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Rare missense variants of neuronal nicotinic acetylcholine receptor altering receptor function are associated with sporadic amyotrophic lateral sclerosis

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Sporadic amyotrophic lateral sclerosis (SALS) is a motor neuron degenerative disease of unknown etiology. Current thinking on SALS is that multiple genetic and environmental factors contribute to disease liability. Since neuronal acetylcholine receptors (nAChRs) are part of the glutamatergic pathway, we searched for sequence variants in *CHRNA3*, *CHRNA4* and *CHRNB4* genes, encoding neuronal nicotinic AChR subunits, in 245 SALS patients and in 450 controls. We characterized missense variants by *in vitro* mutagenesis, cell transfection and electrophysiology. Sequencing the regions encoding the intracellular loop of AChRs subunits disclosed 15 missense variants (6.1%) in 14 patients compared with only six variants (1.3%) in controls ($P = 0.001$; OR 4.48, 95% CI 1.7–11.8). The frequency of variants in exons encoding extracellular and transmembrane domains and in intronic regions did not differ. NAChRs formed by mutant α3 and α4 and wild-type (WT) β4 subunits exhibited altered affinity for nicotine (Nic), reduced use-dependent rundown of Nic-activated currents (I_{Nic}) and reduced desensitization leading to sustained intracellular Ca^{2+} concentration, in comparison with WT-nAChR. The cellular loop has a crucial importance for receptor trafficking and regulating ion channel properties. Missense variants in this domain are significantly over-represented in SALS patients and alter functional properties of nAChR *in vitro*, resulting in increased Ca^{2+} entry into the cells. We suggest that these *gain-of-function* variants might contribute to disease liability in a subset of SALS because Ca^{2+} signals mediate nAChR's neuromodulatory effects, including regulation of glutamate release and control of cell survival.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a devastating disease characterized by degeneration of motor neurons in the cerebral cortex, brain stem and spinal cord leading to progressive paralysis and death within 2–6 years (1). Approximately 5% of ALS cases are familial. Mutations in the cytosolic Cu/Zn superoxide dismutase protein (*SOD1*) are well-established

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causes of ALS, accounting for 1–2% of all cases and 20% of the familial forms. Mutations in genes encoding Angiogenin (2) TDP-43 (3), and FUS (4,5) have been recently found in a small proportion of sporadic as well as familial ALS patients. Thus, sporadic ALS (SALS) may be caused by Mendelian genes with reduced penetrance, and evidence from twin and other studies suggests that SALS shows complex inheritance (6–12).

Increased motor neuron vulnerability to glutamate-induced excitotoxicity currently represents one of the leading hypotheses to explain SALS (13). We focused our attention on genes encoding neuronal acetylcholine receptors (nAChRs) because these receptors are involved in regulation of glutamate release (14–16). Furthermore, nAChRs have been shown to prevent glutamate-induced motor neuronal death in primary cultures of the rat spinal cord (17) and are implicated in naturally occurring programmed motor neuron death in chick and human spinal cord (18).

nAChR subunits share a common topology with a large extracellular N-terminal domain, four α-helical transmembrane domains and a short extracellular C terminus; a large cytoplasmic loop is situated between the third and fourth transmembrane domains (Figs 1A and 2A). Each domain has a different functional role (14,15). The cytosolic loop is an important domain responsible for nAChR receptor assembly and targeting, and influences the ion channel properties (19,20). We therefore searched for sequence variants in genes encoding neuronal AChR subunits, focusing primarily on the cytosolic loop. The properties of identified mutations were characterized *in vitro* by an electrophysiological assay.

RESULTS

Genetic findings

Sequencing the exons of *CHRNA3*, *CHRNA4* and *CHRNB4* encoding the intracellular loop disclosed 15 missense variants in 14 of 245 patients (6.1%) and 6 variants in 450 controls (1.3%) [$P = 0.001$; odds ratio (OR) 4.48, 95% CI 1.7–11.8]. In the extracellular domains, we detected four missense variants in patients compared with five of controls ($P = 0.72$); in transmembrane domains the number of missense variants was zero in patients and three in controls ($P = 0.55$) (Table 1). All the detected variants are reported in Supplementary Material, Table S1.

The cumulative number of all missense variants was 19 of 245 (7.7%) patients and 14 of 450 (3.1%) controls ($P = 0.01$ by Yates Chi-square; OR 2.6; 95% CI 1.28–5.31) (Table 1).

In patients, 15 of 19 (79%) missense variants were found within the intracellular loop, 4 (21%) were in the extracellular domains and none in the transmembrane domains. In controls, 6 of 14 variants (43%) were detected in the loop, 5 (37%) in the extracellular domains and 3 (20%) in the transmembrane domains.

The frequency of rare (<1%) synonymous variants did not differ between patients and controls (2.4% of patients versus 2.2% of controls, $P = 0.94$ by Yates Chi-square) (Supplementary Material, Table S1).

Mutagenesis, cell transfection and electrophysiology

Four of the identified mutations were investigated in details for altering the functional properties of nAChRs and assayed by electrophysiology, using recombinant expression of four distinct α-subunit mutations produced by site-directed mutagenesis, as well as a wild-type (WT) control. To minimize the experimental variability, WT or α-mutant subunits were co-expressed by transient transfection with a common β4 subunit.

