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Nicotinic acetylcholine receptors and epilepsy

Andrea Becchetti [a,*], Laura Clara Grandi [a], Marta Cerina [a], Alida Amadeo [b]

[a] Department of Biotechnology and Biosciences, and NeuroMI (Milan Center of Neuroscience), University of Milano-Bicocca, Piazza della Scienza 2, Milano 20126, Italy
[b] Department of Biosciences, University of Milano, Via Celoria 26, Milano 20133, Italy

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ABSTRACT

Despite recent advances in understanding the causes of epilepsy, especially the genetic, comprehending the biological mechanisms that lead to the epileptic phenotype remains difficult. A paradigmatic case is constituted by the epilepsies caused by altered neuronal nicotinic acetylcholine receptors (nAChRs), which exert complex physiological functions in mature as well as developing brain. The ascending cholinergic projections exert potent control of forebrain excitability, and wide evidence implicates nAChR dysregulation as both cause and effect of epileptiform activity. First, tonic-clonic seizures are triggered by administration of high doses of nicotinic agonists, whereas non-convulsive doses have kindling effects. Second, sleep-related epilepsy can be caused by mutations on genes encoding nAChR subunits widely expressed in the forebrain (*CHRNA4*, *CHRNB2*, *CHRNA2*). Third, in animal models of acquired epilepsy, complex time-dependent alterations in cholinergic innervation are observed following repeated seizures. Heteromeric nAChRs are central players in epileptogenesis. Evidence is wide for autosomal dominant sleep-related hypermotor epilepsy (ADSHE). Studies of ADSHE-linked nAChR subunits in expression systems suggest that the epileptogenic process is promoted by overactive receptors. Investigation in animal models of ADSHE indicates that expression of mutant nAChRs can lead to lifelong hyperexcitability by altering i) the function of GABAergic populations in the mature neocortex and thalamus, ii) synaptic architecture during synaptogenesis. Understanding the balance of the epileptogenic effects in adult and developing networks is essential to plan rational therapy at different ages. Combining this knowledge with a deeper understanding of the functional and pharmacological properties of individual mutations will advance precision and personalized medicine in nAChR-dependent epilepsy.

1. Introduction

Epilepsy comprises a group of syndromes characterized by the chronic occurrence of *seizures*. Following the International League Against Epilepsy, a seizure can be defined as a *transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain* [1]. Epilepsy is a very common disease, affecting about 3% of the human population [2,3]. Although different epileptic

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; ADNFLE, (autosomal dominant) nocturnal frontal lobe epilepsy; ADSHE, (autosomal dominant) sleep-related hypermotor epilepsy; AED, anti-epileptic drug; BDNF, brain-derived neurotrophic factor; BLA, basolateral amygdala; CA1, cornu ammonis 1; ChAT, choline acetyltransferase; *CHRNA2*, gene encoding α2 nAChR subunit; *CHRNA4*, gene encoding α4 nAChR subunit; *CHRNB2*, gene encoding β2 nAChR subunit; *CHRNA7*, gene encoding α7 nAChR subunit; CNS, central nervous system; DHβE, dihydro-β-erythroidine; DR, dorsal raphe; EC, enthorhinal cortex; EC_{50}, half-effective concentration; E_{GABA}, reversal potential of GABAergic currents; EEG, electroencephalography; ErbB4, Erb-B2 Receptor Tyrosine Kinase 4; HDB, horizontal diagonal band nucleus; hiPSC, human induced pluripotent stem cell; ICD, intracellular domain; IP_3, inositol trisphosphate; KCC2, K+ /Cl- cotransporter-2; mAChR, muscarinic ACh receptor; LC, locus coeruleus; LDT, laterodorsal tegmental nucleus; M2/Fr2, secondary motor area/frontal area 2; MLA, methyllycaconitine; MS, medial septal nucleus; NACHO, nAChR chaperon TMEM35a; nAChR, nicotinic ACh receptor; NBM, nucleus basalis magnocellularis; NKCC1, Na+ /K+ /Cl- cotransporter-1; NMDA, N-methyl-D-aspartate; NRG, neuregulin; P_{Ca}, permeability to Ca2 +; P_{Na}, permeability to Na+; PFC, prefrontal cortex; PIP_2, phosphatidyl inositol 4,5 bisphosphate; PLC, phospholipase C; P_o, open probability; PPT, pedunculopontine nucleus; PTZ, pentylentetrazole; PV, parvalbumin; REM, rapid-eye-movements; RTN, reticular thalamic nucleus; SE, status epilepticus; SHE, sleep-related hypermotor epilepsy; SI, substantia innominata; SOM, somatostatin; TC, thalamocortical; TMD, transmembrane domain; TMS, transcranial magnetic stimulation; VAChT, vesicular ACh transporter; VDB, vertical diagonal band nucleus; V_{rest}, resting membrane potential.

* Correspondence to: Department of Biotechnology and Biosciences, University of Milano-Bicocca, Piazza della Scienza 2, 20126 Milano, Italy.
E-mail addresses: andrea.becchetti@unimib.it (A. Becchetti), laura.grandi@unimib.it (L.C. Grandi), m.cerina2@campus.unimib.it (M. Cerina), alida.amadeo@unimi.it (A. Amadeo).

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syndromes share the recurrency of spontaneous seizures, they are otherwise heterogeneous in terms of causes, pathogenetic mechanisms and clinical manifestation [2]. According to the recent classification, one can distinguish focal onset seizures, limited to a restricted area of one hemisphere, from generalized onset seizures, involving both hemispheres. The latter may involve both hemispheres since the beginning ('primary generalized seizures', in the older terminology), or arise from a focal event ('focal to bilateral tonic-clonic' seizures, substituting the older term 'secondary generalization') [4].

As other complex pathologies, epileptic syndromes are determined by a combination of acquired and genetic factors [5]. Nonetheless, a convenient distinction can be drawn between *symptomatic* epilepsies (i.e., caused by identified previous insults) and *idiopathic* epilepsies (i.e., arisen independently of any identifiable lesion or macroscopic abnormality). In the former, seizures are promoted by structural changes induced by stroke, tumor, infection or trauma, or by metabolic or immune disorders [6]. In idiopathic epilepsies, a genetic predisposition seems to be essential [5]. The great recent advances in gene sequencing technology have pointed to a complex genetic architecture in epilepsy, involving poly- or oligogenic risk determined by multiple genes whose cooperating action determines the epileptogenic process [3]. Although single-gene epilepsies are individually rare, overall they offer invaluable information, as they define an ensemble of etiologic factors that point to a spectrum of possible pathogenetic mechanisms. Many epilepsy genes turned out to encode ion channels or other proteins implicated in neuronal excitability and synaptic transmission. In fact, the first gene to be associated to idiopathic epilepsy was *CHRNA4* [7], encoding the α4 subunit of the neuronal nicotinic acetylcholine receptor (nAChR; see *Section 5*).

2. The cholinergic system in epilepsy

Acetylcholine (ACh) is the main excitatory neurotransmitter in the peripheral nervous system and is a major regulator of excitability in the forebrain [8,9]. ACh is synthesized by cholinergic nuclei mainly located in the brainstem and basal forebrain (Fig. 1), as well as by interspersed striatal and cortical cholinergic cells. The brainstem pedunculopontine (PPT) and lateral dorsal tegmental (LDT) nuclei mainly innervate the thalamus, the midbrain dopaminergic areas and the striatal complex [10]. The basal forebrain nuclei give rise to the main projections to the neocortex, hippocampus and amygdala [11]. Interspersed cholinergic interneurons are abundant in the *corpus striatum* [12], but are also present in neocortex [13,14] and, possibly, hippocampus [15]. The cholinergic nuclei are mostly active during wakefulness and the rapid-eye-movement (REM) sleep, but almost silent during non-REM sleep [16,17]. Hence, they constitute major regulators of the cortical tone and thus of arousal, attention, and cognitive performance during wakefulness. ACh brings about its functional effects on target cells by activating both ionotropic nAChRs and metabotropic (muscarinic, mAChRs) receptors. For general overviews of the manifold physiological roles of these receptors in the brain, see refs. [9,18–20].

From an epileptological point of view, both nAChRs and mAChRs may be involved in epilepsy, as hyperstimulation of either can cause seizures. For instance, hyper-activation of mAChRs underlies the classic pilocarpine model of temporal lobe epilepsy. Pilocarpine is a cholinergic agonist with dominant muscarinic action [21]. When applied intra-cerebrally at high concentrations (300–400 mg/Kg, in rats) [22] it causes transient *status epilepticus* (SE) by activation of M1 mAChRs [23]. After a latent period, such treatment leads to chronic epileptic seizures. This important experimental model has been extensively discussed by others [22,24], and will not be recapitulated here. On the other side, nAChR activation can cause acute nicotine-dependent seizures [25]. In addition, nicotine is an efficacious kindling agent at sub-convulsive doses [26]. The cellular mechanisms underlying these effects are still matter of debate and could offer some hints about the causes of tobacco-dependent epilepsy. Specific information about the implication of different nAChR subtypes in epilepsy has come from the identification of mutations in nAChR subunit genes, linked to genetic sleep-related epilepsy [3,7,27]. Altogether, these investigations suggest that nAChRs can produce epileptogenic effects at both developing and adult stages, because they exert regulatory roles on synaptogenesis as well as on the excitability of mature synaptic circuit. Finally, several experimental models of epilepsy show that the cholinergic system may be altered *because* of seizure recurrence, which may lead to cholinergic neuron degeneration accompanied by a deficit of cholinergic innervation. In turn, these alterations modify the expression of cholinergic receptors in target cells [28].

