GABA(A) receptor homeostasis at the C. elegans neuromuscular junction

Alyson L. Sujkowski
The University of Toledo

Follow this and additional works at: http://utdr.utoledo.edu/theses-dissertations

Recommended Citation
Sujkowski, Alyson L., "GABA(A) receptor homeostasis at the C. elegans neuromuscular junction" (2010). Theses and Dissertations. 986.
http://utdr.utoledo.edu/theses-dissertations/986

This Thesis is brought to you for free and open access by The University of Toledo Digital Repository. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of The University of Toledo Digital Repository. For more information, please see the repository's About page.
A Thesis

entitled

GABA\textsubscript{A} Receptor Homeostasis at the \textit{C. elegans} Neuromuscular Junction

by

Alyson L. Sujkowski

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Master of Science in Biology

_________________________________
Dr. Bruce Bamber, Committee Chair

_________________________________
Dr. Richard Komuniecki, Committee Member

_________________________________
Dr. Robert Steven, Committee Member

_________________________________
Dr. Patricia Komuniecki, Dean
College of Graduate Studies

The University of Toledo

August 2010
Schizophrenia, alcoholism, anxiety, insomnia and epilepsy are all associated with abnormalities in GABA neurotransmission. GABA is the principal inhibitory neurotransmitter in the human brain. The strength of GABA synapses is tightly regulated. Prolonged agonist exposure to human GABA receptors promotes receptor downregulation, contributing to serious conditions such as Status Epilepticus. Changes in GABA receptor phosphorylation are proposed to increase receptor endocytosis, however, the underlying mechanisms regulating the status of GABA receptor phosphorylation are not well understood. An attractive hypothesis is that hyperpolarization due to GABA receptor activation initiates the signaling pathway, since cytoplasmic calcium concentrations are expected to sharply drop upon agonist exposure, although this mechanism has yet to be demonstrated. The human brain is composed of billions of neurons and trillions of synapses, making the human brain difficult to study. To examine neuronal circuitry in a simplified yet relevant setting, we employ *C. elegans* as a model.
because the organism is anatomically simple and amenable to genetic analysis. Importantly, the pre-and postsynaptic mechanisms of GABA neurotransmission are both well-characterized and conserved between *C. elegans* and humans.

In *C. elegans*, GABA receptors are expressed in muscles, and are required for coordinated locomotion. GABA receptor activation (binding of GABA or muscimol) promotes receptor conformational change, allowing intracellular chloride influx and cellular hyperpolarization. In *C. elegans* these changes lead to flaccid paralysis because hyperpolarized muscles cannot achieve high intracellular calcium levels required to activate the contractile apparatus. However paralysis is short-lived, because muscimol exposure also stimulates endocytosis and trafficking of GABA receptors to the lysosome, causing robust reduction of GABA and muscimol responsiveness [1], thus bringing the excitation-inhibition balance back toward a normal level. To investigate the intracellular signaling involved, we are evaluating the importance of each of the immediate consequences of GABA receptor activation independently (i.e. receptor conformational change, chloride influx, and hyperpolarization), using a combination of mutant and transgene expression strategies to uncouple these normally interdependent events. Using GABA receptor mutants that cannot bind agonist, we have demonstrated that downregulation is indeed downstream of GABA receptor activation, and not dependent on a separate muscimol receptor. Using temperature-sensitive potassium channels, we have demonstrated that acute hyperpolarization alone is insufficient to induce downregulation, indicating a role for receptor conformational change and chloride influx. Using mutants with chloride transport defects, we have demonstrated that chloride influx and/or hyperpolarization are required, and that receptor conformational change alone is
insufficient. Thus, the simple hypothesis that hyperpolarization elicits downregulation through reduced intracellular calcium is not tenable. Possible approaches for evaluating the importance of chloride influx will be presented to determine whether it acts synergistically with hyperpolarization or receptor conformational change. These experiments will define the inputs to the signaling pathway that stimulate increased GABA receptor endocytosis and reduced GABA synapse strength.
Acknowledgements

This thesis would not have been possible without the education and support of my major advisor, Dr. Bruce Bamber. There have been many ups and downs throughout my education and I would not be where I am today without his patience and understanding. I have learned so much in the past few years and this is largely due to his strength as a scientist, teacher, and mentor.

The science presented in this thesis would not be possible without the contributions of the Bamber lab. I would like to thank all members, both past and present, who contributed to this research. I must also mention the other labs that provided valuable insight and discussion, particularly the Komuniecki, Steven, and Liu labs. I would also like to thank my committee members who have taken the time out of their busy schedules for this defense. Lastly I would like to thank my family for their love, support, and encouragement during my years as a student.
Contents

Abstract .................................................................................................................................... iii

Acknowledgements ................................................................................................................. vi

List of Figures .......................................................................................................................... ix

1. Introduction ......................................................................................................................... 1

GABA\(_A\) Receptors .................................................................................................................. 1

GABA\(_A\) receptor homeostasis ............................................................................................... 2

*C. elegans* as a model organism ........................................................................................... 8

GABA\(_A\)R in *C. elegans* undergoes agonist dependent downregulation ............................. 12

Agonist induced GABA\(_A\)R downregulation is a result of increased lysosomal trafficking ............................................................... 17

2. Materials and Methods .................................................................................................... 21

*C. elegans* strains and methods: ......................................................................................... 21

Locomotion assays ................................................................................................................. 22

Body length assays ................................................................................................................ 22

Temperature assays ............................................................................................................... 22
Developmental assays ............................................................................................................ 23

Immunofluorescence and image analysis ................................................................................. 23

Plasmids ....................................................................................................................................... 24

3. Results ....................................................................................................................................... 25

GABA\textsubscript{A}R activation is necessary for agonist induced receptor downregulation ....... 25

Chloride influx and hyperpolarization are necessary for GABA\textsubscript{A}R downregulation ..... 28

Hyperpolarization is not sufficient to signal post synaptic GABA\textsubscript{A}R downregulation .... 30

Changes in postsynaptic excitability alter presynaptic synapse morphology ....................... 35

4. Discussion ................................................................................................................................. 39

Agonist dependent GABA\textsubscript{A}R downregulation in C. elegans provides a model for synaptic homeostasis ................................................................................................................................. 39

The role of receptor activation and cell excitability on GABA\textsubscript{A} receptor downregulation ................................................................................................................................. 40

Agonist induced receptor downregulation requires GABA\textsubscript{A}R activation ...................... 41

Chloride influx and hyperpolarization are important signals for GABA\textsubscript{A}R downregulation ................................................................................................................................. 43

Hyperpolarization via potassium efflux does not trigger GABA\textsubscript{A}R downregulation ..... 45

Chloride gradients may provide the signal for GABA\textsubscript{A}R internalization .................... 48

A signaling pathway may exist between the pre- and postsynaptic GABA terminals ......... 49

5. References ................................................................................................................................ 51
List of Figures

1.1 Phosphorylation controls GABA<sub>A</sub>R surface expression in vertebrates ..................6
1.2 *unc-49* GABA<sub>A</sub>R is required for coordinated locomotion in *C. elegans* .............11
1.3 *C. elegans* adapt GABA neurotransmission in response to chronic agonist exposure .................................................................14
1.4 Postsynaptic GABA<sub>A</sub>R is reduced following agonist exposure .........................16
1.5 The molecular signal for GABA<sub>A</sub>R is posttranscriptional.................................18
1.6 Agonist induced GABA<sub>A</sub>R downregulation is a result of increased endocytosis....19