Expression of the complexes α3R385Hβ4, α3S388Fβ4, α4R345Cβ4 and α4Q572Rβ4 yielded functional nAChRs in HEK 293 and GH4C1 cells, indicating that these mutations did not prevent the expression of the receptors, at variance with the effect of other mutations in the same region (21–23). Outside-out single channel recordings showed that the mutations in the α3 subunit did not alter the unitary conductance or kinetics of α3β4 nAChR-channels (Fig. 1B and Table 2). In contrast, both mutations in the α4 subunit reduced the burst duration of α4β4 nAChRs, leaving the channel conductance unaffected (Fig. 2B, Table 2 and Supplementary Material, Table S2). Furthermore, we analyzed the Ca^{2+} permeability of mutant nAChR-channels (measured as the fractional Ca^{2+} current, P_f), which was not significantly altered in comparison with the corresponding WT-nAChRs (Table 2).

All α3 subunit mutations altered some properties of the nicotinic responses. Specifically, Nic dose-current response curves showed that both α3R385Hβ4 and α3S388Fβ4 nAChRs had an increased affinity for Nic as compared with WT α3β4 nAChR (Fig. 1C and Table 3). When nAChRs were repetitively activated, the current response evoked by Nic diminished, a phenomenon known as use-dependent current rundown. Using the rundown protocol (see Material and Methods), it was found that the use-dependent rundown of both α3R385Hβ4 and α3S388Fβ4 nAChRs were strongly reduced as compared with WT α3β4 nAChR (Table 3 and Fig. 1D). We also examined the desensitization of nAChRs during sustained applications of Nic, using equivalent concentrations for each receptor variant, namely the EC_{80}. From the raw data it was apparent that the desensitization of α3R385Hβ4 and α3S388Fβ4 nAChRs was slowed, as I_{Nic} reached its half-maximal peak amplitude in a significantly longer time (Table 3). Moreover, analyzing the decay phase of I_{Nic} revealed that both mutations induce the appearance of a non-desensitizing component, which is substantially absent in WT α3β4 nAChR (Fig. 1E and Supplementary Material, Table S3). All these data are predictive of an enhanced and sustained response of the α3R385Hβ4 and α3S388Fβ4 mutant nAChRs to prolonged nicotinic stimulation. Accordingly, Ca^{2+} imaging experiments showed that the intracellular Ca^{2+} concentration remained stably elevated for the entire duration of transmitter application (60 s) in cells expressing α3 mutant nAChRs, but not WT α3β4 nAChR (Fig. 1F and Table 3).

The situation was different for the α4R345Cβ4 and α4Q572Rβ4 mutant nAChRs, which had a lower apparent affinity for Nic, compared with α4β4 nAChR (Fig. 2C and Table 3). However, these mutant nAChRs again had a strongly reduced current rundown (Fig. 2D and Table 3) and a slowed