In the following, we briefly review the main properties of the nAChR subtypes that appear to be more relevant in human epilepsy and in the main experimental models of the disease (*Section 3*). Next, we discuss nicotine-induced seizures (*Section 4*) and the possible mechanisms by which mutant nAChRs can lead to sleep-related epilepsy (*Section 5*). How epileptic seizures may lead to long-term alterations of the cholinergic system is analysed in *Section 6* . Finally, we review some of the

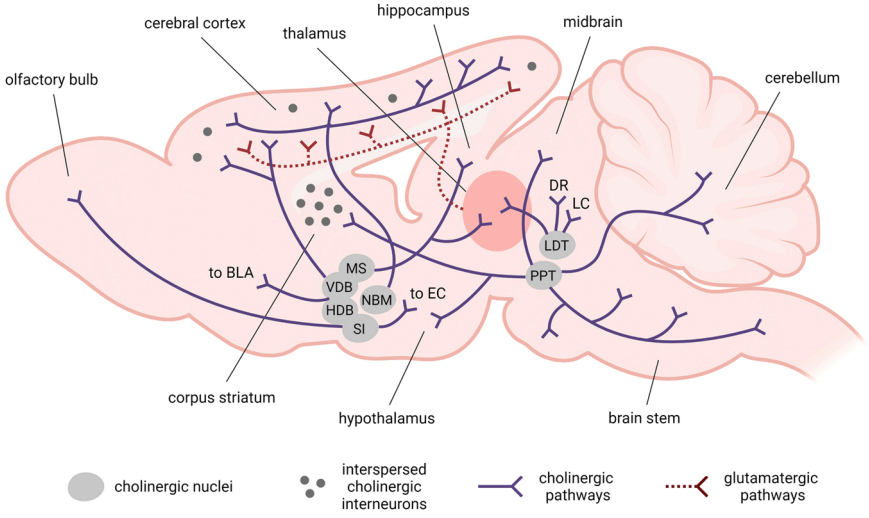

Fig. 1. Cholinergic pathways in murine brain. BLA, basolateral amygdala; DR, dorsal raphe; EC, enthorhinal cortex; HDB, horizontal diagonal band nucleus; LC, locus coeruleus; LDT, lateral tegmental nucleus; MS, medial septal nucleus; NBM, nucleus basalis magnocellularis; PPT, pedunculopontine nucleus; SI, substantia innominata; VDB, vertical diagonal band nucleus. The figure was prepared with *BioRender* (BioRender.com).

pharmacological and therapeutic implications of recent work and point to perspectives for precision and personalized medicine (*Section 7*).

3. Main properties of the nAChR subtypes implicated in epilepsy

The nAChRs are pentameric ion channels permeable to cations, composed of different combinations of α and β subunits, which can assemble in homo- or heteromeric form. The recent availability of high-resolution structures has brought major advances in the comprehension of the heteromeric nAChR organization [29,30]. The nAChR subunits comprise a large extracellular N-terminal domain, four consecutive transmembrane α-helix segments (M1 to M4) that assemble in a bundle to form a bulky transmembrane domain (TMD), and a short variable extracellular C-terminal segment. Fig. 2 offers a simplified scheme of a typical nAChR subunit. The long intracellular loop between M3 and M4 constitutes the intracellular domain (ICD), which regulates channel function and trafficking by post-translational modification and protein-protein interaction [30]. In heteromeric receptors, the main portion of the ligand-binding site is provided by the extracellular domain of a principal α subunit (e.g., α4) and is complemented by a binding surface located on a complementary subunit (e.g., β2) [29,30]. In homomeric receptors, such as $(\alpha7)_5$, the same type of subunit exerts both roles. The number of binding sites thus depends on subunit composition. The five M2 domains line the channel pore and participate in the gating process. By convention, the M2 amino acid residues are numbered from the − 1' residue, which forms the selectivity filter at the cytoplasmic mouth of the pore, to the 20' residue at the extracellular end [31]. Agonist binding drives a complex TMD conformational change that opens the channel (*activation*) by tilting the M2 helices. The resting channel gate is located ~5 nm away from the neurotransmitter binding site and is constituted by a constriction provided by 2 or 3 rings of hydrophobic amino acid residues, and especially a leucine ring at the 9' position [30,31]. Several amino acid loops are critical in transferring the activation signal from the agonist-binding pocket to the activation gate, including 3 loops in the extracellular domain, the pre-M1 linker, and the M2-M3 loop [30]. If the agonist is quickly removed after activation, the ion channel closes (*deactivation*). Otherwise, activation is followed by *desensitization*, a process by which channels enter into a non-conductive state different from the resting closed state. When desensitized, receptors are refractory to activation. The desensitization gate is different from the resting (activation) gate and is thought to be formed by a ring of glutamate residues towards the cytoplasmic end of the pore [30,31]. The desensitization kinetics is a complex process, comprising rapid and slow (up to minutes) components, which differ among nAChR subtypes [31].

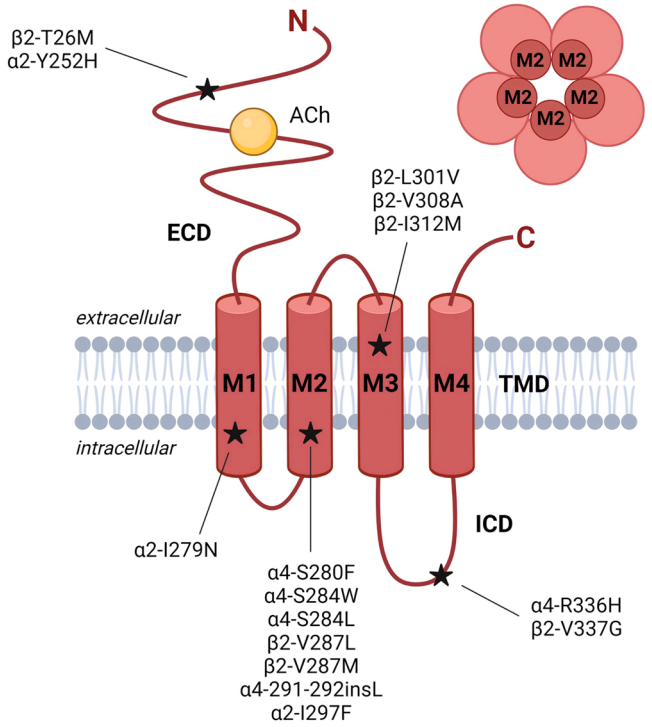

Fig. 2. Main structural domains in nAChR subunits with (AD)SHE mutations. Mutations identified in familial or sporadic SHE are only indicated if functional studies in expression systems are available. References are given in the main text. ECD, extracellular domain; TMD, transmembrane domain; ICD, intracellular domain. The figure was prepared with *BioRender* (BioRender.com).

The homomeric $(\alpha7)_5$ and the heteromeric α4β2 are the main nicotinic subtypes in the mammalian forebrain [32]. The former has low affinity for ACh and nicotine, with half-effective concentration (EC_{50}) of ~100–200 μM, and high permeability to Ca^{2+} (the permeability ratio between Ca^{2+} and Na^{+}, P_{Ca}/P_{Na}, is in the order of 10; [33]). Moreover, α7 receptors undergo rapid desensitization in the presence of the agonist. When activated by 1 mM ACh, the time constant of exponential current decay (τ_d) is lower than 1 ms [34]. In contrast, heteromeric nAChRs generally have a much higher sensitivity to the agonists. The α4β2 receptors expressed in *Xenopus* oocytes and mammalian cell lines display a biphasic concentration-response relationship, with a high-affinity ($EC_{50} \approx 1$ μM) and a low-affinity ($EC_{50} \approx 60$ μM) component [35–38]. These components are attributed, respectively, to the $(\alpha4)_2(\beta2)_3$ and the $(\alpha4)_3(\beta2)_2$ stoichiometries [39]. Moreover, α4β2 nAChRs have an apparent $P_{Ca}/P_{Na} \approx 2$ [40], with higher P_{Ca} in the $(\alpha4)_3(\beta2)_2$ stoichiometry [41]. Finally, desensitization is slower in heteromeric nAChRs. Activation of α4β2 receptors with saturating concentrations of ACh yields τ_d values in the order of 100 ms, in mammalian cell lines [38,42]. The main biophysical features of α4β2 and α7 nAChRs are summarized in Table 1.

The physiological picture is however complicated by the fact that α4β2 receptors can recruit other subunits. One of the most widespread is α5, serving as an accessory subunit (i.e., unable to bind the agonist) in adult and developing forebrain, where it increases α4β2 function without altering the total number of receptors [43–45]. A more restricted pattern of expression is displayed by α6 (found in *striatum*, visual pathway, and mesencephalic dopaminergic structures; [46]). It should be added that α7 and β2 subunits can associate to form functional receptors in expression systems [47] and in the basal forebrain cholinergic neurons [48]. The α7β2 subtype has functional properties closer to those typically found in heteromeric receptors, such as slow desensitization, and it may have a wider distribution than previously thought [49–52]. Whether these observations are relevant in the context of epilepsy is unknown.

