Understanding intercellular communication in the brain: Identified neuromuscular synapses of the fruitfly *Drosophila* serve as a model

The transmission of information between nerve cells in the brain takes place at specialized sites of contact, the synapses. Spatial interactions between synapses and temporal modulation of synaptic efficacy represent basic functional elements required for all higher brain functions including feature abstraction, learning and memory, and cognition. The detailed analysis of the molecular mechanisms which control and modify synaptic transmission is thus of fundamental significance for understanding brain function in health and disease and has therefore become a focal research area in the neurosciences. At chemical synapses, which constitute the predominant type of contacts, electrical signals of the presynaptic neuron cause the release of neurotransmitters and neuromodulators which diffuse to the postsynaptic cell and modify either its membrane potential or intracellular chemical reactions, or both. Structurally, synaptic contact sites are characterized on the presynaptic side by an accumulation of synaptic vesicles and a membrane thickening, often associated with ribbon-shaped specializations, a synaptic cleft that separates pre- and postsynaptic cell, and again a membrane thickening on the postsynaptic side. Depolarization of the presynaptic membrane causes the opening of voltage-sensitive calcium channels which allow calcium to enter the presynaptic terminal. This calcium signal induces the transmitter-loaded synaptic vesicles to fuse with the presynaptic membrane (exocytosis) and to shed their content into the synaptic cleft. Binding of the transmitter molecules to receptor proteins embedded in the postsynaptic membrane modulates ionic currents across the postsynaptic membrane and/or triggers intracellular signalling cascades with a wide range of possible effects on metabolic reactions modifying proteins and altering gene expression. The synaptic vesicle membrane, which by exocytosis had become integrated into the presynaptic membrane, is taken up back into the presynaptic terminal to form new synaptic vesicles by the process of endocytosis (synaptic vesicle recycling). The basic principles involved in synaptic transmission have been known for almost half a century, and a few dozen proteins specifically expressed at the synapse have been identified in recent years. However, the detailed molecular events leading to calcium-triggered exocytosis and endocytotic recycling of synaptic vesicles are still largely unknown.

It is clear that the study of the concerted interactions of protein complexes involved in exo-endocytotic synaptic vesicle cycling greatly profits from the availability of genetic tools. The fruitfly *Drosophila* has been a favourite object of genetic studies for almost a century, main reasons being easy culturing which allow the maintenance of thousands of mutant strains in a small space, short generation period of 10 days, and the giant chromosomes of larval salivary glands. Modern gene technology now permits single proteins to be eliminated by genetic knock-out and to be re-introduced by transgenic techniques after specific modifications by site-directed *in vitro* mutagenesis of the encoding genes. Genetic dissection of the molecular mechanisms involved in synaptic vesicle cycling in fact has made extensive use of *Drosophila* in order to assay the effects of the genetic changes in an otherwise intact organism. In recent years a large collection of mutants has been assembled which affect a wide variety of synaptic proteins and help to shape the evolving picture of the molecular basis of neurotransmitter release and vesicle recycling.

Since a single neuron in the brain often receives synaptic input from hundreds of presynaptic cells and in turn transmits its signals to similar numbers of postsynaptic neurons, vesicle trafficking in these synapses is exceedingly difficult to study. Rather, most information on synaptic function has been obtained by the study of certain identified synapses in model systems such as the frog neuromuscular junction, the mammalian cervical ganglion, the squid giant synapse, and, more recently the larval neuromuscular junction of *Drosophila*. This latter system had been established 25 years ago (Jan and Jan 1976) but has recently become highly attractive by the application of fluorescent dyes and the use of conditional mutations which block synaptic transmission at high but not at low temperature.

When supplied in the extracellular fluid, the fluorescent dye FM1-43 is taken up into synaptic vesicles during endocytosis and thus selectively stains vesicles that have been recycled. These vesicles

destain only after renewed exocytotic fusion (transmitter release). Thus it is possible to image the entire exo-endocytotic synaptic vesicle cycle *in vivo* at high spatial and temporal resolution.

