then included in 5% agarose and cutted with VT1000S vibratome (Leica Microsystems) to obtain 60 µm thick tissue sections. The slices were then incubated in NH₄Cl 0.05 M for 30 min to block the aldehyde groups residual from fixation. After washing with phosphate buffered saline (PBS), they were pre-treated with 0.1% bovine serum albumin (BSA) / 0.2% Triton X-100 in PBS for 30 min, and incubated for 75 minutes with Alexa 488-labeled streptavidin (Molecular Probes Inc.). Images were analyzed with a TCS SP2 AOBS laser scanning confocal microscope (Leica Microsystems). Confocal micrographs were collected and digitized as TIF files with the Leica confocal scanning microscope (Leica Microsystems) and software package (Leica Confocal Software). Digital micrographs were optimized for resolution (final resolution 300 dpi), brightness, and contrast using Adobe Photoshop CS2 9.0 (Adobe Systems, San Jose, CA). Images were not altered in any way, e.g. by removing or adding image details.

Significance Statements

Before starting the results presentation and interpretation, it is necessary to provide some information about the nature of our preparations, the overall PFC circuitry structure and the concept of local circuit. We conducted our experiments on PFC brain slices. These are 300 μm thick coronal tissue sections dissected from a murine living brain. The slicing procedure obviously implies the cutting of the neural afferences coming from structures positioned outside the brain slice. This is an enormous restriction in the total amount of possible modulation of the electrical activity in the dissected area (in our case PFC) produced by the extreme complexity of the whole cerebral organ activity. After dissection, tissue samples remain incubated in an interface chamber with artificial cerebrospinal fluid recirculation and controlled temperature and oxygenation, for at least one hour before electrophysiological investigations (Matthies et al., 1997). During this period the slices are depleted of any kind of neuromodulators in the extracellular compartment, which eliminates every residual trace of the modulation coming from external structures. Nonetheless, after the strong reduction in the electrical activity produced by dissection and washout of the extracellular compartment, brain slices still show residual spontaneous activity. These signals are almost certainly maintained alive by a portion of neurons with pacemaker or reverberating activity. This produces a sort of stable and balanced electrical activity that allows the experimenter to test one circuit element (the targeted neuron) inserted in its network context. If the tissue slice remains viable during the incubation period, it is

possible to obtain reproducible and comparable results between one sample and another. In order to minimize spurious variability, it is necessary to study tissue samples from animals of the same strain and age. In our case, we used FVB mice with an age between P20 and P50, in which most of the circuit maturation is concluded and aging phenomena can be excluded. In fact, the expression of GABAergic markers (Le Megueresse and Monyer, 2013) and nicotinic subunits (Molas and Dierssen, 2014) is reasonably stable after P21. To reduce at the minimal terms the structure variability, a further contribution to the data reproducibility in cortical brain slices is the relatively stereotyped local circuit structure. This is actually composed of a single excitatory unit, the pyramidal neuron with an all-or-nothing possibility of firing, and a conserved structure of surrounding inhibitory cells that modulates its excitability. This modulation is explicated in terms of two well known electrophysiological processes, the feed-back and the feed-forward inhibition. The so composed local circuit can be considered as a single independent unit that fire in concertation forming a network with synchronous activity. This model can only be applicable during experimental conditions in which the stimuli coming from outer structures are completely removed and the remaining network activity is a stable minimum activity elicited by pacemaker cells, perhaps residing in another cortical layer. Moreover, the morphological simplicity and modularity of this structure can also be linked to developmental and morphological evidences all over literature about cerebral cortex (Kandel et al., 2000; Purves et al., 2004). In the light of all these assumptions we here propose an alternative way to interpret patch-clamp recorded activity before and

after pharmacological modulation. Our attempt is to explain the pathophysiological mechanisms produced by hyperfunctional nAChRs activity during development and mature life.

Results

After localization of the putative pyramidal neurons by their well known triangular shape, size (somatic diameter around 20 μM) and laminar location (**Fig. 1A**), we applied a series of 0.5 s depolarizing current steps in order to analyze their firing pattern. Below threshold, cells exhibited a quasi-linear response, whereas above threshold they showed the typical low-frequency firing with no adaptation, and APs with wide spike width followed by slow and round shaped after-hyperpolarization (**Fig. 1B**). In particular, the cells stimulated with 200 pA of injected current showed AP frequencies of 10-15 Hz (**Fig. 1C**), consistently with values found in literature for pyramidal neurons. In order to record EPSCs, the identified pyramidal neurons membrane potential was clamped at -70 mV, close to the GABA reversal potential (E_{GABA}). This procedure allowed us to conduct our experiments near V_{rest} and to minimize the amplitude of inhibitory input signals, avoiding the use of GABA_A receptors inhibitors. EPSCs were measured as inward currents as shown in the left panel of **Figure 1D**. During IPSCs recordings, membrane potential was clamped at +10 mV in order to minimize glutamate receptor currents and, therefore, IPSCs were measured as outward currents (right panel of **Fig. 1D**). Cs^+ was added to the internal solution to replace K^+ ; in this way we were able to minimize the persistent outward potassium current, reducing the time occurred to reach the steady-state (around 100 pA) at approximatively two minutes. This protocol allows the IPSCs isolation avoiding the bath application of glutamate receptors

inhibitors, thus leaving the overall electrical activity of the brain slices unaltered.

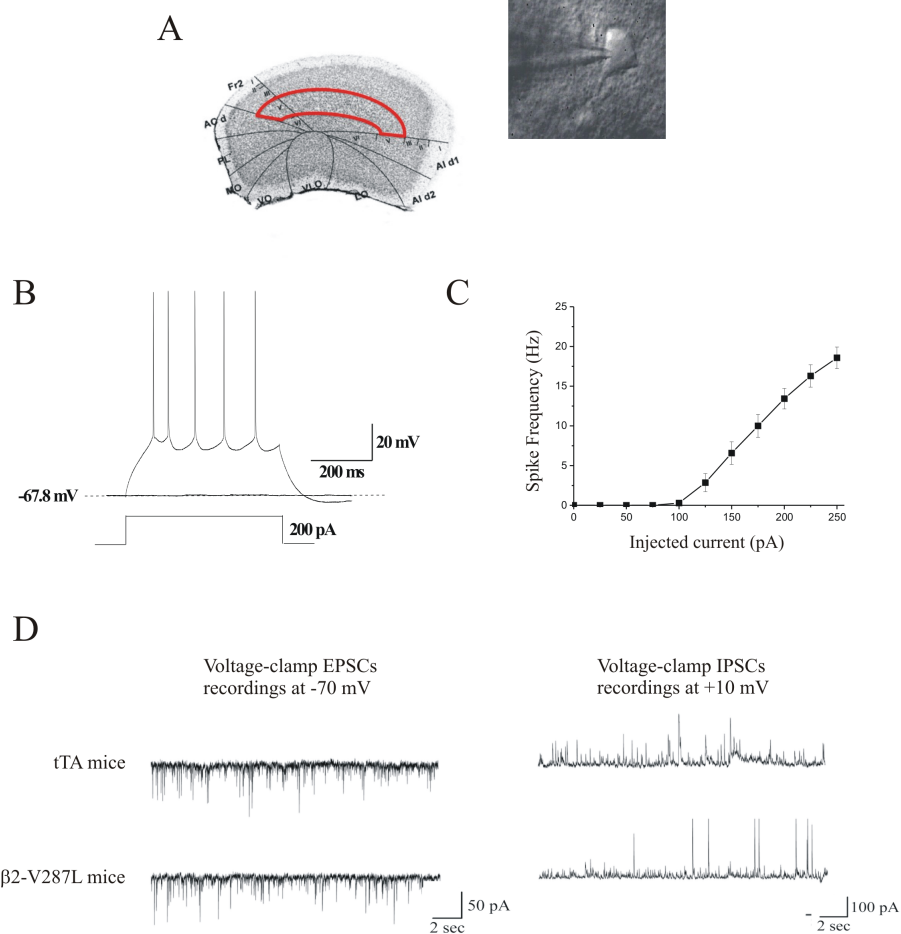


Figure 1. PFC layer V pyramidal neurons recognition and standard EPSCs and IPSCs recordings. *A*, Image of a coronal murine PFC section; the red shape highlights the layer V of the Fr2 region. *Top right*: pyramidal neuron during patch-clamp recording. *B*, Pyramidal neuron response to 200 pA depolarizing currents (500 ms). *C*, The panel plots the average frequency-stimulus relation for twelve pyramidal neurons. *D, left panel*, Typical EPSC traces recorded at -70 mV. *right panel*, Typical IPSC traces recorded at +10 mV.

Nicotine stimulated EPSCs onto pyramidal neurons in both control and $\beta 2$ -V287L mice

To assess the impact of hyperfunctional heteromeric $\beta 2$ -V287L* nAChRs activity on excitatory transmission we simulated an ACh upsurge in our PFC brain slices. As reported in the previous chapter, nicotine was preferred to ACh, to avoid applying muscarinic receptors' inhibitors, which can affect nAChRs at relatively low concentrations (Zwart and Vijverberg, 1997). Nicotine was always applied in the bath at a concentration of 10 μ M. This corresponds to the peak of the steady-state ('window') current for both $\alpha 4\beta 2$ and $\alpha 7$ receptors (Aracri et al., 2010), thus permitting to test the tonic contribution of both nAChR subtypes in conditions that mimic diffuse ACh transmission (e.g., Descarries et al., 1997). **Figure 2A** shows typical recordings in the indicated experimental conditions for tTA mice (upper panel) and $\beta 2$ -V287L mice (lower panel). In both control and double transgenic mice, nicotine consistently increased the spontaneous EPSCs frequency (**Fig. 2C**). The time course of a representative experiment is shown in **Figure 2B**, which plots the number of EPSCs in consecutive 10 s intervals, in the indicated conditions. In a series of similar experiments, nicotine significantly increased the EPSCs frequency in control mice (**Fig. 2C, upper panel**). This effect was particularly relevant in $\beta 2$ -V287L mice, in which the frequency approximately doubled after nicotine administration (**Fig. 2C, lower panel**). The effect was always reversible during washout. For all experiments, full statistics are given in the figure legends.

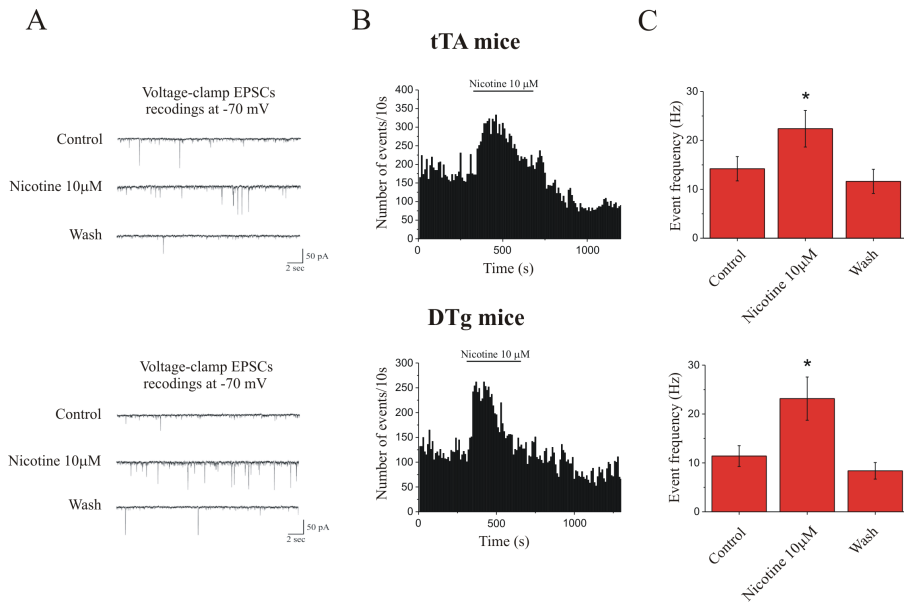


Figure 2. Nicotine stimulates glutamate release onto pyramidal neurons in both control and $\beta 2$ -V287L mice. *A*, Representative EPSCs in the indicated conditions, 10 μ M nicotine reversibly stimulated event frequency (tTA mice *upper panel*; $\beta 2$ -V287L mice, *lower panel*). *B*, Time course of the EPSCs frequency in the same experiment, each bar corresponds to 10 s. *C upper panel*, Average EPSCs frequencies recorded in tTA mice ($n = 9$) were 14.20 ± 2.48 Hz (Control), 22.39 ± 3.74 Hz (Nicotine 10 μ M; $p < 0.05$ compared to control), 11.61 ± 2.46 Hz (Washout of nicotine). *Lower panel*, Average EPSCs frequencies recorded in $\beta 2$ -V287L mice ($n = 12$) were 11.40 ± 2.13 Hz (Control), 23.15 ± 4.41 Hz (Nicotine 10 μ M; $p < 0.05$ compared to control), 8.38 ± 1.69 Hz (Washout of nicotine).