In contrast with the above subunits, the distribution of α2 in the brain significantly differs among mammalian groups. In primates, α2 is widely expressed in the forebrain, where it is thought to mainly form α2β2 receptors [53–55]. However, its expression also largely overlaps with that of β4 [54,56,57], with which it can associate to form functional receptors [58]. In rodents, α2 expression is considerably more restricted [32]. Nonetheless, α2 could exert important regulatory roles even in rodent neocortex, as it is expressed in specific GABAergic cell populations of infragranular neocortex layers [59–61]. These recent

Table 1
Functional properties of the two main nAChR subtypes in the mammalian forebrain.

	Homomeric $(\alpha7)_5$	Heteromeric α4β2
Affinity for ACh and nicotine	EC_{50} ~ 100–200 μM	EC_{50} ~ 1 μM for $(\alpha4)_2(\beta2)_3$ EC_{50} ~ 60 μM for $(\alpha4)_3(\beta2)_2$
Permeability to Ca^{2+}	P_{Ca}/P_{Na} ~ 10	P_{Ca}/P_{Na} ~ 2
Desensitization	Rapid $\tau_d < 1$ ms	Slow τ_d in the order of 100 ms

observations offer some suggestions about how α2 may be involved in epileptogenesis (see *Sections 5 and 8*), although a detailed functional comparison with the human neocortex is lacking.

Nicotinic receptors produce excitatory effects on the target cellular compartments by two mechanisms (Fig. 3). First, channel opening causes membrane depolarization, as the reversal potential of the currents flowing through nAChRs is generally between − 10 and 0 mV [62]. Second, nAChR activation stimulates calcium signals because: i) depolarization can activate voltage-gated Ca^{2+} channels; ii) Ca^{2+} enters the cell through nAChRs [18]; iii) Ca^{2+} influx through these paths can stimulate Ca^{2+} release from intracellular stores [63–65]. The balance of these mechanisms depends on the nAChR subtype involved and can occur at the pre-, post- and extrasynaptic level. Moreover, recent work shows that α7 nAChRs can assume unconventional metabotropic roles, by stimulating heteromeric G proteins [65,66] and regulate IP_3-sensitive Ca^{2+} release [65–68]. Whether the latter mechanism extends to heteromeric nAChRs is unknown (Fig. 3). The net result of nAChR activation on a local circuit depends on the combined actions on excitatory and inhibitory neurons. Nevertheless, the overall effect of ACh throughout neocortex layers appears to be excitatory [69].

4. Nicotine-dependent seizures

Work started in the mid-twentieth century has revealed that in vivo application of high doses of nicotine (higher than 2–3 mg/Kg, in mice) induces tonic-clonic convulsions in mammals [70–72]. Moreover, nicotine overdose induced by self-application of multiple transdermal nicotine patches has been reported to cause seizures in a fraction of human patients, although evidence is still limited [73]. Subsequent results demonstrated that sub-convulsive doses of nicotine are effective as kindling agents, at least in mice [26]. Determining the underlying mechanisms would provide hints about the specific involvement of nAChR subtypes in seizures, and help to understand some of the nicotine-specific effects of tobacco, in which nicotine mixes with thousands of disparate compounds, some of which have pro-seizure effects [25,74]. However, drawing a unified interpretation from in vivo studies and clinical observations is difficult, given the wide range of applied drug concentrations. In acute pro-convulsion tests, nicotine is injected in the cerebral ventricles or peritoneum. Although detailed pharmacokinetic determinations are lacking, micromolar (at least) nicotine concentrations are probably reached in the brain, within the several minutes needed to induce seizures. Similar concentrations are attained by repeated drug administration. For instance, daily intraperitoneal nicotine injections of 2.5–5 mg/Kg in rats yield final concentrations in different brain regions of 5–7 μg/g (or mL) [75], i.e., higher than 10 μM. In contrast, cigarette smoking or single transdermal patches in humans induce intracerebral nicotine concentrations of 50–200 nM [76,77].

To interpret the basis of nicotine-induced seizures and understand the role of different nAChR subtypes during prolonged drug applications, one needs to consider the steady state levels of the applied compounds. At any given time, the average number of open channels equals the product of the total number of channels expressed in the plasma membrane (N) times the probability that a given channel is open (open probability, P_o). P_o depends on ligand concentration and time [62]. At the steady state, P_o is approximated by the product of the experimentally determined fraction of active channels times the fraction of non-desensitized channels. Plotting the resulting P_o against the agonist concentration gives a bell-shaped curve (Fig. 4) that defines the range of concentrations at which the average number of open channels is different from zero. This is sometimes named 'window current', by analogy with voltage-gated channels. At low agonist concentrations, P_o tends to zero, as most nAChRs are closed (resting state). At high agonist concentrations, P_o tends to zero, as nAChRs are mostly desensitized. At intermediate concentrations, $P_o \neq 0$, as a significant fraction of channels are simultaneously active and not desensitized. The precise dependence on concentration depends on receptor subtype. For both α7 and α4β2 nAChRs, the maximal steady state P_o is reached at micromolar nicotine concentrations (e.g., [78,79]). On the other hand, at nanomolar concentrations (up to 100 nM), a significant fraction of the high affinity heteromeric nAChRs are desensitized, while the low affinity α7 receptors are closed (but not desensitized). Finally, long-term exposure to nanomolar concentrations of nicotine produces a long-term increase of nAChR expression (thus changing N, instead of P_o), through different mechanisms [82]. The implications of these notions are discussed below.

4.1. Acute seizures induced by nicotine

In experimental animals, the pro-convulsant effects of nicotine are attributed to nAChR activation, as they are mimicked by a variety of nicotinic agonists and are largely blocked by wide-spectrum nicotinic antagonists, such as mecamylamine [72,81–83]. Work in transgenic mice is broadly consistent with this notion. First, the sensitivity to nicotine-induced seizures is higher in mice carrying mutant α7 receptors with slower desensitization [84,85], or mutant α4 subunits hypersensitive to the agonists [86]. Second, a protective effect is observed in mice deficient for subunits which tend to increase heteromeric receptor's

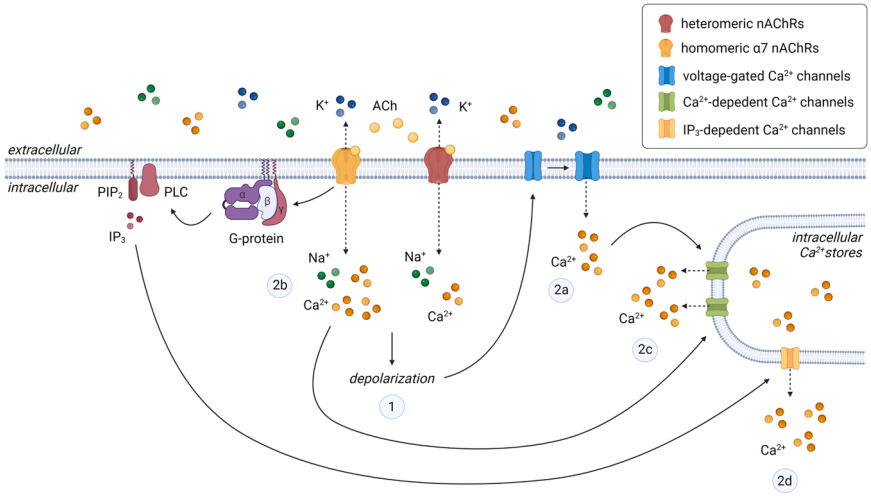

Fig. 3. Mechanisms of nAChR action in the cell. The scheme summarizes the main mechanisms by which homo- and heteromeric nAChRs control V_m and calcium signals. These actions can be exerted at pre-, post- or extra-synaptic level. IP_3, inositol trisphosphate; PIP_2, phosphatidyl inositol 4, 5 bisphosphate; PLC, phospholipase C. Reference are given in the main text. The figure was prepared with *BioRender* (BioRender.com).

