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Free Communications 3: Ligand-Gated Ion Channels

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Chair: Louise Tierney
Analysis of a GABA\textsubscript{A}\(\gamma2\) (R43Q) knock-in mouse model of familial epilepsy

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Epilepsy is a common neurological disorder that causes paroxysmal electrical discharges in the brain. It can be difficult to treat, with up to 30\% of all patients unable to achieve satisfactory pharmacological control of their seizures, and many others living with a host of adverse side effects such as sedation and cognitive impairment. Genetic analysis of familial forms of epilepsy led to the discovery of ion channel gene mutations linked to a range of epilepsy syndromes, thus pointing to changes in ion channel function as the primary causative agent of many common forms of epilepsy. Lessons learned from the analysis of these mutations and how they alter neurobiology and neurophysiology are vital to our gaining knowledge of the fundamental mechanisms of seizure genesis, and provide the key to developing better strategies to control epilepsy. We have begun this process with a detailed examination of an autosomal dominant mutation of a GABA\textsubscript{A}\(\gamma2\) receptor subunit [GABR\(\gamma2\)(R43Q)] found in a large family with GEFS+ (Generalised Epilepsy with Febrile Seizures plus). The study of mouse models harbouring human epilepsy causing mutations is one way in which a more direct link between genes and phenotypes can be made that not only permits the molecular study of mutated genes \textit{in vivo}, but which may also provide a direct link to phenotype. We therefore generated a knock-in mouse model with the GABR\(\gamma2\)(R43Q) mutation, to investigate the \textit{in situ} functional consequences of this genetic lesion on inhibitory synapses, and to assess the potential involvement of this mutation in the development of epilepsy.

Wild type mice (\(\gamma2^{R43/R43}\)) and mice heterozygous for the GABR\(\gamma2\)(R43Q) mutation (\(\gamma2^{R43/Q43}\)) were anaesthetised i.p. with ketamine (100 mg/kg) and xylazine (15 mg/kg), and instrumented for EEG recording. Video and EEG analysis of recordings revealed absence seizures, with correlated 3-7 Hz spike and wave discharges (SWD) in the mutant animals. Human patients carrying the GABR\(\gamma2\)(R43Q) mutation display absence seizures with 3 Hz SWD; recapitulation of the human phenotype in our mouse model is a vital validation step, and suggests that underlying pathological mechanisms may be shared between mouse and human. The heterozygous mutant mice also showed an elevated sensitivity to challenge with the pro-convulsant drug, pentylenetetrazol (40-120 mg/kg, s.c.).

Miniature Inhibitory Postsynaptic Currents (mIPSCs) were analysed in Layer 2/3 cortical neurons using the whole cell patch clamp technique, in acute brain slices obtained from P14-16 mice decapitated after anaesthesia by inhalation of isoflurane. Analysis of wild type, heterozygous and homozygous mutant (\(\gamma2^{Q43/Q43}\)) mice demonstrated a reduction in amplitude (\(\gamma2^{R43/R43} = 67.5 \pm 2.91\) pA, \(2^{R43/Q43} = 58.4 \pm 2.22\) pA, \(2^{Q43/Q43} = 40.0 \pm 2.62\) pA), and a slower rate of decay (\(\gamma2^{R43/R43} = 4.7 \pm 0.15\) ms, \(\gamma2^{R43/Q43} = 5.9 \pm 0.32\) ms, \(2^{Q43/Q43} = 31.4 \pm 2.62\) ms). The heterozygous phenotype was much more subtle than would be expected from a simple gene dosing effect. Interestingly, the frequency of detectable synaptic events was also drastically reduced in the homozygous mutant animals (\(\gamma2^{R43/R43} = 7.17 \pm 0.83\) Hz, \(\gamma2^{Q43/Q43} = 0.85 \pm 0.15\) Hz). We are currently characterising mIPSCs in the thalamocortical relay circuit, which has been implicated in the generation of the SWD of absence epilepsy.