3.1 GABA<sub>A</sub>R ligand binding mutants do not display agonist induced downregulation...27
3.2 Receptor conformational change is not the signal for GABA<sub>A</sub>R downregulation....29
3.3 Increased potassium channel conductance does not trigger GABA<sub>A</sub>R downregulation ........................................................................................................32
3.4 Differences between wild-type and *twk-18(cn110)* UNC-49 GABA<sub>A</sub>R abundance are apparent as early as larval stage L3 .................................................................34
3.5 GABA release is altered in *twk-18(cn110)* ..................................................................36
3.6 GABA release increases in muscimol adapted worms.................................38
4.1 Receptor activation is necessary to trigger downregulation.................................42
4.2 Receptor conformational changes associated with activation are not sufficient for downregulation ..............................................................................................................44
4.3 Hyperpolarization and paralysis independent of Cl- influx are not sufficient for receptor internalization .................................................................47
1. Introduction

GABA<sub>A</sub> Receptors

Inhibitory neurotransmission is vital to maintaining the balance between excitatory and inhibitory inputs in the brain. Defects in inhibitory neurotransmission that disrupt the excitation-inhibition equilibrium are associated with various neurological disease states. For example, too little inhibition may result in anxiety or epilepsy. In contrast, too much inhibition is associated with anesthesia and coma. GABA is the principal inhibitory neurotransmitter in the human brain and acts primarily through GABA<sub>A</sub> receptors (GABA<sub>A</sub>Rs). GABA<sub>A</sub>Rs are cys-loop ligand gated ion channels belonging the superfAMILY that includes nicotinic acetylcholine receptors (nAchRs), glycine receptors (GlyRs), and 5-HT<sub>3</sub> serotonin receptors [2, 3]. Receptors of this superfAMILY contain a large N-terminal extracellular domain, 4 transmembrane regions (M1-M4) and a large intracellular loop between M3 and M4 [4]. 18 GABA<sub>A</sub>R subunits have been identified belonging to 7 subunit classes (α1−6, β1−3, γ1−3, δ, ε, θ, π) [5]. Most synaptic GABA<sub>A</sub>Rs, mediating phasic inhibition, are composed of 2α, 2β, and 1γ subunit. GABA<sub>A</sub>Rs at synapses have low GABA sensitivity and fast desensitization. Relatively low GABA sensitivity and fast desensitization are appropriate functional characteristics as synaptic GABA<sub>A</sub>Rs are exposed to high GABA concentrations [6]. In contrast, tonic
inhibition is controlled by extrasynaptic GABA_A Rs that are usually composed of 2α, 2β and 1δ subunit. Extrasynaptic GABA_A Rs have relatively high GABA sensitivity and no desensitization, and are activated by dilute GABA spillover from nearby synapses [7]. Because GABA_A R subtype composition determines the function of the receptor, developing subtype-specific drugs has become an important area of pharmacological research [8]. γ2 containing GABA_A Rs are sensitive to benzodiazepines, important to the treatment of anxiety and epilepsy [8]. Previous studies using α1 knock-in mice show that the anxiolytic action of benzodiazepines is mediated by α1, α2, α3, and α5, and the α5 subunit has also been liked to learning and memory [9, 10]. α1 containing receptors are sedative hypnotic targets, while agonists to α2/α3 have anxiolytic properties [6, 9, 11]. Further studies in mice show that point mutations in β2/β3 subunits render insensitivity to anesthetics [12, 13]. Subtype specific drugs are used to pharmacologically manipulate the strength of GABA synapses and treat pathological conditions resulting from defects in inhibitory neurotransmission. Neural circuit function is therefore critically dependent on the mechanisms that establish and maintain GABA synapses.

**GABA_A receptor homeostasis**

Homeostatic plasticity stabilizes neural activity at a set-point in response to changes in excitability. The balance between neuronal excitation and inhibition can be modified by changes in cell size, synapse number or strength [14]. Set-point levels of activity are established during neural development. The size and complexity of the nervous system increases during development, and plasticity is vital to the prevention of neurological disease [15]. For example, studies in rat forebrain show that glutamate receptors have an
early postnatal overshoot followed by a gradual decline. NMDA and AMPA receptor (NMDAR, AMPAR) expression peaks postnatally and synaptic excitation predominates over inhibition. GABA inhibition is established later in development, contributing to an increased susceptibility to seizures postnatally [16]. In a second study, GABA\(_A\)R subunit \(\alpha_1\) is expressed at low levels at birth and increases upon maturation of cultured cortical neurons. Changes in \(\alpha_1\) expression modify receptor kinetics and benzodiazepine sensitivity, demonstrating altered response of the neonatal brain to anti-epileptics. Therefore, drugs that enhance GABA\(_A\)R function may not be appropriate treatments early in development [17]. During development, such dynamic changes in the expression of excitatory and inhibitory neurotransmitter receptors (NTRs) establish set-point levels of activity vital to normal nervous system function.

Homeostatic plasticity is also important in maintaining the stability of mature synapses. In the excitatory system, AMPAR accumulation is bidirectionally altered in response to changes in neural activity. Studies in cultured spinal neurons show that inhibiting excitatory neurotransmission increases mEPSC amplitudes and synaptic AMPAR expression. In contrast, mEPSC amplitudes and synaptic AMPARs decrease upon amplification of excitatory neurotransmission [18]. Additional studies have shown that postsynaptic ion channel, excitatory and inhibitory NTR abundance is altered synergistically upon chronic neural suppression [19]. Furthermore, presynaptic neurotransmitter release increases to achieve normal depolarization when postsynaptic NTR levels are impaired. This suggests that a transsynaptic signal monitors neural activity [20]. Neuronal circuits therefore employ an array of homeostatic mechanisms to achieve relatively constant synapse strength.
In the GABA system, synapse strength is established and maintained homeostatically, dependent primarily on receptor surface expression. GABA$_A$R surface expression is dependent on multiple molecular mechanisms. A key determinant of GABA synapse strength and receptor surface expression is synaptic receptor stability. Newly synthesized GABA$_A$Rs are trafficked to the cell membrane. Assembled receptors may be recruited to synaptic clusters or extrasynaptic populations subject to lateral diffusion. Previous studies in mammalian systems have found that receptors are recruited to synaptic sites based on the excitation state of the cell. For example, increased excitation causes increased lateral diffusion of GABA$_A$R and decreased confinement at the synapse [21]. In a second study, pharmacological activity deprivation with TTX decreased mIPSC amplitude and reduced synaptic GABA$_A$R expression. Diminished GABA$_A$R number and function suggests postsynaptic plasticity [22].

GABA$_A$R surface expression and synapse strength is also dependent on subunit composition. Altered GABA$_A$R subunit expression is linked to various pathological conditions. An arginine to glutamate substitution at residue 43 (R43Q) of the $\gamma$2 subunit has been identified in epileptic patients. The R43Q mutation generates a hyperexcitable state by slowing receptor deactivation and increasing desensitization. GABA mediated inhibition is compromised by an accumulation of desensitized synaptic receptors [23]. In a second study, the R43Q mutation of $\gamma$2 abolishes benzodiazepine sensitivity, altering treatment options for familial epilepsy [24]. Defects in GABA$_A$R subunit expression have also been linked to post-partum depression. During pregnancy neurosteroid levels increase, potentiating GABA function. GABA$_A$R subunit $\gamma$2 and $\delta$ expression decreases to compensate for the elevated inhibition. At parturition neurosteroid levels rapidly
decline and the γ2 and δ subunits must rebound immediately. Failure to restore δ subunit expression is linked to abnormal maternal behavior in mice [25]. Considering the importance of GABA_A receptor homeostasis, it is necessary to understand the principal molecular mechanisms involved.