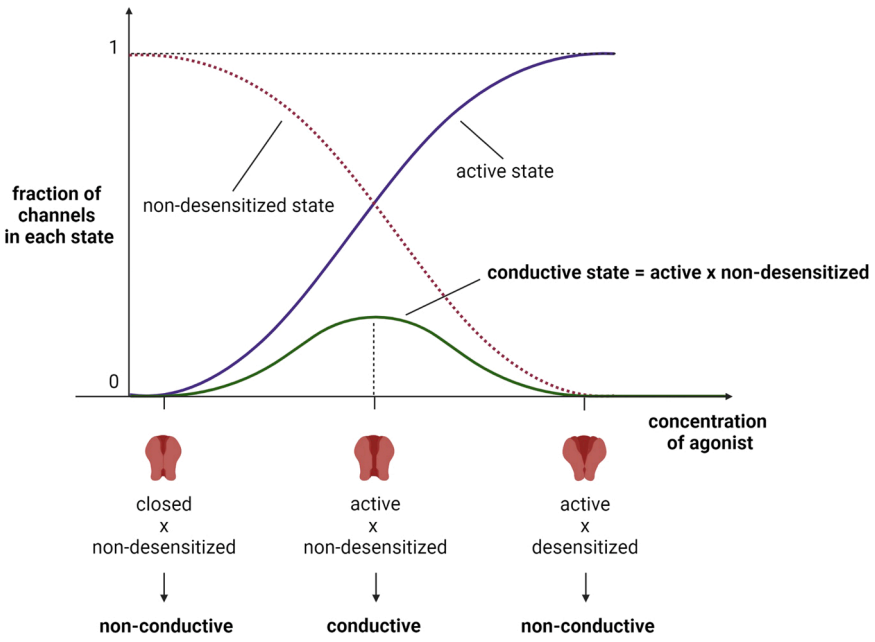

Fig. 4. Steady state activation and desensitization of ligand-gated channels against the agonist concentration. The graph is meant as a help to intuition and does not faithfully represent in a quantitative way the steady state properties of any specific nAChR subtype. The figure was prepared with *BioRender* (BioRender.com).

function, such as α5 [87], β4 [88], or both [88]. Such evidence also excludes that a major role in nicotine-dependent seizures is exerted by spurious effects of high concentrations of nicotinic ligands, such as direct or indirect modulation of K^+ channels [89,90].

And yet, unequivocally defining the contribution of different nAChR subunits in specific brain regions, in wild type condition, turned out to be challenging. Early electrophysiological and electroencephalographic (EEG) analyses in rats and rabbits suggested that nicotine-induced seizures had hippocampal origin [91–94]. As is well known, hippocampus is particularly susceptible to develop seizures. The CA1 region is liable to be entrained in seizure activity, although not generating it, because of a weaker efficacy of local inhibition [95]. Indeed, in mouse hippocampal slices, epileptiform activity in CA1 pyramidal cells can be induced by nicotine [96], but only at concentrations (0.8 mM) much higher than those likely to be reached in vivo, which suggests that the epileptogenic effect in vivo may depend on the integrity of the cerebral network that regulates hippocampal activity. In fact, the hippocampus expresses high amounts of nAChRs, in both pyramidal and GABAergic neurons, at pre- and postsynaptic sites [97,98]. Such a complex pattern of expression would be consistent with a special sensitivity to nicotine but makes it difficult to ascertain the cellular basis of nicotine-dependent seizures. Studies with specific nAChR antagonists and murine knockout models aimed to better define the relative importance of individual nAChR subunits. Nicotine-dependent seizures are more effectively inhibited by the α7 blocker methyllycaconitine (MLA) than by the α4-containing nAChR antagonist dihydro-β-erythroidine (DHβE) [72,81,83,85]. This led to initially surmise that seizures were caused by hyperactivation of α7 receptors located on glutamatergic terminals, which could boost excitatory activity in the hippocampus [72]. However, the above-mentioned work in murine strains expressing α4 subunits hypersensitive to the agonists [86], or defective for α5 or β4 [87,88], points to heteromeric nAChRs as major players in the development of nicotine-induced seizures. What is more, in α7-null mice the sensitivity to nicotine-induced seizures is unchanged, and no concomitant compensatory increase of other nicotinic subunits is observed [84,99]. Hence, evidence in genetically modified mice supports the hypothesis that heteromeric nAChRs give a significant contribution to nicotine-dependent seizures, which is consistent with the overall pharmacological results in vivo. First, as discussed earlier, the nicotine concentrations used in acute applications in vivo are likely to engage both hetero- and homomeric nAChR. In genetically modified animals, the pro-convulsant effect would be facilitated when the 'window current' (Fig. 4) of either type of receptor is widened. Second, the α4β2 agonist ABT-418 effectively induces seizures [81]. Third, although MLA blocks nicotine-induced seizures, this drug may cross-react with α4β2 receptors, at the used concentrations (in the micromolar range) [100]. Somewhat more puzzling is the observation that high doses of nAChR antagonists are pro-convulsive *in the absence* of administered agonists [81,94].

A possible explanation that reconciles these complex results implicates the details of the nicotinic regulation of hippocampal GABAergic neurons [81], where α7 and α4β2 receptors are found on both cell bodies and synaptic terminals [97,98]. In principle, either excitation or inhibition of these neurons could lead to seizures, in the proper physiological context. A classic mechanism foresees that decreasing the excitability of GABAergic neurons (mainly parvalbumin-expressing, PV+, basket cells) impairs recurrent inhibition thus promoting seizures by hampering surround inhibition of pyramidal neurons [101]. A decrease of basket cell activity could arise by inhibiting nAChRs in the cell soma, which could be reinforced by inhibition of nAChRs regulating the glutamatergic drive on these GABAergic cells. This would explain the seizure facilitation obtained by i) application of nAChR inhibitors, and ii) nAChR desensitization produced by nicotinic agonists [25,42,100]. An alternative mechanism implies GABAergic barrages in stimulating seizures by synchronizing local populations of pyramidal cells through rebound inhibition [102]. This mechanism could be promoted by transient overstimulation of the nAChRs expressed by GABAergic cells.

In conclusion, to further define the cellular mechanisms underlying nicotine-dependent seizures it will be necessary to achieve precise control of the spatiotemporal application of agonists and antagonists on local networks. Importantly, these experiments should be extended to brain regions that appear to be implicated in nicotine-dependent seizures, beyond the hippocampus. First, the genetic forms of nAChR-dependent sleep-related epilepsy suggest involvement of the cerebral cortex and the thalamocortical system (see *Section 5*). Second, recent

work in rodents shows that convulsive doses of nicotine produce maximal neuronal activation in the piriform cortex, amygdala, medial habenula, paratenial thalamus, anterior hypothalamus and solitary nucleus (as measured by c-fos expression; [83]), which delineates a pattern of activation in subcortical and limbic regions. Such work indicates that amygdala neurons may be a major focus of seizures [83], in agreement with previous studies with the rapid electrical kindling model in amygdala [103].

4.2. The kindling effect of nicotine

Kindling was originally described as the progressive development of epilepsy in animals electrically stimulated by electrodes implanted in limbic structures [104]. Subsequently, other kindling procedures were introduced, and particularly chemical kindling. Most of the effective compounds, such as picrotoxin, bicuculline [105], and pentylenetetrazole (PTZ) [106,107], are thought to act by blocking $GABA_A$ receptors. Nevertheless, in mice, nicotine is also an efficacious kindling agent, when repeatedly administrated at initially sub-convulsive doses [26]. The underlying mechanisms appear to be different from those operating in acute nicotine-induced seizures, as nicotine-kindled seizures are much more sensitive to antiepileptic compounds such as levetiracetam, tiagabine and phenytoin [26]. Peculiarities are also observed with respect to other forms of chemical kindling. For instance, the pattern of c-fos expression is different from the one observed after PTZ-kindling, as it shows less activation in limbic regions [26]. Once again, the contribution of specific nAChR subtypes in nicotine kindling is uncertain. As discussed earlier, prolonged application of nicotine has a biphasic effect. After nAChR desensitization, steady exposure to nicotine stimulates the expression of both homo- and heteromeric nAChR subunits [80]. Regardless of the mechanistic details, an interesting sex-dependency is observed in nicotine kindling, as female rats are more sensitive to nicotine, at least in adolescence [108]. The effect is attributed to sex-related differences in oxidative balance, and particularly a lesser availability of antioxidant defences in females [108]. The hypothesis relies on the fact that epileptic seizures are often associated with oxidative stress [109], largely because of glutamatergic, and especially NMDA receptor, overactivation [110]. Hence, nAChR stimulation during nicotine kindling would be particularly effective at increasing glutamate release [111] as well as favour NMDA receptor function by postsynaptic depolarization (which relieves Mg^{2+} block). At the present stage, however, the contribution of other mechanisms to the sex-dependency of nicotine kindling, such as the possible role of hormonal factors, remains to be determined.

5. nAChR subunits in sleep-related epilepsy

Mutations on genes encoding subunits of heteromeric nAChRs are frequently associated to sleep-related epilepsy. The first evidence traces back to 1995, when a missense mutation on *CHRNA4* ($\alpha4^{S248F}$) was found to be linked to Autosomal Dominant Nocturnal Frontal Lobe Epilepsy (ADNFLE) [7]. We note that different authors have used different codon numbering for α4. For instance, $\alpha4^{S248F}$ refers to the original *Torpedo* sequence, which corresponds to $\alpha4^{S280F}$ of the Gene Bank human sequence (which we will use here). For clarity, Table 2 lists the different numbering, also referring to murine clones. ADNFLE is the Mendelian form of NFLE, a focal epilepsy characterized by hypermotor seizures or tonic-dystonic postures mainly occurring during non-REM sleep (frequently, in stage 2). Seizures last on average ~30 s and may be preceded by abrupt arousals. Longer events are nonetheless possible and may take the form of nocturnal wanderings. Often, but not necessarily, the disease arises in childhood or adolescence [112,113]. Because of lingering disagreements about the definition and the characteristics of NFLE, a consensus conference held in 2016 aimed to better define the disorder and the diagnostic criteria [113]. (AD)NFLE was thus renamed (AD)SHE, or (Autosomal Dominant) Sleep-related Hypermotor Epilepsy, which conveys that sleep-related seizures are not necessarily nocturnal and highlights that the motor aspects are an essential feature of the pathology. Moreover, seizures do not necessarily origin in the frontal lobes. Nonetheless, extra-frontal seizures propagate to frontal cortex, and the ensuing generation of hypermotor seizures suggests that a common cortico-subcortical circuit is implicated in the motor events [113].