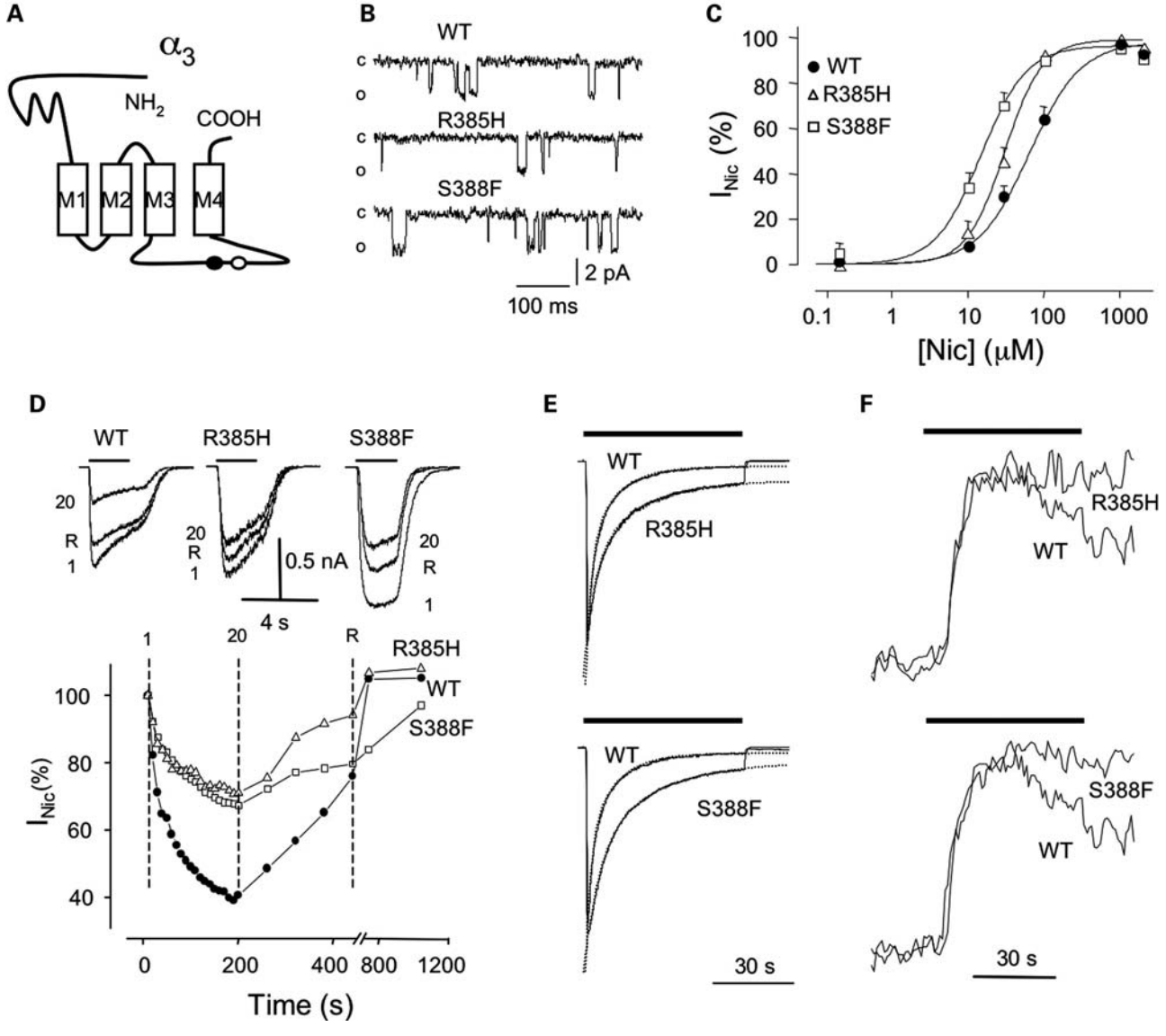

Figure 1. Functional characterization of mutant α3β4 nAChRs. (**a**) Structure of the α3 subunit, indicating the R385H (black) and S388F (white) mutations. (**b**) Unitary events evoked by Nic (1 μM, −80 mV) in outside-out patches from GH4C1 cells expressing wild-type (WT), R385H and S388F α3β4 nAChRs. (**c**) Nic dose-I_{Nic} response curves recorded in HEK cells (7–10 cells per data point). Solid lines represent best fitting Hill curves, error bars represent s.e.m. (**d**) Run-down and recovery of I_{Nic} for WT and mutant nAChRs in HEK cells. *Top*, representative whole-cell responses evoked by the first (1) and 20th (20) high-frequency Nic applications and their partial recovery 4 min after switching to the low-frequency stimulation (R). Horizontal bars (in here and thereafter), Nic applications. *Bottom*, time course of the relative peak amplitude of I_{Nic}, averaged over 4 (R385H) or 5 cells (WT, S388F). S.E.M. (<5% at each point) omitted for clarity. Dotted vertical lines indicate the time points of the top traces. EC80 Nic concentrations were: WT, 180 μM; R385H, 40 μM; S388F, 60 μM. (**e**) Decay of I_{Nic} during sustained Nic (EC_{80} concentration, −50 mV) applications, for mutant versus. WT-nAChRS. Responses recorded from 7 cells for each nAChR type, scaled and averaged. Dotted lines represent the exponential curve best fitting the decay phase (parameters are listed in Supplementary Material, Table S3). (**f**) Decay of Ca^{2+} transients during sustained Nic (at EC_{80} concentration) applications in HEK cells loaded with Fura 2 AM. Responses recorded from 11 to 15 cells for each nAChR type, scaled and averaged.

desensitization (Fig. 2E and Table 3), with an increased non-desensitizing component (Fig. 2E and Supplementary Material, Table S3). As expected, intracellular Ca^{2+} concentration remained high during sustained Nic applications to cells expressing α4 mutant nAChRs, but not WT α4β4 nAChR (Fig. 2F and Table 3).

We also analyzed the desensitization of nAChRs containing the most frequently observed mutation, β4R349C, co-expressed with WT α3, WT α4 or mutant α4R487Q (which resembles the double mutation identified in one patient) and α4R495Q or α4R487Q subunits co-expressed with WT β4. In accordance with data reported above, these five mutant nAChRs show a slowed desensitization (data not shown) suggesting that a slowed desensitization is a feature common to SASL-associated mutant in the intracellular loop of nAChRs. The mutant β4M456V co-expressed with WT α4 did not behave differently from WT α4β4.

