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A mouse model of the slow channel myasthenic syndrome: Neuromuscular physiology and effects of ephedrine treatment

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ABSTRACT

In the slow channel congenital myasthenic syndrome mutations in genes encoding the muscle acetylcholine receptor give rise to prolonged ion channel activations. The resulting cation overload in the postsynaptic region leads to damage of synaptic structures, impaired neuromuscular transmission and fatigable muscle weakness. Previously we identified and characterised in detail the properties of the slow channel syndrome mutation εL221F. Here, using this mutation, we generate a transgenic mouse model for the slow channel syndrome that expresses mutant human ε-subunits harbouring an EGFP tag within the M3–M4 cytoplasmic region, driven by a ~1500 bp region of the *CHRNB* promoter. Fluorescent mutant acetylcholine receptors are assembled, cluster at the motor endplates and give rise to a disease model that mirrors the human condition. Mice demonstrate mild fatigable muscle weakness, prolonged endplate and miniature endplate potentials, and variable degeneration of the postsynaptic membrane. We use our model to investigate ephedrine as a potential treatment. Mice were assessed before and after six weeks on oral ephedrine (serum ephedrine concentration 89 ± 3 ng/ml) using an inverted screen test and in vivo electromyography. Treated mice demonstrated modest benefit for screen hang time, and in measures of compound muscle action potentials and mean jitter that did not reach statistical significance. Ephedrine and salbutamol show clear benefit when used in the treatment of *DOK7* or *COLQ* congenital myasthenic syndromes. Our results highlight only a modest potential benefit of these β2-adrenergic receptor agonists for the treatment of the slow channel syndrome.

Introduction

The congenital myasthenic syndromes (CMS) are inherited disorders of neuromuscular synaptic transmission. Mutations in many different genes associated with the neuromuscular junction have been shown to underlie these syndromes through a variety of molecular mechanisms (Chaouch et al., 2012; Engel, 2012; Palace and Beeson, 2008). The mechanisms, amongst others, include kinetic abnormalities of the acetylcholine receptor (AChR), reduced numbers of endplate AChR, severely reduced endplate acetylcholinesterase (*COLQ* mutations) and mutations in the *AGRIN-MUSK-DOK7* AChR clustering pathway that are thought to impair stability of the synaptic structure.

The slow channel congenital myasthenic syndrome was first described in 1982 (Engel et al., 1982). It is the only dominant form of CMS, and is due to mutations within the AChR subunits that cause prolonged activation of the AChR ion channel (Ohno et al., 1995; Sine et al., 1995). Weakness of cervical, scapular and finger extensor muscles is often an early feature. The disorder is commonly slowly progressive affecting respiratory, limb and bulbar muscles (Engel, 2012). On electromyography (EMG) patients usually show a decrement of the compound muscle action potential (CMAP) at 3 Hz stimulation, and in vitro microelectrode studies show prolongation of the endplate potentials (EPP) and currents (EPC), and miniature endplate potentials (MEPP) (Engel et al., 1982). Ultrastructural studies show what is termed an 'endplate myopathy' with areas of degenerating junctional folds thought to result from synaptic exposure to high intracellular Ca^{2+} concentrations. Neuromuscular transmission may also be impaired by depolarization block due to temporal summation of endplate potentials and the increased propensity for mutant AChR harbouring slow channel syndrome mutations to enter the desensitised state (Engel et al., 2010). At least 20 different mutations responsible for SCS have been published (Engel et al., 2010). Mutations fall into two broad, though not exclusive, categories: those that result in stabilisation of the open state of the channel, usually

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occurring in the pore-lining M2 domain of the AChR subunits, or those that increase the affinity of the receptor for acetylcholine (ACh) and prolong association time. The traditionally effective therapy for myasthenic disorders of anticholinesterase medication is usually detrimental in slow channel syndrome, and alternative treatments that block the AChR ion channel when it is in the open state, such as fluoxetine or quinidine, are used (Engel, 2007).

Ephedrine was reported to benefit patients with myasthenia gravis in the 1930s (Edgeworth, 1930, 1933), but its use was superseded by anticholinesterases and corticosteroids. A series of recent reports have highlighted the beneficial effects of ephedrine or salbutamol, both β2-adrenergic receptor agonists, in treating defined CMS subgroups due to mutations in *DOK7* or *COLQ* (Beeson et al., 2006; Bestue-Cardiel et al., 2005; Chan et al., 2012; Lashley et al., 2010; Mihaylova et al., 2008; Schara et al., 2009). An additional report also found salbutamol of benefit in some patients with AChR deficiency (Sadeh et al., 2011). Although the benefit is clear for patients with *DOK7* or *COLQ* mutations, the molecular pathway for this effect is not known. *In vitro* studies suggest β2-adrenergic receptor agonists can have an effect on quantal release of acetylcholine or on AChR kinetics, but not at the levels that would be present in the serum of treated patients (Milone and Engel, 1996; Sieb and Engel, 1993; Wood and Slater, 2001).

Previously we have characterised the kinetic properties of the εL221F slow channel syndrome mutation in detail (Croxen et al., 2002; Hatton et al., 2003). Here, we generate and characterise a transgenic mouse model of the slow channel syndrome that harbours an EGFP marker within the mutant AChR ε-subunit εL221F that replicates the fatigable weakness and neurotransmission defects of the human disease. We show that ephedrine enhances neuromuscular transmission in this transgenic mouse model of slow channel syndrome, and so maybe an appropriate additional therapy for this disorder.