Table 2
Codon numbering of different ADSHE mutations.

Human		Rat		Mouse	
Torpedo-based	Gene Bank	*Torpedo*-based	Gene Bank	*Torpedo*-based	Gene Bank
$\alpha4^{S248F}$	$\alpha4^{S280F}$	$\alpha4^{S252F}$		$\alpha4^{S252F}$	
$\alpha4^{776ins3}$	$\alpha4^{291–292insL}$	$\alpha4^{+L264}$		$\alpha4^{+L264}$	
$\alpha4^{S252L}$	$\alpha4^{S284L}$	$\alpha4^{S256L}$	$\alpha4^{S286L}$		
	$\beta2^{V287L}$	$\beta2^{V262L}$	$\beta2^{V286L}$		$\beta2^{V287L}$
	$\beta2^{V287M}$	$\beta2^{V262M}$	$\beta2^{V287M}$		$\beta2^{V287M}$

After the identification of $\alpha4^{S280F}$, other *CHRNA4* mutations were found in ADSHE families [114,115], and *CHRNB2* was soon also associated to the disease [116,117]. A few years later, *CHRNA2* was found to be linked to a sleep-related epilepsy affine to ADSHE [118]. The spectrum of ADSHE mutations identified on nAChR subunits is now increasing every year (e.g., [119,120]). Fig. 2 shows the subunit variants for which functional studies are available. Recent evidence suggests that mutant nAChR subunits could be implicated in other forms of epilepsy. For instance, *CHRNB2* and *CHRNA4* variants were identified in patients with sleep-related insular epilepsy [121]. Furthermore, ADSHE families carrying *CHRNA4* and *CHRNB2* mutations display cognitive deficits, mental retardation, and schizophrenia-like symptoms more frequently than initially reported, although no clear association has been so far observed between individual mutations and specific neurological symptoms [122,123]. Overall, only 10–12% of the ADSHE families carry mutations on nAChR genes [112], but the discovery of further ADSHE genes had to wait for the development of more efficient sequencing methods. Evidence is now strong for *KCNT1*, encoding the sodium-gated K^+ channel $K_{Na}1.1$ [124], which underlies a severe form of ADSHE, and *DEPDC5,* encoding dishevelled egl-10 and pleckstrin domain-containing protein 5, which is thought to be implicated in brain morphogenesis [125–127]. Also implicated in ADSHE appear to be *CRH*, encoding the corticotropin releasing hormone [128], *NPRL2* and *NPRL3,* encoding the regulators of mammalian target of rapamycin [129]. For a brief overview of ADSHE genes, see ref. [130]. Several lines of functional evidence support the hypothesis that mutations of nAChR subunits do cause ADSHE. The study of nAChRs carrying these mutations in cellular expression systems reveals complex biophysical and pharmacological alterations (*Section 5.1*). Moreover, studies in animal models of ADSHE show that expression of these mutations can reproduce some of the characteristics of the human pathology (*Section 5.2*).

5.1. Studies on mutant nAChR subunits in expression systems

The first ADSHE mutations were localized on the functionally critical M2 segment of either α4 or β2. To define the relative biophysical alterations, mutant and wild-type subunits were expressed in *Xenopus laevis* oocytes, for electrophysiological and pharmacological testing. *Xenopus* oocytes are greatly efficient for membrane protein expression, although their huge size limits the possibility of refined kinetic studies by fast agonist application, at least in whole-oocyte mode. Subsequently, mammalian cell lines (especially human embryonic kidney cells) have been also employed, which offer complementary advantages and disadvantages compared to *Xenopus* oocytes. Unfortunately, probably because of the difficulty of obtaining nAChR expression in mammalian cells at levels compatible with reliable investigation by patch-clamp recording, virtually no information is available about the mutant

subunit properties in neurons. The first studies focused on *CHRNA4* mutations in homozygous condition, which revealed complex and partially conflicting functional alterations. Overall, these alterations were interpreted as leading to decreased functionality of α4β2 receptors, because of a lower P_{Ca}, accompanied or not by quicker desensitization [113, 131–134], or decreased potentiation by extracellular Ca^{2+} [135]. A second phase in ADSHE studies started around 2000. First, several *CHRNB2* mutations were identified and included in these studies [79, 116–117, 136–137], which also led to localize ADSHE mutations in other TMD portions, such as M3 [79,137]. Second, because in rare genetic diseases such as ADSHE all patients carry the mutant gene in heterozygous condition, more effort was devoted to determining the functional features of nAChRs in the simulated heterozygotes [116–117, 136, 138–139]. The second wave of studies modified the initial interpretation of the possible pathogenetic mechanism. Given the pleiotropic action produced by ADSHE mutations on the receptor's properties (e.g., on channel gating *and* permeability), the balance of the ensuing effects can depend on the number of mutant subunits in each receptor. Indeed, it turned out that heterozygous ADSHE mutations prevalently cause a gain of receptor function, often produced by increasing the sensitivity to the agonists, accompanied or not by altered desensitization. Instead, major alterations of channel permeability appear to be uncommon in the heterozygous state. Two general explanations may account for the higher apparent affinity for the agonists. First, mutations could enhance ligand binding or facilitate channel opening, as suggested by the prevalent location of the initially identified mutations on M2 (Fig. 2), which is essential in transmitting the conformational rearrangement between the ligand-binding site and the channel gate [29,30]. Second, ADSHE mutations could favor membrane expression of the high-affinity $(\alpha4)_2(\beta2)_3$ stoichiometry [140] and, at least in the case of $\beta2^{V287L}$, facilitate recruitment of α5 into α4β2 receptors [141]. The two mechanisms are not mutually exclusive (e.g., [141]) and the balance of the contribution of either is currently uncertain [141,142]. In particular, to define how different mutations affect distinct channel properties requires detailed single-channel investigation, preferably conducted on different stoichiometric forms. For instance, two (AD)SHE mutations fall on the conserved S284 residue, located at 10' of M2, adjacent to the activation gate. Both mutations, $\alpha4^{S284L}$ [134] and $\alpha4^{S284W}$ [120], cause prominent functional alterations. Extensive single-channel analysis is available for $\alpha4^{S284W}$, showing that the amino acid substitution promotes channel opening in both $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$ stoichiometries [120]. The effect is attributed to steric interference among the bulky tryptophan residues of the different subunits, which stabilizes the open channel state. This functional enhancement largely exceeds the opposite effect produced by a small decrease of single-channel conductance [120]. Extensive single-channel evidence is also available for two mutations identified outside M2-M3, in non-familial SHE: $\alpha4^{R336H}$ [143] and $\beta2^{V337G}$ [144]. These are located in the initial portion of the ICD following M3 and potentiate receptor's function through complex alterations on both $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$ [145]. The main results are destabilization of the desensitized state, in $\alpha4^{R336H}$, and stabilization of the open state, in $\beta2^{V337G}$ [146].

A strong increase in the apparent affinity for the agonists was also observed in the first *CHRNA2* mutation ($\alpha2^{I279N}$), in both homo- and heterozygous condition [118,147]. Differently from the other mutations known at the time, $\alpha2^{I279N}$ was located on the M1 segment, in a TMD portion likely to be directly involved in transducing the ligand-dependent conformational transition between the extracellular domain and M2 [118,148]. This suggests that $\alpha2^{I279N}$ may alter the balance of the close to open transitions, although single-channel measurements are lacking. However, in contrast to the above pattern, the *CHRNA2* mutations linked to typical ADSHE studied until now cause a 'loss-of-function' phenotype, even in heterozygous condition [149,150]. A possible interpretation of these observations calls into play the specific pattern of α2 expression in neocortex GABAergic cells (see *Section 8*).

The discovery of novel variants in ADSHE and other genetic epilepsies by the current gene sequencing technology is quickly outpacing the possibility of carrying out detailed functional studies. Nonetheless, the known molecular mechanisms by which ADSHE mutations alter the nAChR function already suggest possible pharmacological strategies for personalized medicine (*Section 7.1*). Yet, fully understanding how mutant nAChRs can determine the pathogenesis of ADSHE requires considering the mutant subunits in the context of the relevant neural circuits, in adult and developing brains. One must clarify how mutant subunits determine cortical hyperexcitability, how interaction with the thalamic input and the ascending arousal system determines the sleep-related features of ADSHE, and which subcortical structures may contribute the hypermotor events [27,151]. This rationale drove the third wave of studies in ADSHE, i.e. the generation of animal models of the disease.

