The JBS-Associated E3 Ligase UBR-1 Maintains Synaptic Glutamate Homeostasis and Regulates Neurotransmitter Balance

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

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Abstract

Johanson-Blizzard Syndrome (JBS) is a rare, sometimes fatal autosomal recessive multi-systemic congenital genetic disorder, characterized by pancreatic exocrine insufficiency and mental retardation. JBS results from mutations in the gene UBR1, an E3 ubiquitin ligase that specifically targets proteins for proteasomal degradation. There are multiple UBR1 homologs in humans; however, mutations in UBR1 alone lead to JBS. Although it is known that loss-of-function mutations in UBR1 cause JBS, the cellular and molecular mechanisms through which UBR1 underlie normal or pathological development remain elusive.

In this study, I describe the identification and characterization of UBR-1, the sole UBR1 homologue in the nematode Caenorhabditis elegans (C. elegans). In the
first data chapter, I show that UBR-1 functions through a simple premotor interneuron circuit to regulate body bending during backward locomotion. I find that the absence of UBR-1 disrupts glutamate homeostasis, and further identified three components of a UBR-1-regulated signaling pathway - a glutamate metabolic enzyme GOT/GOT-1.2, a vesicular glutamate transporter VGlut3/EAT-4, and a glutamate-gated chloride channel GluCl/AVR-15 - all involved in glutamatergic synaptic transmission in this premotor circuit. These findings suggest that UBR-1 maintains synaptic glutamate homeostasis in this premotor circuit to control locomotion.

In the second data chapter, I demonstrate a role of UBR-1-mediated glutamate homeostasis in GABAergic neurons. A simple GABAergic motor circuit controls *C. elegans* defecation. *ubr-1* mutants exhibit defecation defects, coinciding with reduced activity of the GABAergic motor neurons, as well as reduced GABA signaling. I show that the reduced neuronal activity is augmented by removing the glutamate metabolic enzyme GOT-1, whereas reduced GABA signaling is rescued by removing the vesicular glutamate transporter EAT-4, both function in the GABAergic motor neurons. These findings implicate a requirement of UBR-1 in maintaining the balance between inhibitory (GABA) and excitatory (glutamate) neurotransmitters, and of glutamate homeostasis in regulating the activity of GABAergic neurons. Finally, I propose that dysregulation of glutamate homeostasis mediated by the functional loss of UBR1 may underlie systemic and neurodevelopmental defects in JBS patients.
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List of Specific Contributions

I was solely responsible for experiments, data collection and figure preparation for all figures in Chapters 2-4, unless specified as otherwise.

Figure 5. Images in Panel J were taken by Wesley Hung.

Figure 9. Experiments were performed with Wesley Hung and Maria Lim.

Figure 10. Experiments were performed with Wesley Hung and Maria Lim.

Figure 15. Panel D: The mass spectrometry experiments were performed with the assistance of Anas M. Abdel Rahman.

Figure 16. All mass spectrometry experiments were performed with the assistance of Anas M. Abdel Rahman.

Figure 18. Images were taken by Wesley Hung.

Figure 20. Panel B: Images were taken by Wesley Hung.

Figure 21. Panel C: data were generated with Maria Lim. Panel D: Images were taken by Maria Lim and Wesley Hung.
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Chapter 1 Introduction
1.1 Selective Protein Degradation by the Ubiquitin-proteasome System

Protein degradation is an essential function of all cells, and degradation of proteins is carried out by two main proteolytic systems, lysosomes and proteasomes. Lysosomes are membrane-bound organelles; they contain hydrolytic enzymes capable of breaking down macromolecules. Lysosomes regulate cellular homeostasis, such as the localization of plasma membrane receptors in response to ligand binding (Boya, 2012; Haglund and Dikic, 2012). Proteasomes are multi-subunit protein complexes and participate in regulated breakdown of proteins that are modified by covalently bound ubiquitin (Adams, 2003; Voges et al., 1999). The Ubiquitin-proteasome system (UPS) is central to the regulation of almost all cellular processes, including antigen processing, apoptosis, biogenesis of organelles, cell cycle and division, DNA transcription and repair, differentiation and development, immune response and inflammation, neural and muscular degeneration, morphogenesis of neural networks, modulation of cell surface receptors, ion channels and the secretory pathway, response to stress and extracellular modulators, ribosome biogenesis, and viral infection (Ciechanover, 1998; Haglund and Dikic, 2012; Pickart and Eddins, 2004; Schwarz and Patrick, 2012).

1.1.1 The Ubiquitin Pathway

Targeted proteolysis eliminates misfolded proteins, generates antigens, and controls cell signaling by restricting the concentration, localization or subunit
composition of regulators. In eukaryotes, selective degradation of intracellular proteins is mainly carried out by the UPS. This pathway involves two discrete and successive steps: 1) selectively tagging proteins through covalent attachment of a single or multiple copies of a 76 amino acid protein called ubiquitin, and 2) degradation of selectively tagged proteins by the 26S proteasome complex with the release of free and reusable ubiquitin.

