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Regulated lysosomal trafficking as a mechanism for regulating $GABA_A$ receptor abundance at synapses in *Caenorhabditis elegans*

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ABSTRACT

$GABA_A$ receptor plasticity is important for both normal brain function and disease progression. We are studying $GABA_A$ receptor plasticity in *Caenorhabditis elegans* using a genetic approach. Acute exposure of worms to the $GABA_A$ agonist muscimol hyperpolarizes postsynaptic cells, causing paralysis. Worms adapt after several hours, but show uncoordinated locomotion consistent with decreased GABA signaling. Using patch-clamp and immunofluorescence approaches, we show that $GABA_A$ receptors are selectively removed from synapses during adaptation. Subunit mRNA levels were unchanged, suggesting a post-transcriptional mechanism. Mutants with defective lysosome function (*cup-5*) show elevated $GABA_A$ receptor levels at synapses prior to muscimol exposure. During adaptation, these receptors are removed more slowly, and accumulate in intracellular organelles positive for the late endosome marker GFP-RAB-7. These findings suggest that chronic agonist exposure increases endocytosis and lysosomal trafficking of $GABA_A$ receptors, leading to reduced levels of synaptic $GABA_A$ receptors and reduced postsynaptic GABA sensitivity.

Introduction

$GABA_A$ receptor plasticity plays an important role in modulating GABA synapse strength, and shaping the excitation–inhibition balance in the brain. One physiological function of this plasticity is to maintain neuronal homeostasis in the face of changing network excitability. Experimental perturbations of overall network excitability induce global compensatory changes in postsynaptic $GABA_A$ receptor expression that scale GABA synapse strength, returning excitability to a pre-set level (Davis, 2006; Kilman et al., 2002; Nelson and Turrigiano, 2008). $GABA_A$ receptor plasticity can serve an adaptive physiological role: for example, during pregnancy and parturition, $GABA_A$ receptor levels decrease to compensate for increased levels of neurosteroids that potentiate $GABA_A$ receptor function. Failure to correctly modulate receptor levels leads to post-partum depression and defective maternal behavior in mice (Maguire and Mody, 2008). On the other hand, $GABA_A$ receptor plasticity can contribute to disease progression: epileptic seizures sometimes lead to reduced $GABA_A$ receptor surface levels and the development of status epilepticus, a potentially fatal condition characterized by prolonged unremitting seizures (Goodkin et al., 2008; Naylor et al., 2005; Terunuma et al., 2008). This downregulation may be homeostatic at a local level, since excessive activity of inhibitory neurons leads to excess GABA release, and prolonged GABA exposure can downregulate $GABA_A$ receptor levels *in vitro* and *in vivo* (Lyons et al., 2001; Maloteaux et al., 1987; Naylor et al., 2005; Tehrani and Barnes, 1988).

The signaling and trafficking pathways that modulate $GABA_A$ receptor levels in response to altered excitability are not fully understood. Regulated receptor endocytosis plays a central role, and subunit phosphorylation is critical for determining whether receptors remain on the cell surface, or bind the AP2 clathrin adaptor protein and internalize (Jacob et al., 2008; Kittler et al., 2005; Smith et al., 2008). We do not fully understand how this mechanism is integrated into overall pathways of activity-dependent $GABA_A$ receptor regulation. For example, in some systems, GABA itself induces downregulation of $GABA_A$ receptors (Lyons et al., 2001; Maloteaux et al., 1987; Naylor et al., 2005; Tehrani and Barnes, 1988), while in other systems it does not (Goodkin et al., 2008). In some cases, increased calcium entry and calcineurin activation stimulate $GABA_A$ receptor downregulation (Bannai et al., 2009; Lyons et al., 2001; Wang et al., 2003), while in other cases, decreased intracellular calcium may be the key signal (Terunuma et al., 2008). One factor that complicates the study of $GABA_A$ receptor plasticity is the complexity of mammalian neuronal networks. Neurons typically receive inputs from many different excitatory and inhibitory synaptic partners, so it is difficult to know whether an experimental manipulation such as incubating with

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GABA, or altering network excitability globally will affect $GABA_A$ receptors cell—autonomously, or through interactions with synaptic partners.

An alternative strategy is to study $GABA_A$ receptors in a model organism with a simpler nervous system, such as the nematode *Caenorhabditis elegans*. The molecular components of GABA neurotransmission are highly conserved between *C. elegans* and mammals, including a $GABA_A$-like receptor encoded by the *unc-49* gene (Bamber et al., 1999; Schuske et al., 2004). This receptor functions in the body-wall muscles to mediate muscle relaxation, which is necessary for coordinated locomotion. These muscles receive inputs from identified excitatory acetylcholine neurons and inhibitory GABA neurons (White et al., 1986). The anatomy and development of these cells are stereotyped and fully described, and gene expression can be manipulated precisely and independently in the pre- and postsynaptic cells. By studying *C. elegans*, it is possible to examine $GABA_A$ receptor regulation in a defined, native system *in vivo* using a genetic approach. Studies of GABA signaling at the *C. elegans* neuromuscular junction have already identified important genes required for GABA neurotransmission, such as the GABA vesicular transporter (McIntire et al., 1997), and provided evidence for broad conservation in the mechanisms of GABA signaling between *C. elegans* and mammals (Vashlishan et al., 2008). To begin investigating $GABA_A$ receptor plasticity in *C. elegans*, we characterized changes in $GABA_A$ receptor expression and function in worms exposed to the $GABA_A$ agonist muscimol. Muscimol treatment initially paralyzes worms by chronically activating the GABA receptors (McIntire et al., 1993). However locomotion recovers over a time course of several hours because postsynaptic $GABA_A$ receptor abundance and GABA responsiveness are downregulated as the result of increased $GABA_A$ receptor trafficking to the lysosome.