5.2. Animal models of ADSHE

Since 2006, several murine lines have been produced expressing mutant α4 or β2 subunits linked to ADSHE (Table 3). First, knock-in adult mice (C57BL/6 strain) expressing heterozygous α4 mutations corresponding to the human $\alpha4^{S280F}$ and $\alpha4^{291–292insL}$ showed recurrent seizures and increased nicotine-dependent GABA (but not glutamate) release on pyramidal neurons in layers II/III of frontal cortical areas [152]. These results suggested that hyperactivation of inhibitory neurons by mutant nAChRs could lead to synchronize pyramidal neurons by rebound inhibition, in proper physiological circumstances [102,152]. However, the phenotype resulted to be different when $\alpha4^{S280F}$ was expressed in CD1–129/Sv background. In this case, a nicotine-induced dystonic arousal complex was observed, but no spontaneous seizures [153]. Hence, although nAChR mutations alter the physiology of the sleep-wake transitions, the specific phenotypes and the pro-seizure effect appear to depend on the genetic background. This is in line with previous work showing that the sensitivity to nicotine-induced seizures in mice is strain-dependent [81]. This notion, although complicating the functional study of ADSHE mutations, could have important implications for the human pathology, as it suggests that the work on penetrance and expressivity of ADSHE mutations in relation to the human genetic background should be deepened [154].

Another possibility is that mice present limitations as animal models of human epilepsy, as is partly suggested by the fact that most of literature on experimental epileptology concerns rats. Transgenic rats expressing $\alpha4^{S286L}$ (corresponding to the human $\alpha4^{S284L}$) were in fact generated by Zhu et al. [155], which did display seizures during slow-wave sleep, accompanied by cortical network disinhibition because of attenuated GABAergic transmission before seizures, and abnormal glutamate release at seizure onset [155]. Neocortex overactivation is also indicated by higher expression of the astroglial connexin-43, preceded by increased phosphorylated extracellular signal-regulated kinase (pERK), before epilepsy onset [156]. Moreover, multiprobe microdialysis was applied in these rats to investigate neurotransmitter release in the thalamocortical and corticostriatal pathways [156–158], especially focusing on the secondary motor area (M2/Fr2) which could be potentially relevant for the hypermotor symptoms of ADSHE, because of its pattern of connections [159,160]. These analyses demonstrate functional abnormalities in thalamocortical motor and cognitive pathways [157] (see *Section 5.2.2*), which may contribute to explain the motor symptoms associated with ADSHE and some aspects of cognitive impairment.

A second line of studies with animal models focused on $\beta2^{V287L}$. Knock-in of $\beta2^{V287L}$ in C57BL/6 mice induced a disturbed sleep pattern and abnormal excitability in response to nicotine, but no overt epileptiform activity, although experiments in synaptosomes indicated a larger nicotine-induced GABA release [161,162]. However, in transgenic rats expressing the same mutation, spontaneous epileptic seizures were also observed [163]. The other related transgenic model was generated in mice to regulate the timing of $\beta2^{V287L}$ expression, by conditionally

Table 3
Animal models in ADSHE.

Model	*Mutation (human sequence)*	*Phenotype*	*Reference*
Knock-in Mouse (C57BL/6)	$\alpha4^{S280F}$	Increment of slow wave in background EEG (0.5–4 Hz) and spontaneous epileptic in seizures during wakefulness Enhanced sensitivity to nicotine application Increase of nicotine-evoked GABAergic activity in cortical layer II/III pyramidal cells.	[152]
Knock-in Mouse (C57BL/6)	$\alpha4^{291-292insL}$	Similar to $\alpha4^{S280F}$	[152]
Knock-in Mouse (CD1/ 129 Sv) Also mated with C57BL/6, to obtain different genetic backgrounds	$\alpha4^{S280F}$	Nicotine-induced dystonic arousal complex. No spontaneous seizures Lower sensitivity to nicotine-induced seizures in all genetic backgrounds	[153]
Transgenic Rat (Sprague Dawley)	$\alpha4^{S284L}$	Seizures during slow wave sleep, arising in sensorimotor cortex Attenuation of cortical GABAergic transmission and abnormal glutamate release during slow wave sleep. Nocturnal wanderings and paroxysmal arousals (detected by video monitoring) Functional abnormalities in TC motor and cognitive pathways, increased connexin-43. Impaired intrathalamic inhibition Decreased KCC2/NKCC1 ratio in frontal cortex at 8 weeks of age	[156–158, 187]
Transgenic conditional (TET-off) Mouse (FVB)	$\beta2^{V287L}$	Spontaneous seizures during periods of increase delta wave activity The phenotype is only observed if $\beta2^{V287L}$ is expressed until P15. Decreased glutamatergic drive on neocortex fast-spiking neurons. Higher nAChR currents in SOM+ neurons. Delayed GABAergic switch. Decreased KCC2 in mature RTN	[164–165, 185]
Knock-in Mouse (C57BL/6)	$\beta2^{V287L}$	Altered sleep pattern and circadian rhythm Anxiety behaviours and abnormal natural reward Nicotine induced dystonic arousal complex without spontaneous seizures Larger nicotine-induced GABA release from synaptosomes	[161–162]
Transgenic Rat (Sprague-Dawley)	$\beta2^{V287L}$	Spontaneous seizures during the light phase (resting period for rats) similar to paroxysmal arousals observed in ADSHE patients Higher sensitivity to nicotine-induced seizures	[163]

modulating the transgene under control of the tetracycline promoter (TET-off system; [164]). Mice expressing $\beta2^{V287L}$ displayed spontaneous seizures during periods dominated by increased EEG *delta* wave activity, which is typical of slow-wave sleep [8]. The difference between the knock-in and the transgenic models could be due to the different gene dosage, which is a general issue with transgenic animals. However, in both transgenic rats and the conditional mouse model, no change was observed in membrane expression of β2-containing and α7 nAChRs, nor in the relative subunit composition [163–165]. Hence, a direct effect of gene dosage on the modulation of synaptic circuit seems unlikely. Nevertheless, the possible early effects of the transgene on the β2-dependent modulation of the neocortex transcriptional program [166] merits a deeper study.

In Manfredi's conditional model, seizures did not develop if $\beta2^{V287L}$ was silenced by doxycycline until postnatal day 15, whereas silencing the transgene at later stages had no effect on the epileptic phenotype [164]. These findings suggest that $\beta2^{V287L}$ permanently alters the neocortex network at early stages. In agreement with the results obtained in the other animal models of ADSHE [152–153, 155, 161, 163], no major changes in brain structure were observed in these mice, except for a ~10% decrease of prefrontal cortex (PFC) thickness [165], which has some resemblance with the PFC thinning observed in young patients of frontal epilepsy [167–169]. Moreover, expression of $\beta2^{V287L}$ did not alter the mesopontine and forebrain cholinergic nuclei, nor the main GABAergic cell populations in neocortex, leaving the cholinergic and GABAergic innervation of PFC unchanged [165]. However, these mice display a permanent prefrontal disinhibition in layer V of M2/Fr2 region, because of a decreased glutamatergic input on fast-spiking PV+ GABAergic neurons. Moreover, they showed increased somatic nicotinic currents in somatostatin-expressing (SOM+) GABAergic cells. A summary of the nAChR expression in the main neuronal types in layer V of M2/Fr2 is reported in Fig. 5. Somatic expression of nicotinic currents in fast-spiking neurons tends to decrease after the first postnatal month [170]. Thus, in the mature awake brain, activation of $\beta2^{V287L}$-containing nAChRs by the high cholinergic tone may counteract hyperexcitability by promoting local inhibition by SOM+ cells and partly rescuing the decreased glutamatergic drive of fast-spiking

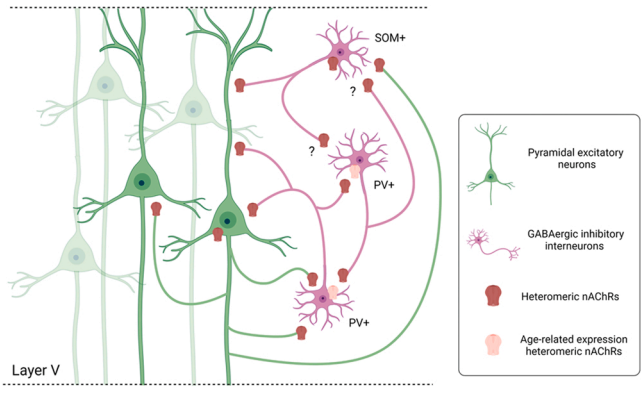

Fig. 5. Heteromeric nAChRs in layer V of murine premotor cortex. Summary of the heteromeric nAChR localization in the main cell types (pyramidal, fast-spiking PV+ and regular spiking SOM+ GABAergic cells) in layer V of the murine premotor M2/Fr2 region. Figure summarizes references [27,159,160, 165,170]. The figure was prepared with *BioRender* (BioRender.com).

GABAergic neurons [165]. Such interpretation suggests that seizures are rare in wakefulness because of the high cholinergic tone [8] and nicotine can decrease symptoms in ADSHE patients (*Section 7*) by partially rescuing the mutation effect.

