NEUROBIOLOGY

Synapses take the rap

Stanley C. Froehner and Justin R. Fallon

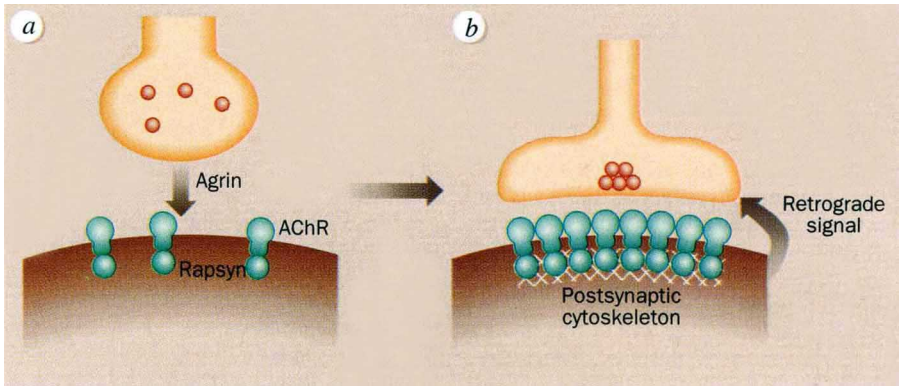
ANY savvy investor knows that what really counts is not what you make but where you put it. In the realm of cell–cell communication, synapses are the premier example of deploying resources for maximal return. The gold standard of synapses, the neuromuscular junction, covers only about 1/10,000 of the muscle cell surface, but all of the molecular components necessary for synaptic transmission, including the postsynaptic nicotinic acetylcholine receptor (AChR), are clustered at this site[1]. A central issue in neurobiology is understanding the mechanisms responsible for assembling these remarkable specializations. Now, results from a collaboration between the laboratories of Josh Sanes and the late John Merlie (page 232 of this issue[2]) show that rapsyn, a protein closely associated with nicotinic acetylcholine receptors, is not only involved in receptor clustering but may also be important for molecular specializations on both sides of the synapse.

The differentiation and alignment of the pre- and postsynaptic machinery is a cooperative process requiring the continuous exchange of information between the two cells (see figure). Signals from the nerve induce the formation of the postsynaptic apparatus. Two such nerve-derived signals are responsible for the localized accumulation of AChRs. Acetylcholine-receptor-inducing activity (ARIA) promotes synthesis of AChRs[3] and may mediate the selective activation of receptor genes in synaptic nuclei[4]. Independently, a protein called agrin initiates AChR aggregation by binding to its own receptor and activating an intracellular cascade culminating in the clustering of AChRs as well as several other synaptic molecules[5]. The mechanisms mediating presynaptic differentiation are poorly understood, but undoubtedly involve information feedback from the postsynaptic cell.

For some time, it has been thought that AChRs are anchored at the synapse by interaction with cytoskeletal and peripheral membrane proteins, and that agrin induces clustering at least in part by promoting the assembly of such postsynaptic cytoskeletons. Rapsyn (receptor-associated protein at the synapse, also known as the 43K protein) is a major component of the submembrane complex[6]. First identified in *Torpedo* postsynaptic membranes, rapsyn is a peripheral membrane protein closely associated with the nicotinic receptor. Evidence that it participates in clustering comes from coexpression studies in heterologous cells[7,8]. AChRs expressed alone are diffusely distributed over the cell surface, but become organized into discrete clusters when coexpressed with rapsyn; indeed, rapsyn has inherent clustering activity, and can form membrane-associated clusters even in the absence of the AChRs. But although rapsyn has the expected profile of an anchoring protein, its function at the synapse, and whether or not it is part of the agrin pathway, was unknown.