Ubiquitination of proteins is achieved through a cascade of enzymatic reactions involving the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2, also referred to as ubiquitin carrier protein or UBC) and the ubiquitin ligase (E3) (Figure 1) (Ciechanover and Iwai, 2004; Ciechanover et al., 2000; Hershko and Ciechanover, 1998; Varshavsky, 1996). Conjugation of ubiquitin to protein substrates begins with the formation of a thioester bond between E1 and ubiquitin in an ATP-dependent manner. E2 transfers the activated ubiquitin, which involves the formation of another transient thioester bond between E2 and ubiquitin, to a substrate that is recruited by an E3 ubiquitin ligase. Ubiquitin forms a covalent bond with a Lysine residue on the substrate (Figure 1).

The E3 ubiquitin ligases recruit substrates via protein-protein interaction (Ciechanover and Iwai, 2004; Ciechanover et al., 2000; Hershko and Ciechanover, 1998; Varshavsky, 1996). The interactive motifs in the substrates, recognized by the specific E3 ubiquitin ligases, are called degradation signals (degrons). E3 ligases are divided into three classes based on the conserved structural domains and the mechanism by which ubiquitin is transferred from the E2 to the substrate: RING (The really interesting new
gene), U-boxes, and HECT (Homologous with E6-associated protein C-terminus) E3s (Metzger et al., 2012). RING and U-box family E3s catalyze direct transfer of ubiquitin from E2 to the substrate, by simultaneously binding the substrate and an E2 bound with the ubiquitin (Berndsen and Wolberger, 2014; Budhidarmo et al., 2012; Deshaies and Joazeiro, 2009; Hatakeyama et al., 2001). The HECT E3 ligases are unique among the E3s in that they possess intrinsic catalytic activity (Bernassola et al., 2008). The HECT domain binds to E2 and forms a ubiquitin-thioester intermediate with a ubiquitin moiety and then directly transfers the ubiquitin to a targeted substrate (Bernassola et al., 2008; Huibregtse et al., 1995).

E3 ligases thus play a major role in increasing the rate of ubiquitin transfer by bringing together the right E2 with the right substrate (Berndsen and Wolberger, 2014). Multiple rounds of ubiquitination results in the attachment of multi-ubiquitin chains to the substrates, which can be recognized by the 26S proteasome complex and degraded. Mono ubiquitination can lead the protein to a number of different fates including endocytosis, protein sorting, DNA damage response and transcriptional regulation (Hicke, 2001; Sadowski and Sarcevic, 2010). Following degradation of the substrate, short peptides derived from the substrate and reusable ubiquitin are released (Ciechanover and Iwai, 2004).
Figure 1: The Ubiquitination Pathway

A schematic representation of the process of protein ubiquitination. The ubiquitin activating enzyme (E1) activates a ubiquitin (Ub) through a thiol-ester bound in an ATP dependent reaction. Activated ubiquitin is transferred to the ubiquitin conjugating enzyme (E2), which recruits the activated Ub to a ubiquitin ligase (E3). The E3 ubiquitin ligase recruits protein substrates, and brings them in close proximity to the E2. Ubiquitin is transferred to the substrate through a covalent bond with the substrate’s lysine side chain. When the process is repeated, a poly-ubiquitin chain is formed on the substrate. Poly-ubiquitinated proteins are recognized and targeted by the proteasome for degradation (not shown in the drawing).
1.1.2 The N-end Rule Pathway

The RING family E3 ligases recognize substrates with either an internal degron or a degron in the N-terminus of the substrate. One such degron, N-degron, is a destabilizing N-terminal amino acid residue of a protein (Arginine, Arg; lysine, Lys; histidine, His; Leucine, Leu; phenylalanine, Phe; tryptophan, Trp; tyrosine, Tyr and isoleucine, Ile are destabilizing N-degrons) (Sriram et al., 2011; Tasaki et al., 2012; Varshavsky, 1996). N-degrons are targeted by the N-end rule pathway, which defines the stability of a protein based on its identity and post-translational modification of its N-terminal amino acid (Varshavsky, 1996). The N-end rule pathway functions in organisms from bacteria, to plants and animals including mammals (Gibbs et al., 2014).