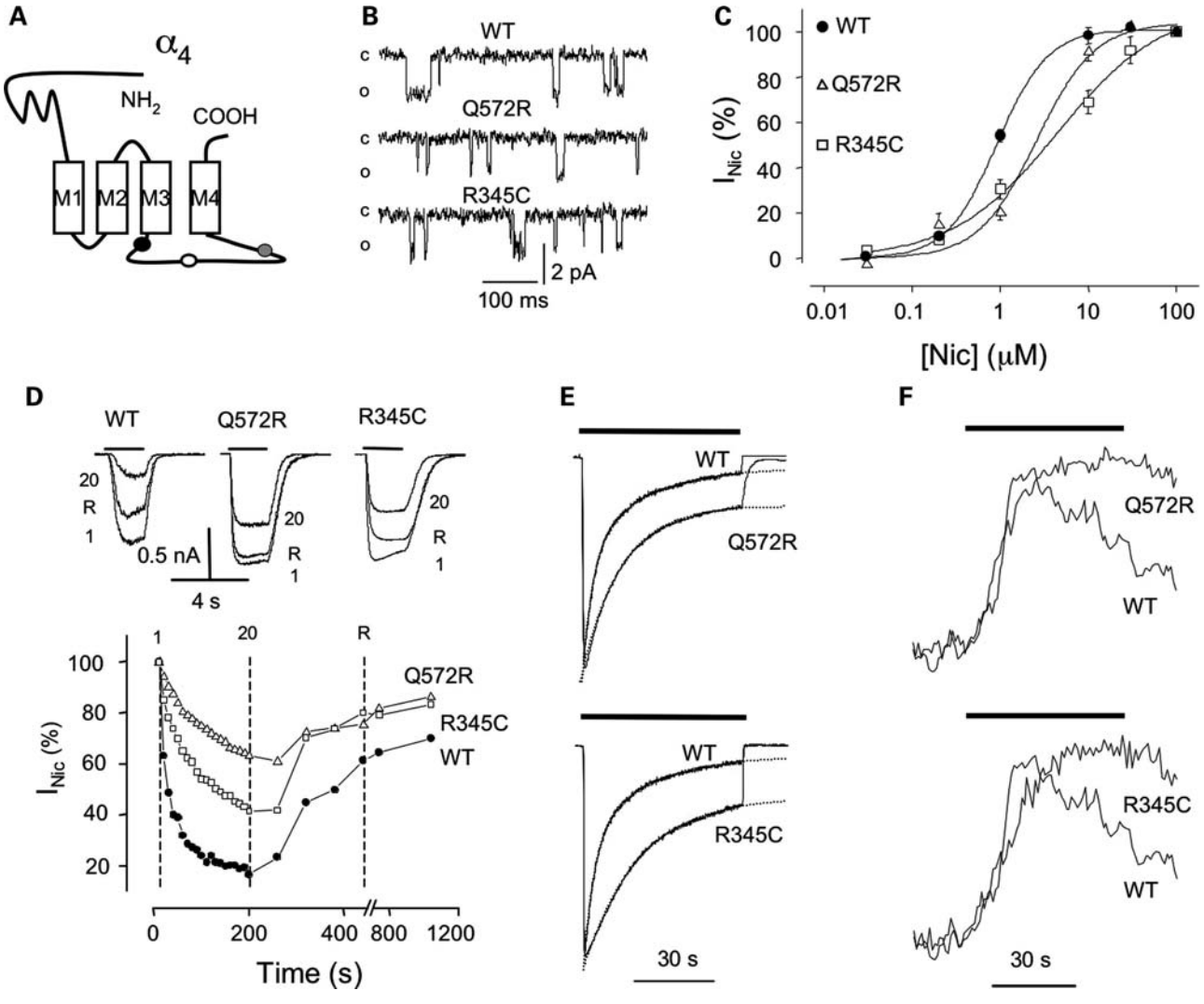

Figure 2. Functional characterization of the mutant α4β4 nAChRs. (**a**) Structure of the α4 subunit with the R345C (black), R495Q (white) and Q572R (gray) mutations indicated. (**b**) Single channel currents evoked by Nic (20 nM, −60 mV) in outside-out patches from GH4C1 cells expressing WT, Q572R and R345C mutant α4β4 nAChRs (**c**) Nic dose-I_{Nic} response curves as in (c). (**d**) Run-down of I_{Nic} obtained as in (d). *Top*, representative whole-cell currents as in (d). Holding potential, −50 mV. *Bottom*, time course of the relative peak amplitude of INic, averaged over 6 (Q572R) or 9 cells (WT and R345C); dotted vertical lines as in (d). EC_{80} Nic concentrations were: 2 μM (WT), 5 μM (Q572R), 15 μM (R345C). (**e**) Decay of I_{Nic} during sustained Nic (at EC_{80} concentration, −50 mV) applications, scaled, averaged and fitted as in (e). Data were recorded from 8 cells for each type of receptor. (**f**) Comparison of Nic-evoked Ca^{2+} transients in cells expressing WT and mutant nAChRs, recorded and processed as in (f).

DISCUSSION

We have found that the cumulative frequency of rare missense variants detected in the regions encoding the intracellular domain of *CHRNA3*, *CHRNA4* and *CHRNB4* were significantly higher in a cohort of 245 SALS patients than in 450 controls (6.1 versus 1.3%, $P = 0.001$; OR 4.48, 95% CI 1.7–11.8).

The accumulation of rare, mildly deleterious mutations in an individual human genome is likely to be an important contributor to complex diseases, including SALS (24–26). These mutations are weakly evolutionarily deleterious and their low frequency in the human population is the result of mutation-selection balance. The low allele frequency of an amino acid variant can, by itself, serve as predictor of its functional significance, with lower frequency indicating greater pathogenicity (24). According to the mutation-selection model, the weak purifying selection against susceptibility alleles is likely to result in extensive allelic heterogeneity, with low frequency mutations at many different sites (26). Thus the cumulative frequency of rare mutations rather than their individual frequencies is considered a suitable method for candidate gene investigations (24).

We therefore analyzed the cumulative frequency of rare variants detected in genes encoding subunits of nAChRs. Extending the target of mutational study to a group of genes involved in multisubunit proteins or in the same metabolic pathway, increases the probability of detecting association. Thus the combined data of three genes, encoding for α3, α4 and β4 subunits, are reported here.