Whole-cell nicotinic currents in pyramidal neurons

During data analysis, we noticed that the increase in EPSCs wasn't the only excitatory effect produced by nicotine administration. At -70 mV, nicotine activated typical slowly desensitizing inward current, in a fraction of the tested pyramidal neurons (**Fig. 3A and 3B**). These results are summarized in **Figure 3C**, showing that 50 to 60% of our pyramidal neurons displayed whole-cell currents elicited by nicotine in both control and double transgenic mice. Moreover, the average current density was almost doubled in DTg mice (**Fig. 3D**). We already encountered the very same phenomenon during IPSCs recordings in Wt FS interneurons after nicotine administration (see Chapter 2). In that occasion, we used DH β E to demonstrate that $\alpha 4\beta 2^*$ nAChRs were completely responsible for that process. In this case we cannot totally exclude $\alpha 7^*$ nAChRs contribution, however, the sensitivity to 10 μ M nicotine and the slowly desensitizing kinetics strongly suggest $\alpha 4\beta 2^*$ nAChRs activation. We conclude that pyramidal neurons can also express functional nAChRs on the somatic compartment, in layer V of murine Fr2 PFC. This indicates that both somatic and presynaptic components can contribute to the excitatory effect.

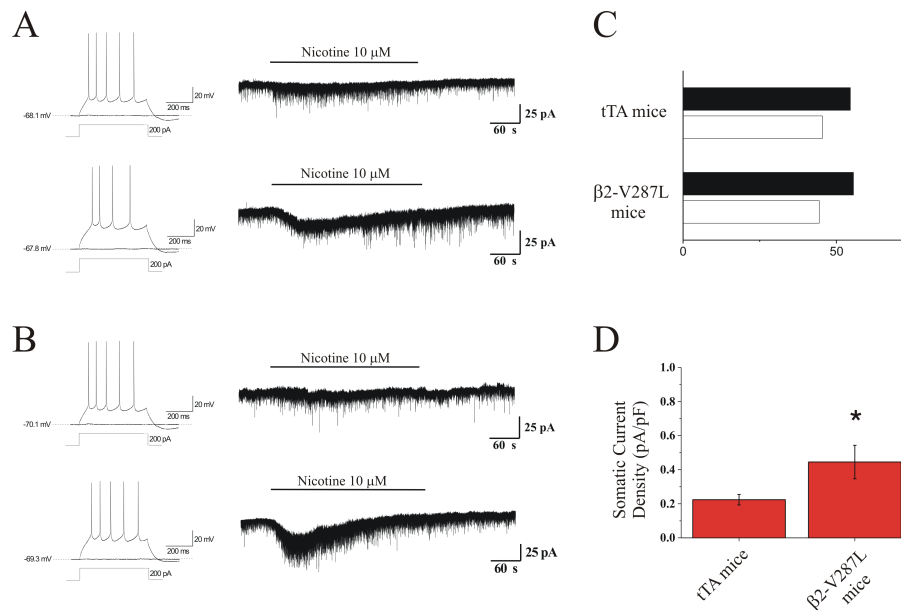


Figure 3. Nicotine application stimulates slow desensitizing somatic currents onto pyramidal neurons in both control and $\beta 2$ -V287L mice. *A*, Experiments conducted on tTA mice. The left panels illustrate, for each experiment, the corresponding action potential firing pattern (only the response to a 200 pA current step is displayed). After testing cell firing, each cell was voltage-clamped at -70 mV, and nicotine was added. In the *lower panel* a slow somatic current is represented *B*. $\beta 2$ -V287L mice pyramidal neuron response to 200 pA depolarizing currents (500 ms) and relative voltage-clamp trace at -70 mV representing nicotine application. In the *lower panel* a slow somatic current is shown. *C*. In about 55% of the tested cells (n=20), nicotine elicited an inward slowly desensitizing current (*black bars*) in both control and double transgenic littermates. *D*. Average somatic current density stimulated by nicotine application; tTA mice (n=6) 0.22 ± 0.03 pA/pF and $\beta 2$ -V287L mice (n=5) 0.44 ± 0.09 pA/pF (p < 0.05 compared to tTA mice, non-paired t-test).

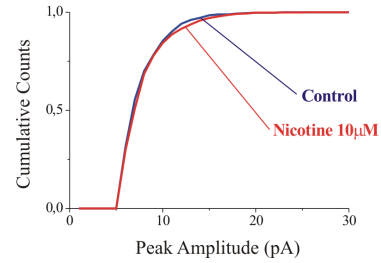
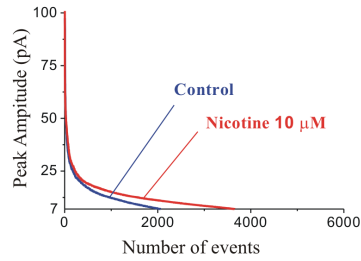
We next directed our attention on the amplitude of the recorded signals. In **Figure 4A** (tTa mice) and **Figure 5A** (DTg mice) we show the peak amplitude distribution for a representative experiment. The analyzed events recorded during two minutes of the steady-state, in control conditions and 10 μ M nicotine application, are plotted in the

descending amplitude order. In this way, we can link informations on peak amplitude (seen as a shift of the curve down or up-ward), total event number (seen at the interception between curves and x axis) and quantitative peak amplitude distribution (seen as changes in curve slope). In view of the presence of the nicotinic somatic current in almost a half of the recorded pyramidal neurons, we decided to separately analyze the collected data on spike amplitude for these two populations. Interestingly, if we compare the neurons that did not show any somatic current presence (*top panels*), in which all excitatory signals recorded are due to the EPSCs, we observe a very similar behaviour between control and affected mice. Moreover, the cumulative counts (**Figure 4B and 5B**) plotting the amplitude distributions unrelated to event frequencies, showed an almost perfect overlap between control conditions and agonist application. We can conclude that, from a presynaptical point of view, the presence of hyperfunctional mutant receptors does not alter the amplitude of the excitatory input signals. If we now look at the counterpart, the neurons that actually express somatic nAChRs (*bottom panels*), we can observe an increase in peak amplitude compared to the cells that do not express them. Moreover, the increase is more pronounced in double transgenic animals. This effect is probably due to the hyperfunctionality of mutant receptors expressed on the soma of pyramidal neurons in $\beta 2$ -V287L mice.

A

B

tTA mice pyramidal neurons non-expressing somatic current



tTA mice pyramidal neurons expressing somatic current

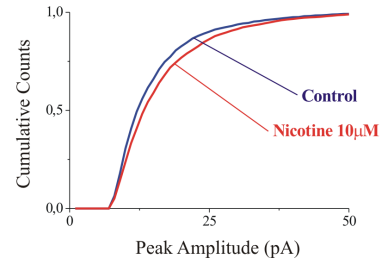
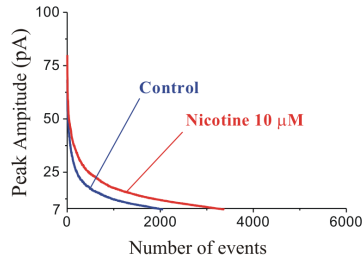
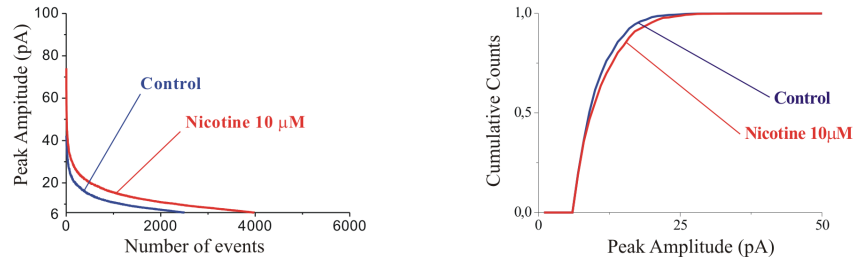


Figure 4. In tTA mice, $\alpha 4\beta 2$ nAChRs activation produced similar effects on both frequency and amplitude of the recorded signals in pyramidal neurons showing or not somatic whole cell currents. *A*, Plot showing ordered EPSCs amplitude during steady-state conditions in a representative experiment. Nicotine increased the number of events but not their amplitude in both neurons presenting or not somatic currents (*respectively left and right panels*). *B*, Amplitude distribution of the EPSCs recorded for 2 minutes in the indicated conditions, at the steady-state. In pyramidal neurons non-expressing somatic currents nicotine did not produce any effect on event amplitude. On the contrary, in 5 out of 6 neurons expressing somatic currents we found a small increase in peak amplitudes (KS test).

A

B

DTg mice pyramidal neurons non-expressing somatic current



DTg mice pyramidal neurons expressing somatic current

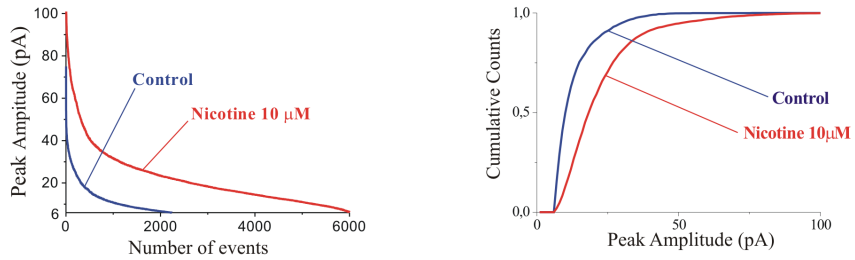


Figure 5. In β 2-V287L mice, nicotine application produced stronger effects on both frequency and amplitude of the recorded signals in pyramidal neurons showing whole-cell somatic currents. *A*, Plot showing ordered EPSCs amplitude during steady-state conditions in a representative experiment. In every case, nicotine increased the number of events, especially in pyramidal neurons presenting somatic currents (bottom panel), in which a strong increase in peak amplitude is also appreciable. *B*, Amplitude distribution of the EPSCs recorded for 2 min in the indicated conditions at the steady-state. In pyramidal neurons non-expressing somatic currents nicotine produced no significant effects on event amplitude. On the contrary, in all the neurons expressing somatic currents ($n=5$) we found a strong increase in peak amplitudes (KS test).

$\alpha 4\beta 2$ nAChRs activity regulates GABA release onto pyramidal neurons in both control and $\beta 2$ -V287L mice

We used the experimental protocol described in the previous section to stimulate nAChRs activity during IPSCs recordings on both tTA and $\beta 2$ -V287L mice. **Figure 6A** shows typical recordings in the indicated experimental conditions for tTA mice (*upper panel*) and $\beta 2$ -V287L mice (*lower panel*). In both control and double transgenic mice, nicotine significantly increased the steady-state IPSCs frequency (**Fig. 6C**). In contrast to what we observed during EPSCs recordings, the stimulatory effect of nicotine on inhibitory transmission was equal in both control and affected animals. The time course of a representative experiment is shown in **Figure 6B**, which plots the number of IPSCs in consecutive 10 s intervals, in the indicated conditions. In addition, after nicotine exposure, we applied 1 μ M DH β E, a selective $\alpha 4^*$ nAChRs antagonist, to distinguish the contribution of $\alpha 7$ homomeric nAChRs and $\alpha 4\beta 2$ heteromeric nAChRs to the observed effect. As expected, in the presence of DH β E, the stimulatory effect of nicotine on spontaneous IPSCs frequency was abolished. We conclude that the tonic effect of nicotine on the spontaneous IPSCs recorded on pyramidal neurons was largely mediated by $\alpha 4^*$ nAChRs.