The N-end rule pathway was discovered in *Saccharomyces cerevisiae*, by Varshavsky and colleagues, following the initial observation that an unidentified protein with E3 ubiquitin ligase activity (N-recognin) had a high affinity for proteins with a free α-amino group at the N-terminus (Bachmair et al., 1986). A series of elegant genetic analyses led to the identification of Ubr1 as the N-recognin that differentially degrades proteins bearing different N-terminal moieties (N-end rule pathway) in *Saccharomyces cerevisiae* (Bartel et al., 1990; Varshavsky, 1996). Substrates of the N-end rule pathway are generated by proteolytic cleavage by endopeptidases including caspases, calpains, separases and secretases that expose destabilizing N-terminal amino acids (Piatkov et al., 2014; Sriram et al., 2011; Tasaki et al., 2012). N-degrons can also be generated through post-translational modifications that convert stabilizing pro-N-degrons to destabilizing N-degrons. Such modifications include oxidation, arginylation, leucylation,
Interestingly, prokaryotes, which lack the ubiquitin proteasome system, also exhibit the N-end rule activity. In bacteria, an ATP-dependent Clp protease complex (ClpAP) acts as the protease, analogous to the 26S proteasome in eukaryotes, to selectively degrade substrates bearing different N-terminal amino acids (Tobias et al., 1991). Amino acyl-tRNA transferases attach N-terminal Arginine (Arg), lysine (Lys) and methionine (Met) to the primary destabilizing residues Leucine (Leu), phenylalanine (Phe), tryptophan (Trp) and tyrosine (Tyr), as the N-degrons (Leibowitz and Soffer, 1971; Ninnis et al., 2009; Shrader et al., 1993; Watanabe et al., 2007). ClpS, the N-recognin binds these substrates and delivers them to the ClpAP protease complex for degradation (Erbse et al., 2006; Roman-Hernandez et al., 2009).

In eukaryotes, there are two main branches of the N-end rule pathway: the Arg/N- and Ac/N-end rule pathways (Sriram et al., 2011) (Figure 2). Substrates for the Arg/N-end rule pathway arise from either endonuclease-processed products, or, proteins that are post-translationally modified by the Met-amino peptidase (MetAP) and Arginyl-transferase (ATE1) to replace the N-terminal Met with Arg (Kwon et al., 1999). ATE1-deficient mice die as embryos with defects in cardiac and vascular development, establishing the physiological importance of protein arginylation (Kwon et al., 2002). The E3 N-recognins of this pathway specifically target the un-acetylated basic N-terminal residues (Arg, Lys and His; Type I substrates) and bulky hydrophobic N-terminal
residues (Leu, Phe, Trp, Tyr and Ile; Type 2 substrates) (Sriram et al., 2011; Tasaki et al., 2012; Varshavsky, 1996). The Arg/N-end rule pathway has been implicated in a wide range of cellular functions, such as DNA repair, chromosome segregation, meiosis, spermatogenesis, metabolism, dipeptide import, apoptosis, neurogenesis and neuronal aging, oxygen sensing and cardiovascular development (An et al., 2006; Byrd et al., 1998; Kwon et al., 2003; Piatkov et al., 2012; Rao et al., 2001; Tasaki et al., 2012; Varshavsky, 2011).

The second branch, the Ac/N-end pathway, targets nascent proteins through N-terminally acetylated (Nt-acetylated) residues. Nt-acetylation is carried out by ribosome-associated Nt-acetyl transferases (NATs), which transfer the acetyl group from the acetyl-coenzyme A to the N-terminal α-amino group (Tabor et al., 1953). In eukaryotes, Nt-acetylation is a mostly irreversible modification that occurs both during translation and post translationally to a majority of newly synthesized proteins (Choudhary et al., 2009; Starheim et al., 2012). Nt-acetylation was generally considered to protect nascent proteins from degradation, but it was recently shown to function as the N-degron.Degradation of Alpha 10 (DOA10), an endoplasmic reticulum (ER) transmembrane E3 ligase, targets proteins with Nt-acetylated Met, Ala, Val, Ser, Thr and Cys for ubiquitination (Hwang et al., 2010; Kim and Hwang, 2014; Shemorry et al., 2013).
Figure 2: The N-end Rule Pathway

A. The list of amino acid residues that render artificial substrates to be unstable when present at the N-terminus (N-degrons). B. Two branches of the N-end rule pathway. Top panel, in the Arg/N-end rule pathway substrates that bear N-terminal Met are post-translationally modified by the Met-amino peptidase and Arginyl-transferase to replace Met with a N-terminal Arg. The resulting protein is then recognized by UBR1 for degradation. Bottom panel, in the Ac/N-end rule pathway Nt-acetyl transferases (NATs) catalyze the transfer of acetyl groups to the N-terminal of the substrates. The resulting protein is then recognized by DOA10 for degradation.
It was recently reported that the Ac/N-end E3 ligase, yeast Ubr1 and its mouse homologues UBR1 and UBR2, can also directly target substrates with un-acetylated N-terminal methionine if it is followed by a bulky hydrophobic residue Leu, Phe, Trp, Tyr and Ile (denoted by Φ) for ubiquitination. Hence the Arg/N- and Ac/N-end rule pathways converge, and co-regulate the stability of proteins bearing Met at the N-terminus (Gibbs et al., 2014; Kim et al., 2014) (Figure 2).