Endosomal trafficking is a primary determinant of GABA_A receptor surface expression and synapse strength. The GABA_A receptor β3 subunit contains an atypical basic patch motif subject to phosphorylation by protein kinase C (PKC). The basic patch of β3 has been found to interact with the clathrin adaptor protein AP2, targeting the receptor for internalization. Phosphorylation of β3 on conserved residues S408/9 inhibits AP2 binding, decreasing endocytosis and increasing GABA_A receptor surface expression (Figure 1.1) [26]. Similarly, a second phosphodependent binding motif has been identified on the GABA_A receptor γ2 subunit. γ2 contains a conserved Yxxφ motif subject to phosphorylation by the SRC tyrosine kinase. Phosphorylation inhibits the interaction between AP2 and γ2, increasing synaptic GABA_A receptor [27]. Following clathrin mediated internalization endocytosed GABA_A receptors undergo one of two events. Receptors may enter into a subsynaptic pool, available for interaction with recycling machinery and reentry into the plasma membrane. Constitutive endocytic recycling establishes basal levels of GABA_A receptor insertion and removal [28]. Recycling proceeds at a slow rate based on studies on recombinant and native GABA_A receptors [29, 30]. Alternatively, endocytosed GABA_A receptors may be targeted for degradation. Specifically, the γ2 subunit contains conserved lysine residues subject to ubiquitination. A lysine to arginine mutation prevents ubiquitination, slows lysosomal targeting, and increases synaptic GABA_A receptor abundance and function [31].
Figure 1.1 Phosphorylation controls GABA\textsubscript{A}R surface expression in vertebrates.

GABA\textsubscript{A}R subunit $\beta$3 contains a basic patch subject to phosphorylation by PKC. In dephosphorylated $\beta$3, the basic patch is exposed, allowing the clathrin adaptor protein AP2 to bind. AP2 binding initiates receptor internalization, decreasing GABA\textsubscript{A}R abundance and function.
The previous examples display the importance of GABA\textsubscript{A}R plasticity on both activity-dependent adaptation and pathophysiology. Status epilepticus (SE), a state of prolonged, unremitting seizure activity, is of particular significance. The progression of SE has been linked to defects in inhibitory neurotransmission leading to a hyperexcitable postsynaptic cell. The biochemical pathways leading to diminished GABA\textsubscript{A}R are not well understood. Reduced GABA\textsubscript{A}R surface expression is related to SE. Prolonged seizure activity causes increased intracellular GABA\textsubscript{A}R accumulation, specifically a reduction in \(\beta2/3\) and \(\gamma2\) subunits at the synapse. Decreased \(\beta2/3\) and \(\gamma2\) subunit expression is also observed, suggesting a transcriptional mechanism of receptor downregulation [32]. A second study identifies increased excitation, calcium and calcineurin as the underlying molecular signals. Excessive \(\text{Ca}^{2+}\) influx as a result of increased depolarization activates the \(\text{Ca}^{2+}\)-sensitive phosphatase calcineurin. Pharmacological inhibition of calcineurin results in a reduction in GABA\textsubscript{A}R surface expression [21]. In contrast, a third study identifies GABA release as the precipitating factor. In this example SE increases GABA release resulting in desensitization and internalization of GABA\textsubscript{A}R. Increased GABA\textsubscript{A}R removal from the synapse causes failed inhibition and self-sustaining seizure [33]. This study suggests that agonist dependent GABA\textsubscript{A}R downregulation may provide insight into SE progression. GABA\textsubscript{A}R posttranslational modification has also been found to contribute to the development of SE. SE selectively decreases phosphorylation of the GABA\textsubscript{A}R subunit \(\beta3\) at residues S408/9. The basic patch AP2 binding motif of the \(\beta3\) subunit is unmasked, enhancing
endocytosis. Furthermore, the PKC-β3 interaction is reduced and protein phosphatase 2A (PP2A)-β3 interaction is enhanced. [34].

Understanding GABA<sub>A</sub>R homeostasis provides important information regarding development and maintenance of synapses at a set-point level of activity. Deviation from the set-point may result in developmental defects in synapses and cell excitability levels. Determination of how inhibitory neural circuits are established during development is therefore significant. Insight into how mature synapses adapt and respond to modifications in neural activity is also important. Comprehension of the molecular mechanisms involved in GABA synapse strength will identify new pharmacological targets for the initiation, progression and maintenance of disease. This study will explore changes in both presynaptic neurotransmitter (NT) release and GABA<sub>A</sub>R internalization postsynaptically. The molecular signals initiating GABA<sub>A</sub>R internalization and the underlying endocytic pathways will be investigated. The experiments in this thesis will identify how cellular excitation state modulates synaptic GABA<sub>A</sub>R abundance and function.

**C. elegans as a model organism**

While studies in mammalian systems have provided valuable insight into GABA plasticity and homeostasis, there are many accompanied caveats. The human nervous system contains billions of neurons and trillions of synapses. 18 mammalian GABA<sub>A</sub>R subunits have been identified yielding thousands of possible subtypes, further complicating investigation. The GABA synapse is also difficult to study in vivo in mammalian systems. In vitro studies require dissociated cultures, disrupting network
connectivity. Alternative approaches employ post-mortem brain slices, further disturbing neural circuitry. We therefore use the model organism *C. elegans*.

*C. elegans* have a simple, uniform nervous system consisting of 302 neurons and about 5000 synapses. The genetic sequence is known and various mutations have been identified allowing rapid genetic screening, RNA interference (RNAi), and transgene insertion. In addition, *in vivo* studies of an intact nervous system are possible using behavior, imaging, pharmacology, and electrophysiology. Furthermore, the genetic sequence of the *C. elegans* GABA\(_\text{A}\)R is known. *C. elegans* has a single GABA\(_\text{A}\)R required for coordinated locomotion. Sinusoidal motion is achieved by a simple cross-inhibitory circuit between excitatory acetylcholine receptors (AchRs) and inhibitory GABA\(_\text{A}\)Rs at the neuromuscular junction (NMJ) (Figure 1.2 A,B) [35, 36]. Defects in GABA neurotransmission can therefore be quickly identified by uncoordinated phenotypes. *unc-49* encodes the *C. elegans* GABA\(_\text{A}\)R. The genetic sequence consists of a single copy of the N-terminus including the intracellular loops and ligand-binding domain. The N-terminal sequence is followed by 3 tandem copies of the C-terminus. The N-terminus is alternatively spliced to a single C-terminal exon, thus encoding 3 subunits with a single genetic sequence (Figure 1.2 C) [37]. Northern blots show that UNC-49A mRNA is barely detectible in *C. elegans* while UNC-49B and UNC-49C are expressed. UNC-49B is sufficient to form a homomeric GABA\(_\text{A}\)R but is also able to form an UNC-49B/C heteromer. UNC-49C cannot form a homomorphic receptor. UNC-49B is responsible for synaptic localization and channel gating while UNC-49C is involved in rapid desensitization and shaping mIPSPs. The UNC-49B/C heteromer has reduced GABA sensitivity compared to the UNC-49B homomer. In addition the UNC-
49B/C heteromer is sensitive to pregnenolone sulfate (PS) and resistant to picrotoxin (PTX). These findings identified UNC-49B/C as the in vivo receptor in C. elegans [38]. Interestingly, the UNC-49B/C heteromer is approximately 35-45% similar to mammalian α, β, γ, and ρ subunits, validating the C. elegans GABAₐR as a reasonable experimental model [39].
Figure 1.2 unc-49 GABA$_A$R is required for coordinated locomotion in *C. elegans*. A) Dorsal and ventral body wall muscles are contacted by ACh and GABA motor neurons in a cross-inhibitory circuit B) GFP-tagged UNC-49 GABA receptors cluster at NMJs [40] C) unc-49 encodes three GABA$_A$R subunits, including a common N-terminus and three C-termini. Complex alternative splicing generates three separate subunits [37].
**GABA\(_A\)R in *C. elegans* undergoes agonist dependent downregulation**