Table 1. Rare missense variants

Domains	Amino acid substitution	Patients (245)		Control Individuals ($N = 450$)		*P*-value	Functional study
		Number	%	Number	%		
Extracellular	α4 T32N	0	–	1	0.22		nt
	β4 R96S	1	0.4	2	0.44		nt
	β4R136W[a]	1	0.4	0	–		Affinity[c]
	β4R136Q[b]	2	0.8	0	–		nt
	β4M186V	0	–	1	0.22		nt
	β4Q496H	0	–	1	0.22		nt
Subtotal		4	1.6	5	1.1	0.72	
Loop	α4R336C[b]	1	0.4	0	–		nt
	α4R345C	1	0.4	0	–		Desensitization
	α4P451L[b]	1	0.4	1	0.22		nt
	α4P446L	0	–	1	0.22		nt
	α4G454S	1	0.4	1	0.22		nt
	α4R487Q	1	0.4	0	–		Desensitization
	α4R495Q	1	0.4	0	–		Desensitization
	α4Q572R	1	0.4	1	0.22		Desensitization
	α3R385H	1	0.4	0	–		Desensitization affinity
	α3S388F	1	0.4	0	–		Desensitization affinity
	β4R349C[b]	5	2.04	2	0.44		Desensitization
	β4M456V	1	0.4	0	–		No effect
Subtotal		15	6.1	6	1.3	0.001	
Transmembrane	β4M467V[a]	0	–	3	0.67		Affinity[c]
subtotal		0	0	3	0.7	0.55	
Total		19	7.7	14	3.1	0.01	

nt = not tested; Desensitization = reduced receptor desensitization; Affinity = increased agonist affinity.
[a]Not reported in NCBI SNP Database and in Ensembl Database. Only reported by Liang *et al*. (32).
[b]Rare variants reported in NCBI SNP Database Ensembl Database. No validation studies.
[c]Described by Liang *et al*. (32).

Table 2. Channel functional properties of WT and mutant nAChRs

Receptor	Conductance (pS)	τ_b (ms)	P_f (%)
WTα3β4	32.80 ± 0.60 (4)	6.3	3.0 ± 0.5 (5)
α3R385Hβ4	31.90 ± 0.43 (8)	5.1	2.5 ± 0.5 (5)
α3S388Fβ4	33.00 ± 0.42 (6)	4.2	2.7 ± 0.5 (5)
WTα4β4	30.62 ± 0.47 (10)	20.6	1.6 ± 0.2 (5)
α4R345Cβ4	31.64 ± 0.41 (5)	12	1.2 ± 0.5 (5)
α4Q572Rβ4	30.19 ± 0.45 (8)	11.2	1.5 ± 0.3 (5)

Number of cells in brackets; all *P*-values versus WT, >0.05; at the exception of *P*-values for mutants α4 τ_b, <0.01.

Neuronal nAChRs are pentameric ligand-gated channels, consisting of different combinations of α2–10 and β2–4 subunits. They are widely expressed in several regions of the central and peripheral nervous system (14–16). In the spinal cord nAChRs are present on presynaptic terminals of dorsal and ventral horns and of central regions (27). Furthermore, expression of nicotinic subunits has been demonstrated also on postsynaptic sites, including spinal (18,27,28) and brainstem (29) motor neurons. nAChR play a role in many modulatory functions including regulation of glutamate release, neuroprotection and control of cell death (16). nAChRs dysfunction may have a role in neurological disorders including dementia, schizophrenia, Parkinson disease and attention-deficit/hyperactivity disorder (14,15,30).

Mutations in *CHRNA2*, *CHRNA4* and *CHRNB2* genes are responsible for the rare Autosomal Dominant Nocturnal Frontal Lobe Epilepsy (ADNFLE) (31). Most ADNFLE mutations are located in transmembrane domains which anchor the proteins in the plasma membrane and contribute to channel kinetics and ion selectivity. No human diseases have been associated so far with mutations within the extracellular domain and the intracytoplasmic loop of neuronal nAChRs. Some forms of congenital myasthenic syndromes are caused by mutations within the cellular loop of ε subunit of muscle nAChRs, resulting in changes of the receptor expression and function (23).

We planned to study the cellular loop of nAChRs because this domain is important for interaction with intracellular proteins, receptor trafficking and influencing single-channel conductance and receptor desensitization (19,20). Point mutations in this domain have been reported to affect the functional properties of neuronal nAChRs (22).

The frequency of rare variants detected in the cellular loop was significantly higher than in controls. In contrast, the number of missense variants in the regions encoding extracellular domains did not differ between patients and controls and no variant was observed in transmembrane domains of patients.

To explore the functional significance of the identified missense variants in the loop, we characterized in details four of them by mutagenesis, cell transfection and electrophysiology.