1.1.3 The UBR Family E3 Ligases

In yeast, Ubr1 functions with the Ubc2/Rad6 E2 to target proteins with both Type 1 (Arg, Lys and His) and Type 2 (Leu, Phe, Trp, Tyr and Ile) N-degrons for ubiquitination (Bartel et al., 1990; Varshavsky, 1996). Ubr1 contains an N-terminal UBR box (70-amino acid zinc finger like domain) that binds Type 1 substrates, a N-domain (80-amino acid domain similar to ClpS in bacteria) that binds the Type 2 substrates, and an internal module consisting of a region enriched for basic amino acids (BRR) and a RING finger that binds the E2 (Tasaki et al., 2012) (Xie and Varshavsky, 1999).

The first physiological substrate of yeast Ubr1 identified was a subunit of the cohesion complex SCC1, which holds the sister chromatids together during metaphase. Sepaerin protease cleaves SCC1 when the cell cycle progresses from metaphase to anaphase, creating an Arg N-degron that is targeted by Ubr1 for degradation, allowing efficient chromatin separation (Rao et al., 2001). In yeast, other physiological Met-Φ-N-end rule substrates were identified including a transcriptional activator Msn4, a 3-
hydroxyaspartate dehydratase Sry1, a Golgi-associated cytosolic GTPase Arl3 and a subunit of 20S proteasome Pre5 (Kim et al., 2014).

In mammals, there are seven UBR-box containing proteins, UBR1-UBR7 (Kwon et al., 1998; Tasaki et al., 2005). Among them, UBR1-UBR3 share overall structural organization with yeast Ubr1, which includes the N-terminal UBR box, the N-domain, and the BRR/RING finger region. UBR4-UBR7, on the other hand, share only the UBR box with UBR1-3, and with each other. The functional classification of UBR1-7 is a bit confusing: UBR1, UBR2, UBR4 and UBR5 were found to exhibit N-degron recognition activity, whereas UBR3, UBR6 and UBR7 did not (Kwon et al., 1998; Tasaki et al., 2005).

There are only a few known physiological substrates for the mammalian N-end rule pathway. This includes the Arg/N-end rule physiological substrates, the GTPase activators RGS4 and RGS5 that inactivate the G protein complex, and rapidly switch off G-protein coupled receptor (GPCR) signaling pathways. Degradation of RGS proteins by UBR1 is inhibited by the lack of oxygen or nitric oxide (Hu et al., 2005; Lee et al., 2011; Xia et al., 2008b). Other targets of the N-end rule pathway include key disease-associated proteins such as the breast cancer-related tumor-suppressor BRCA1 (Xu et al., 2012) and Parkinson’s disease-associated PINK1 (PTEN induced putative kinase 1) (Yamano and Youle, 2013).

Another interesting puzzle about the UBR family proteins is the lack of substrate
conservation. The mammalian homologues of yeast Ubr1 substrates do not harbor N-degrons and *vice versa* for mouse homologues. Multiple lines of evidence indicate that both yeast and mammalian Ubr1 may also recognize substrates utilizing mechanisms beyond the ‘N-end rule’. Yeast Ubr1 was recently reported to function as an additional E3 ligase in the ER-associated protein degradation (ERAD) pathway that targets cystic fibrosis transmembrane conductance regulator (CFTR) for proteasome degradation (Stolz et al., 2013). Several known Ubr1 substrates harbor internal, instead of N-degrons, including a homeodomain protein, Cup9, a transcriptional repressor of the Ptr2 peptide transporter (Byrd et al., 1998; Turner et al., 2000), a Gα-subunit GPA1 (Madura and Varshavsky, 1994), and an O6-alkylguanine-DNA alkyl transferase Mgt1 (Hwang et al., 2009).  

1.1.4 Functional Loss of Human UBR1 Causes Johanson-Blizzard Syndrome (JBS)  

JBS is a rare, clinically distinct, sometimes fatal autosomal recessive multisystemic congenital malformation syndrome. The clinical hallmarks of JBS are exocrine pancreatic insufficiency, nose wing hypo/aplasia, mental retardation, hearing loss, hypothyroidism, scalp, dental defects, growth failure, and urogenital and anorectal malformations (Johanson and Blizzard, 1971; Zenker et al., 2005). In the exocrine pancreas, a gradual destruction of the pancreatic acinar cells, with no evidence of apoptosis, underlies pathological development (Zenker et al., 2005).  

Loss-of-function (*lf*) mutations in human UBR1 cause Johanson-Blizzard
Syndrome (JBS) (Zenker et al., 2005). JBS patients harbour homozygous or compound heterozygous if mutations in human UBR1 gene. Pathogenic mutations in JBS patients comprise nonsense and frame shift mutations that lead to premature stop codons, splice site mutations, missense mutations and small in frame deletions affecting conserved amino acid residues. Mutations are spread throughout the protein, and patients with nonsense and frame shift mutations that lead to protein truncations exhibit 100% hearing impairment, mental retardation and growth impairment. Individuals with non-truncating mutations show combinatory features at a significantly lower frequency. But irrespective of the type of mutations, all patients share severe exocrine pancreatic insufficiency, nose wing hypo/aplasia and dental defects (Sukalo et al., 2014). Among the multiple human UBR1 homologues, mutations in UBR1 alone lead to JBS. How UBR1 regulates development and underlies JBS pathophysiology remains elusive.