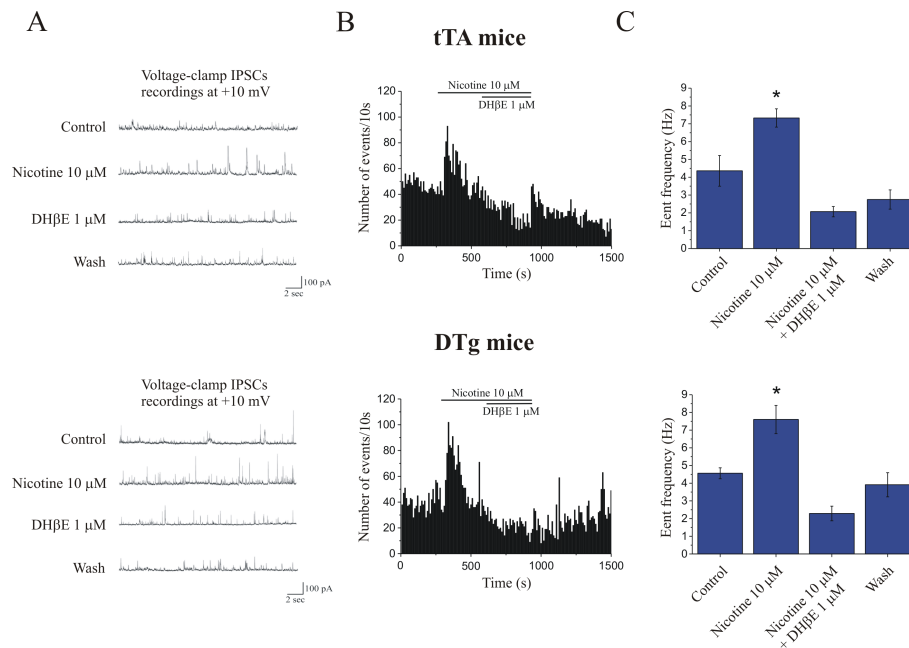


Figure 6. $\alpha 4 \beta 2$ nAChRs activity regulates GABA release onto pyramidal neurons in both control and $\beta 2$ -V287L mice. *A*, Representative IPSCs in the indicated conditions; 10 μ M nicotine increased their frequency, while DH β E 1 μ M suppressed this effect (tTA mice *upper panel*; $\beta 2$ -V287L mice, *lower panel*). *B*, Time course of the IPSCs frequency in the same experiment, each bar corresponds to 10 s. *C upper panel*, Average IPSCs frequencies recorded in tTA mice (n=8) were: 4.56 ± 0.30 Hz (Control), 7.60 ± 0.79 Hz (Nicotine 10 μ M; $p < 0.05$ compared to control), 2.28 ± 0.41 Hz (Nicotine 10 μ M + DH β E 1 μ M), 3.91 ± 0.68 Hz (Washout of nicotine). *Lower panel*, Average IPSCs frequencies recorded in $\beta 2$ -V287L mice (n=8) were: 4.35 ± 0.86 Hz (Control), 7.32 ± 0.51 Hz (Nicotine 10 μ M; $p < 0.05$ compared to control), 2.06 ± 0.28 Hz (Nicotine 10 μ M + DH β E 1 μ M), 2.75 ± 0.53 Hz (Washout of nicotine).

Mutant $\alpha 4\beta 2$ nAChRs expression does not affect the inhibitory tone sensed by pyramidal neuron in layer V PFC

We focused our attention on amplitude distribution of recorded IPSCs during steady state conditions after drug application (**Fig. 7A**). The panels show two representative experiments during which 10 μ M nicotine application produced a sizable increase in IPSCs frequency (tTA mice, *upper panel*; DTg mice, *lower panel*). This effect was totally abolished by 1 μ M DH β E administration, eliciting a strong reduction in events frequency. In **Figure 7B** the relative amplitude distributions for the same two representative experiments are shown, indicating that nicotinic stimulation is unable to impact on the amplitude of the recorded IPSCs. Summarizing, no clear differences were found, between control and DTg mice, either in recorded IPSCs amplitude or in their frequency, indicating that heteromeric nAChRs stimulation increases GABA release on pyramidal neurons but hyperfunctional receptors expression is not able to alter the total inhibitory tone sensed by them.

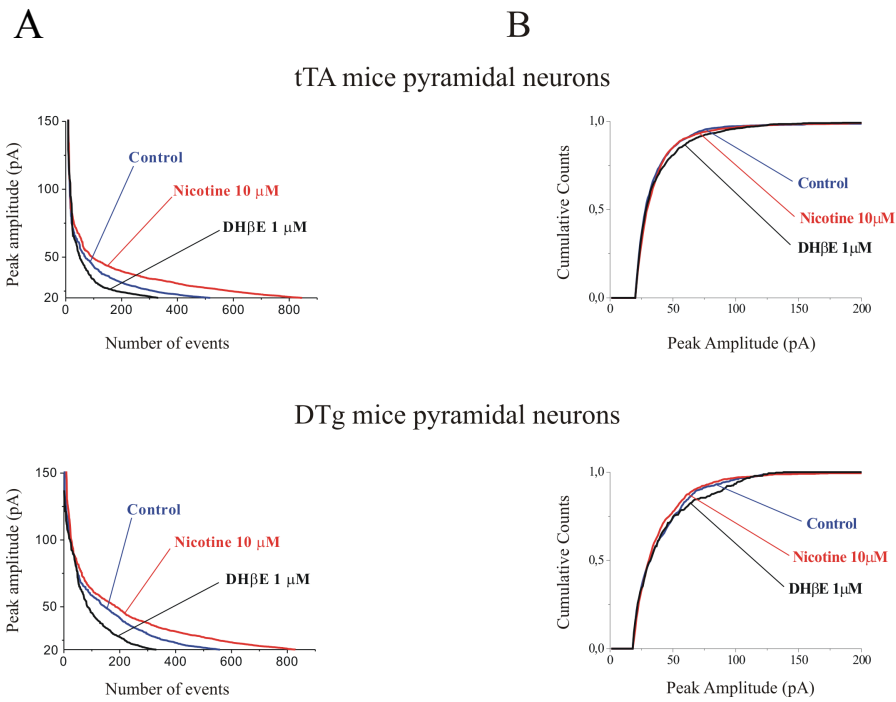


Figure 7. Hyperfunctional $\alpha 4 \beta 2$ nAChRs activity doesn't affect GABA release onto pyramidal neurons in $\beta 2$ -V287L mice. *A*, Plot showing ordered IPSCs amplitude during steady-state conditions in a representative experiment. In every case, nicotine increased the number of events while DH β E reverted this effect. Peak amplitude also follows the same behaviour in both control (*upper panel*) and double transgenic mice (*lower panel*). *B*, Amplitude distribution of the IPSCs recorded for 2 min in the indicated conditions, at the steady-state. Neither in tTA mice nor in double transgenic mice nicotine produced appreciable effects on event amplitude (KS test).

Mutant $\alpha 4\beta 2$ nAChRs expression is able to alter the excitatory/inhibitory ratio in $\beta 2$ -V287L mice pyramidal neurons during nicotinic stimulation

We then calculated the relative increase of both EPSCs and IPSCs frequencies in layer V pyramidal neurons produced by 10 μ M nicotine administration on our preparations. This analysis allowed us to verify that, while the IPSCs increase is around 66% in both our experimental groups, the EPSCs increase shows two different behaviours between tTA and $\beta 2$ -V287L mice (**Figure 8A**). In fact, in control mice, we observed a similar increase in IPSCs frequency compared to the EPSCs. This indicates that nicotine administration is able to unbalance the excitatory/inhibitory ratio in favour of inhibition. Conversely, in double transgenic mice, we reported a relative frequency increase 50% higher in EPSCs than for IPSCs, leading toward excitation (**Figure 8B**). This can be seen as a possible mechanism through which mutant heteromeric nAChRs expression can promote hyperexcitability in layer V pyramidal neurons of the PFC Fr2 region.

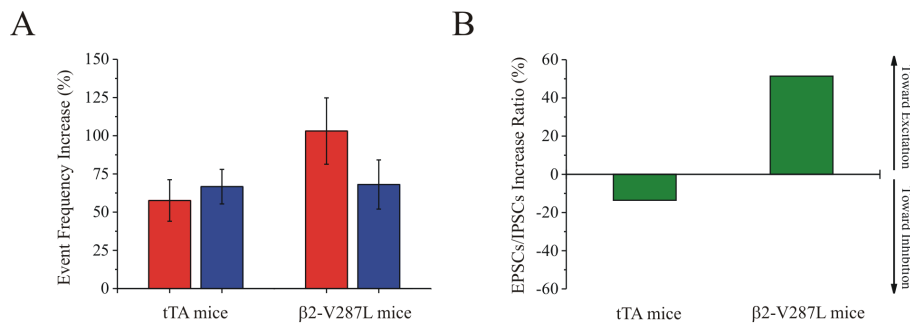


Figure 8. Mutant $\alpha 4\beta 2$ nAChRs expression is able to alter the excitatory/inhibitory ratio in $\beta 2$ -V287L mice pyramidal neurons during nicotinic stimulation. **A**, Plot showing the relative increase of EPSCs and IPSCs frequencies after nicotinic stimulation in our two experimental groups: 57.50% $\pm$ 13.62% (tTA mice EPSCs, n=9), 66.67% $\pm$ 11.34% (tTA mice IPSCs, n=8), 103.05% $\pm$ 21.75% ($\beta 2$ -V287L mice EPSCs, n=12), 68.06% $\pm$ 16.1% ($\beta 2$ -V287L mice IPSCs, n=8). **B**, Plot representing relative increases ratios (EPSCs/IPSCs): -13.6% $\pm$ 0.43% (tTA mice) and 51.4 $\pm$ 0.96 ($\beta 2$ -V287L mice)

Interneuronal populations in layer V Fr2 region of PFC

We next focused on the inhibitory part of the cortical network, the interneurons. These are universally recognised as short-axon neurons that use GABA as neurotransmitter. Although these cells have been widely studied for almost a century, their heterogeneity still prevents an exhaustive classification (De Felipe, 2002; Markram, 2004). In fact, several morphological, electrophysiological and molecular criteria have been recognised to distinguish them but no clear non-overlapping features have been found yet (Cauli et al., 1997; De Felipe et al., 2013;). We chose to conduct our experiments on two largely studied cellular subpopulations that together account for the great majority of cortical inhibitory neurons, the Fast Spiking (FS) interneurons and the Regular Spiking Non Pyramidal (RSNP) neurons. The former group is composed of basket cells and chandelier cells expressing parvalbumin (PV+); these neurons typically project their axons horizontally and target the pyramidal neurons somata or axon initial segment (Kawaguchi et al., 1993; Kubota et al., 2014). From an electrophysiological point of view, their evoked firing pattern shows a high-frequency train of action potentials with little adaptation and short spike width followed by deep after-hyperpolarization (Connors and Gutnick 1990; Bacci et al., 2003b; Okaty et al., 2009; Tai et al., 2014). In particular, the cells studied in the present work had AP frequencies of 80-100 Hz (with 200 pA stimulus), first spike width at half-maximal amplitude of 0.68 ± 0.03 s (n=16), and after-hyperpolarization of -13.7 ± 0.64 mV (n=16). The other major population of interneurons we focused on, the RSNP neurons, showed AP frequencies of 30-40 Hz (with 200 pA stimulus), more pronounced

adaptation compared to FS cells, spike widths larger than 1 ms and shallow AHPs (**Fig. 9**). These cells have been recognised in literature as mainly somatostatine expressing (SOM+) neurons and comprehend bipolar interneurons, Martinotti cells and large bitufed cells that usually project their axons vertically towards upper layers (Kawaguchi et al., 2003). To better identify this type of cells, during some of our recordings, neurobiotin was added to the internal solution to mark the soma and the dendritic and axonal arborizations. Further incubation with streptavidin conjugated with green fluorescent protein allowed us to confirm the bipolar morphology reported in literature for this interneuronal population (**Figure 10**).

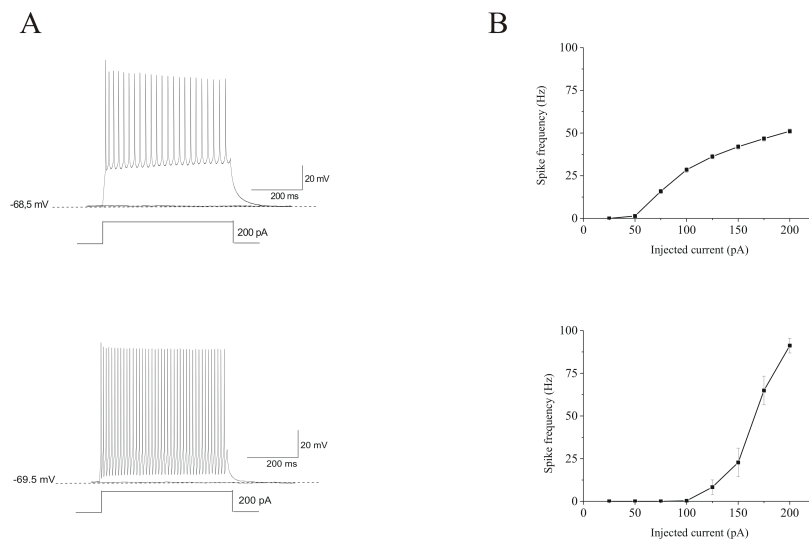


Figure 9. PFC layer V RSNP neurons and FS interneurons recognition through evoked firing patterns. *A*, RSNP neuron (*upper panel*) and FS interneuron (*lower panel*) response to 200 pA depolarizing currents (500 ms). *B*, The panels plot the average frequency-stimulus relation for twenty-nine RSNP neurons (*upper panel*) and twenty-seven FS interneurons (*lower panel*).