In vitro studies showed that the variants identified in the α3 subunit of SALS patients result in a definite gain-of-function of the nAChR. This effect bears a strong analogy with that observed in slow-channel congenital myasthenic syndromes,

Table 3. Functional properties of Nic-evoked whole-cell responses of WT and mutant nAChRs

Receptor	EC_{50} (μM)	n_H	I_{Nic} rundown I_{20}/I_1 (%)	I_{Nic} desensitization $T_{0.5}$ (s)	Ca^{2+} response $T_{0.2}$ (s)
WTα3β4	58 ± 4 (7)	1.2 ± 0.1	40 ± 5 (5)	6.2 ± 1.9 (7)	44 ± 5 (12)
R385Hα3β4	15 ± 2 (10)**	1.4 ± 0.2	71 ± 4 (5)**	9.8 ± 1.2 (6)**	>>60 (11)
S388Fα3β4	30 ± 3 (10)**	1.8 ± 0.1*	67 ± 5 (4)**	12.3 ± 2.1 (7)**	>>60 (16)
WTα4β4	0.9 ± 0.1 (6)	1.5 ± 0.2	13 ± 4 (9)	8.7 ± 2.3 (8)	35 ± 6 (11)
R345Cα4β4	4.4 ± 0.1 (5)**	0.7 ± 0.1*	42 ± 5 (9)*	18.8 ± 1.9 (8)*	>>60 (15)
Q572Rα4β4	2.5 ± 0.2 (6)*	1.3 ± 0.1	63 ± 3 (6)**	25.6 ± 2.2 (8)*	>>60 (14)

Numbers in brackets, number of cells. *$P < 0.05$ and **$P < 0.01$ versus the corresponding WT values. I_1, I_{20}: amplitude of the current evoked by the first and the 20th Nic application. $T_{0.5}$: Time necessary for I_{Nic} to decay by 50% during continuous Nic application (EC_{80} concentration, −50 mV). $T_{0.2}$: Time necessary for fluorescence signal to decay by 20% during Nic applications lasting 60 s (EC_{80} concentration).

Table 4. Clinical and genetic features of patients with missense variants within the intracellular loop

Patient	Region of origin	Mutated subunit	Sex	Age of onset	Site of onset	Phenotype	Follow-up*	Outcome	Cigarette smoke	Variant	Aminoacid substitution
1 (A.C.)	Lazio	α3	F	45	Spinal	p-UMN	34	Tracheostomy	No	1154 G>A	R385H
2 (S.L.)	Toscana	α3	M	59	Spinal	Flail Arm	47	Alive	No	1163 C>T	S388F
3 (M.F.)	Lazio	α4	F	70	Bulbar	p-UMN	32	Dead	Yes	1033 C>T	R345C
4 (P.P)	Calabria	α4	M	54	Spinal	p-UMN	34	Dead	No	1484 G>A	R495Q
5 (P.A.L.)	Lazio	α4	M	31	Spinal	p-UMN	10	n.a.	Yes	1715 A>G	Q572R
6 (M.L.)	Molise	α4	M	62	Spinal	p-UMN	75	Alive**	Yes	1352 C>T	P451L
7 (T.C.)	Lazio	α4	M	62	Bulbar	Bulbar frontal dementia	28	Alive	Yes	1460 G>A	R487Q
		β4								1045 C>T	R349C
8 (P.G.)	Lazio	α4	M	64	Bulbar	Classic	33	Dead	Yes	1360G>A	G454S
9 (S.A.)	Lazio	α4	F	68	Bulbar	Classic	35	Dead	No	1006C>T	R336C
10 (R.M.)	Marche	β4	F	63	Spinal	Classic	10	n.a.	n.a.	1366 A>G	M456V
11 (I.M.)	Lazio	β4	M	51	Spinal	Classic	27	Tracheostomy	Yes	1045 C>T	R349C
12 (Sb.A.)	Umbria	β4	F	65	Bulbar	Classic	32	Tracheostomy	No	1045 C>T	R349C
13 (Se.A.)	Calabria	β4	M	56	Spinal	Flail	48	Dead	Yes	1045 C>T	R349C
14 (C.M.)	Lazio	β4	M	71	Spinal	Classic	27	Alive	Yes	1045 C>T	R349C

*: months; **: non-invasive ventilation; n.a.: not available.

in which gain-of-function mutations of the endplate nAChRs, even if not accompanied by an enhanced Ca^{2+} permeability, cause cationic overloading and Ca^{2+}-induced toxicity (23). Mutations in the α4 subunit have a more complex impact, as the lower affinity for the agonist and the shorter burst duration may dampen nAChR responses. However, the reduced desensitization and use-dependent run-down of the mutant α4β4 nAChRs prolong their responsiveness during sustained stimulation, with a concomitant maintained increase of intracellular Ca^{2+} concentration. A slowed desensitization was also observed for five other nAChRs harbouring mutant subunits identified in SALS patients (Table 1). Further studies of all identified rare missense variants are needed to elucidate their functional consequences. Remarkably, the β4R136W and β4M467V polymorphisms, falling outside the intracellular loop, and detected in healthy controls (32), but also in one of our patients, have been reported to accelerate nAChR desensitization (32). There is a growing body of evidence that within the central nervous system nAChRs are involved in a form of non-synaptic transmission, much slower than normal synaptic transmission, which contributes to shape Ca^{2+} accumulation in target cells (33). In addition, the high levels of nicotine chronically present in the brains of smokers are likely to cause desensitization of nAChRs (34), so that ACh- and nicotine-induced desensitization of central nAChRs may have a profound impact on cholinergic neurotransmission in the brain (35).