Two in vivo models, yeast and mouse, have been used to explore the cellular functions and mechanisms of UBR1. Yeast Ubr1 is non-essential; Ubr1 null yeast exhibit only subtle phenotypes - a slightly longer doubling time coinciding with slightly increased susceptibility of chromosomal loss during cell division (Varshavsky, 1996). Mice have at least four Ubr1 functional homologues as N-recognins, UBR1, UBR2, UBR4, and UBR5 (Tasaki et al., 2005). UBR1<sup>−/−</sup> mice are viable and fertile, and do not show any obvious symptoms or malformations reminiscent of JBS, except being slightly underweight (Balogh et al., 2002) and a mild impairment in pancreatic enzyme production and increased susceptibility to experimentally induced pancreatitis (Zenker et al., 2005). UBR2<sup>−/−</sup> mice die as embryos with defects in homologous chromosome pairing
during meiotic prophase I (Kwon et al., 2003). \textit{UBR1}^{−/−}; \textit{UBR2}^{−/−} double knockout mice, on the other hand, die at midgestation with defects in neurogenesis and cardiovascular development (An et al., 2006). How the yeast and mouse phenotypes relate to JBS remains to be examined.
1.2 Overview of Glutamatergic Signalling in Neuronal and Non-neuronal Tissues

Glutamate is the primary neurotransmitter at most excitatory synapses in the vertebrate central nervous system (CNS) (Ottersen and Storm-Mathisen, 1984). Glutamate is the sole precursor for another neurotransmitter γ-amino butyric acid (GABA) in GABAergic neurons (Costa et al., 1979). As a neurotransmitter, glutamate plays a major role in mediating sensory information, motor coordination, emotions, and learning and memory (Mattson, 2008; Shepherd and Huganir, 2007).

Glutamate is also one of the most abundant amino acids in all cells, playing a central role in cell metabolism as a basic metabolite. Glutamate and its derivatives are at the center of amino acid homeostasis, carbohydrate metabolism, energy production (TCA cycle), and nitrogen metabolism (Kelly and Stanley, 2001). In glial cells, glutamate is the sole precursor for glutamine (Brosnan et al., 1996; Dejong et al., 1996; Norenberg et al., 1997; Sonnewald et al., 1997). It is also a precursor to glutathione (Meister and Larsen, 1995), a major defense against oxidative stress in all cells. Primary but intriguing studies imply that glutamate may also influence cellular activity in a range of tissue types, in a fashion similar to a cytokine.

Glutamate signaling has to be tightly regulated. Disturbances in glutamatergic signaling have been implicated in the pathophysiology of several metabolic (Kelly and Stanley, 2001) and neurodegenerative disorders (Siegel and Sanacora, 2012).
1.2.1 Glutamate Biosynthesis

In the CNS, glutamate has to be locally synthesized, as it cannot cross the blood-brain barrier (Hawkins, 2009). A prevailing view on the maintenance of neurotransmitter glutamate is through the glutamate-glutamine cycle, the metabolic trafficking of glutamate and glutamine between neurons and astrocytes (Norenberg and Martinez-Hernandez, 1979; Palmada and Centelles, 1998; van den Berg and Garfinkel, 1971; Waniewski and Martin, 1986). Briefly, the neurotransmitter glutamate is synthesized from glutamine by the mitochondrial glutaminase (Hamberger et al., 1979). Glutamate released at nerve terminals is taken up by astrocytes, and in astrocytes, glutamine synthetase converts glutamate to glutamine. Neurons take up glutamine released from astrocytes, and the phosphate-activated glutaminase (PAG), converts it to glutamate.

Maintenance of the neurotransmitter glutamate has long been believed to depend on de novo glutamine synthesis in astrocytes. This hypothesis predicts a constant supply of glutamine from astrocytes to keep up with glutamate release at the nerve terminal. Growing evidence indicates that the neurotransmitter glutamate may not necessarily be derived from glutamine. Instead, glutamine may serve as a nutrient for neurons. Glutamine is mostly metabolized to CO₂ when administered to the intact brain, supporting its role in the supply of neuronal energy (Bradford et al., 1978; Hassel and Sonnewald, 1995; Zielke et al., 1998). Furthermore, PAG, the enzyme proposed to deamidate glutamine to glutamate, is not expressed in some populations of glutamatergic neurons (Laake et al., 1999; Ottersen et al., 1998).
An alternative pathway for synthesizing brain glutamate is from glucose and amino acids such as leucine, isoleucine, and valine that can cross the blood-brain barrier. The carbon backbone of glutamate is derived from glucose via the tricarboxylic acid (TCA) cycle. Briefly, glucose is broken down via glycolysis into pyruvic acid, which enters the TCA cycle and generates α-ketoglutarate (α-KG) (Hassel and Brathe, 2000a; Hassel and Brathe, 2000b). Because uptake of amino acids into the brain is much less than uptake of glucose, the amino group of glutamate must be recycled in the brain when glucose breaks down to CO₂ and water. Aspartate is one important amino group reservoir for glutamate synthesis.