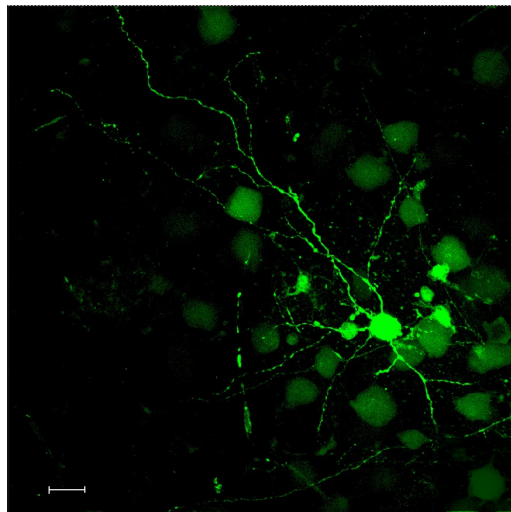


Figure 10. RSNP neurons morphology. Picture representing a RSNP neuron in the V layer of the Fr2 region of PFC made with neurobiotin-streptavidin system. The green fluorescent signal enlightens the typical bipolar shape reported in literature for RSNP neurons. Scalebar lenght: 20 μ m.

Nicotine exposure produced an increase in glutamate release onto FS interneurons in both control and double transgenic mice

We then studied the nicotinic control of one of the major type of interneuron found in layer V PFC, the FS cells. Once again, we tested the excitatory response to 10 μ M nicotine exposure (**Fig. 11A**). Our frequency analysis showed a transient increase of the EPSCs in both tTA and β 2-V287L mice. This effect was always reversed on washout (**Fig. 11B and 11C**). Interestingly, we found that the average spontaneous EPSCs frequency, recorded during control steady-state conditions, was nearly halved in double transgenic mice compared to the control littermates (respectively 22.03 ± 6.51 Hz and 43.82 ± 5.64 Hz).

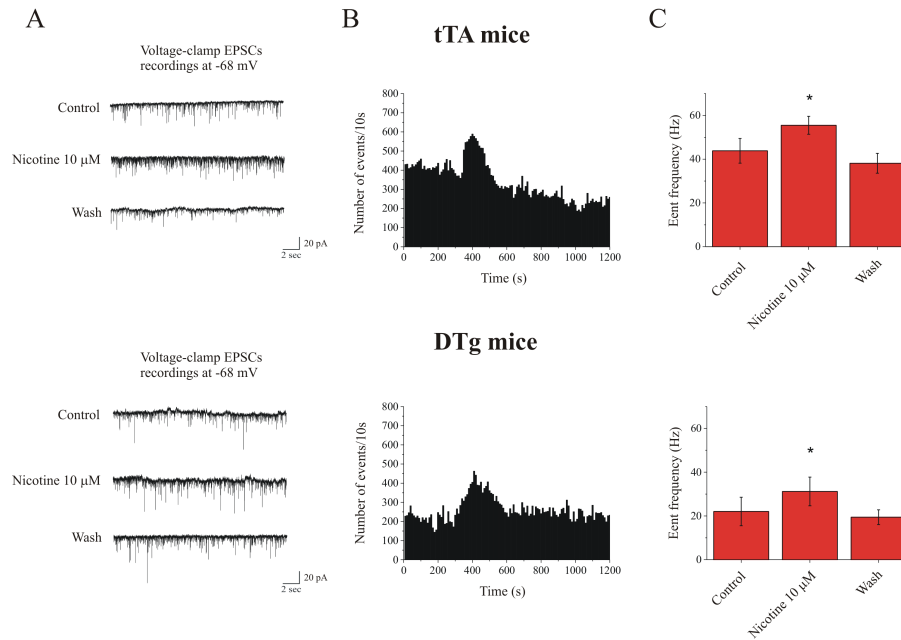


Figure 11. Nicotine stimulates glutamate release onto FS neurons in both control and $\beta 2$ -V287L mice. *A*, Representative EPSCs in the indicated conditions, 10 μ M nicotine reversibly stimulated their frequency (tTA mice *upper panel*; $\beta 2$ -V287L mice, *lower panel*). *B*, Time course of the EPSCs frequency in the same experiment, each bar corresponds to 10 s. *C upper panel*, Average EPSCs frequencies recorded in tTA mice (n=6) were 43.82 ± 5.64 Hz (Control), 55.48 ± 4.10 Hz (Nicotine 10 μ M; $p < 0.05$ compared to control), 38.12 ± 4.54 Hz (Washout). *Lower panel*, Average EPSCs frequencies recorded in $\beta 2$ -V287L mice (n=5) were 22.03 ± 6.51 Hz (Control), 31.2 ± 6.55 Hz (Nicotine 10 μ M; $p < 0.05$ compared to control), 19.43 ± 3.36 Hz (Washout).

During our analysis, we also noticed the presence of a slowly desensitizing somatic current in a fraction of the tested cells after nicotine exposure (**Fig. 12A and 12B**). More precisely we found current positive the 50% of FS interneurons recorded in control mice and the 33.33% of the same population in double transgenic mice (**Fig. 12C**). The difference between these two percentages is probably due to the small cohorts of tested cell (n=6) and not to a different

expression of somatic nAChRs in our two murine strains. Moreover, we observed no significant difference in the average densities of the nicotinic somatic currents recorded in tTA and $\beta 2$ -V287L mice (respectively 0.24 ± 0.06 pA/pF and 0.26 ± 0.07 pA/pF) (**Fig. 12D**).

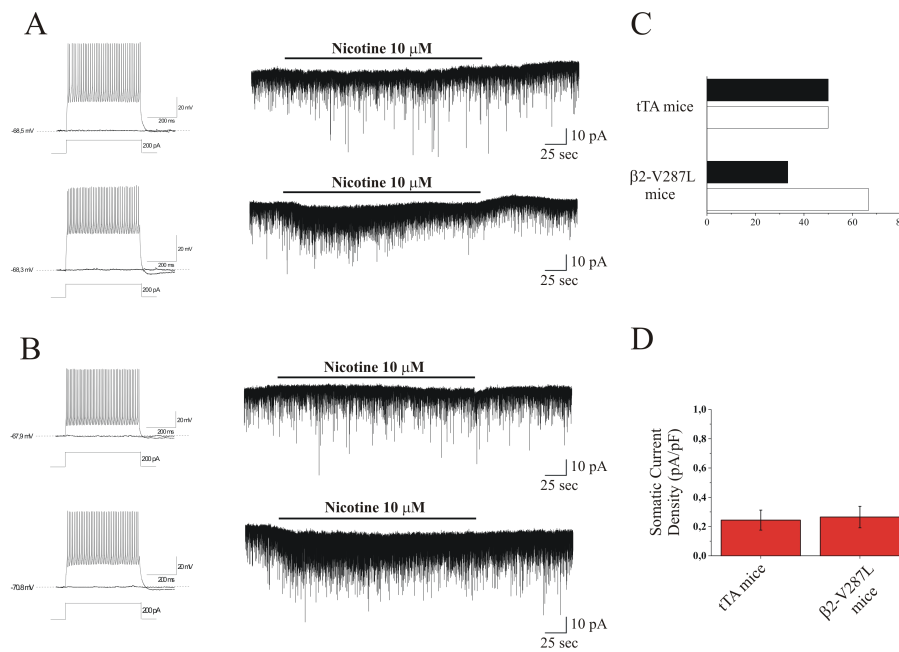


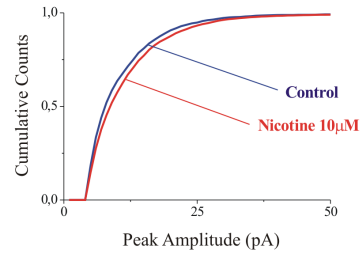
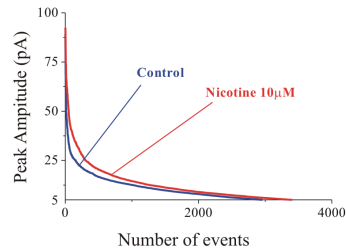
Figure 12. Nicotine application stimulates slow desensitizing somatic currents onto FS interneurons in both control and $\beta 2$ -V287L mice. *A*, Experiments conducted on tTA mice. The left panels illustrate, for each experiment, the corresponding action potential firing pattern (only the response to a 200 pA current step is displayed). After testing cell firing, each cell was voltage-clamped at -70 mV, and nicotine was added. In the *lower panel* a slow somatic current is represented *B*, $\beta 2$ -V287L mice FS interneurons response to 200 pA depolarizing currents (500 ms) and relative voltage-clamp trace at -70 mV representing nicotine application. In the *lower panel* a slow somatic current is shown. *C*, In tTA mice, in about 50% of the tested cells (n=6), nicotine elicited an inward slowly desensitizing current (*black bars*), while in double transgenic animals, only two over six cells showed the same behaviour (33,33%). *D*, Average somatic current densities stimulated by nicotine application; tTA mice (n=6) 0.24 ± 0.06 pA/pF and $\beta 2$ -V287L mice (n=4) 0.26 ± 0.07 pA/pF.

Amplitude analysis carried on these recordings showed no significant increase in event amplitude after nicotine administration, especially in small scale events (**Fig. 13A**). The low amplitude of the nicotinic somatic currents observed in only a fraction of the tested cells, suggest a poor expression of somatic nAChRs in FS interneurons. Therefore, we conclude that the potentiating effect elicited by nicotine on excitatory transmission is mainly produced through an increase of presynaptic release of glutamate onto FS interneurons. These findings are in agreement with our previous results on miniature IPSCs in FS cells (Aracri et al., 2010). Moreover, the relative distribution of peak amplitudes, shown in **Figure 13B**, also confirms the absence of any effect produced by nicotine administration on this feature.

A

B

tTA mice FS interneurons



DTg mice FS interneurons

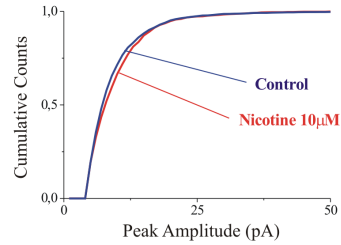
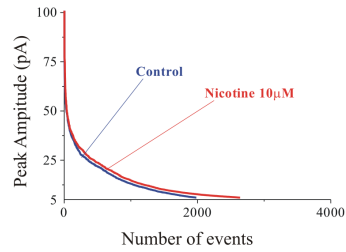


Figure 13. Nicotine application produced similar effects on the excitatory transmission sensed by FS interneurons in control and double transgenic mice.

A, Plot showing ordered EPSCs amplitude during steady-state conditions in a representative experiment. Nicotine poorly increased the number of events and their amplitude in both control and double transgenic mice. **B**, Amplitude distribution of the IPSCs recorded for 2 minutes in the indicated conditions, at the steady-state. Neither in tTA mice nor in double transgenic mice nicotine produced significant effects on event amplitude (KS test).

$\alpha 4\beta 2$ nAChRs activity produced opposite effects on GABA release onto FS interneurons in control and double transgenic mice

We tried to evaluate the effects of nicotinic stimulation on inhibitory transmission towards FS interneurons. Therefore, we administered once again 10 μ M nicotine to simulate a cholinergic upsurge on the neuronal network and subsequently 1 μ M DH β E to confirm the $\alpha 4\beta 2$ heteromeric nAChRs contribution to the observed effect. During our experiments we applied saturating concentration of AP5 and CNQX to completely abolish the glutamatergic currents. **Figure 14A and 14B** show the typical voltage clamp recordings in the indicated conditions and the IPSCs frequency trend of two representative experiments. Surprisingly, control IPSCs frequencies were very different in tTA and double transgenic mice. In fact, in control mice, we observed a spontaneous IPSCs frequency of 2.73 ± 0.50 Hz that, after nicotine application, doubled reaching 5.20 ± 0.67 Hz. The heteromeric nAChRs antagonist DH β E completely abolished this effect and IPSCs frequency returned to control values after washout. In $\beta 2$ -V287L mice we observed a control IPSCs frequency of 7.70 ± 1.44 Hz, approximatively three-folds higher than what we recorded in tTA mice. Moreover, nicotine application did not produce any increase of IPSCs frequencies while, on the contrary, it was able to nearly halve that value (4.02 ± 1.16 Hz). In this case, 1 μ M DH β E did not reverse this effect, producing a further decrease in IPSCs frequency that remained stable after washout (**Fig. 14C**).