Chronic exposure to the GABA\(_A\)R agonist muscimol causes flaccid paralysis in *C. elegans*. Muscimol activates GABA\(_A\)R by interacting with the ligand-binding site. GABA\(_A\)R undergoes a conformational change, opening chloride (Cl\(^-\)) channels. Cl\(^-\) influx causes postsynaptic membrane hyperpolarization and paralysis. Body bend assays are used to quantify muscimol paralysis. Body bends, measured from the center of the worm, are counted for five seconds after tapping the animal on the nose. Wild-type (N2) animals perform 4-5 body bends during this five second interval. Body bends in N2 worms decrease to less than one during acute muscimol exposure. *unc-49(e407)* mutants do not express GABA\(_A\)R and are insensitive to GABA as well as muscimol. Body bends in *unc-49(e407)* animals average 1.5 prior to and following acute muscimol exposure. Interestingly, long-term agonist exposure yields an adaptive phenotype. N2 worms chronically exposed to agonist regain locomotion, suggesting GABA synapse modification. Both N2 and *unc-49(e407)* animals average 1-2 body bends following long-term agonist exposure (Figure 1.3 A,B). Normal locomotion, agonist dependent paralysis and adaptation can therefore be distinguished. In addition, muscimol adapted N2 worms display the shrinker phenotype characteristic of GABA defective worms. Defects in GABA neurotransmission cause muscle cells to contract without coordinated cross-inhibition. The worm shrinks following nose touch due to bilateral body wall muscle contraction. This uncoordinated phenotype is observed in animals defective in both GABA release and GABA\(_A\)Rs. Patch-clamp electrophysiology was used to identify the basis of adaptation in GABA neurotransmission. The GABA sensitivity of postsynaptic
GABA\textsubscript{\textalpha}Rs is reduced six fold following chronic muscimol exposure (Figure 3.1 C). The reduction in current amplitude suggests that adaptation is a result of decreased number or function of postsynaptic GABA\textsubscript{\textalpha}Rs.
Figure 1.3 *C. elegans* adapt GABA neurotransmission in response to chronic agonist exposure. A) Wild type animals propagate sinusoidal motion before muscimol exposure. Acute agonist exposure paralyzes worms. Locomotion resumes after 15 hours. *unc-49(e407)* are muscimol resistant. B) Quantitative analysis of body bends shows initial paralysis and recovery in response to muscimol. C) Whole cell patch clamp electrophysiology displays six-fold reduction in GABA sensitivity, quantified in the right panel [1].
An anti-UNC-49 polyclonal antibody was generated to further explore decreased postsynaptic GABA sensitivity. Confocal microscopy was used to examine permeabilized and stained whole worms. Images were then quantified to determine postsynaptic GABA_A R abundance. Animals showed a six-fold decrease in GABA_A R after ten hours of agonist exposure (Figure 1.4). Receptor abundance and GABA sensitivity decreased in parallel.
Figure 1.4 Postsynaptic GABA$_A$R is reduced following agonist exposure. Anti-UNC-49 immunostain reveals ventral cord GABA$_A$Rs. Anti-UNC-49 localizes opposite SNB-1-GFP expressed in GABA neurons. Quantification of confocal sections shows agonist-induced GABA$_A$Rs decrease.
**Agonist induced GABA\(_A\)R downregulation is a result of increased lysosomal trafficking**

Muscimol may downregulate GABA\(_A\)R by one of two mechanisms. Transcription of GABA\(_A\)Rs may decrease in response to chronic hyperpolarization. Alternatively, receptors may undergo enhanced degradation. Two experimental methods were used to identify the mechanisms involved. First, *unc-49* mRNA levels were measured, and found to remain unchanged. This observation rules out a mechanism whereby muscimol exposure reduces GABA\(_A\) receptor levels by decreasing *unc-49* transcription rate.

An alternative hypothesis was then explored: muscimol adaptation may be the result of increased receptor internalization. Lysosomal degradation was inhibited with the *cup-5*(ar465) mutant to test this hypothesis. If adaptation to muscimol exposure is due to enhanced GABA\(_A\)R degradation, then inhibiting lysosomal function should slow or prevent the elimination of receptors. Anti-UNC-49, prior to muscimol exposure, revealed a slight increase in receptor at the synapse. This observation suggests that receptor is actively being degraded in adult *C. elegans* to maintain normal synaptic levels (Figure 1.5 A-F). Following muscimol exposure, *cup-5*(ar465) mutants showed slightly slowed removal of receptor downregulation, but strikingly, prominent accumulation within an intracellular compartment (Figure 1.6 A-C). Furthermore, this internalized GABA\(_A\)R colocalized with RAB-7-GFP, a late endosome marker (Figure 1.6 D). Colocalization suggests that GABA\(_A\)Rs accumulate in the late endosome. Taken together these findings indicate that muscimol dependent downregulation is a result of increased endocytosis and lysosomal degradation [1].
**Figure 1.5** The molecular signal for GABA_\textsubscript{A}R downregulation is posttranscriptional.

A) Anti-UNC-49 immunostain at the wild type GABA synapse B) *cup-5(ar465)* Anti-UNC-49 immunostain shows slight but significant increase in postsynaptic receptor, no intracellular accumulation. C) Quantification of immunofluorescence, P<0.05, n=10 for each data point. (D-F) Frequency distribution of total fluorescence, average pixel intensities, and volumes of individual GABA receptor clusters (P<0.001 for cluster volume and total fluorescence between wild-type and *cup-5(ar465)*. P>0.05 at day 2 and P<0.05 at day 3 for average fluorescent intensity, Kolmogorov-Smirnov test) [1].
Figure 1.6 Agonist induced GABA$_{A}$R downregulation is a result of increased endocytosis. A) Muscimol causes decreased GABA receptor immunofluorescence in wild type and cup-5(ar465) mutant worms B, C) Anti-UNC-49 antibody stain shows intracellular GABA receptor accumulation in cup-5 mutants after 6h muscimol exposure. Intracellular accumulation is absent in wild type animals. D) Intracellular GABA receptor immunofluorescence co-localizes with GFP-RAB-7, a late endosome marker, in lysosomal mutants exposed to muscimol [1].
In the present study, I am using GABA\textsubscript{A} receptor plasticity in \textit{C. elegans} as a model system to understand how GABA\textsubscript{A} receptor levels are regulated in general, and more specifically, how GABA\textsubscript{A} receptors become downregulated in response to agonist exposure. By exploiting the anatomical simplicity of \textit{C. elegans} and the availability of a variety of relevant mutant backgrounds, I can probe this question at a level of detail not possible in more complex mammalian systems. My specific objective is to determine which of the events associated with GABA\textsubscript{A} receptor activation stimulate receptor internalization and lysosomal trafficking. Three key events take place upon agonist binding to GABA\textsubscript{A} receptors: First, the receptors undergo a conformational change to open the channel pore; second, chloride flows into the cell though the channel; and third, the cell hyperpolarizes. I have used mutant backgrounds that uncouple these events to assess their impact on GABA\textsubscript{A} receptor levels independently of one another. Finally by examining the distribution of the synaptic vesicles in the presynaptic GABA neurons, I have begun to address whether GABA synapses in \textit{C. elegans} show the coordinated pre- and postsynaptic changes typical of synaptic homeostasis.
2. Materials and Methods