Hans Krebs established the role of glutamate in metabolism way before glutamate was identified as an excitatory neurotransmitter. Krebs’ first observed that in the absence of glucose, extracellular glutamate was metabolized and converted into aspartate by transamination in rat brain homogenates. He further demonstrated that the addition of glucose and pyruvate inhibited transamination and subsequent synthesis of aspartate in rat brain (Haslam and Krebs, 1963; Krebs, 1935). The transamination activity was shown to result mainly from two transaminases, glutamate oxaloacetate transaminase (GOT) and alanine aminotransferase (ALT). GOT catalyzes the reversible transfer of an α-amino group between aspartate (Asp) and α-ketoglutarate (α-KG), and converts them to oxaloacetate (OAA) and glutamate (Glu), respectively, whereas ALT catalyzes the reversible transfer of an α-amino group between alanine (Ala) and α-ketoglutarate (α-KG), and converts them to pyruvate (Pyr) and glutamate (Glu), respectively (Haslam and Krebs, 1963; Hayashi et al., 1990; Hirotsu et al., 2005; Stern et al., 1949) (Figure 3).
GOT and ALT hence establish the homeostasis among three abundant amino acids, glutamate, alanine and aspartate. GOT activity is found in the liver, heart, skeletal muscle, kidney, brain, pancreas, lung, leukocytes and erythrocytes, and through α-KG, a precursor for nucleotides, an important enzyme for amino acid and nucleotide metabolism.

Glutamate can also be synthesized from α-ketoglutarate and ammonia by glutamate dehydrogenase (GDH). GDH catalyzes the reversible inter-conversion of glutamate to α-ketoglutarate (Figure 3). The specific role of GDH in the brain is not clear, but it is an important enzyme in protein degradation (McKenna et al., 2000).

A recent study breaches the separate regimes for metabolic versus synaptic glutamate synthesis. It showed that synaptic vesicles are endowed with GOT, and GOT synthesizes glutamate from aspartate directly at the synapse (Takeda et al., 2012).
Figure 3: A Diagram of Glutamate Metabolic Pathways.

GOT/AST, glutamate oxaloacetate transaminase; ALT, alanine aminotransferase; GDH, glutamate dehydrogenase; GAD, glutamic acid decarboxylase.
1.2.2 Glutamate Release

In glutamatergic neurons, nerve terminals contain the highest concentrations of glutamate. At synaptic terminals, glutamate is packaged into synaptic vesicles by vesicular glutamate transporters (VGLUTs). VGLUTs are multimeric protein complexes that are proton/glutamate antiporters. VGLUT-mediated vesicular uptake of glutamate is independent of sodium and potassium, and stimulated by low chloride concentration (Bellocchio et al., 2000; Juge et al., 2006; Lewis and Ueda, 1998; Tabb and Ueda, 1991; Takamori et al., 2000). In synaptic vesicles, VGLUTs are driven by the membrane potential of the electrochemical proton gradient across the vesicle membrane, which is generated by vacuolar H\(^+\)-dependent ATPase. Synaptic vesicle-bound glycolytic ATP-synthesis systems, the glyceraldehyde phosphate dehydrogenase/3-phosphoglycerate kinase complex and pyruvate kinase provide the ATP required for \(v\)-H\(^+\)-ATPase activity (Ikemoto et al., 2003; Ishida et al., 2009). To date, three VGLUTs have been identified - VGLUT1, VGLUT2 and VGLUT3. VGLUT1 and VGLUT2 are primarily expressed in different populations of glutamatergic neurons; VGLUT3, on the other hand, has been reported to be present in GABAergic, cholinergic and monoaminergic neurons, in addition to glutamatergic neurons (Fremeau et al., 2002; Fremeau et al., 2004). The physiological function of VGLUT3 in non-glutamatergic neurons is not well established. Glutamate is released into the synaptic cleft by exocytosis of synaptic vesicles in a Ca\(^{2+}\)-and soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-dependent process (Pang and Sudhof, 2010; Sudhof and Rothman, 2009).
Although most work has focused on glutamate release by synaptic vesicle exocytosis, several non-vesicular, non-exocytotic mechanisms have been documented in both neurons and astrocytes. One such mechanism is through swelling-induced anion channel efflux (Kimelberg et al., 1990; Kimelberg and Mongin, 1998; Levi and Raiteri, 1993; Longuemare and Swanson, 1995; Wang et al., 2013a). The second mechanism is through the reversed operation of glutamate transporters (EAATs) at the plasma membrane, upon changes in ion gradients (Chao et al., 2010; JABAUDON et al., 2000; JENSEN et al., 2000; Longuemare and Swanson, 1995; ROETTGER and Lipton, 1996; ROSSI et al., 2000). The third mechanism is through the activity of a cystine-glutamate exchanger (xCT); cystine-glutamate exchangers carry cystine into the cell in exchange for internal glutamate in a 1:1 ratio (Bannai, 1986). Extracellular glutamate inhibits uptake of cystine, and uptake of cystine through cystine-glutamate exchangers causes glutamate release (BRIDGES et al., 2012; Sato et al., 1999; Sontheimer, 2008; Williams and Featherstone, 2014).