Ca^{2+} signals mediate the neuromodulatory effects of nAChRs, including regulation of cell survival and control of glutamate release. Reduced desensitization of presynaptic nAChRs at glutamatergic terminals might result in excessive excitatory glutamate release, and potentiated activity of postsynaptic nAChRs might bring Ca^{2+} entry into neurons to neurotoxic levels (16). In the case of SALS, both excess of glutamate and Ca^{2+} overloading of motor neurons may have a role in disease initiation or spread (13,36).

Clinically, the 14 patients with missense variants in the loop had different phenotypes (Table 4). Though the majority of patients with *CHRNB4* variants had a classic phenotype and most patients with *CHRNA3* and *CHRNA4* variants had a predominant Upper Motor Neuron (p-UMN) form, no clear genotype–phenotype correlation could be established. SALS is actually a spectrum of conditions, showing wide variability with respect to age of onset, duration of the disease, topographic distribution of the clinical signs and amount of UMN versus lower motor neuron impairment (1,37). Phenotypic heterogeneity of ALS could reflect genetic heterogeneity. One patient, harbouring mutations in both *CHRNB4* and

CHRNA4 genes, presented with a severe bulbar involvement associated with frontal dementia. Interestingly, a possible involvement of nAChR in the pathogenesis of dementia has been suggested (14,15,38) and cognitive impairment is reported in *CHRNA4* mutations causing *ADNFLE* (31).

SALS is considered a complex genetic disease resulting from the interaction between environmental factors and specific susceptibility genes. Several models have been suggested to explain the genetics of SALS, including the liability threshold model for a discrete trait with non-Mendelian familial clustering (6). Our *in vitro* studies showed that all the analyzed mutations are not neutral but result in a gain-of-function of the nAChRs which might have a mild deleterious effect. Missense variants predisposing to complex diseases are expected to be found in controls, although at significantly lower frequency than in patients, as in our study, with an OR in the range we have detected (39). Thus, the observed variants may act as predisposing factors for ALS, but disease develops only once a critical threshold of liability is crossed, due to the cumulative contribution of environmental agents or of other genes.

The hypothesis that environmental factors may concur in altering neuronal nAChRs function is plausible since these receptors are the target of several substances, including tobacco nicotine, cyanobacterial alkaloid neurotoxin anatoxin-a (40) and organophosphate insecticides (41). Though nicotine may have neuroprotective activity (17), overstimulation of nAChRs with high dose nicotine has toxic effects on neurons, due to excessive entry of calcium into cells (38). Notably, an evidence-based review of exogenous risk factors in ALS concluded that cigarette smoking was a probable ('more likely than not') risk factor for SALS, based on two high quality studies (42). Eight of our patients harbouring nAChRs mutations were heavy cigarette smokers thus suggesting the possible additional contribution of nicotine in altering the receptor function.

On the other hand the possibility that disease development is the result of the cumulative effects of variants at multiple genes is suggested by the detection of two missense variants, in α4 and in β4, in one of our patients. Since nAChRs have a pentameric structure with subunit stoichiometry usually of $(\alpha_x)_2(\beta_y)_3$, the effects of these variants are likely to summate.

In conclusion, we observed that rare missense variants within the intracellular loop of three nAChRs subunits are significantly over-represented in SALS patients and alter receptor functions. We hypothesize that the here reported nAChR variants represent a predisposing factor for a subset of SALS and that cholinergic dysfunction is a putative pathogenetic mechanism for motor neuron degeneration. Further studies are needed at both genetic and functional level to confirm this hypothesis.

MATERIAL AND METHODS

We carried out sequence analysis of exons and flanking intronic regions of *CHRNA3*, *CHRNA4* and *CHRNB4* genes encoding α3, α4 and β4 nAChR subunits, respectively, in 245 Italian SALS patients and 450 control individuals matched by age (mean age difference: 5 years), sex and ethnic origin. These subunits were selected for being the most abundant ones in mammalian brain and spinal cord (14,15). On the basis of the critical role of the large intracellular loop for receptor functions (19,20), we planned to search for sequence variants in the regions encoding this domain. In a second phase, we analyzed also the extracellular and transmembrane domains.

Patients

Genomic DNA from peripheral blood leukocytes was obtained from patients and from controls after informed consent. Both patients and controls were from the Centre and the South of Italy. Diagnosis of ALS was made according to revised El Escorial/Arlie House Criteria (43). None of the affected individuals had a family history of ALS. Mutations of *SOD1* were excluded in each patient. ALS patients were subdivided into three clinical phenotypes: p-UMN, classic and Flail Arm. Included in the p-UMN group were those patients whose clinical manifestations were dominated by pyramidal signs, mainly severe spastic paraparesis (37). All these patients showed, by definition, clear signs of lower motor neuron impairment from the beginning of the disease. The classic group was defined by the presence of prevailing lower motor neuron signs associated with slight to moderate pyramidal signs. The Flail Arm phenotype was characterized by symmetric, predominantly proximal, wasting and weakness of both arms, leading to severe functional disability in the initial phase of the disease (44).