5.2.1. Effect of ADSHE mutations on maturation of neocortex synaptic circuit

How $\beta2^{V287L}$ could alter the balance of excitatory synapses targeting pyramidal and fast-spiking neurons by acting during PFC maturation is unclear. The β2 subunit expression peaks between the first and the second postnatal week [171] and β2-containing nAChRs regulate the formation and distribution of dendritic spines in neocortex and hippocampus [172,173]. In layer VI, the effect is modulated by α5 incorporation [174]. However, little is known about whether and how heteromeric nAChRs regulate the formation of excitatory synapses on GABAergic neurons. The latter process is thought to be controlled by the postsynaptic tyrosine kinase receptor ErbB4, and its presynaptic secreted ligands, especially neuregulin 1 (NRG1) [175,176] and 3 (NRG3) [177]. Some recent results suggest that both homo- and heteromeric nAChRs contribute to stimulate ErbB4 in GABAergic neurons, probably by calcium signals that proteolytically activate presynaptic NRG3 [178]. Thus, overstimulation of the NRG/ErbB4 pathway by mutant nAChR during synapse formation could cause a functional unbalance of glutamatergic synapses on fast-spiking PV+ cells. Moreover, $\beta2^{V287L}$ could affect the maturation of glutamatergic transmission by delaying the developmental GABAergic switch. In rodents, the reversal potential of $GABA_A$ currents (E_{GABA}) hyperpolarizes during the 2nd postnatal week [179]. This leads to the typical inhibitory function of GABA in the adult brain and is correlated with GABAergic neuron maturation [180]. The GABAergic switch is attributed to increased expression of the Cl^- extruder K/Cl cotransporter-2 (KCC2) [181], as compared to the Na/K/2Cl cotransporter-1 (NKCC1), and is also observed in human brain, around birth [182]. During development, NKCC1 is thought to absorb Cl^-, although the flux direction generally depends on ionic and V_m conditions and the role of NKCC1 in the mature brain is debated [183,184]. In PFC layer V of mice expressing $\beta2^{V287L}$, the KCC2/NKCC1 ratio around the 8th postnatal day is lower than in the controls and E_{GABA} is more depolarized, which points to a delay of GABAergic switch [185]. Prolonging the early depolarizing effect of GABA could alter the formation and distribution of glutamatergic synapses by modulating Ca^{2+} signals [173]. Moreover, altered KCC2 expression could affect the cytoskeleton of dendritic spines by non-ionic mechanisms [186]. Once again, little is known about these mechanisms in GABAergic cells.

A decreased KCC2/NKCC1 ratio and E_{GABA} depolarization were also observed in frontal cortex of rats expressing $\alpha4^{S286L}$ [187]. Here, however, the effect arose at 8 weeks, concomitant to seizure onset. A late effect seems consistent with the fact that α4, differently from β2, does not present a postnatal expression peak [171]. Whether in this case the altered Cl^- homeostasis is cause or effect of epileptogenesis is unclear. Nonetheless, seizure onset was prevented by blocking NKCC1 with furosemide [187], which adds to the growing literature suggesting that modulating Cl^- cotransporters could have prophylactic action in epilepsy and other neurologic diseases [186].

5.2.2. Mutant nAChRs and thalamocortical transmission

Given the sleep-related nature of ADSHE, the effects of mutant nAChRs on the thalamocortical system remain somewhat neglected. ADSHE seizures often occur during stage 2 of non-REM sleep [112,113], probably during arousal events [151,188], which are typically accompanied by bouts of ACh release [8]. An EEG hallmark of stage 2 of sleep is the occurrence of sleep spindles. These are waxing and waning 10–15 Hz oscillations lasting 0.5–3 s, followed by a refractory period of 5–10 s, and generated by a complex thalamocortical interplay. For a complete and updated review, see ref. [189]. In brief, spindles are paced by the reticular thalamic nucleus (RTN), which provides the main GABAergic inhibitory input to the thalamic relay nuclei. Burst activity in RTN cells mainly occurs at spindle frequencies and is initiated by descending corticothalamic glutamatergic input. RTN cell activity, in turn, determines rhythmic hyperpolarization of neurons in thalamocortical (TC) cells of primary thalamic nuclei. These are progressively engaged into burst activity, which further synchronizes RTN neurons by excitatory feedback. During sleep, spindle interruption and the subsequent refractory period depend on burst-dependent progressive hyperpolarization of RTN cells and afterdepolarization in TC cells, aided by desynchronizing input from neocortex and locus coeruleus [189]. On arousal, sleep spindles are interrupted by input from the ascending reticular activating system.

Previous clinical evidence by Picard and colleagues suggests that ADSHE seizures preferentially arise during ongoing frontal sleep spindles, whose duration thereby lengthens, and may facilitate seizure onset [190]. Although none of the patients recruited in this study carried any mutation in nAChR subunit genes, it was hypothesized that overactive nAChRs could also lead to longer spindles and promote seizures [190]. This hypothesis agrees with recent results indicating that nicotine application stimulates spindle activity in humans [191]. Moreover, in a sample of patients carrying ADSHE mutations on either α4 or β2, heteromeric nAChR expression increased in the mesencephalic regions that innervate the cholinergic LTD nucleus. The latter regulates the mediodorsal thalamic nucleus, which provides the main projections to the PFC [192]. An overactive mediodorsal thalamic nucleus could hamper the normal arousal-induced interruption of the spindle oscillations, thus transforming them into pathological thalamocortical oscillations that might trigger seizures [192].

What do animal models of ADSHE suggest about the thalamic implication in ADSHE? Transgenic mice bearing $\beta2^{V287L}$ display a lower KCC2 level in the mature RTN, but not at early postnatal stages [185]. RTN neurons express a particularly high density of postsynaptic heteromeric nAChRs, which can trigger action potentials on activation of the cholinergic afferents, an effect possibly facilitated by the presence of $\beta2^{V287L}$-containing receptors [193]. Thus, the low KCC2 amount may reflect RTN hyperactivity, as KCC2 downregulation is known to occur in overactive cells, in which increased calcium mobilization stimulates calpain-dependent signals [185]. Whether and how this may affect spindle regulation remains to be determined by experiments in vivo or in thalamocortical slices.

Finally, altered physiology of thalamic pathways was found in transgenic rats expressing $\alpha4^{S284L}$ [157,158], in which enhanced transmission in thalamocortical motor pathway and attenuated transmission in thalamocortical cognitive pathway were observed by studying release of glutamate and GABA with multiprobe microdialysis [157]. Moreover, the thalamic hyperdirect pathway (connecting motor thalamus to basal ganglia) was also found to be activated. Such a mechanism could contribute to the nocturnal paroxysmal dystonia of ADSHE patients bearing this mutation [158]. In addition, the impaired action of mediodorsal thalamus and orbitofrontal cortex (cognitive pathway) could contribute to the cognitive deficit and schizophrenia-like symptoms observed in several ADSHE families.

6. The cholinergic system in experimental models of acquired epilepsy

The alterations produced on the cholinergic system in the animal models of acquired epilepsy are debated. These models especially focus on limbic seizures, as temporal lobe epilepsy is the most frequent epileptic form in humans and one of the most refractory to pharmacological treatment [194]. In brief, systemic or intracerebral administration of certain convulsant molecules leads to series of closely spaced seizures or a condition of SE, which is followed by chronic seizure recurrence after a variable latent period [195]. The most studied chemo-convulsants are pilocarpine, which produces overstimulation of M1 mAChRs [23,196], and kainic acid, which activates ionotropic

glutamate receptors [197]. In general, establishment of chronic seizures is accompanied by SE-induced secondary cell loss not only in hippocampus and other limbic structures, but also neocortex and thalamus [198–202]. Whether and how such treatments alter the cholinergic nuclei and particularly those of the basal forebrain is less clear, as different studies have applied considerably variable methods using different markers, such as Nissl staining for cell bodies, or acetylcholinesterase (AChE), choline acetyltransferase (ChAT) and vesicular ACh transporter (VAChT) for cholinergic structures. A recent critical discussion is provided by Giorgi and colleagues [202]. Briefly, considering the most recent studies carried out with precise cell counts with stereological methods [203–205], it appears that when SE is elicited systemically, cells expressing VAChT and ChAT increase in the medial septum, which is accompanied by a denser cholinergic innervation in the dentate molecular layer at 120 days [204], but not 60 days [205], following SE. In contrast, focal induction of SE from the limbic structures leads to decrease of cholinergic cells and cell damage affecting all cholinergic nuclei of basal forebrain at varying degrees, depending on the severity and duration of SE, but generally at 5 days after SE [205]. Considering also previous results indicating a marked neuronal loss [198–206], these results suggest that early cell loss could be followed by compensatory changes [202]. Unfortunately, very little is known about the contribution of specific ACh receptors, and especially nAChRs, in the above experimental models, except that a decrease of α7 expression is observed in human samples from patients of post-traumatic or post-hemorrhage epilepsy [207]. Nonetheless, the evidence summarized in the present section points to the possibility of modulating the cholinergic system as an add-on therapy in epilepsy [208].

7. Antiepileptic treatment: modulating nAChRs and the cholinergic pathways

7.1. Modulating nAChRs

The evidence discussed in the previous sections suggests that targeting nAChRs could be an effective antiepileptic strategy in ADSHE. Moreover, modulating nAChRs may have broader applications in epilepsy, as nAChR antagonists can attenuate epileptiform activity in different experimental seizure models [209–211], and may be combined with blockade of glutamate receptors [209]. More specifically, targeting specific nAChR subtypes or mutant ADSHE subunits could allow personalized treatment in ADSHE patients.