1.2.3 Glutamatergic Neurotransmission

Glutamate released into the synaptic cleft activates both pre- and post-synaptic receptors (GluRs). The physiological effect of glutamate release on postsynaptic termini is determined by the nature of receptor subtypes, their subcellular localization, as well as their interactions with scaffolding and signaling proteins in the post-synaptic density (Meldrum, 2000). There are three families of glutamate receptors: ionotropic, metabotropic and delta glutamate receptors (Traynelis et al., 2010). Ionotropic receptors
are cation (Ca\(^{2+}\), Na\(^+\)) channels; upon agonist binding, a conformational change leads to opening of the channel. Metabotropic receptors do not conduct ions; they instead activate or inhibit second messenger systems through G proteins upon glutamate binding (Mayer and Armstrong, 2004). Delta glutamate receptors exhibit sequence similarity to ionotropic receptors, but do not appear to exhibit channel activities (Lomeli et al., 1993; Schmid and Hollmann, 2008; Yamazaki et al., 1992).

### 1.2.3.1 Ionotropic Glutamate Receptors

Ionotropic receptors have high affinities for glutamate and can be further categorized into three sub-classes based on their selectivity for agonists, N-methyl-D-aspartate receptor (NMDA), α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate (KA). The NMDA-type glutamate receptors (consisting of NR1, NR2A-D, and NR3A-B) are among the most tightly regulated glutamate receptors; they are slowly inactivating cation channels (Cull-Candy and Leszkiewicz, 2004; Dingledine et al., 1999; Hansen and Traynelis, 2014; Paoletti, 2011; Traynelis et al., 2010). NMDA receptor activation uniquely requires co-agonists and directly causes membrane depolarization. There are at least six binding sites for endogenous ligands to regulate the probability of ion channel opening: two recognition sites for co-ligands glutamate and glycine/D-serine (Kleckner and Dingledine, 1988; Wolosker et al., 1999), a polyamine regulatory site that promotes receptor activation, and a separate cation recognition site (Mg\(^{2+}\), H\(^+\)) that inhibits ion flux (Nowak et al., 1984; Traynelis et al., 1995). *in vitro* ligands that activate NMDA receptors are short chain dicarboxylic amino acids, including
glutamate, aspartate and NMDA. *in vivo*, NMDA receptor activation requires not only agonists, but also removal of cations blocking the channel upon postsynaptic membrane depolarization (Blanke and VanDongen, 2009; Danbolt, 2001).

AMPA receptors are found in many parts of the brain and are the most commonly found glutamate receptor in the nervous system (Belachew and Gallo, 2004; Wisden and Seeburg, 1993). Kainate receptors have both pre and post-synaptic functions and have more limited distribution in the brain compared to NMDA and AMPA receptors (Contractor et al., 2011). Post-synaptic kainate receptors are involved in excitatory neurotransmission (Contractor et al., 2011). On the other hand pre-synaptic kainate receptors are implicated in controlling inhibitory neurotransmission by modulating the release of inhibitory neurotransmitter GABA (Hansen and Traynelis, 2014; Lourenco et al., 2010; Rodriguez-Moreno and Sihra, 2011). AMPA/kainate receptors mediate fast excitatory neurotransmission in response to glutamate binding (Palmer et al., 2005).

AMPA/kainate receptors are of low affinity (Patneau and Mayer, 1990) and desensitize quickly upon exposure to AMPA/kainate (Meyerson et al., 2014; Sun et al., 2002; Tang et al., 1989; Trussell and Fischbach, 1989). GLUR1-GLUR4 are AMPA receptor subunits (Borges and Dingledine, 1998; Dingledine et al., 1999); kainate receptor subunits are called GLUR5-GLUR7, KA1, KA2 (Chittajallu et al., 1999).