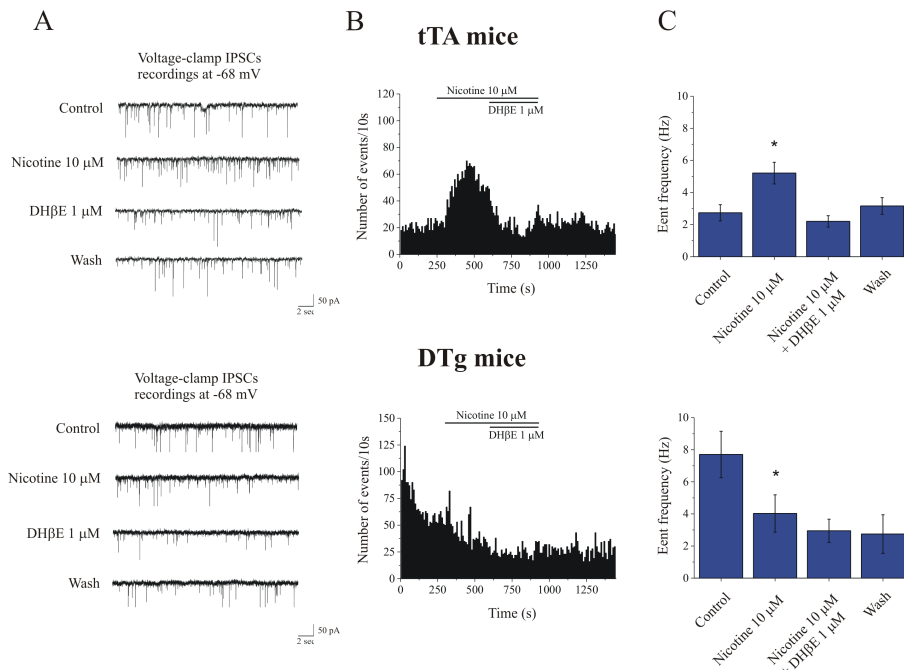


Figure 14. Nicotine stimulates GABA release onto FS neurons in control mice but not in β 2-V287L mice. *A*, Representative IPSCs in the indicated conditions, 10 μ M nicotine reversibly stimulated their frequency in tTA mice (*upper panel*). In β 2-V287L mice, nicotine application decreased IPSCs frequency (*lower panel*). *B*, Time course of the IPSCs frequency in the same experiment, each bar corresponds to 10 s. *C upper panel*, Average IPSCs frequencies recorded in tTA mice (n=11) were: 2.73 ± 0.50 Hz (Control), 5.20 ± 0.67 Hz (Nicotine 10 μ M; $p < 0.05$ compared to control), 2.20 ± 0.75 Hz (DH β E 1 μ M), 3.16 ± 0.51 Hz (Washout). *Lower panel*, Average IPSCs frequencies recorded in β 2-V287L mice (n=5) were: 7.70 ± 1.44 Hz (Control), 4.02 ± 1.16 Hz (Nicotine 10 μ M; $p < 0.05$ compared to control), 2.94 ± 0.72 Hz (DH β E 1 μ M), 2.74 ± 1.20 Hz (Washout).

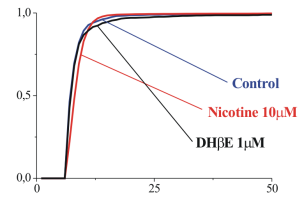
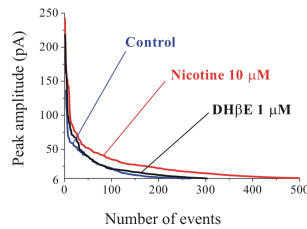
Nicotinic stimulation increased the inhibitory transmission on FS interneurons in tTA mice but had opposite effects in $\beta 2$ -V287L mice

Amplitude analysis conducted on the voltage-clamp recordings obtained in FS interneurons confirmed our observations during frequency analysis. In tTA mice, nicotine administration produced an increase in frequency and also a small increase in event amplitude (**Fig. 15A and 15B**). This effect was completely reversed by 1 μ M DH β E application. On the contrary, in double transgenic mice, nicotine didn't produce any appreciable effect on IPSCs amplitude while DH β E was able to reduce it. Therefore, as we saw in frequency analysis, the initial high inhibitory tone on FS interneurons observed in $\beta 2$ -V287L animals resulted more than halved by nicotinic stimulation and further reduced by DH β E administration.

A

B

tTA mice FS interneurons



DTg mice FS interneurons

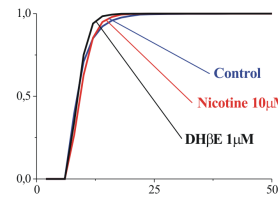
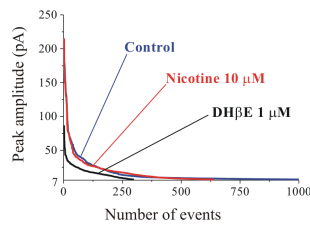


Figure 15. Nicotine administration produced opposite effects on the inhibitory tone sensed by FS interneurons in control and $\beta 2$ -V287L mice. *A*, Plot showing ordered IPSCs amplitude during steady-state conditions in a representative experiment. In tTA mice, nicotine increased the number of events while DH β E reverted this effect. Peak amplitude also follows the same behaviour (*upper panel*). In double transgenic mice (*lower panel*), nicotine didn't alter event amplitude. *B* Amplitude distribution of the IPSCs recorded for 2 minutes in the indicated conditions, at the steady-state. Neither in tTA mice nor in double transgenic mice nicotine produced appreciable effects on event amplitude (KS test).

Nicotine administration produced a strong increase in EPSCs frequency in RSNP neurons in both control and double transgenic animals

In RSNP neurons, in control conditions, we observed a low excitatory tone represented by small and rare spontaneous EPSCs (**Fig. 16A**). In these cells, 10 μ M nicotine bath application produced a very strong increase in excitatory signals, raising the average event frequency from 3.72 ± 1.28 Hz to 49.84 ± 8.06 Hz in tTA mice and from 2.26 ± 0.56 Hz to 52.17 ± 6.01 Hz in $\beta 2$ -V287L mice. This effect was only partially reversed after 10 minutes from agonist removal, showing an almost 60% reduction in the average spike frequency. (**Fig. 16B and 16C**).

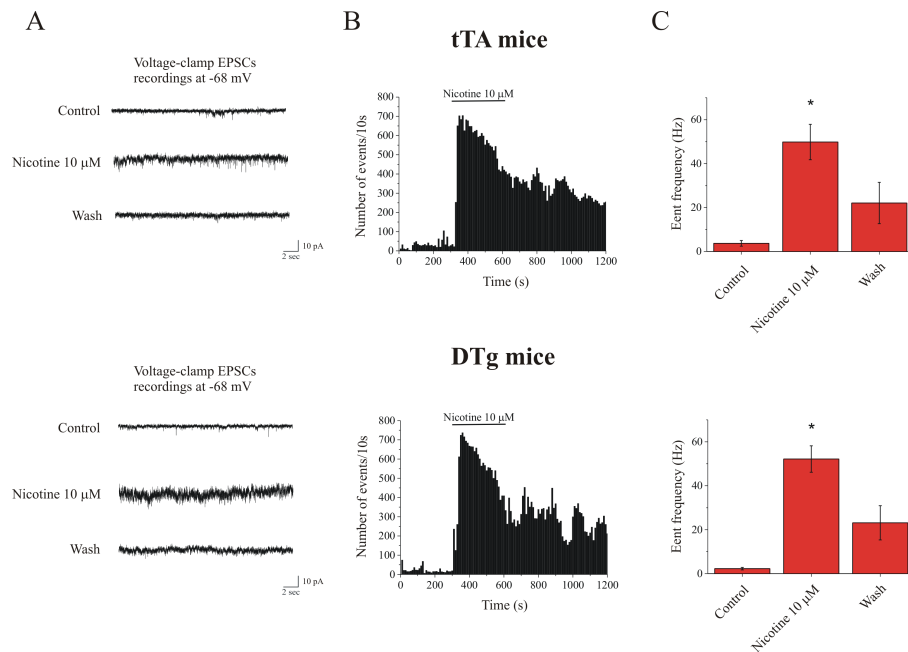


Figure 16. Nicotine strongly stimulates glutamate release onto RSNP neurons in both control and $\beta 2$ -V287L mice. *A*, Representative EPSCs in the indicated conditions, 10 μ M nicotine reversibly stimulated their frequency (tTA mice **upper panel**; $\beta 2$ -V287L mice, **lower panel**). *B*, Time course of the EPSCs frequency in the same experiment, each bar corresponds to 10 s. *C* **upper panel**, Average EPSCs frequencies recorded in tTA mice (n=9) were 3.72 ± 1.28 Hz (Control), 49.84 ± 8.06 Hz (Nicotine 10 μ M; $p < 0.05$ compared to control), 22.08 ± 9.39 Hz (Washout). **Lower panel**, Average EPSCs frequencies recorded in $\beta 2$ -V287L mice (n=8) were 2.26 ± 0.56 Hz (Control), 52.17 ± 6.01 Hz (Nicotine 10 μ M; $p < 0.05$ compared to control), 23.12 ± 7.77 Hz (Washout).

Moreover, in every RSNP neuron studied during our recordings, nicotine produced a slowly desensitizing somatic current (**Fig. 17A and 17B**). The average current density was estimated in 0.31 ± 0.04 pA/pF in control mice and 0.53 ± 0.15 pA/pF in double transgenic mice (**Fig. 17C**). This difference is probably caused by the hyperfunctionality of mutant $\alpha 4\beta 2$ heteromeric nAChRs expressed on the somatic compartment of RSNP neurons.

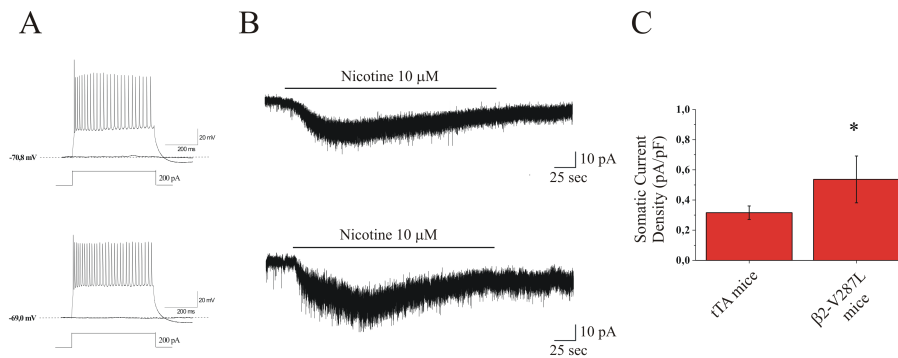


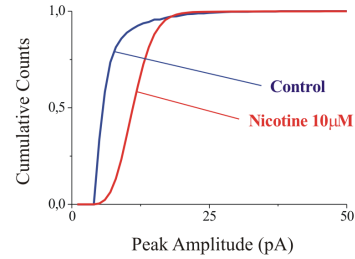
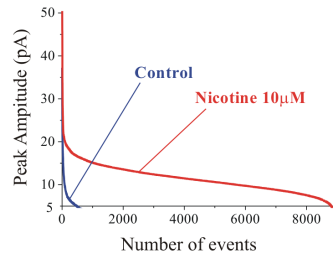
Figure 17. Nicotine application stimulates slow desensitizing somatic currents into RSNP neurons in both control and $\beta 2$ -V287L mice. *A*, The panels illustrate, for each experiment, the corresponding action potential firing pattern (only the response to a 200 pA current step is displayed). *B*, After testing cell firing, each cell was voltage-clamped at -70 mV, and nicotine was added. In every single case, RSNP showed a slow desensitizing somatic current elicited by nicotine application (*upper panel*, tTA mice; *lower panel*, double transgenic mice). *C*, Average somatic current density stimulated by nicotine application; tTA mice (n=13) 0.31 ± 0.04 pA/pF and $\beta 2$ -V287L mice (n=14) 0.53 ± 0.15 pA/pF (p < 0.05 compared to tTA mice, non-paired t-test).

During nicotine administration, the massive raise in event frequency was always accompanied by an increase in peak amplitude (**Fig. 18A and 18B**). Taken together, these data suggest that, besides the usual effect of nicotine on glutamate release, a significant postsynaptic component is present in RSNP neurons, because of the somatic nAChRs stimulation. Therefore, it is possible that the effect on EPSCs amplitude depends on signalling mechanisms triggered by the Ca^{2+} influx through nAChRs.

