## 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/$\mu m^2$; 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 two-fold 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.

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