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PHARMACOLOGY MEETS VESICULAR TRAFFICKING AT A CNS SYNAPSE:  
PREGABALIN EFFECTS ON SYNAPTIC VESICLE CYCLING IN HIPPOCAMPAL  
NEURONS

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## ABSTRACT

The contribution by Micheva et al., (2006) in the current issue adds to our understanding of the action of pregabalin, a drug used for treatment of partial seizures and neuropathic pain. The authors examine the effects of pregabalin on presynaptic function of cultured hippocampal neurons using a powerful technique to follow the trafficking of synaptic vesicles in individual boutons. The study revealed that pregabalin reduces the readily releasable pool of synaptic vesicles in an NMDA receptor-dependent manner.

Pregabalin is a recently approved anticonvulsant and analgesic drug for use in the treatment of partial seizures and in the treatment of neuropathic pain due to diabetic and post-herpes neuropathies. Its structure, function and therapeutic uses are similar to those of gabapentin. Although both drugs were originally developed with the notion of specifically modulating GABAergic transmission, their biological and therapeutic effects were found to be independent of GABA receptors (both types A and B) and GABA uptake into neurons. Biochemical and electrophysiological studies found that the drugs caused small reductions in stimulated glutamate, norepinephrine, and peptide release from neurons, suggesting a basis for their therapeutic effects. Unexpectedly, both drugs were found to bind with high affinity to the auxiliary  $\text{Ca}^{2+}$  channel subunit  $\alpha_2\text{-}\delta$  (type 1 or type 2) (Piechan *et al.*, 2004;Gong *et al.*, 2001), raising the possibility that these agents act through modulation of voltage sensitive  $\text{Ca}^{2+}$  channels. Indeed, in some but not in all studies, pregabalin and gabapentin cause partial inhibition of  $\text{Ca}^{2+}$  currents.

The contribution by (Micheva *et al.*, 2006) in the current issue takes a fresh look at the action of pregabalin. The authors examine the effects of pregabalin on presynaptic function of cultured hippocampal neurons using a powerful technique to follow the trafficking of synaptic vesicles in individual boutons. The method uses amphipathic fluorescent dyes (FM dyes) that label endocytic vesicles in living neurons (Betz and Bewick, 1992;Rizzoli and Betz, 2005) . When added to the extracellular medium, these membrane-impermeant probes equilibrate between the aqueous phase and the outer leaflet of the plasma membrane in which they become highly fluorescent (Figure 1 C1). Upon endocytosis that follows exocytosis, the labeled

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membrane is internalized. When removed from the extracellular medium, the dye is retained by the endocytic vesicles in the bouton but is lost from the plasma membrane (Figure 1 C2). Endocytic vesicles are transformed within approximately one minute into synaptic vesicles containing the dye. Most importantly, these recycled synaptic vesicles lose the probe upon subsequent exocytosis when the vesicle membrane is exposed to the extracellular medium (Figure 1 C3).

This technique has permitted the dynamics of the exocytic/endocytic cycle of synaptic vesicles to be investigated at individual boutons. In cultured hippocampal neurons used in the present study, each bouton contains approximately 200 synaptic vesicles (Figure 1A). Half of these vesicles recycle upon stimulation at 37°C and are labeled upon incubation with FM dye (Figure 1D) (Micheva and Smith, 2005). By labeling boutons by prior electrical stimulation in the presence of FM dye, and then washing the cultures of extracellular dye, the subsequent exocytosis of synaptic vesicles can be followed by measuring the decrease in bouton fluorescence.

The present study has made use of the unique strengths of this system to provide new insights into the action of pregabalin. By measuring fluorescence intensity changes in hundreds of individual boutons per experimental group with a time resolution of seconds, mechanistic insights were achieved despite the intrinsic variability of synaptic release. In fact, the remarkable statistical precision of the experiments in the hands of these investigators permitted the discovery of heretofore unappreciated aspects of pregabalin effects.

The study revealed a small inhibition of synaptic vesicle exocytosis at high stimulus frequency in both glutamatergic and GABAergic neurons at clinically relevant concentrations of pregabalin. Unexpectedly, the investigators found that pregabalin targets a subpopulation of

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recycling vesicles, the readily releasable pool. This pool of vesicles is reduced by approximately 25%. Vesicles in the readily releasable pool are the first to be released upon electrical stimulation in the presence of  $\text{Ca}^{2+}$  and are specifically released in a  $\text{Ca}^{2+}$ -independent manner in response to hypertonic sucrose (Rosenmund and Stevens, 1996). Consistent with these results is the reduction in spontaneous synaptic vesicle exocytosis, which is known to be independent of  $\text{Ca}^{2+}$  and probably comes from the same readily releasable pool. These effects are unlikely to be caused by inhibition of voltage sensitive  $\text{Ca}^{2+}$  channels. They probably reflect a new action of pregabalin on the vesicular trafficking pathway in neurons, perhaps mediated by an unknown function of the auxiliary  $\text{Ca}^{2+}$  channel subunit  $\alpha_2\text{-}\delta$ . The effect of pregabalin to reduce the readily releasable pool is likely to have important functional implications. The reduction is predicted to decrease the probability of synaptic vesicle fusion even during a single action potential (Rosenmund and Stevens, 1996).

Another intriguing aspect of the study was the finding that a NMDA antagonist prevented the pregabalin-induced inhibition of transmission. Although NMDA receptors are primarily postsynaptic, there is also compelling evidence for presynaptic NMDA receptors (MacDermott *et al.*, 1999). Thus, the NMDA receptor-mediated effect could originate either postsynaptically through a retrograde signaling pathway (Micheva *et al.*, 2003) or presynaptically. It will be important in future studies to determine the mechanism by which NMDA receptor activation participates in pregabalin action.

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Figure 1. **Vesicular trafficking in hippocampal presynaptic boutons.** A. Electron micrograph of a bouton of a cultured hippocampal neuron. Arrowheads show edges of an active zone where exocytosis occurs. Black arrows indicate docked synaptic vesicles (SV), which are thought to represent the readily releasable pool. White arrow is a synaptic vesicle that is probably not docked. [reprinted from (Rizzoli and Betz, 2005) and (Murthy *et al.*, 2001) with permission from Elsevier and Nature Publishing Group]. B. Horse radish peroxidase (HRP)-labeled structures in a bouton after electrical stimulation. HRP was present during electrical stimulation (10 Hz for one min) and for one min afterwards (protocol is identical to that used for FM 4-64 labeling). HRP was detected by the dark appearance of reaction product from 3,3'-diaminobenzidine. Numerous structures were labeled including synaptic vesicles, clathrin coated vesicles and large vesicular structures. Quantitation of these micrographs as well as FM 4-64 labeling of living cells indicates that ~50% of the 200 SV in boutons recycle at 37°C [reprinted from (Micheva and Smith, 2005) with permission from the Society for Neuroscience, copyright 2005]. C) Protocol for FM 4-64 staining of synaptic vesicles (adapted from (Holz and Fisher, 2006)). D) Summary of different synaptic vesicle (SV) pools in cultured hippocampal boutons at 37°C. Data from (Micheva and Smith, 2005).