Gautam *et al.*[2] have tackled these problems using mice in which rapsyn expression was eliminated by targeted disruption of its gene. First, the obvious question — are postsynaptic specializations affected in these mutants? The answer is clearly yes. Although homozygous mutant mice showed no gross abnormalities, they had impaired movement and died within hours of birth. Upon examination of neuromuscular junctions, the reason for these defects became clear — the AChR clustering so readily apparent at normal nerve–muscle contacts was absent in the mutant. Second, rapsyn is required for agrin-induced AChR clustering on cultured muscle cells, and thus must lie directly in the signalling pathway activated by agrin. In contrast, the selective transcription of AChR genes at synaptic nuclei is unaffected in the mutant. So the agrin/rapsyn clustering pathway must be distinct from the signalling events that regulate AChR expression.

These results are clear evidence that rapsyn is essential for AChR clustering at synapses, but the mutants also reveal that rapsyn is much more widely involved in structuring postsynaptic specializations. Among the proteins concentrated in the postsynaptic membrane are dystrophin and utrophin[9]. Utrophin may be especially important for AChR clustering, as it is restricted to the crests of the postjunctional folds along with rapsyn and the AChR; it is also associated with dystroglycan, a receptor for agrin[10], and the syntrophins, one isoform of which is synapse-specific[11]. The synaptic concentration of each of these components is dramatically reduced or even eliminated in the mutant. So rapsyn must interact, directly or indirectly, with components of the utrophin complex. These *in vivo* observations agree with results of coexpression studies showing that dystroglycan targets to recombinant rapsyn-induced clusters[12], although the biochemical interactions required for targeting are not yet known. Finally, effects of the rapsyn mutation extend to basal lamina, where the synaptic form of acetylcholine esterase is greatly reduced. In all, then, organization of much of the postsynaptic apparatus depends on the proper expression of rapsyn.



Steps in synaptic differentiation. *a*, Agrin secreted by motor neurons induces clustering of nicotinic acetylcholine receptors (AChRs) and organization of the utrophin/dystrophin cytoskeleton in a rapsyn-dependent mechanism. *b*, The organized postsynaptic apparatus in turn generates signals that promote presynaptic differentiation. The components of this hypothetical retrograde pathway are unknown, but at least some of them are also rapsyn-dependent.

A completely unexpected consequence of the rapsyn mutation was aberrant presynaptic differentiation. Normal and mutant terminals were quite similar, exhibiting close apposition of pre- and postsynaptic membranes, an intervening basal lamina and typical patterns of synaptic vesicles. But nerve endings in mutant mice formed long branches that lacked the distinct arbours characteristic

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of normal terminals, suggesting defects in expression or organization of muscle-cell adhesion or signalling molecules. Rapsyn thus emerges in a new role and in largely uncharted territory — the mechanisms guiding the precise alignment of pre- and postsynaptic membranes. It has long been speculated that nerve-induced postsynaptic specializations in turn generate retrograde signals required for furthering presynaptic differentiation (see figure). The placement of rapsyn in this chain of events was unexpected but opens the way for a molecular attack on this problem.

What next? One outstanding question is the identity of rapsyn's cytoskeletal/signalling partners. Although rapsyn is characterized by a zinc finger and a leucine zipper, which are probably sites for protein–protein interactions, it shares no convincing homology with any known proteins. So further examination of the synaptic actions of rapsyn, including the identification of proteins associated with it, might provide information on signalling pathways that regulate both postsynaptic and presynaptic events. Given the diverse consequences of the rapsyn mutation, we can look forward to the unveiling of other classes of signalling molecules that also rely on being in the right place to give maximal return. □

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NITRIC OXIDE

No endothelial NO

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THE more the biological functions of nitric oxide are investigated, the more of them emerge. The stream of new findings has continued unabated for some eight years, sometimes delivering surprises with clinical implications. The latest turn of events appears on page 239 of this issue[1], where Huang *et al.* describe the consequences of knocking out the mouse gene encoding nitric oxide synthase in endothelial cells — nitric oxide produced by such cells is a potent vasodilator, thought to be involved in the regulation of blood pressure and local blood flow, and abnormalities in its production occur in atherosclerosis, diabetes and hypertension.