To determine whether the altered characteristics of the inhibitory currents were due to a pre- or post-synaptic perturbation, immunohistochemical analysis was performed in brain slices obtained from P12-P16 mice killed after pentobarbitone anaesthesia (100 mg/kg, i.p.). In homozygous mutant mice, punctate staining of glutamic acid decarboxylase (GAD), the marker of GABAergic synaptic inputs, was unaltered compared to wild type controls. However, in contrast, immunoreactivity for GABA\textsubscript{A}\(\alpha1\) and \(\gamma2\) subunits was virtually absent from most brain regions, including the cortex, thalamus and cerebellum. The distribution of \(\gamma2\) subunits was investigated further in cortical neurons, maintained in primary cultures for 14-16 days. Pregnant female mice were killed by cervical dislocation, and cortices dissected from decapitated E15-E16 embryos. To determine the location of GABA\textsubscript{A} receptors subunits in cultured neurons, extracts of total cellular proteins and cell surface proteins were prepared: the cell surface proteins were biotinylated in living cultures, and subsequently extracted on an immobilised avidin column. In heterozygous and homozygous mutant animals, the \(\gamma2\) subunit was detected in extracts of total cellular protein, but its expression was reduced in cell surface protein extracts. Our studies suggest that a reduction in cell surface expression of the \(\gamma2\) subunit, along with a reduction in amplitude and altered deactivation kinetics of mIPSCs, are the key molecular deficits responsible for absence epilepsy in this model.
The receptor-associated protein, rapsyn, and regulation of postsynaptic acetylcholine receptor packing density and turnover at the neuromuscular synapse
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Rapsyn is a membrane-associated protein that binds the cytosolic M3-M4 loop domain of the nicotinic acetylcholine receptor (AChR). During embryogenesis, neural agrin (a proteoglycan secreted by the nerve terminal) is thought to coordinate the spatially-appropriate activation of Muscle Specific Kinase (MuSK). This initiates divergent intracellular pathways that result in 1) formation of an AChR cluster (a process that depends upon rapsyn) and 2) possibly also the transcriptional activation of synaptic genes. The precise signaling pathways for these effects remain ambiguous (Bezakova & Ruegg, 2003). We have been studying the role of rapsyn in the homeostasis of the established synapse. Rapsyn was tagged by fusing enhanced green fluorescent protein (EGFP) to its C-terminus (Gervásio & Phillips, 2005). Rapsyn-EGFP functioned like unmodified rapsyn since it assembled into AChR clusters when cultured myotubes were treated with neural agrin. To study its role in vivo we anaesthetized 4-week old female FVB mice with 5µl/g I.P. of a mixture of ketamine (10mg/ml) and xylazine (10mg/ml). The tibialis anterior muscle was exposed and electroporated with expression plasmid for rapsyn-EGFP, followed by subcutaneous injection of 30 µl of buprenorphine (300µg/ml). Rapsyn-EGFP targeted to the dispersed Golgi elements in the muscle fibre where it may normally assemble with the newly synthesized AChR. Rapsyn-EGFP also targeted directly to the postsynaptic AChR cluster where it increased the stoichiometry of rapsyn to AChR. This was associated with a slowing in the metabolic turnover of synaptic AChR (Gervásio & Phillips, 2005). Rapsyn-AChR stoichiometry can also be increased by neural agrin treatment, suggesting a possible physiological mechanism that might regulate retention of AChRs within the postsynaptic AChR cluster.

What do we mean when we speak of a postsynaptic receptor cluster? These are often defined in papers merely as bright spots or puncta of anti-receptor antibody staining. Intracellular receptors are often located immediately beneath the postsynaptic membrane. These may be confused with functional, surface-exposed receptors in routine immunostaining. In the case of the neuromuscular synapse, small <1µm AChR clusters are found at the lips of the post-junctional membrane infoldings. Here AChRs are packed tightly together (10,000/µm²; Salpeter & Harris, 1983) and mediate the postsynaptic current. Clusters interdigitate with post-junctional membrane in-foldings in which a reserve of AChRs are present, but at 10-fold lower density. While exposed to the extracellular fluid, these ‘unclustered’ AChRs are unlikely to contribute greatly to the normal postsynaptic current. Unclustered AChRs seem to be in continual interchange with the neighboring, clustered AChRs. Alterations in the efficiency of rapsyn-mediated AChR clustering might change the fraction of ‘synaptic’ AChRs that are engaged in the postsynaptic AChR cluster, and thereby the amplitude of the postsynaptic current. To gauge the efficiency of postsynaptic AChR clustering we have developed a Fluorescence Resonance Energy Transfer (FRET) technique. FRET is a sensitive method for detecting situations where two fluorescently labeled proteins come within 10nm of each other. AChRs are labeled with a mixture of TRITC-α-bungarotoxin (FRET donor) and Alexafluor647-α-bungarotoxin (acceptor). These are allowed to bind randomly to the AChR. The two binding sites on each AChR channel are separated by about 8nm and yield only weak intramolecular FRET. FRET efficiency increases 5-fold due to intermolecular FRET when AChRs come together in a cluster. FRET efficiency was low at synapses in newborn mice but increased approximately twofold during postnatal development, coincident with a similar increase in the rapsyn-AChR stoichiometry at the synapse. This suggests that the efficiency with which AChRs are partitioned within postsynaptic AChR clusters may be regulated by rapsyn-AChR stoichiometry.