Results

C. elegans adapts to GABA agonist exposure

Worms become paralyzed when exposed to the $GABA_A$ receptor agonist muscimol. Muscimol was previously used to classify mutants defective in GABA synaptic transmission. Mutants defective in postsynaptic GABA receptor function are resistant, while worms defective in presynaptic GABA synthesis or release are sensitive (McIntire et al., 1993). We placed wild-type young adult worms on NGM plates containing 10 mM muscimol, and observed flaccid paralysis within 30 min. This concentration seems high, but it is generally recognized that drugs penetrate the *C. elegans* cuticle poorly, and the active concentration within the worm is probably much lower. Muscimol-exposed worms stopped moving, lost their normal sinusoidal posture, and showed the rubberband phenotype typical of mutants expressing overactive inhibitory ion channels in muscle (Greenwald and Horvitz, 1986; Greenwald and Horvitz, 1980). *unc-49(e407)* mutants, which carry a null mutation in the muscle GABA receptor, were unaffected by muscimol (Fig. 1A), confirming that muscimol paralysis is dependent on GABA receptor activation (McIntire et al., 1993). However, worms did not remain paralyzed during continuing exposure to muscimol. After overnight exposure (15 h), wild-type worms regained normal body posture, and resumed foraging. To control for the possibility that the observed adaptation was due to degradation of the muscimol in the plate, we replaced the exposed worms on the muscimol plate with naïve worms, and observed the same acute flaccid paralysis (not shown). These observations indicate that worms adapt behaviorally to long-term muscimol exposure.

Adapted worms did not move normally, but instead showed the 'Shrinker' phenotype characteristic of worms with defective GABA neurotransmission. Normally, acetylcholine released from motor neurons stimulates muscle contraction on one side of the body, and simultaneously activates an inhibitory motor neuron that projects to the opposite side of the body to release GABA. GABA elicits muscle relaxation, facilitating a smooth body bend. In the absence of GABA neurotransmission, muscles on both sides of the body contract simultaneously, causing the body to shorten, or 'shrink' (Schuske et al., 2004). We developed a simple locomotion assay to distinguish between acute muscimol paralysis, the Shrinker phenotype, and normal locomotion. Because the Shrinker phenotype is particularly pronounced when backward motion is initiated, we counted body bends in the first 5 s after gentle stimulation to the nose (see Experimental methods). Wild-type worms not exposed to muscimol produced 4–5 body bends, GABA receptor null mutants produced 1–2 body bends, and muscimol-paralyzed worms only occasionally produced a body bend in the first 5 s after stimulation. Within several hours, muscimol-exposed worms gradually increased their body bend frequency to levels equivalent to the GABA receptor deficient mutants (Fig. 1B). These results suggest that adaptation to muscimol may be the result of downregulation of GABA receptors in the body-wall muscles.

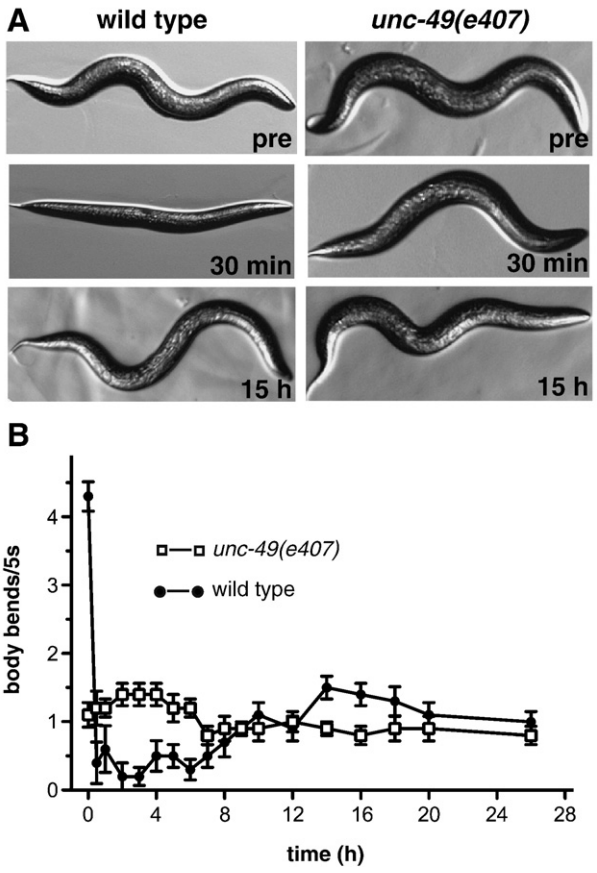

Fig. 1. Behavioral adaptation of *C. elegans* to muscimol exposure. A) Young adult worms rapidly undergo acute flaccid paralysis when placed on plates containing 10 mM muscimol (left panel, top and middle). After longer exposures, worms adapt, regaining normal posture and resuming locomotion (left panel, bottom). *unc-49* null worms, lacking the muscle GABA receptor, are unaffected by muscimol treatment at any time (right panels). B) Quantitative analysis of locomotion during the progression of muscimol exposure. *Y* axis shows the number of body bends at the vulva in the first 5 s following gentle tap to the nose.

GABA receptors are downregulated in muscimol-adapted worms

To begin analyzing the physiological basis for adaptation to muscimol in *C. elegans*, we examined the GABA responsiveness of body-wall muscles in muscimol-exposed worms using whole-cell patch-clamp electrophysiology. Worms exposed to muscimol between 3 and 15 h showed a 3.5- to 5-fold reduction in GABA current amplitudes (Fig. 2). By contrast, responsiveness to levamisole, a