In a *Drosophila* mutant (*shibire-ts*) that had been isolated due to its reversible paralysis at high temperatures the GTPase enzyme dynamin is modified. Dynamin is involved in endocytotic vesicle recycling. In the mutant endocytosis is blocked at non-permissive temperatures. Using this mutant the dynamics of vesicle depletion and replenishment in the synapses of the larval neuromuscular junction has now been studied quantitatively in a series of elegant experiments (Estes *et al* 1996; Koenig and Ikeda 1996; Kuromi and Kidokoro 1998, 1999, 2000; Delgado *et al* 2000).

The larval body wall of *Drosophila* contains sets of 30 muscles stereotypically repeated from body segment to body segment. In a larva cut open along the dorsal midline, the muscle pairs 6/7 and their innervating motorneurons are easily recognized and accessible to optical and electrophysiological analysis. Muscles 6/7 are innervated by 2–4 motorneurons whose terminal branches invade the muscle and form characteristic varicose thickenings along their length, termed synaptic boutons. These boutons contain several presynaptic specializations (membrane thickening with T-shaped ribbon surrounded by a cluster of synaptic vesicles) adding up to a total of about 550 transmitter release sites on each muscle (Atwood *et al* 1993). By two-electrode voltage clamp recordings from the muscle and stimulation of the motor neuron it has recently been possible to follow the time course of vesicle fusion during prolonged stimulation (Delgado *et al* 2000).

In a first experiment the authors decreased external calcium concentrations to such low values that the muscle responds only to a small percentage of the stimuli. Under these conditions most responses that are obtained will represent the effect of the fusion of a single vesicle. In this way the electric charge entering the muscle in response to the transmitter content of a single vesicle (unitary stimulus) can be determined. By dividing the total charge transfer across the muscle membrane due to a stimulus under normal calcium conditions by this unitary charge, the number of vesicles released by such a normal stimulus can be calculated.

When a long train of stimulus pulses is applied, the number of vesicles released per pulse at first decreases sharply, then more slowly, and finally a steady state is reached where the number of released vesicles equals the number of recycled vesicles. This number depends on the stimulus frequency for low stimulus rates but saturates at about 1000 vesicles per s at 10 Hz indicating that the maximum recycling rate at each release site is about 1·8 vesicles per s. The data demonstrate the existence of three distinct pools of synaptic vesicles, a small pool that is immediately releasable (IRP), an intermediate readily releasable pool (RRP), and a reserve pool (RP) that is activated only during high-frequency stimulation. Using the *shi-ts* mutant at non-permissive temperature (no recycling) the capacities of these pools has been determined. The IRP is depleted to 1/e (37%) after 2–4 stimuli, the RRP after 100 to 200 stimuli, while the RP reaches this level after 1500–2500 stimuli. At high stimulus frequencies the RRP and RP are depleted by fewer stimuli possibly due to some process of facilitation. From these experiments the total number of synaptic vesicles within the boutons on muscle 6 or 7 has been calculated to be about 90,000.

Using the fluorescent dye FM1-43 the position of the vesicle pools within each synaptic bouton could be identified. The vesicles of the RRP locate to the periphery of the boutons while the RP is concentrated towards the centre of the bouton. The rates of destaining of the bouton periphery corresponded well to the electrophysiologically determined depletion of the RRP while the destaining of the centre of the bouton reflected the depletion of the reserve pool. By these experiments it was verified that endocytosis is blocked in *shi-ts* mutants at non-permissive temperatures. In addition it was demonstrated, however, that translocation of vesicles from the releasable pools to the reserve pool becomes negligible under these conditions. The reverse, recruitment of reserve pool vesicles to the RRP, can be largely inhibited by treating the preparation with cytochalasin-D, a drug that blocks actin polymerization. Using this drug the authors could estimate that the RRP consists of about 14% to 20% of the total number of vesicles.

From these experiments the observed quantal content of each of a train of repetitive stimuli could be calculated by a simple sum of three exponential functions. It will be interesting to see if the predictive power of the equations also hold true for less regular (more natural) stimulus volleys. As is generally the case in biology, one also has to expect further complications due to the actual complexity of the system. There are at least three types of boutons on muscles 6 or 7, with differences in structure,

vesicle content, and protein composition. It is unlikely that these differences should play no role in over-all synaptic function. Nontheless the experiments described represent an important step towards quantitative modelling of synaptic transmission which eventually, when similar data become available for the considerably more heterogeneous interneuronal synapses (Stevens and Wesseling 1998), may lead to a better understanding of the behaviour of networks of neurons in the brain.

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