A

B

tTA mice RSNP neurons



DTg mice RSNP neurons

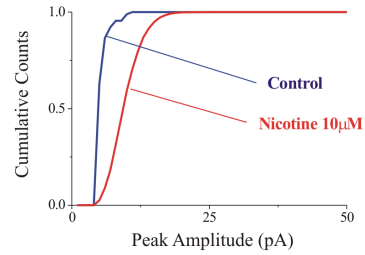
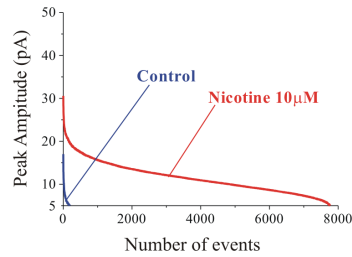


Figure 18. Nicotine application produced a dramatic increase of frequency and amplitude of excitatory signals in both control and double transgenic mice. *A*, Plot showing ordered EPSCs amplitude during steady-state conditions in a representative experiment. Nicotine strongly increased the number of events and their amplitude in both control and double transgenic mice. *B*, Amplitude distribution of the EPSCs recorded for 2 minutes in the indicated conditions, at the steady-state. In every RSNP neuron tested, in both tTA mice and double transgenic mice, nicotine produced a strong increase in event amplitude (KS test).

Nicotinic stimulation produced a strong increase of IPSCs frequency on RSNP neurons in both control and double transgenic mice

As described in the previous sections, after 5 minutes of spontaneous IPSCs recordings on RSNP neurons, we applied 10 μ M nicotine to stimulate nAChRs activation. Also in this case, saturating concentrations of AP5 and CNQX were used to abolish glutamatergic currents recording during our experiments. In **Figure 19A and 19B**, voltage-clamp recordings in the indicated conditions and a time course plot of IPSCs at 10 s intervals of two representative experiments are respectively shown. As a result of nicotine administration, we observed an approximate three-fold increase in events frequency in both control (*upper panel*) and double transgenic mice (*lower panel*) at steady-state conditions. This effect was largely reversed by 1 μ M DH β E (**Fig. 19C**). After washout, IPSCs frequency was usually restored as in control conditions. Full statistics are reported in figure legends.

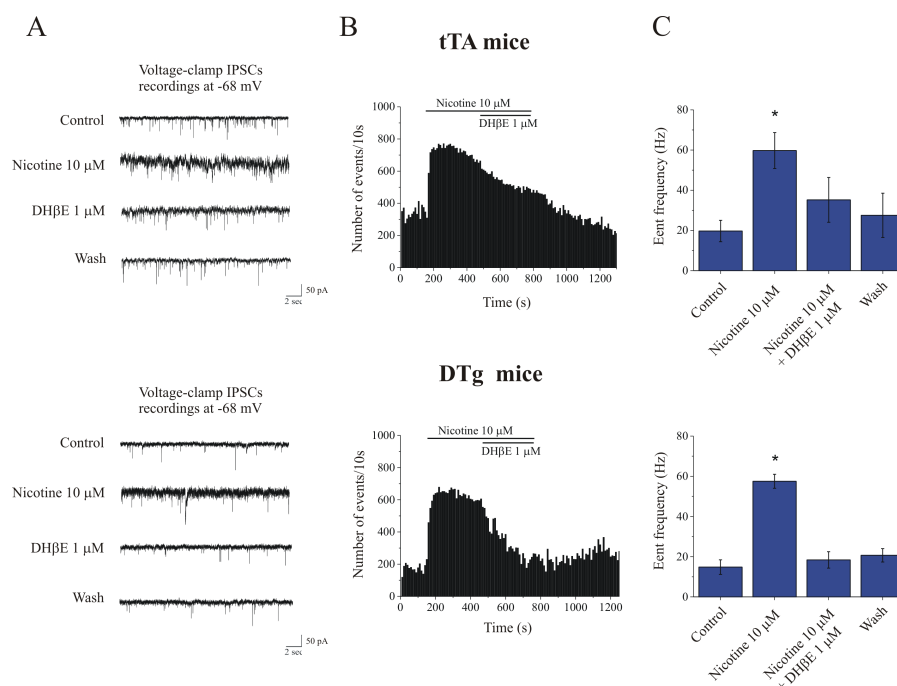


Figure 19. Nicotine strongly stimulates GABA release onto RSNP neurons in both control and β 2-V287L mice. *A*, Representative IPSCs in the indicated conditions, 10 μ M nicotine reversibly stimulated their frequency (tTA mice *upper panel*; β 2-V287L mice, *lower panel*). *B*, Time course of the IPSCs frequency in the same experiment, each bar corresponds to 10 s. *C upper panel*, Average IPSCs frequencies recorded in tTA mice (n=5) were 19.71 ± 5.32 Hz (Control), 59.75 ± 8.93 Hz (Nicotine 10 μ M; $p < 0.05$ compared to control), 35.21 ± 11.13 Hz (DH β E 1 μ M), 27.52 ± 11.0 Hz (Washout). *Lower panel*, Average IPSCs frequencies recorded in β 2-V287L mice (n=5) were 14.81 ± 3.61 Hz (Control), 57.5 ± 3.47 Hz (Nicotine 10 μ M; $p < 0.05$ compared to control), 18.39 ± 4.07 Hz (DH β E 1 μ M), 20.67 ± 3.31 Hz (Washout).

After events frequency analysis we focused our attention on the amplitude distributions of the IPSCs recorded in layer V RSNP neurons (**Fig. 20A**). Compared to controls, nicotine application approximately doubled the GABAergic currents amplitude recorded during steady-state conditions in tTA mice and produced also a sizable increase in β 2-V287L mice. 1 μ M DH β E was always able to

reverse this effect (**Fig. 20B**). As a result, the inhibitory tone sensed by RSNP neurons appears to be slightly depressed in double transgenic mice, compared to control littermates.

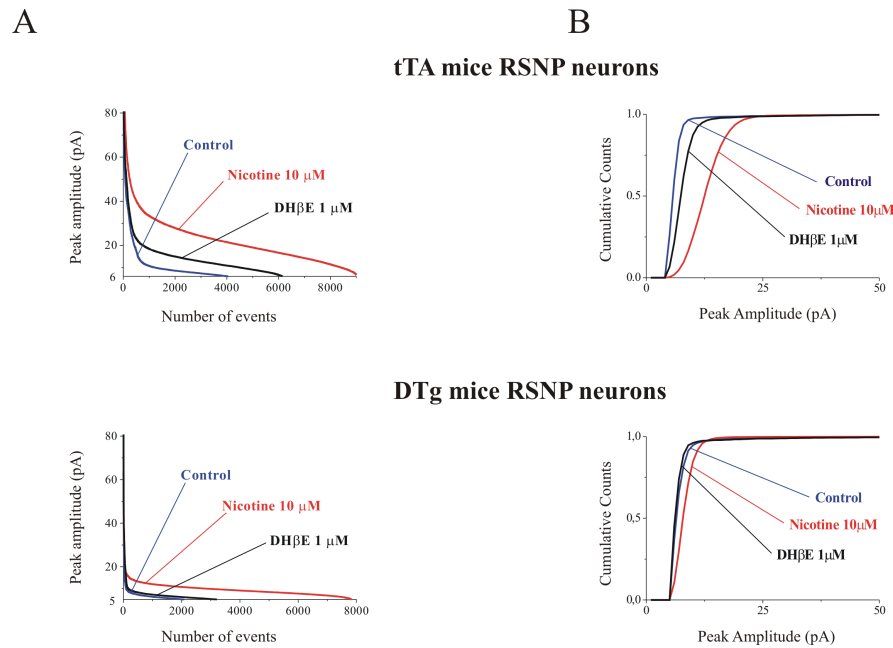


Figure 20. Nicotine administration produced a strong increase of frequency and amplitude of the IPSCs in both control and double transgenic mice. A, Plot showing ordered IPSCs amplitude during steady-state conditions in a representative experiment. Nicotine increased the number of events while DH β E reverted this effect. Peak amplitude also follows the same behaviour in both control (*upper panel*) and double transgenic mice (*lower panel*). **B** Amplitude distribution of the IPSCs recorded for 2 minutes in the indicated conditions, at the steady-state. In tTA mice, nicotine administration produced a strong increase in event amplitude and 1 μ M DH β E largely reversed this effect. In double transgenic mice, in 3 out to 5 neurons, nicotinic stimulation also produced an appreciable increase in event amplitude while 1 μ M DH β E completely abolished this effect (KS test).

Discussion

Here we show that, in layer V PFC, nAChRs activity is deeply involved in the regulation of circuits excitability. In fact, in pyramidal neurons, they have been found not only at the presynaptical level, where they can enhance glutamate release, but also on the soma of at least the 50% of the cells, where they can produce a slow desensitizing whole-cell current able to strongly depolarize them (Zolles et al., 2009). As expected, in $\beta 2$ -V287L mice these effects were amplified due to the presence of mutant hyperfunctional nAChRs. Interestingly, when we studied GABA release onto pyramidal neurons, we found that, although the nAChRs activity strongly increased the inhibitory tone, the effect was similar in control and double transgenic mice, showing a well balanced behaviour. In these experiments, 1 μ M DH β E completely abolished this effect, thus indicating that activation of heteromeric $\alpha 4\beta 2$ nAChRs accounts for the entire effect. These results show that, in double transgenic mice, the net effect of activating nAChRs amounts to an overall increase in excitatory activity. We then focused our attention on the two major types of cells that compose the interneuronal compartment, the RSNP neurons and the FS interneurons. Here we found that, in RSNP neurons, nAChRs are highly expressed on the soma producing a whole-cell depolarizing current. This phenomenon, summing to spontaneous EPSCs, is able to strongly increase the small sized events thus masking the synaptic communication. This is particularly evident in RSNP neurons in which, in control conditions, we found a low spontaneous excitatory post-synaptic transmission while, after

nicotine exposure, we observed a huge increase in frequency of small sized events. This may make these interneurons very sensitive to volume transmission during periods of cholinergic stimulation. Moreover, in RSNP neurons, we observed a powerful increase in IPSCs frequency after the application of 10 μ M nicotine. Also in this case, there were no major differences between control and double transgenic mice, however, looking at the amplitude of these signals, we attested a slight depression of the inhibitory transmission in affected animals. In FS interneurons, nicotine administration elicited a small transient increase in EPSCs frequency in both control and double transgenic animals. However, during control conditions, we observed an halved excitatory transmission in β 2-V287L mice compared to controls. Focusing on GABA release, we found an opposite behaviour between tTA and β 2-V287L mice. In the former, nAChRs stimulation produced an almost doubling of the recorded inhibitory signals, whereas, in double transgenic mice, we observed an initial high IPSCs frequency during control conditions that was halved by nicotine administration. All together, these data suggest the presence of an altered structure of synaptic connections between interneurons.

Framing our results in the context of the cortical microcircuit

By looking at our steady-state results, we were able to obtain a global overview on the excitability of different points of the network during a given experimental conditions. Moreover, we schematized these results in the context of the local cortical microcircuit (**Fig. 21**). We thus considered the output excitatory unit of the cortical microcircuit, the V layer pyramidal neuron, surrounded by interneurons. Following morphological criteria, we connected the FS interneurons exclusively to the perisomatic region of pyramidal neurons, whereas RSNP neurons were connected to the dendritic arborizations of pyramidal neurons and other interneurons. We also inserted in our scheme another interneuron identified by a question mark (?) to represent the other types of interneurons that we disregarded during our study, but surely participate in the overall structure. After drawing the microcircuit, we inserted our results relative to EPSCs and IPSCs frequency. In particular, we focused on control steady-state conditions because they highly resemble the physiological conditions observed during the stage II of sleep. In fact, literature reports a low cholinergic tone in PFC during this period (Jones, 2008). The resulting schemes is represented in **Figure 21**. In both tTA and double transgenic mice pyramidal neurons, we observed minor differences in the EPSCs frequencies between cells expressing or not somatic $\alpha 4\beta 2^*$ nAChRs. We then looked at the overall inhibitory tone sensed by pyramidal neurons during control conditions and we observed a perfect balance between tTA and double transgenic mice (respectively 4.56 ± 0.3 Hz

and 4.36 ± 0.86 Hz). These data seem to indicate that, in layer V pyramidal neurons, the basal excitatory/inhibitory ratio is scarcely altered in $\beta 2$ -V287L animals. However, in RSNP neurons and FS interneuron the situation was different. During control steady-state conditions, we recorded a very low excitatory input signal in RSNP neurons and, also in this case, in $\beta 2$ -V287L animals we found a little depression in these signals if compared to controls (respectively 2.26 ± 0.56 Hz and 3.72 ± 1.28 Hz). However, looking at the inhibitory counterpart, tTA mice RSNP neurons sensed an average signal that was 19.71 ± 5.32 Hz in frequency, while double transgenic littermates showed an IPSCs frequency of 14.81 ± 3.61 Hz. As a result, this lack of inhibition shifted the excitatory/inhibitory balance making RSNP neurons overactive in $\beta 2$ -V287L mice. On the contrary, in FS interneurons the excitatory inputs were nearly halved in double transgenic mice (tTA mice 43.82 ± 5.64 Hz and double transgenic mice 22.03 ± 6.51 Hz). Furthermore, in these cells we also observed a more than doubled inhibitory transmission in $\beta 2$ -V287L mice (7.7 ± 1.44 Hz) compared to controls (2.98 ± 0.76 Hz). One possibility is that expression of $\beta 2$ -V287L alters the number of reciprocal synaptic connections between the different populations of GABAergic interneurons, e.g. FS-FS cells or RSNP-FS cells, or the interneuronal populations not considered in this study (marked with ? symbol). The decrease of FS activity would make the local network hyperexcitable. At the present state, this is an hypothesis based on an incomplete knowledge of the precise structure of the local circuit and lacking some of the neurons participating to this architecture, as minor interneuronal subpopulations (Irregular Spiking, Late Spiking, Low

Threshold Spiking, represented in the scheme with a question mark). Nonetheless, some alterations in the reciprocal inhibition between interneurons appeared to be present in double transgenic mice. Overinhibition of FS interneurons, responsible for feed-forward inhibition, would well fit the ADNFLE symptoms. In fact, during stage II of sleep, the PFC is transiently stimulated by high frequency trains of action potentials (*sleep spindles* and *K-complexes*) coming from the thalamus (Helou et al., 2008). In this condition, an enhanced feed-back inhibition would only partially counteract the excess of excitatory activity and the depressed feed-forward inhibition would not probably be sufficient to stop these phenomena, letting them spread towards other cortical areas (epileptic seizures).