*C. elegans* strains and methods:

*C. elegans* were grown on NGM agar plates using standard techniques. Animals were fed using *E. Coli* strains OP50 and NA22, depending on population size. Wild type was Bristol N2. Mutant strains were *unc-49(e407)*, *cup-5(ar465)*, *unc-49(e382)*, *abts-1(ok1566)*, and *twk-18(cn110)*. Transgenic strains were *punc-25:snb-gfp*, *pmyo-3:rab-7-gfp*, and *twk-18(cn110);punc-25:snb-gfp*. *pmyo-3:rab-7-gfp* was constructed using Gateway cloning technology (Invitrogen) and the pKS11 destination vector, courtesy of Dr. Barth Grant (Rutgers University). Expression vector was injected into *lin-15(n765ts)* at 10ng/µl. The plasmid pEK1, containing the wild-type *lin-15* gene was co-injected as a transformation marker. Progeny of injected animals were raised at 20°C and transgenic strains identified by their non-Muv phenotype. *twk-18(cn110);punc-25:snb-gfp* was produced via genetic cross. Wild-type N2 males were crossed with *twk-18(cn110)* hermaphrodites to produce *twk-18(cn110)* males. These males were crossed with *punc-25:snb-gfp* hermaphrodites to produce *twk-18(cn110)+/punc-25:snb-gfp* heterozygotes. 12 of these animals were cloned and their progeny screened for double mutants. These animals were easily identified by SNB-GFP expression and flaccid paralysis upon shifting to 30°C. For muscimol exposure, worms were placed on 10mM muscimol plates.
(Ascent Scientific) and incubated at 20°C. *E. Coli* strain NA22 was present on muscimol plates throughout the exposure.

**Locomotion assays**

Locomotion was assessed using a simple nose-touch assay. An eyebrow hair was used to touch the nose of the experimental animal and body-bends were counted for a period of 5 seconds. One body bend was defined as the transition from convex to concave. Typically, 5-10 animals were observed per assay.

**Body length assays**

To determine the extent of body wall muscle contraction or paralysis, body length was analyzed. Worms under conditions of altered muscle excitability were observed on an Olympus MVX10 dissection microscope and photographed using Microsuite SEV software (Olympus). Images were analyzed Volocity software (Improvision, Lexington, MA). A “spine” was superimposed along the center of each worm, allowing consistent quantification of body length.

**Temperature assays**

*twk-18(cn110)* animals exhibit flaccid paralysis at 30°C. Because *C. elegans* cannot be raised at this elevated temperature animals were exposed only for periods up to 6 hours. Animals were raised at 20°C until experimentation, at which time they were placed at the experimental temperature. Animals assayed were immediately fixed using Ruvkun fixation solution and flash frozen in a dry ice and ethanol bath until antibody staining could be performed. To analyze the acute effects of temperature, NGM plates were pre-
warmed in a 30°C incubator and twk-18(cn110) animals transferred individually to heated plates. Those undergoing paralysis were then photographed using an Olympus MVX10 dissection microscope.

**Developmental assays**

Although the *C. elegans* life cycle has been well studied, variations in larval molts may occur in mutated strains. In order to circumvent this problem, animals in developmental assays were observed and fixed every 4 hours for initial experiments. Once an appropriate developmental cycle had been established, wild-type and experimental animals of equal stage were compared. Determination of L1 through L3 animals was based on body length. L4 animals were identified by the unique appearance of the vulval primordium prior to oocyte formation and egg laying. Day 1 adults were observed to have approximately 10 eggs in the gonad with few laid eggs present. At day 2 many eggs had been laid per plate with the occasional presence of second generation L1 animals.

**Immunofluorescence and image analysis**

Rabbit anti-UNC-49 antibodies were prepared using amino acids 23-67 of UNC-49. Antibody was prepared using the antigen and purification methods first described by Gally and Bessereau [41]. Antibody staining produced a punctate pattern in *C. elegans* dorsal and ventral nerve cords. unc-49(e407) null animals showed no staining by the antibody and were used as a negative control throughout the study. Whole mount immunostaining was performed according to the Ruvkun method [40, 42]. Stained worms were imaged using an Olympus IX70 inverted confocal microscope with a 63x objective lens and Fluoview 5.0 software. Generally, hundreds of stained *C. elegans*
were mounted on a single slide. Worms with their ventral cord oriented toward the objective were selected for imaging. Apart from this orientation criterion, selection of worms for imaging was random, and was not influenced by perceived staining intensity. The anterior region of the ventral cord (from the retrovesicular ganglion to the vulva) was imaged. The full height of the ventral cord was captured using 24 or fewer 0.4µM optical slices. Laser intensity and PMT were adjusted to prevent fluorescence saturation. Images were analyzed using Volocity software as previously described [1].

**Plasmids**

The pKS11 plasmid, obtained from Dr. Barth Grant, was used to construct several plasmids used to express the MOD-1 serotonin-gated chloride channel in muscle to test for heterologous cross-regulation of UNC-49. pKS11 is a Gateway (Invitrogen) compatible destination vector characterized by a 5’ myo-3 promoter sequence, gateway cassette, gfp sequence, and the let-58 untranslated region. gfp sequence was removed via PinAI and HindIII restriction digest. A mod-1 entry clone was constructed using standard PCR according to the Gateway manual. LR recombination reactions produced pmyo-3::mod-1 expression vectors used for muscle specific Cl⁻ channel expression. There are five serotonin receptors present in *C. elegans*. The pmyo-3::mod-1 plasmid was therefore injected into quintuple mutant *C. elegans* strains lacking all of these receptors to prevent the possible effects of serotonin on GABA_A receptor levels through endogenous receptors from interfering with the assay. Coelomocyte GFP was used as a coinjection marker.
3. Results

The GABA agonist muscimol causes flaccid paralysis in *C. elegans*. Muscimol binds GABA receptor, inducing a conformational change that opens ligand-gated chloride channels. Chloride enters the cell, hyperpolarizing the postsynaptic membrane. Worms eventually adapt to muscimol paralysis, exhibiting the shrinker phenotype characteristic of GABA-defective mutants. Adaptation results from a 10-fold reduction in GABA\textsubscript{A} receptor at the synapse proportional to a reduction in GABA sensitivity. *unc-49* mRNA levels were not reduced, suggesting a post-transcriptional mechanism of agonist-dependent downregulation. I hypothesize that the signal for receptor downregulation is triggered by one of the following: Receptor activation and conformational change, Cl\textsuperscript{-} entry, or hyperpolarization and paralysis. I have uncoupled each of these events and tested them individually.