A first issue is whether well-known AEDs, besides their effects on voltage-gated channels, also modulate nAChRs. Early work in expression systems has shown that carbamazepine, a first-line AED in ADSHE that inhibits voltage-gated Na^+ channels, also produces open channel block of heteromeric nAChRs, at therapeutic concentrations [212,213]. The same applies to oxcarbazepine, a less toxic analog of carbamazepine that mainly acts through its metabolite 10-hydroxycarbamazepine [213]. Moreover, several ADSHE mutations alter the nAChR sensitivity to these AEDs [147, 212–213], which would further justify the special efficacy of these compounds on ADSHE. The capability of blocking nAChRs is also found in lamotrigine [214]. Thus, it is urgent to better define the spectrum of effects produced by widely used as well as novel AEDs on nAChRs, to better plan the selective use of these compounds in epilepsy.

Second, following up the initial studies [215], increasing clinical effort has aimed to determine whether nicotine can exert efficacious antiepileptic action in ADSHE. Clinical studies carried out by Brodtkorb and colleagues in 4 children carrying $\alpha4^{S280F}$ [216] and 17 patients with a broad age spectrum, 12 of whom carried $\alpha4^{S280F}$ [217], revealed a dramatic reduction in seizure frequency, especially when nicotine was applied through transdermal patches. Another recent study reported favorable effects of nicotine in 3 out of 4 ADSHE patients carrying different variants (not all characterized functionally) in either α4, α2 or β2 nAChRs. Notably, the strongest effects were observed in the patient carrying $\alpha4^{S284L}$ [218], whose biophysical properties have been fully characterized. These results are generally attributed to the desensitizing effects of steady nicotine applications, or to the possible normalizing effect of nicotine on the proportion of high- and low-affinity stoichiometries of α4β2 nAChRs [140]. Another possibility, suggested by work in animal models of ADSHE (*Section 5.2*), is that nicotine sustains the GABAergic control of local neocortex circuits, which may be permanently hampered by developmental effects of the ADSHE subunits [165]. These and other hypotheses will require further substantiation. Nonetheless, the clinical results suggest that nicotine treatment merits much wider consideration in ADSHE.

Application of nicotine in ADSHE patients would represent a first step towards precision therapy, which is still inadequate in epilepsy [219]. The next step in precision medicine approaches would be to specifically target individual mutations. Current evidence about the biophysical properties of mutant subunits suggests different possible pharmacological approaches. First, in the presence of mutations that facilitate channel opening [118,120,146], an open channel blocker would be effective in counteracting nAChR overactivity. Second, certain amino acid residues, such as tryptophan in $\alpha4^{S284W}$, may offer the possibility of developing compounds to specifically target the mutant nAChR form in the open state [120]. Third (*Section 5.1*), some ADSHE mutations affect the proportion of α4β2 nAChR stoichiometric forms [140], or display stronger functional effects on one stoichiometric form [145,146]. In these cases, the epileptogenic effect could be modulated by regulating the relative proportion of the main stoichiometries, by using chaperon molecules [80]. Heteromeric nAChR assembly and membrane expression in the CNS are modulated by the endoplasmic reticulum protein NACHO [220] and the cytoplasmic protein 14–3–3 [221]. It has been recently found that NACHO selectively promotes expression of the high-affinity $(\alpha4)_2(\beta2)_3$ nAChR, whereas 14–3–3η (the cortical isoform of 14–3–3) has a similar effect on the low-affinity $(\alpha4)_3(\beta2)_2$ nAChR [222]. Thus, these molecular chaperons appear to constitute potential molecular targets to control the balance of stoichiometric forms in ADSHE [222].

These approaches may also suggest how to circumvent the possible long-term effects of nicotine, especially in children and adolescents. We notice that, considering the pathogenetic hypotheses delineated in *Section 5* , it is far from being obvious whether a nicotine-induced long-term increase of nAChR expression would be harmful or beneficial to patients. To increase the relevance of preliminary testing of different compounds on specific ADSHE mutations, it would be advisable to express these mutations in reconstituted human neocortex networks. Recent success obtained with human induced pluripotent stem cell (hiPSCs) differentiated into cortical neurons, yielding a quasi-physiological balance of excitatory and GABAergic cells [223], encourages proceeding along this way.

7.2. Modulating the cholinergic pathways

Irrespective of the specific involvement of nAChRs, stimulating the cholinergic pathways could have beneficial effects as anti-epileptic treatment through recovery of the normal cholinergic signaling [202, 208]. A recent example is provided by Wang and colleagues, who found that optogenetic stimulation of the direct cholinergic pathway between the medial septum and the hippocampus has antiseizure effect in temporal lobe epilepsy, through activation of the SOM+ GABAergic neurons [208]. In principle, a similar rationale could be applied to human patients by transcranial magnetic stimulation (TMS). Although TMS still needs a complete assessment, its capability to both modulate and detect changes in cortical excitability potentially offers considerable advantages in epilepsy therapeutics [224]. For instance, in rats with vascular dementia, TMS treatment for 30 days significantly increases the AChE and ChAT activity and the density of cholinergic neurons. This effect has been attributed to the restoration of cholinergic activity in hippocampal CA1 region, driven by BDNF expression [225]. Moreover, by coupling electrical peripheral stimulation with TMS of the motor cortex, the

central cholinergic circuits can be functionally evaluated. This induces an effect named short-latency afferent inhibition, which is produced by inhibitory interactions in the neocortex, and is reduced in cholinergic forms of dementia, such as Alzheimer's disease and dementia with Lewy bodies [226].

8. Conclusions

Long-course studies in genetic epilepsies and pharmacologically induced seizures highlight the role of heteromeric nAChRs in epileptogenesis. This is in line with the important role of these receptors in regulating brain excitability, and is consistent with their physiological properties, especially the relatively slow kinetics and the high sensitivity to ACh. The involvement of *CHRNA7,* and thus α7-containing receptors, in epilepsy is uncertain. This may be partly due to the difficulty of fully testing the role of this gene in animal models, as deleting *CHRNA7* produces no clear pathological alteration in mice [100, 227–228]. In humans, a genetic predisposition to idiopathic generalized epilepsy [154,229] and neurodevelopmental disorders accompanied by seizures [154, 230–231] has been associated to sporadic and familial microdeletion of the chromosome region 15q13.3, which comprises *CHRNA7*. These phenotypes are probably determined by the combination of *CHRNA7* deficiency with the deletion of other genes located in 15q13.3, as microdeletion of this region in mice leads to schizophrenia- and epilepsy-related alterations [232]. Hence, although α7 nAChRs are thought to exert a significant functional role during maturation of glutamatergic synapses [171], the manifold roles of *CHRNA7* in neural development and the possible implications for epilepsy remain to be clarified.

In general, despite great advances in the genetics of epilepsy, including ADSHE, large gaps remain in our comprehension of the biological mechanisms leading to the epileptic phenotype, which often involve developmental alterations that cause permanent dysregulation of the cerebral networks. Studies of ADSHE-linked nAChR mutations in murine models point to complex alterations of the GABAergic transmission, in the neocortex and thalamus. Facilitation of seizures during sleep appears to arise from permanent hyperexcitability of the cortical circuits, in a context of altered thalamic input. Further work is necessary to decipher the functional role of nAChR subtypes in specific GABAergic populations, which could explain the role of individual mutant subunits in epilepsy. For instance, α2 seems to be mainly implicated in the physiology of SOM+ GABAergic cells in deep cortex layers [61,165], which offers hints on how the study of its peculiar implication in ADSHE can be approached. Moreover, the implication of subcortical structures in hyperkinetic seizures and their interplay with the cortical input are unclear.

For both genetic epilepsy and nicotine-induced seizures, a full understanding of the underlying cellular mechanisms will require: i) precise spatiotemporal modulation of the relevant mature circuits, including the thalamus, and ii) deepening the nAChR roles in synaptic maturation. Finally, little is known about these matters in human brain or networks reconstituted from human tissue. A first step towards this goal would be expressing mutant receptors in neurons differentiated from hiPSCs [223,233]. Evidence that ADSHE mutations can produce some of their effects on cultured synaptic networks is already available in mouse [234]. These approaches will be made easier by the recent advances in the multi-electrode array technology, which allows profiling the physiological and pharmacological properties of neuronal networks [235], for comparison with the information obtained at a local level by the patch-clamp method. A more rapid application of these methods to individual ion channel mutations is offered by the availability of the modern targeted mutagenesis methods. Combining these approaches may lead to a great extension of precision and personalized medicine approaches in the genetic epilepsies caused by mutations in nAChRs or other ion channels.

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CRediT authorship contribution statement

Andrea Becchetti: Writing – original draft, Visualization, Conceptualization, Writing – review & editing. **Laura Clara Grandi:** Visualization, Conceptualization, Writing – review & editing. **Marta Cerina:** Visualization, Conceptualization, Writing – review & editing. **Alida Amadeo:** Writing – original draft, Visualization, Conceptualization, Writing – review & editing.

Declaration of competing interest

The authors declare no competing interest.

Data Availability

No data was used for the research described in the article.

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