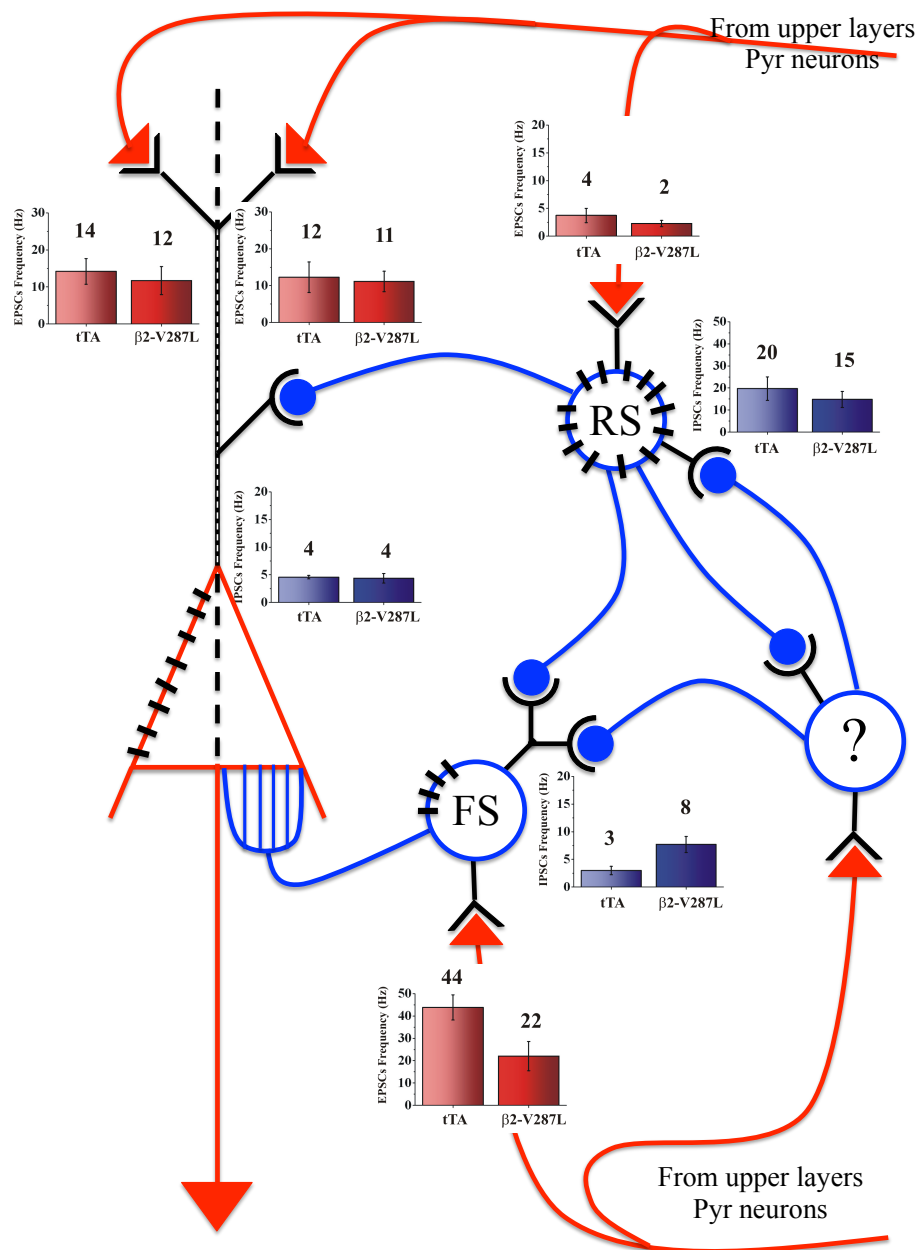


Figure 21. Local circuit scheme representing tTA mice and $\beta 2$ -V287L mice excitability during control conditions. Red plots represent EPSCs average frequencies (Hz) while blue plots represent IPSCs average frequencies (Hz). Black bars represent inactive somatic nAChRs. FS: Fast Spiking interneurons; RS: RSNP neurons.

Figure 22 illustrates the same microcircuit with the results obtained on synaptic transmission in the presence of 10 μ M nicotine. This condition mimics what may happen during bouts of ACh release in NREM 2 sleep. We focused on the excitatory transmission on pyramidal neurons that does not express somatic nAChRs and we found that in double transgenic mice these signals are unchanged as we saw in control condition. However, looking at neurons that express somatic nAChRs, we found the opposite situation and this is probably due to the effect of the somatic current that is doubled in β 2-V287L mice (0.22 ± 0.03 pA/pF), compared to control animals (0.44 ± 0.09 pA/pF). Moreover, comparing pyramidal neurons excitability, we observed a quite stable situation in tTA mice between cell expressing or not somatic nAChRs, while in double transgenic mice half of the cells showed a higher transmission. As observed in control conditions, the total amount of inhibition on pyramidal neurons is well balanced between the two animal cohorts tested (tTA 7.6 ± 0.79 Hz; β 2-V287L 7.32 ± 0.51 Hz). In RSNP neurons we found an excitatory transmission quite similar between control and double transgenic mice (respectively 49.84 ± 8.06 Hz and 52.17 ± 6.01 Hz) and this is probably due to the fact that these signals are almost exclusively produced by somatic nAChRs activation. This produced slow desensitizing somatic currents whose average current densities were 0.31 ± 0.04 pA/pF in tTA mice and 0.53 ± 0.15 pA/pF in β 2-V287L mice. Focusing on inhibitory transmission in these cells, we found very similar values for event frequencies (respectively 57.5 ± 3.47 Hz and 59.75 ± 8.93 Hz). In FS interneurons, we observed an excitatory transmission almost halved in β 2-V287L mice (31.3 ± 6.55 Hz)

compared to control littermates (55.49 ± 4.1 Hz). These results are probably poorly influenced by the small somatic currents observed in a fraction of FS interneurons (tTA mice 0.24 ± 0.06 pA/pF; $\beta 2$ -V287L mice 0.26 ± 0.07 pA/pF). The inhibitory transmission on these cells showed similar values in IPSCs frequencies in double transgenic mice compared to controls (respectively 4.02 ± 1.16 Hz and 3.84 ± 0.53 Hz).

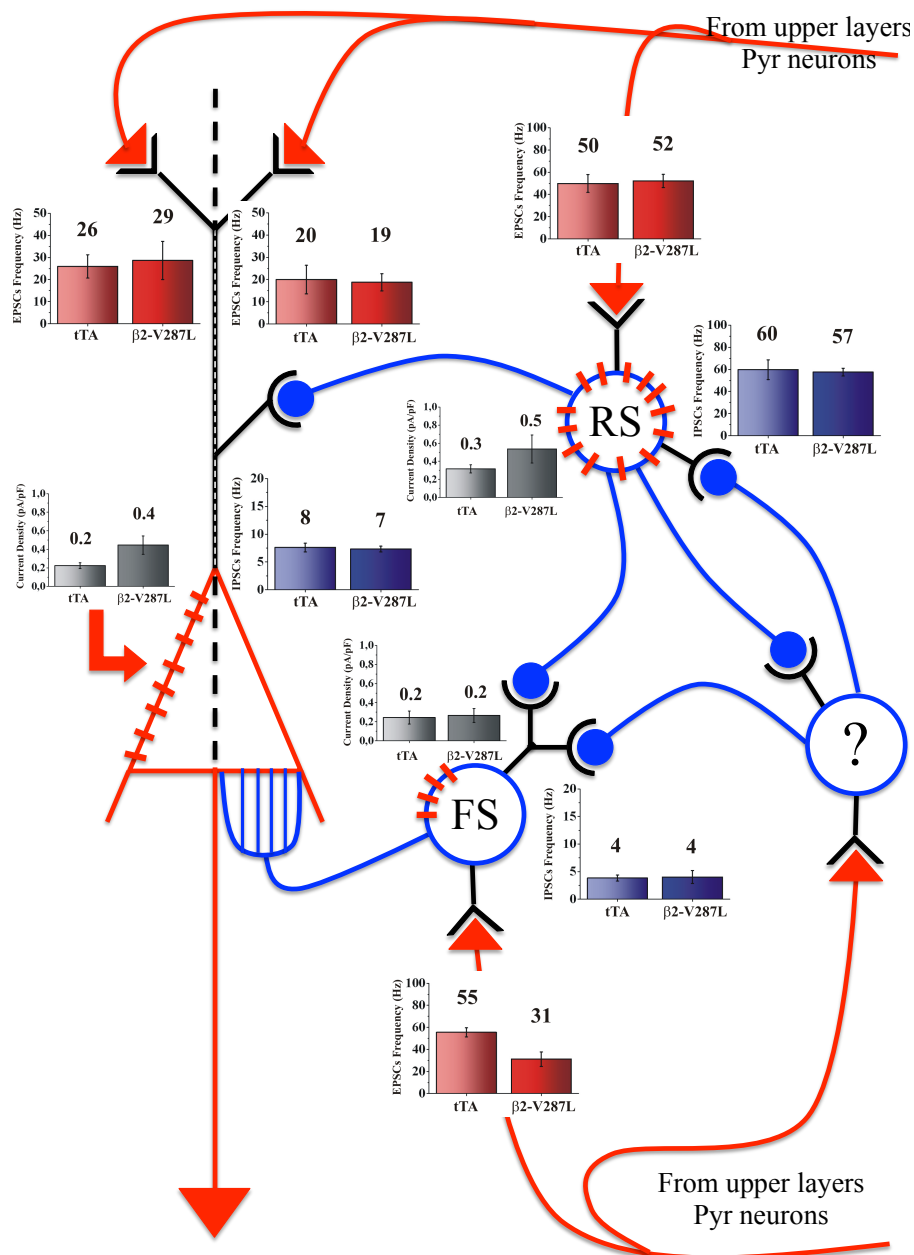


Figure 22. Local circuit scheme representing tTA mice and $\beta 2$ -V287L mice excitability during 10 μ M nicotine administration. Red plots represent EPSCs average frequencies (Hz) while blue plots represent IPSCs average frequencies (Hz). Grey plots represent the average somatic currents amplitudes (pA). Red bars represent activated somatic nAChRs. FS: Fast Spiking interneurons; RS: RSNP neurons.

nAChRs activity strongly influences glutamatergic and GABAergic transmission in both mature circuits and during neocortex development