**GABA\textsubscript{A}R activation is necessary for agonist induced receptor downregulation**

Muscimol causes paralysis in *C. elegans* by binding and activating receptor, opening Cl\textsuperscript{-} channels, and hyperpolarizing the muscle cells, which eventually result in increased GABA\textsubscript{A}R trafficking from the postsynaptic membrane to the lysosome. First, it was important to determine if activation of GABA\textsubscript{A}R, and not an alternate receptor, activates this pathway. *unc-49(e382)* carries an allele with a point mutation in the ligand binding domain. It is reasonable to assume that inactivation of the ligand binding domain will
also inhibit muscimol initiated receptor activation. *unc-49(e382)* receptors will not undergo conformational change, postsynaptic Cl⁻ influx, or hyperpolarization-stimulated paralysis. Behaviorally, *unc-49(e382)* animals exhibit the shrinker phenotype characteristic of GABA defective mutants and are muscimol resistant. Anti-UNC-49 antibody staining showed no agonist dependent downregulation following muscimol exposure. In addition, untreated *unc-49(e382)* animals displayed decreased baseline levels of GABAₐR (Figure 3.1). I expect that decreased receptor is due to a mutation dependent defect in synaptic insertion. These findings support the hypothesis that GABAₐR activation is required for agonist dependent downregulation.
Figure 3.1 GABA$_A$R ligand binding mutants do not display agonist induced downregulation. A) *unc-49(e382)* animals have reduced GABA$_A$R (top panel) and do not downregulate upon agonist exposure. B) Quantification of wild type and *unc-49(e382)* UNC-49 receptor levels during a ten hour muscimol exposure time course.
**Chloride influx and hyperpolarization are necessary for GABA\textsubscript{A}R downregulation**

GABA\textsubscript{A}R activation opens ion channels, allowing Cl\textsuperscript{-} influx at the *C. elegans* neuromuscular junction. The Cl\textsuperscript{-} transport mutant *abts-1(ok1566)* was used to test whether receptor conformational change alone is the signal for postsynaptic GABA\textsubscript{A}R downregulation. In these mutants, the loss of a chloride/bicarbonate cotransporter that normally pumps chloride out of the cytoplasm causes intracellular Cl\textsuperscript{-} levels to be abnormally high [43]. When GABA/agonist binds postsynaptic GABA\textsubscript{A}R, the ligand-gated Cl\textsuperscript{-} channel opens and Cl\textsuperscript{-} exits, rather than enters the cell. This presumably causes GABA action to be excitatory. If receptor conformational change were sufficient for GABA\textsubscript{A}R downregulation, one would expect that agonist exposure would cause increased GABA\textsubscript{A}R trafficking away from the synapse. Body length assays on wild-type animals showed body wall muscle relaxation following muscimol exposure. The *abts-1(ok1566)* mutant displayed body wall muscle contraction, validating excitatory GABA action in these animals (Figure 3.2 A). UNC-49 GABA\textsubscript{A}R does not downregulate following agonist exposure as evidenced by anti-UNC-49 immunofluorescence (Figure 3.2 B-D). These findings suggest that receptor conformational changes associated with receptor activation are not sufficient for downregulation. Relevant signaling therefore requires Cl\textsuperscript{-} influx and/or hyperpolarization for activation of the receptor downregulatory pathway.
Figure 3.2 Receptor conformational change is not the signal for GABA$_A$R downregulation. A) Body length assay performed on wild type and $abts$-$1$(ok1566) strains prior to and following 10mM muscimol exposure. B) Quantification of anti-UNC-49 antibody staining in C, D. C.) Confocal image of ANTI-UNC-49 immunofluorescence of ventral $abts$-$1$(ok1566) animal prior to and D.) following 10h application of GABA agonist muscimol.
Hyperpolarization is not sufficient to signal post synaptic GABA$_A$R downregulation

The previous experiment suggested that the conformational changes associated with muscimol binding and channel activation were not sufficient to induce UNC-49 downregulation, indicating that the subsequent events were important. To specifically test whether hyperpolarization was important, I tested whether potassium channel activation could elicit UNC-49 downregulation. The rationale was that activating potassium channels effectively uncouples the hyperpolarization and Cl$^-$ influx that normally occur simultaneously when UNC-49 is activated by muscimol. TWK-18 is a twin pore K$^+$ channel expressed on C. elegans body wall muscles. The twk-18(cn110) mutation encodes a K$^+$ channel with steep temperature dependence of activity [44]. K$^+$ conductance at the NMJ is increased at all temperatures in twk-18(cn110). At 20ºC increased K$^+$ conductance has no effect on behavior. However, shifting the temperature to 30ºC paralyzes the worm due to hyperpolarizing K$^+$ efflux. I was therefore able to determine if hyperpolarization and paralysis, independent of Cl$^-$ influx, are sufficient to induce GABA$_A$R downregulation. Anti-UNC-49 immunofluorescence showed no GABA$_A$R downregulation after shifting the animals to 30ºC for six hours. This result suggests that hyperpolarization alone is insufficient to initiate the UNC-49 downregulation process. To control for the possibility that endocytic trafficking in twk-18(cn110) is defective, thus occluding UNC-49 downregulation, I also exposed twk-18(cn110) worms to muscimol. This experiment demonstrated that GABA$_A$R downregulation in twk-18(cn110) animals can still take place normally (Figure 3.3). These results imply either that Cl$^-$ influx is the principal determinant of GABA$_A$R
downregulation, or that downregulation cannot be elicited by any single event, but requires the combination of receptor conformational change, chloride influx, and hyperpolarization.
Figure 3.3 Increased potassium channel conductance does not trigger GABA$_{	ext{A}}$R downregulation. A) *twk-18(cn110)* mutants anti-UNC-49 staining patterns are similar at 20º and 30ºC B) Absolute synaptic fluorescence is not significantly different upon temperature shift. C) Anti-UNC-49 antibody stain shows decreased synaptic staining in K$^+$ mutants following muscimol exposure D) Quantification of GABA$_{	ext{A}}$R downregulation in wild type and *twk-18(cn110)* animals.
It is interesting to note that increased K$^+$ conductance does not result in an overt behavioral phenotype at 20°C even though they presumably have chronically elevated levels of potassium efflux, and mild hyperpolarization due to their hyperactive TWK-18 channels. One possible explanation is that these mutants compensate by expressing reduced levels of other inhibitory ion channels during development. Comparison of UNC-49 levels in developing twk-18(cn110) mutants and wild type animals confirmed this possibility. Wild-type and mutant strains were raised at 20°C and examined as larval stages L1-L4, young adult, and day 2 adult animals. Anti-UNC-49 staining showed decreased postsynaptic GABA$\_\alpha$R as early as larval stage L3 (Figure 3.4 A, B). To better characterize these differences, I analyzed the properties of the individual synapses. Although twk-18(cn110) animals have less overall GABA$\_\alpha$R staining at the neuromuscular junction, the intensity of staining remains constant (Figure 3.4 C). Synaptic volume analysis mirrors total synaptic fluorescence (Figure 3.4 D). This suggests that the packing density of GABA$\_\alpha$R remains relatively constant. By contrast, the volumes of the synapses are reduced, in a manner that mirrors the changes in overall receptor abundance. Therefore, I conclude that the size of the postsynaptic receptor field is regulated developmentally, in response to the expression of other ion channels, but the packing density of GABA$\_\alpha$ receptors within those synapses is unaffected.
Figure 3.4 Differences between wild-type and twk-18(cn110) UNC-49 GABA<sub>A</sub> R abundance are apparent as early as larval stage L3 A) twk-18(cn110) mutants anti-UNC-49 staining is decreased in comparison to wild type B) Quantification of postsynaptic fluorescence shows differences as early as L3 C) Fluorescent intensity is unchanged throughout developmental series D) Synapse by synapse analysis of volume parallels total fluorescence
Changes in postsynaptic excitability alter presynaptic synapse morphology