Material and methods

Plasmid construction

Human AChR ε-subunit cDNA (Beeson et al., 1993a) was amplified and cloned into the *Bam*HI site of pcDNA3.1hygro(+) making pcDNA-hu-ε. Point mutagenesis of c.661C>T to generate the εL221F mutation was carried out using Quikchange (Stratagene) and confirmed by sequencing. A wild type human ε subunit (*CHRNE*) genomic clone (Beeson et al., 1993b) was sub-cloned via PCR into pGEM-T Easy, with the forward primer for this reaction containing an *Nhe*I site. cDNA from the start up to exon 10 of the human AChR ε-subunit cDNA was removed from pcDNA-hu-ε using *Nhe*I and *Sfi*I, and was replaced by genomic sequence (containing the mutation) that was excised using the *Nhe*I site in the forward primer and the first *Sfi*I site in the genomic sequence within exon 10. The resulting vector is termed pcDNAεL221F. EGFP was amplified with primers containing *Sfi*I restriction sites and was cloned into the *Sfi*I site in exon 10 of the ε subunit in pcDNAεL221F, making pcDNAεL221F-EGFP. The promoter of the human AChR β subunit was chosen to provide muscle-specific expression of the transgene. The CMV promoter in pcDNAεL221F-EGFP was excised using *Ssp*I and *Nhe*I and was replaced by residues −1493 to +18 (where +1 is the transcription start site) of the human AChR β-subunit gene, giving the final construct cassette βpromεL221F-EGFP.

Transfections

TE671 cells and HEK 293 cells were purchased from ATCC and were maintained in DMEM (Sigma-Aldrich) supplemented with 10% FCS (TCS Cellworks Ltd) and 100 units/ml each of penicillin G and streptomycin (PS) purchased from Invitrogen. Cells in 6-well plates were transfected with 3 μg DNA/well using calcium phosphate precipitation (TE671 cells) or PEI (HEK 293 cells). 48 h following transfection expression of the EGFP-tagged AChR εL221F subunit was visualised using an Axiovert 200 inverted Zeiss fluorescent microscope. For electrophysiology cDNA encoding AChR subunits α:β:δ:ε were used in the ratio of 2:1:1:1.

Generation of mutant mice

The transgene expression cassette containing the AChR β-subunit promoter and DNA encoding human εL221F-EGFP was excised from the βpromεL221F-EGFP plasmid using *Ssp*I and *Avr*II. Transgenic mice were generated by microinjection of the purified expression cassette into the pronucleus of F2 hybrid oocytes from C57BL/6 × CBA/CA parents. Transgenic mice that were positive for both the human ε subunit and EGFP were identified by analysis of PCR amplicons from genomic DNA. Oligonucleotide primers specific for exon 2 of human AChR ε-subunit were 5′-CAATGACCCTTCCTTCTTGACC-3′ and 5′-AGATGAGG GTGGGGGTAGCTT-3′, and EGFP-specific primers were 5′-GGCCCCCC GGGCCCCACCGGTCGCCACCATGGTGAG-3′ and 5′-GGCCCGGGGGGCC TTGTACAGCTCGTCCATGCCGAGAGTG-3′. Mice with targeted disruption of the mouse AChR ε-subunit gene (Missias et al., 1997) were kindly provided by Professor J. Sanes when at Washington University, St Louis, USA.

Mouse breeding and genotyping

Mice that contained the transgene (εL221F-EGFP$^{+/-}$) were crossed with mice heterozygous for the mouse AChR ε-subunit knock-out mutation (mε$^{+/-}$). Progeny with genotype εL221F-EGFP$^{+/-}$ε$^{+/-}$ was mated with siblings of the same genotype to generate litters containing mice that were homozygous for the εL221F-EGFP transgene and heterozygous for the mouse AChR ε-subunit knock-out mutation (εL221F-EGFP$^{+/+}$/mε$^{+/-}$). These were then mated with siblings of the same genotype to produce litters containing mice that were homozygous for εL221F-EGFP in a mouse ε-subunit knock-out background (εL221F-EGFP$^{+/+}$/mε$^{-/-}$). Mouse genotyping was performed by analysis of PCR amplicons from genomic DNA. Human ε-specific and EGFP-specific primers are described above. The presence of the neomycin resistance gene indicated targeted disruption of the mouse ε-subunit and was detected by using primers 5′-CAACAGACAATCGG CTGCTCT-3′ and 5′-GAATGGGCAGGTAGCCGGAT-3′. Mouse ε-subunit was detected using primers 5′-CTCTTCGACAATTATGATCCAGA-3′ and 5′-GAGCCGATAGTCGTGCCAGT-3′ that amplify between exons 2 and 4.

RT-PCR

Total RNA was extracted from leg muscle using RNA-Bee (TEL-TEST Inc., Friendswood, TX, USA), and first-strand cDNA was synthesised using a RETROscript kit (Ambion). EGFP was detected using the primers described above, and mouse AChR α-subunit was detected using primers 5′-AGTCCAATAACGCCGCTGAG-3′ and 5′-TTTCCTAGCGATGGCTATGG.

Electron microscopy

Anaesthetised mice were transcardially perfused with PBS followed by 2.5% glutaraldehyde fixation buffer. The tibialis anterior muscles were post-fixed with 1% osmium tetroxide, dehydrated through a graded ethanol series, and embedded in epoxy resin. Ultra-thin sections from selected areas were contrasted with uranyl acetate and lead citrate and viewed with a Philips CM 100 electron microscope (Eindhoven, The Netherlands). At least five endplate regions were photographed from each muscle.