First, some background. The three principal forms of nitric oxide synthase (NOS) are the neuronal (nNOS or NOS-1), the macrophage or inducible (iNOS or NOS-2) and the endothelial (eNOS or NOS-3). The names can be misleading (see table). For example, iNOS not only occurs in macrophages but in several cell types including hepatocytes, chondrocytes, endothelial cells and fibroblasts. eNOS is not restricted to the endothelium of blood vessels but exists in the epithelium of several tissues, including the bronchial tree; it has also been localized to neurons in the brain, especially the pyramidal cells of the hippocampus, where it may function in long-term potentiation, a model of learning and memory[2]. nNOS also occurs in skeletal muscle[3], where Bredt and associates[4] have found it complexed with dystrophin, and to be absent in Duchenne's muscular dystrophy, which perhaps accounts for symptoms of the disease.

The biological functions of nitric oxide have been clarified considerably by the use of mice with various forms of NOS inactivated through gene knockouts created by homologous recombination. Considering nitric oxide's ubiquitous roles, the viability of such knockouts was questionable: not least because nNOS occurs in the nerves that mediate penile erection, and NOS inhibitors block erection[5], it was thought that null mutant nNOS ($nNOS^-$) mice would not procreate. As it turned out, however, $nNOS^-$ animals do breed and appear to be generally normal[6]. But they do have dilated stomachs with a constricted pyloric sphincter, and so provide a valuable model for the clinical disease infantile hypertrophic pyloric stenosis[6]. $nNOS^-$ mice are resistant to brain damage caused by vascular strokes, confirming that nitric oxide is crucial in mediating stroke damage[7]. And, earlier this year, studies of $nNOS^-$ mice established the importance of this form of the enzyme in behaviour[8] — $nNOS^-$ males are highly aggressive towards other males, to the extent that they will kill their wild-type littermates if left unattended, and they display similarly striking inappropriate and excessive sexual behaviour.

$iNOS^-$ mice have clarified the participation of nitric oxide in inflammatory responses[9,10]. Nitric oxide made by macrophages had long been implicated in the tumoricidal and bactericidal actions of these cells, but studies with NOS inhibitors had been inconclusive. $iNOS^-$ mice have markedly reduced defences against microorganisms such as *Listeria*[9] and *Leishmania*[10], and against the proliferation of lymphoma tumour cells. On the other hand, these animals are resistant to carrageenan inflammation[10] and hypotension elicited by endotoxin[9,10].

Now, Huang *et al.*[1] report on $eNOS^-$ mice. That eNOS is involved in regulating blood pressure had long been implied by the ability of NOS inhibitors to raise blood pressure. But most NOS inhibitors are nonspecific. Conceivably, their inhibition of nNOS, not eNOS, raises blood pressure, because nNOS occurs in neurons in the adventitial layer of vessels. Many different mediators have effects on the cardiovascular system, so one would not be surprised if knocking out eNOS had no effect on blood pressure.

The $eNOS^-$ mice of Huang *et al.* are viable, reproduce normally and are largely indistinguishable from wild-type animals in general appearance and behaviour. The classic role of nitric oxide as 'endothelium-derived relaxing factor' is substantiated by these mice, whose aortic rings display no relaxation in response to acetylcholine and are unaffected by treatment with the NOS inhibitor L-nitroarginine. Their vascular smooth muscle is normal (norepinephrine constriction of blood vessels and its reversal by sodium nitroprus-

Phenotypes of mice deficient in nitric oxide synthase (NOS)

NOS subtype	Phenotype
Neuronal (nNOS, type 1)	Pyloric stenosis[6]
	Resistant to vascular stroke[7]
	Inappropriate, excessive sexual and aggressive behaviour[8]
	Normal hippocampal long-term potentiation[2] and cerebellar long-term depression[14]
Inducible (iNOS, type 2)	More susceptible to *Listeria*[9] and *Leishmania*[10] infection and lymphoma cell proliferation[9]
	Resistant to endotoxin hypotension[9,10] and carrageenan inflammation[10]
Endothelial (eNOS, type 3)	Deficient acetylcholine vasodilation[1]
	Elevated mean blood pressure[1]
	L-nitroarginine-induced hypotension[1]