Previous studies in *C. elegans* demonstrate that GABA_{A}Rs cluster opposite GABA release sites at neuromuscular junctions. Clustering is not dependent on presynaptic GABA release, as *unc-25* mutants, in which GABA synthesis is defective, appear wild type [45]. Synaptic homeostasis often results in changes both pre-and postsynaptically, so I looked at a presynaptic protein that is indicative of presynaptic release efficiency. Analysis of GABA presynaptic terminals using synaptobrevin-GFP fusion protein was performed to test this hypothesis. Punc-25::SNB-GFP (*juIs1*) animals showed no change in total synaptic fluorescence prior to and following 10h muscimol exposure (Figure 3.5 A, B). However, synapse by synapse analysis revealed SNB-GFP clusters increase in volume and decrease in fluorescence intensity (Figure 3.5 C, D). This finding is consistent with a change in the GABA release machinery in muscimol-adapted worms, possibly reflecting increased GABA release. Although this alteration is opposite what one might expect in reaction to chronic postsynaptic hyperpolarization, it may reflect long-term presynaptic adaptation to reduced postsynaptic GABA_{A} receptor levels. Thus, the homeostatic regulation observed postsynaptically by measuring GABA_{A} receptor levels may be part of a larger regulatory reaction to chronic muscimol exposure that includes both presynaptic and postsynaptic pathways.
Figure 3.5 GABA release increases in muscimol adapted worms A) pUNC-25::SNB-GFP fluorescence prior to and following 10h muscimol exposure B) Total SNB-GFP fluorescence C) Synapse by synapse analysis shows increased synapse volume D) and decreased fluorescent intensity after 10 mM muscimol exposure.
Because short-term muscle hyperpolarization seemed to affect the presynaptic GABA release machinery, I also wanted to determine whether the same effect could be observed as the result of long-term developmental hyperpolarization. Therefore I examined whether the elevated postsynaptic K+ conductance in \textit{twk-18(cn110)} also caused a change in SNB-GFP distribution in the GABA neurons. In the \textit{Punc-25::SNB-GFP;twk-18(cn110)} double, I observed increased fluorescence intensity, volume, and overall fluorescence levels (Figure 3.6). This constellation of changes in the SNB-GFP pattern has not been previously described in the literature, and its functional significance is still unclear. However, the alteration of presynaptic structure by a defect in postsynaptic muscle cell excitability suggests the possibility that a retrograde transsynaptic developmental signal patterns the \textit{C. elegans} inhibitory neuromuscular junction, since \textit{twk-18} is expressed in muscles and not neurons.
Figure 3.6 Effect of the *twk-18(cn110)* mutation on presynaptic structure  
A) pUNC-25::SNB-GFP fluorescence in *twk-18(cn110)* animals  
B) Quantification of total presynaptic fluorescence  
C) Synapse by synapse analysis shows increased synapse volume  
D) and increased fluorescent intensity in response to altered postsynaptic K+ conductance.
4. Discussion

Currently, the GABA_A receptor is a pharmacological target in treating diseases such as alcoholism, anxiety, epilepsy, insomnia and schizophrenia. However, the underlying mechanisms regulating GABA neurotransmission in normal human brain function is largely uncharacterized. Examination of receptor activation following agonist exposure, alterations in GABA_A receptor expression and changes in cellular excitability, during both development and adulthood, will provide a better understanding of GABA synapse homeostasis in the model organism C. elegans.

**Agonist dependent GABA_A R downregulation in C. elegans provides a model for synaptic homeostasis**

The GABA_A R is pharmacologically targeted in the treatment of various neurological disorders. Unfortunately, GABA_A R desensitization, or reduction of receptor protein levels, is a common theme following drug treatment. To overcome GABA_A R desensitization, a regimen of increasing drug doses are prescribed, eventually rendering the drug ineffective. The mechanisms responsible for desensitization are not well characterized. We are studying the biochemical signals triggering the desensitization pathway in the model organism C. elegans.

In C. elegans, GABA_A R agonist exposure causes flaccid paralysis of body wall muscles followed by eventual recovery. Our data indicates that downregulation of
GABA\textsubscript{A}R at the neuromuscular junction is a result of chronic hyperpolarization. Downregulation of GABA\textsubscript{A}R significantly reduces GABA sensitivity and providing a mechanism for behavioral adaptation. Adaptive mechanisms can be examined using behavioral assays, antibody staining, and electrophysiology.

Anti-UNC-49 antibody staining revealed a decrease in GABA\textsubscript{A}R levels at the neuromuscular synapse following chronic agonist exposure. Further examination suggested that the reduction in functional synaptic GABA\textsubscript{A}Rs resulted from increased trafficking to the lysosome, and subsequent degradation. However, reduced levels of synaptic GABA\textsubscript{A}Rs could also result from a decrease in receptor synthesis. If receptor synthesis was inhibited, one could conclude that receptor turnover is high and a normal synapse maintains a constant flux of newly synthesized receptor at the neuromuscular junction. In contrast, our results agree with the first hypothesis, suggesting that decreases in GABA\textsubscript{A}R levels at the neuromuscular synapse are a result of receptor internalization followed by lysosomal degradation. In mutants with impaired lysosome function, an intracellular accumulation of receptor was only observed following muscimol application, suggesting that synaptic GABA\textsubscript{A}R is stable in the absence of agonist, having a low rate of turnover at the neuromuscular junction. Agonist exposure triggers a biochemical signaling pathway increasing GABA\textsubscript{A}R internalization from the synapse into the lysosomal pathway.

**The role of receptor activation and cell excitability on GABA\textsubscript{A} receptor downregulation**

Although our data suggests a potential mechanism for GABA\textsubscript{A}R downregulation, the biochemical signaling pathways involved is unknown. Muscimol binding GABA\textsubscript{A}R
causes a conformational change allowing Cl- to enter the cell. Hyperpolarization of the postsynaptic membrane causes flaccid paralysis followed by eventual recovery of sinusoidal motion in animals. Observations suggest that recovery is due to GABA\textsubscript{A}R trafficking away from the synapse. I hypothesize that agonist-induced GABA\textsubscript{A}R downregulation is triggered by one of the following events: 1.) Receptor conformational change 2.) Chloride influx or 3.) Hyperpolarization and paralysis. Our experiments were aimed at testing each of these variables, individually and in combination, to determine the biochemical signals at work.

**Agonist induced receptor downregulation requires GABA\textsubscript{A}R activation**

I first sought to determine if GABA\textsubscript{A}R activation was integral to downregulation. The *unc-49(e382)* mutant was exposed to muscimol and examined. A point mutation in the ligand binding domain of this mutant prevents GABA binding to receptor. In this experiment, ligand binding, receptor conformational change, chloride influx, hyperpolarization and paralysis will not occur. The data show that without these events GABA\textsubscript{A}R downregulation is absent (Figure 4.1). The receptor must be functional, ruling out the possibility that muscimol acts through an alternate receptor.
Figure 4.1 Receptor activation is necessary to trigger downregulation The receptor on the left undergoes activation and conformational change allowing Cl\(^-\) influx and hyperpolarization of the postsynaptic membrane, leading to receptor internalization. The mutant *unc-49(e382)* receptor fails to undergo any of the 3 precipitating events and internalization does not occur.
**Chloride influx and hyperpolarization are important signals for GABAAR downregulation**

The next aim was to observe the effect of GABAAR activation without Cl⁻ influx or hyperpolarization. The *abts-1(ok1566)* mutant was examined to determine if conformational change alone is sufficient to trigger GABAAR downregulation. In *abts-1(ok1566)* mutant animals, Cl⁻ gradient is reversed and GABA activation is excitatory. If GABAAR is internalized following agonist exposure in *abts-1* animals, then receptor conformational change is necessary and sufficient for receptor downregulation.