Functional consequences of clustering GABA$_A$ receptors

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Inhibitory signals in mammalian brains are mediated primarily by $\gamma$-aminobutyric acid type A receptors (GABA$_A$R). Different subtypes of these receptors, distinguished by their subunit composition, are either concentrated at postsynaptic sites where they mediate phasic inhibition or found at non-synaptic (extrasynaptic) sites where they mediate tonic inhibition. Neurons, therefore, require discrete trafficking mechanisms to regulate the subcellular distribution of GABA$_A$R subtypes. Although its precise role in vivo is yet to be clearly defined, the GABA$_A$ receptor-associated protein GABARAP has been shown to participate in trafficking and membrane fusion events that underlie organisational processes at GABAergic synapses (Kittler et al., 2001; Kneussel, 2002). Co-expression of GABARAP has been shown to cluster recombinant GABA$_A$ receptors (Chen et al., 2000; Everitt et al., 2004) and, as a consequence of this ordered packing arrangement, the recombinant GABA$_A$ receptors function differently. At the single-channel level we have shown that GABA$_A$ receptors co-expressed with recombinant GABARAP in L929 cells may display high conductances (>40 pS) (Everitt et al., 2004) which is in stark contrast to the conductance of $\alpha\beta\gamma$ receptors expressed without GABARAP (<40 pS) (Luu et al., 2005). Single-channel amplitude distribution histograms and open probabilities were analysed to examine the effects of drugs such as GABA and diazepam. The presence of the soluble intracellular protein GABARAP influences recombinant GABA$_A$ channels such that they may open to multiple discrete states and the maximum single-channel conductance is dependent on the effective GABA concentration. It remains a fundamental issue as to what these multiple conductance states represent. Is it a single channel or multiple channels opening in concert? An important conclusion is that recombinantly expressed $\alpha\beta\gamma$ receptors behave more like native receptors when cotransfected with GABARAP.

The charge of the P-loop glutamate controls cation-anion selectivity in CNG channels

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Cyclic-nucleotide-gated (CNG) channels play a critical role in olfactory and visual transduction. We have used site-directed mutagenesis and inside-out patch-clamp recordings to investigate ion permeation and selectivity in two mutant homomeric rat olfactory CNGA2 channels expressed in HEK 293 cells. We showed that a single point mutation of the negatively-charged pore-loop (P-loop) glutamate to either a positively-charged lysine or arginine did produce functional channels, which consistently responded to either cGMP or cAMP, although the currents were extremely small. We found that the concentration-response curve of the lysine mutant channel was very similar to that of wild-type (WT) channels, suggesting no major structural alteration to the mutant channels. Reversal potential measurements, during cytoplasmic NaCl dilutions, showed that both the lysine and the arginine mutations switched the selectivity of the channel from cations (P_{Cl}/P_{Na} = 0.07 [WT]) to anions (P_{Na}/P_{Na} = 15 [Lys] or 10 [Arg]). In addition, we showed that these mutant channels seem to have a extremely small single-channel conductance, measured using noise analysis to be about 1 pS, compared to a WT value of about 25 pS. Our results indicated that it is predominantly the charge of the E342 residue in the P-loop, rather than the pore helix dipoles, which controls the cation-anion selectivity of this channel. However, the outward rectification displayed by both mutant channels in symmetrical NaCl solutions suggests that the negative ends of the pore helix dipoles probably play a role in reducing the outward movement of Cl- ions through these anion-selective channels. Such a postulated mechanism is also supportive of the mutations only causing local effects within the selectivity filter region of the channel. These results may have general implications for the determinants of anion-cation selectivity in the large family of P-loop containing channels.

We also showed from measurements of reversal potentials, with different halide ion substitutions, that the relative permeability of the halide ions increases with ionic radius in these E342K and E342R mutant CNG channels. Since ionic radius is inversely related to hydrated ion size, this result indicates that it is dehydration of these ions, as they pass through the selectivity region of the mutant channel, that is the major factor determining their relative permeability, as is also observed in anion-selective GABA_A and glycine receptor channels (Fatima-Shad & Barry, 1993).

Structure and dynamics of the periplasmic loop of the MscL mechanosensitive channel studied by electron paramagnetic resonance spectroscopy

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The periplasmic loop of the bacterial mechanosensitive channel of large conductance (MscL) is one of the five structural domains of the channel, which has been suggested to play a significant role in gating of the channel by mechanical force (Ajouz et al., 2000; Maurer & Dougherty, 2003). The structure of the loop has however, not been fully characterised. After the structural details of the MscL transmembrane helices, TM1 and TM2, were determined by crystallography and electron paramagnetic resonance (EPR) spectroscopy (Chang et al., 1998; Perozo et al., 2001), a model of the complete structure of MscL of E. coli was proposed (Sukharev et al., 2001). The model provided basis for characterisation of the MscL gating by molecular dynamics (MD) simulations (Gullingsrud et al., 2001). Recent MD simulations (Meyer et al., 2004) suggested further an important role for the periplasmic loop in the MscL channel gating.

Using the methods of cysteine scanning mutagenesis, spin labelling and EPR spectroscopy on MscL reconstituted into liposomes, we carried out an initial study towards characterisation of the structural dynamics of the loop. The EPR spectra recorded from the channel in its closed configuration and in the open state induced by lysophosphatidylcholine (LPC) (Perozo et al., 2002), indicated that significant structural rearrangements in the loop region occurred during channel opening. Our results thus appear consistent with the findings of the MD simulation studies of the structural dynamics of the channel. Future experiments are aiming to provide a complete structure of the periplasmic loop in the open and closed states of the MscL channel, which should allow obtaining a more accurate model of the gating mechanism of this channel.