The nervous system must balance excitatory and inhibitory input to constrain network activity levels within a proper dynamic range. This is a demanding requirement during development, when networks form and throughout adulthood as networks respond to constantly changing environments. Defects in the ability to sustain a proper balance of excitatory and inhibitory activity are characteristic of numerous neurological disorders such as epilepsy, schizophrenia, and autism (Rubenstein et al., 2003; Kehrer et al., 2008; Lewis et al., 2012). A variety of homeostatic mechanisms appear to be critical for balancing excitatory and inhibitory activity in a network. These operate at the level of individual neurons, regulating their excitability by adjusting the numbers and types of ion channels, and at the level of synaptic connections, determining the relative numbers of excitatory and inhibitory connections. Nicotinic cholinergic signaling is well positioned to contribute at both levels because it appears early in development, extends across much of the nervous system, and modulates transmission at many kinds of synapses (Mechawar et al., 2000). Further, it is known to influence the ratio of excitatory-to-inhibitory synapses formed on neurons during development. GABAergic inhibitory neurons are likely to be key for maintaining network homeostasis (limiting excitatory output), and nicotinic signaling is known to prominently regulate the activity of several GABAergic neuronal subtypes (Ge et al., 2006). Here we showed that

nAChRs can regulate excitability through enhancement of both glutamate and GABA release in the Fr2 region layer V prefrontal cortex. This mechanism is exerted by heteromeric nAChRs positioned at presynaptical level in two principal ways: first, their activation promotes Ca^{2+} influx which can directly stimulate the release of neurotransmitter vesicles; second, the membrane depolarization produced by cations entering through nAChRs can induce other voltage gated calcium channels opening and the further release of Ca^{2+} from intracellular storages (Marchi and Grilli, 2010). Moreover, in more than a half of the recorded neurons, a slow desensitizing inward current was found. This suggests a conspicuous expression of heteromeric nAChRs on the somatic compartment of all the three neuronal types considered in this study (Pyramidal neurons 55%, RSNP neurons 100% and FS interneurons 50%). This phenomenon has a dualistic effect. On one side, somatic membrane depolarization can facilitate reaching the firing threshold. On the other side, the raising of $[\text{Ca}^{2+}]_i$ may induce the activation of other ion channels or modulate transcription through second messenger cascades. Activation of nAChRs on the somatic compartment is not a rare event because the extracellular concentration of ACh is not only produced by neurotransmitter spillover from synapses. In fact, it has been reported that, in the neocortex, a good portion of cholinergic innervation is non-synaptic, which causes significant paracrine transmission (Agnati et al., 1995; Sarter et al., 2009). All these mechanisms are able to reinforce neuronal activity in a short-term way, conferring to ACh the role of booster. Experiments conducted in heterologous systems expressing mutant $\beta 2\text{-V287L}^*$ nAChRs have

revealed altered electrophysiological properties. In fact, mutant receptors are more sensitive to the agonist, thus increasing the probability of their opening at relatively low concentrations of ACh, as in volume transmission. Moreover, a slower desensitization kinetics have been reported for $\beta 2$ -V287L nAChRs compared to the Wt counterpart (De Fusco et al., 2000) at least in expression systems. This may amplify both the cation influx and calcium concentration in the cytoplasm of double transgenic mice neurons. It has also been reported that nAChRs containing mutant $\beta 2$ -V287L subunits are less sensitive to $[Ca^{2+}]_o$, thus promoting their activation during periods of intense neuronal activity (Rodrigues-Pinguet et al., 2003, 2005). All these alterations of the electrophysiological features of heteromeric nAChRs produced by missense mutation make them hyperfunctional. These observations well fit some of our results, like the generally enhanced neurotransmitter release at synaptical level and the increased whole-cell nicotinic currents at somatic level observed in $\beta 2$ -V287L mice compared to controls. However, other findings emerging from the network interpretation of our data (as diffuse reduction of the excitatory tone and the dramatical decrease of FS interneurons excitability in control conditions) suggest the occurrence of extensive rearrangements of neuronal connectivity which cannot be directly explained by the expression of hyperfunctional nAChRs alone. Several mechanisms through which nAChRs can reshape neuronal connections in a long-term way have been reported in literature. For example, homomeric $\alpha 7^*$ nAChRs have been found to be more permeable to calcium than NMDA receptors and insensitive to voltage dependent Mg^{+} blockade (e.g. Castro and Albuquerque, 1995; Rogers

and Dani, 1995), thereby providing a major route of Ca^{2+} entry at rest or hyperpolarized membrane potentials (Mulle et al., 1992; Rathouz and Berg, 1994). These features confer to homomeric receptors the ability to induce long-term potentiation (LTP) of glutamatergic transmission, a possible mechanism through which higher Ca^{2+} influx through our hyperfunctional receptors could exert its effect (Role and Berg, 1996; John and Berg, 2015; Molas and Dierssen 2014;). Moreover, $\beta 2^*$ -nAChRs are considered essential for acquisition of normal numbers of dendritic spines during development. Mice constitutively lacking the $\beta 2$ -nAChR gene have fewer dendritic spines than do age-matched wild-type mice. Activation of $\beta 2^*$ -nAChRs by nicotine either *in vivo* or in organotypic slice culture quickly elevates the number of spines. Absence of $\beta 2^*$ -nAChRs *in vivo* causes a disproportionate number of glutamatergic synapses to be localized on dendritic shafts, rather than on spines as occurs in wild type. This shift in synapse location is found both in the hippocampus and cortex (Lozada et al., 2012). The experiments conducted by Giorgio Casari and his colleagues in our murine model indicated that the silencing of mutant receptors expression after P15 is not sufficient to recover the pathological phenotype (Manfredi et al., 2009). Therefore, transgene expression during early stages of neuronal circuitry development is needed to induce permanent network alterations which are essential to trigger epileptiform activity. Although the specific nAChR roles during neural circuit wiring are still largely unknown, the spontaneous nAChR activity was reported to regulate the developmental switch between the excitatory and inhibitory roles of GABA, a process considered pivotal in the shaping of neuronal connections and

excitatory/inhibitory balance establishment (Liu et al., 2006). The latter transition depends on the progressive substitution of the transporter NKCC1, which absorbs Cl^- and is mainly expressed in early stages, with KCC2, which extrudes Cl^- and is expressed at later stages (Rivera et al., 1999; Ben-Ari et al., 2012). Both homo- and heteromeric nAChR activity seem to regulate expression of these transporters (Liu et al., 2006). Precise timing of early GABAergic excitation is important for early neuronal development and integration into circuits (Ben-Ari et al., 2012; Ge et al., 2006). Summarizing, the heteromeric nAChRs seem to be deeply involved in developmental processes leading to neuronal circuitry maturation and synapse stabilization as well as direct modulators of both excitatory and inhibitory transmission in the PFC. The overall interpretation of these mechanisms is not straightforward and its far from being fully elucidated. Further experiments will be necessary to shine a light on the many-sided role of mutant nAChRs in the pathogenesis of ADNFLE. Hopefully, this would provide molecular targets to develop a pharmacological line of intervention capable to counteract long-term effects of hyperfunctional $\beta 2$ -V287L nAChRs expression during development.

Future perspectives

The results shown in the present work suggest that the expression of hyperfunctional mutant $\beta 2$ -V287L nAChRs produces morphological alterations in the excitatory/inhibitory ratio, during early stages of development. However, further experiments could be conducted to better characterize the interneuronal populations involved in these effects. Moreover, electrophysiological investigations at different levels, from local circuit patch-clamp recordings to field potentials recordings, could be conducted on brain slices after induction of epileptiform activity with pharmacological agents. This could confirm if the mutation can produce effects on feed-back and feed-forward inhibition, thus promoting the insurgence or facilitating the spreading of epileptiform events. To this purpose, two different approaches will be attempted. One strategy consists in the administration of non-saturating doses of GABA_AR antagonists to induce disinhibition. In this way, modulation with nicotine or ACh would show possible variations of the triggering threshold for epileptiform activity in affected animals. In this context, extracellular field potentials recordings could be performed to correlate the paroxysmal activity to the results obtained with the whole-cell patch-clamp technique. As for the second approach, magnesium free external solution would be used to remove voltage dependent block from glutamate NMDA receptors, thus stimulating excitatory transmission without altering the GABAergic signals. In these conditions, whole-cell recordings would be performed on layer V pyramidal neurons, clamping membrane potential at -30 mV. This value is half-way between the GABA_A and

glutamate reversal potentials, and would permit the identification of inhibitory barrages preceding spontaneous ictal discharges (Trevelyan et al., 2007). In transgenic animals, this approach would highlight possible decrease in feed-forward inhibition, a crucial mechanism to restrain epileptic activity spreading. Other efforts could be addressed to understand how much of the pathogenic process is due to the direct effect of the impaired cholinergic transmission, or to possible alterations in the circuit maturation during early developmental stages. In this direction, we could seek to dissect the complex consequences of $\beta 2$ -V287L nAChRs expression isolating the direct effect produced by the gain of receptor function from the alterations occurring during circuit maturation. To this purpose, exploiting the conditional expression model, we would administer doxycycline to double transgenic mice from birth to postnatal day 15. This approach would generate another category of subject animals composed by healthy adult mice with the pathological expression of mutant nAChRs but no morphological alterations in the cytoarchitecture and synaptical connections of local circuits. In this context, patch-clamp recordings would provide a quantification of the direct impact of $\beta 2$ -V287L nAChRs expression on excitatory and inhibitory transmission. These findings, compared to the results obtained from untreated mice, would help us understand the real weight of the rearrangements occurring during synapse stabilization, unraveling any developmental alteration in excitatory and inhibitory connections. Interdisciplinary approaches, in collaboration with other research groups, will be directed to determine any modification of the well-known switching process of GABA neurotransmitter from excitatory to inhibitory function, a

crucial process for the correct neuronal network connectivity development (Kaila et al., 2014; Li and Xu, 2008; Ge et al., 2006; Chamma et al., 2012; Li et al., 2007). For example, Western-blot analysis could be performed to track the levels of expression of NKCC1 and KCC2 Cl^- co-transporters during the first two postnatal weeks in both control and $\beta 2$ -V287L mice. Moreover, neuroimaging confocal microscopy techniques could be also used to follow the process of down- and up-regulation of the expression of Cl^- co-transporters in the Fr2 region of developing control and affected mice. Immunostaining of PFC brain slices with antibodies targeting NKCC1 and KCC2 proteins could be overlapped with other markers specific for the major neuronal populations, i.e. glutamate and GABA vesicular transporters, heteromeric nAChRs, parvalbumin, somatostatin and others. This approach could superimpose the temporal evolution of the functional switch of GABA neurotransmission with precise informations on the neuroanatomical remodeling of the cortical networks, thus providing valuable insights on these crucial developmental stages. Hopefully, this combined approach would also enlighten possible morphological alterations involving the cytoarchitecture of layer V PFC in $\beta 2$ -V287L transgenic animals. Particular care would be addressed to the distribution of excitatory and inhibitory synapical contacts in order to correlate anatomical informations with electrophysiologic data.

Proximity to cure

Nowadays, the only pharmacological strategy to treat ADNFLE patients consists in administration of classical antiepileptic drugs like carbamazepine (Picard et al., 1999) and oxcarbazepine (Di Resta et al., 2010) to prevent hyperexcitability. Unfortunately, these compounds can only counteract the insurgence of seizures, thus leaving the psychiatric symptoms and mental disabilities unaltered (Derry et al., 2008). Moreover, this line of intervention is ineffective in a large portion of the ADNFLE patients. Our data suggest the occurrence of cortical circuitry alterations in layer V of the Fr2 region (PFC) in mutant mice expressing $\beta 2$ -V287L* nAChRs. Moreover, in this conditional murine model, the suppression of the transgene expression after P15 is not able to revert the epileptic phenotype (Manfredi et al., 2009). These findings narrow the temporal range for a preventive pharmacological intervention to a couple of weeks after birth in mice and a few months in humans. In recent years, Gullo et al. (2014) established an *in vitro* model of ADNFLE using the multi-electrode array (Gullo et al., 2014). This latter is a high-throughput electrophysiological technique that allows the long-term recording of the spontaneous electrical activity of primary cultured cortical neurons. In particular, neocortical cultures from mice expressing $\beta 2$ -V287L display chronic spontaneous epileptiform activity, not requiring pre-treatment with convulsants. This opens the way to study *in vitro* the role of $\beta 2$ -V287L or other ADNFLE subunits on synaptic formation and how it can be pharmacologically modulated to attempt preventive therapeutic approaches to this and other epileptic syndromes. In this

direction, some possible candidates could be modulators of the expression of chloride cotransporters. In fact, the administration of furosemide during the first postnatal weeks, in a rat model carrying an ADNFLE $\alpha 4$ -linked mutation, has shown positive results preventing idiopathic epileptic activity (Yamada et al., 2007). Another strategy could involve the administration of low doses of heteromeric nAChRs agonists in order to promote their desensitization. This approach would compensate the gain of receptor function avoiding possible side effects in other tissues. In case of identification of promising compounds deriving from *in vitro* experimentation, the administration of therapeutical doses of the drugs can be performed during the first two postnatal weeks on both control and affected mice. Electrocorticography could be used to monitor the neuronal activity of treated animals in order to ascertain the possible onset of ictal activities.

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