Experiments in *abts-1* animals show no agonist induced GABAAR internalization (Figure 4.2).
Figure 4.2 Receptor conformational changes associated with activation are not sufficient for downregulation. The left-hand receptor shows events leading to receptor internalization in agonist-exposed wild type animals. The arrow represents the direction of wild type Cl- gradients. The right-hand receptor represents the abts-1 mutation and corresponding reversal of Cl- gradient. Relevant signaling requires Cl- influx and/or hyperpolarization for activation.
The absence of GABA\textsubscript{A}R downregulation following muscimol exposure implicates Cl\textsuperscript{−} as a relevant signal in this pathway. However, further confirmation of this finding is necessary. One can employ Ca\textsuperscript{2+} imaging to study this mutant. Similarly, a cross between the \textit{abts-1(ok1566)} and a strain expressing Cameleon on its body wall muscles will be examined [46]. In a wild type animal, GABAergic inhibition will cause Cl\textsuperscript{−} influx, preventing Ca\textsuperscript{2+} influx, and keeping intracellular Ca\textsuperscript{2+} levels low. In \textit{abts-1}, GABA application should make Ca\textsuperscript{2+} rise because the cell will depolarize. Reversal of this observation and increased intracellular Ca\textsuperscript{2+} levels will confirm the excitatory action of GABA in \textit{abts-1(ok1566)} animals, validating our findings.

**Hyperpolarization via potassium efflux does not trigger GABA\textsubscript{A}R downregulation**

Chronic hyperpolarization of the postsynaptic muscle cell causes paralysis in \textit{C. elegans}. When the UNC-49 receptor is activated, hyperpolarization is a result of Cl\textsuperscript{−} influx. A similar behavioral phenotype is observed during excessive K\textsuperscript{+} efflux. I therefore sought to determine whether hyperpolarization by K\textsuperscript{+} could trigger GABA\textsubscript{A}R downregulation. If hyperpolarization and paralysis were necessary and sufficient for receptor internalization, then GABA\textsubscript{A}R levels would decrease under conditions of elevated K\textsuperscript{+} conductance. In \textit{twk-18(cn110)} animals, increased potassium conductance does not cause GABA\textsubscript{A}R downregulation (Figure 4.3). Furthermore, a behavioral phenotype is not observed until the animals are shifted to 30°C, even though K\textsuperscript{+} conductance is increased at all temperatures. I also observe decreased levels of postsynaptic GABA\textsubscript{A}Rs during
development, providing a possible explanation for wild-type behavior of the mutant. These observations indicate that GABA<sub>А</sub>R homeostasis is not simple; that is to say many factors are acting synergistically. Some combination of receptor conformational change, Cl<sup>-</sup> influx, and hyperpolarization and paralysis may be required to initiate GABA<sub>А</sub>R trafficking from the synapse. The 3 previous experiments collectively suggest Cl<sup>-</sup> influx as a major determinant of GABA<sub>А</sub>R stability.
Figure 4.3 Hyperpolarization and paralysis independent of Cl- influx are not sufficient for receptor internalization. The receptor on the left signifies wild type receptor downregulation. In *twk-18* mutants the GABAAR is not activated, and hyperpolarization occurs due to K+ efflux as a result of a twin-pore potassium channel. Receptor internalization does not occur, strongly implicating Cl- as a principle determinant.
Chloride gradients may provide the signal for GABA\(_A\)R internalization

Two additional experiments will be performed to determine if Cl\(^-\) influx and hyperpolarization provide the molecular trigger for GABA\(_A\)R downregulation. I have constructed a plasmid in which the MOD-1 Cl\(^-\) channel is expressed on *C. elegans* body wall muscles. MOD-1 is a serotonin gated Cl\(^-\) channel allowing Cl\(^-\) influx and hyperpolarization without GABA\(_A\)R activation [47]. The plasmid was injected into quintuple mutant *C. elegans* strains, as serotonin modulates 4 additional receptors. Behavioral analysis shows flaccid paralysis when transgenic animals are exposed to NGM plates containing 30mM serotonin. I intend to subject these animals to Anti-UNC-49 staining and observe for GABA\(_A\)R downregulation. If postsynaptic GABA\(_A\)R is reduced, Cl\(^-\) influx will be identified as an important molecular signal leading to receptor internalization.

A second experiment will be performed in a strain expressing LGC-55 under control of the *myo-3* promoter. This Cl\(^-\) channel is activated by tyramine [48]. Similar experiments will further support or refute Cl\(^-\) entry and hyperpolarization as sufficient for GABA\(_A\)R downregulation. I will also test hyperpolarization and muscimol exposure synergistically. If the previous methods do not cause downregulation alone but are sufficient together, one can conclude that although Cl\(^-\) hyperpolarization is important, it is not sufficient to induce receptor trafficking.
A signaling pathway may exist between the pre- and postysnaptic GABA terminals

Homeostasis in general involves both sides of the synapse [15]. Presynaptic GABA release is not required for postsynaptic GABAₐR clustering [45]. I examined the presynaptic GABA terminal following agonist exposure to test for possible homeostatic mechanisms. Our initial studies show no change in total synaptic fluorescence of vesicular contents, presumably GABA, following agonist application. Synapse by synapse analysis yields interesting observations, however. Chronic muscimol exposure causes SNB-GFP clusters to increase in volume and decrease in fluorescent intensity suggesting increased GABA release into the synapse. The increase in volume of SNB-GFP clusters may be a result of increased vesicle fusion with the synaptic membrane. Similarly, GFP intensity decreases as vesicle contents are released into the synapse. I have observed decreased postsynaptic GABAₐR in response to agonist exposure. It is possible that the presynaptic terminal upregulates GABA release in an attempt to compensate for decreased postsynaptic receptor levels.

The same experiment performed on twk-18(cn110) mutants yields interesting results. The elevated K⁺ conductance in these animals also causes the postsynaptic membrane to become hyperpolarized. In these animals SNB-GFP clusters increase in fluorescent volume and intensity. This finding is interesting because it suggests a novel retrograde signaling mechanism between muscles and neurons. TWK-18 is expressed in muscle cells, while SNB-GFP is expressed in GABA neurons under the control of UNC-25. This suggests that changes in the muscle cell initiate a biochemical signal capable of modifying neuron-specific factors [49].
This study seeks to understand the biochemical pathways involved in GABA$_A$R homeostasis. *C. elegans* provides an intact system to examine these mechanisms *in vivo*. I have determined that agonist exposure causes increased GABA$_A$R internalization from the postsynaptic membrane. Endocytosed receptor enters the lysosomal pathway and is targeted for degradation. Further analysis is needed in order to fully understand the biochemical mechanisms at work. Studies in mammalian systems have implicated clathrin-mediated endocytosis as a major determinant of synapse strength. Similar assays in the model organism *C. elegans* will provide valuable insight into mechanisms that may be conserved. Observation of endocytic compartments and posttranslational modifications targeting the receptor for internalization is necessary. The data suggest that Cl$^-$ influx and hyperpolarization are major determinants of GABA$_A$R homeostasis and cellular excitability.
5. References


52