In our ALS cohort the male to female ratio was 1.9 : 1; the age of onset ranged from 22 to 83 years with a mean of 58.6. The median survival was 38 months (range 5–124). Seventy-four patients had the p-UMN phenotype, 13 had the Flail arm variant and the remaining 158 had the classic type. One hundred and sixty patients, including 15 with young-adult p-UMN form, 5 with young-adult classic form, 29 adult-onset p-UMN type and 116 with adult-onset classic ALS were previously described (37).

Sequencing analysis of genes *SOD1, CHRNA3 CHRNA4* and *CHRNB4*

Primers for amplification of all coding exons and flanking intronic regions of the listed genes were created with Primer Express Version 1.5 (Applied Biosystems) according to the Ensembl sequences (Accession number SOD1: ENSG00000 142168; *CHRNA3*: ENSG00000080644; *CHRNA4*: ENSG 00000101204; *CHRNB4*: ENSG00000117971). PCR amplifications were performed according to standard protocols. Primers and annealing temperatures are listed in Supplementary Material, Table S4. In some reactions dimethylsulphoxide 5% or glycerol (1%) were added to favour DNA denaturation or to improve the reaction specificity, respectively. PCR products were then purified (using Exonuclease I and Shrimp Alcaline Phosphatase, GE Healthcare) and sequenced bidirectionally using the Big Dye terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and a capillary automated sequencer (ABI 3130) (Applied Biosystems), according to manufacturer's instructions.

Generation of nicotinic receptor subunits mutants

The cDNAs encoding the human α3 nicotinic receptor subunit (NCBI accession number: NM000743) and the α4 nicotinic receptor subunit (NCBI accession number: NM000744) were kindly provided by Dr P. Verhassen (Belgium), both cloned in pcDNA3 vector. Point mutations were all generated with Gene Tailor Site-Directed Mutagenesis System (Invitrogen, Italy), following the manufacturer's instructions. Briefly, the cDNAs encoding the human WT α3 and α4 subunits were methylated on cytosine residues for 1 h at 37°C; products obtained were amplified in the mutagenesis PCR reactions using two overlapping primers for each mutagenesis, one of which contained the target mutation (forward).

Cell culture and transfection

The human retroviral packaging cell line HEK293 (HEK) was grown (5% CO_2, 37°C) in Dulbecco's modified Eagle's medium with *Glutamax-I* plus 10% fetal calf serum and 1% penicillin/streptomycin. Plating density was 10^4 cells/cm^2. Rat pituitary GH4C1 cells were grown in HAM F10 nutrient mixture plus 10% fetal calf serum and 1% penicillin/streptomycin. Cell transfection was performed with Lipofectamine (Invitrogen, Italy) adding 2 μg of cDNA for each subunit per Petri dish. HEK cells were mechanically dissociated and replated 24 h before measurements.

Electrophysiology

Whole-cell currents were recorded from HEK cells 1–3 days after transfection. Cells were continuously superfused using a gravity-driven fast exchanger system (RSC-100, Bio-Logic, France), which allowed complete solution exchange in ∼50 ms, a time adequate for the kinetic analysis of the main events addressed in this study. Dose-response curves were constructed plotting current peak amplitude values obtained at different Nicotine (Nic) concentrations, after normalization. The non-linear fitting routine of Sigma Plot software (Jandel Scientific, CA, USA) was used to fit the data to the Hill equation: $I = 1/(1 + (EC_{50}/[A])^{nH})$, where I is the normalized current amplitude induced by Nic at concentration $[A]$; n_H, the Hill coefficient; and EC_{50} the concentration inducing half-maximum response. The protocol for current rundown consisted of 20 applications of Nic (2 s duration) every 10 s, followed by a recovery period when Nic was applied every 60 s. Nic was applied at the concentration yielding 80% of the maximal response (EC_{80}), derived by interpolation of dose-response curves for each nAChR type.

Outside-out single-channel data were recorded from GH4C1 cells. Channel conductance was obtained dividing the unitary amplitudes by the pipette potential, assuming a current reversal potential of about 0 mV for all the receptors (45,46). Only the main conductance state was considered in the analysis. Openings to lower conductances were observed in some patches, for both WT and mutant nAChRs, but their frequency was too low for an adequate analysis. After patch excision, channel activity showed a marked rundown, as previously reported (45,47), and burst analysis could not be properly performed in most cells. Data from different cells were therefore pooled and analyzed together following previously reported criteria (48).

Ca^{2+} measurements

The methods for P_f determinations have been previously described (49).

Data analysis and statistics

Data are reported as means ± SEM, and statistical significance tested using ANOVA ($P < 0.05$). The F/Q ratio values used in the P_f determinations were obtained as linear regressions of the data, using Sigma Plot software.

AUTHOR CONTRIBUTIONS

M.S., M.Z. and F.E. designed and supervised the study and contributed in the writing of the manuscript. G.N. and P.T. were involved in the design of the study. G.N. and A.Al-C. interpreted genetic data and contributed to the writing of the manuscript. A.C., F.M., M.L., A.D.G. recruited patients, collected clinical data and provided DNA samples. I.M., S.L., M.M., D.O. and G.M. performed mutational analysis. C.L., F.T. and F.S. performed subunits mutagenesis and cell expression. S.D.A. performed whole-cell recordings. F.G., A.D.C., C.M. performed single channel recordings. S.F. performed calcium P_f determinations. All authors participated in the critical revisions of the manuscript and approved its contents.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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