Together with NMDA receptors, AMPA receptors play a critical role in long-term potentiation (LTP) (Whitlock et al., 2006). Excitatory postsynaptic potentials (EPSPs) are generated by the release of glutamate into the synaptic cleft that binds to
both the AMPA and NMDA receptors. Ligand binding causes postsynaptic membrane depolarization via Na\(^+\) conductance of AMPA receptors. Membrane depolarization and glutamate binding together activate the NMDA receptor and Ca\(^{2+}\) entry. Ca\(^{2+}\) entry triggers an up-regulation of AMPA receptor levels at membranes, resulting an increase in EPSP size that underlies LTP (Lu et al., 2001; Park et al., 2004). Time course of EPSPs at synapses is controlled by rapid desensitization of AMPA receptors. The prolonged activation of NMDA receptors, by contrast, allows for the temporal and spatial summation of multiple inputs (Grosshans et al., 2002; Niciu et al., 2012; Turrigiano and Nelson, 2004).

### 1.2.3.2 Metabotropic Glutamate Receptors

Unlike ionotropic glutamate receptors that conduct cation influx, metabotropic glutamate receptors exert their effects via activation of intracellular G-proteins and their downstream signaling pathways (Bonsi et al., 2005). Eight-metabotropic glutamate receptors are divided into three subclasses, group I (mGluR1 and mGluR5), group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7 and mGluR8) (Endoh, 2004; Kim et al., 2008). Upon glutamate binding, group I receptors activate phospholipase C. Activation of phospholipase C leads to the formation of inositol-1, 4, 5-triphosphate (IP\(_3\)), which triggers Ca\(^{2+}\) release from cytoplasmic ER stores. (Bonsi et al., 2005; Chu and Hablitz, 2000; Endoh, 2004; Shigemoto et al., 1997). The activation of group II and group III receptors by glutamate results in the inhibition of adenylyl cyclase/protein kinase A activity, which is mediated by inhibitory G-proteins that
decrease intracellular cyclic adenosine monophosphate (cAMP) (Conn and Pin, 1997; Endoh, 2004; Pin and Acher, 2002). Inhibition of cAMP-PKA pathway via mGluR II/III activates Src family kinases and potentiates NMDA currents (Trepanier et al., 2013).

Postsynaptic activation of metabotropic receptors modulates several ligand-gated ion channels including the NMDA, AMPA, kainate, dopamine, GABA_A and norepinephrine receptors (Beaulieu and Gainetdinov, 2011; Connelly et al., 2013; Lorrain et al., 2003; Smith et al., 2009; Wang et al., 2013c; Xi et al., 2011). Whether activation of metabotropic receptors leads to an inhibition or potentiation of ligand-gated channels depends on the downstream signal transduction mechanism, and this effect is often tissue-specific. Activation of all three classes of metabotropic receptors has been shown to inhibit L-type voltage-dependent Ca^{2+} channels, and both group I and group II metabotropic receptors can inhibit N-type Ca^{2+} channels (Choi and Lovinger, 1996). Metabotropic receptors are also expressed on presynaptic terminals in the CNS, and activation of these receptors has been demonstrated to decrease both excitatory glutamatergic and inhibitory GABAergic synaptic neurotransmission (Endoh, 2004; Hermes and Renaud, 2011; Schoepp, 2001; Wang et al., 2012).

1.2.3.3 Delta Glutamate Receptors

Delta glutamate receptors, GluD1 and GluD2, display sequence homology to ionotropic glutamate receptors, but no ligand-gated ion channel activity has yet been assigned (Lomeli et al., 1993; Schmid and Hollmann, 2008; Yamazaki et al., 1992). The
endogenous ligand for these receptors is unknown, but they do not seem to bind glutamate or other glutamate agonists (Williams et al., 2003). GluD1, while found only in low amounts in the adult brain, is abundant in the developing brain, particularly in the caudate putamen. It is also expressed in the inner ear, and is required for high frequency hearing (Gao et al., 2007; Safieddine and Wenthold, 1997). GluD2 is selectively and predominantly expressed in purkinje cells in the cerebellum (Araki et al., 1993). These proteins with sequence similarity to GluR subunits play a role in synaptogenesis, synaptic plasticity and motor coordination, as well as in apoptosis (Araki et al., 1993; Lomeli et al., 1993; Williams et al., 2003).

1.2.3.4 Intracellular Effects of Glutamate Receptor Activation

Both ionotropic and metabotropic glutamate receptors interact with post-synaptic proteins through their intracellular C-termini. Interactions with several scaffolding proteins anchor glutamate receptors at postsynaptic termini. For example, a scaffold protein of the post-synaptic density (PSD), PSD95, has been implicated in trafficking and stabilization of NMDA, AMPA and kainate receptors (Futai et al., 2007; Irie et al., 1997; Levinson et al., 2005; Nam and Chen, 2005; Schapitz et al., 2010; Suzuki et al., 2012). However, recent studies using hippocampal pyramidal cells have demonstrated that direct interaction with PSD-95 specifically mediates stabilization of AMPA synapses at PSD (Yudowski et al., 2013). PSD95 facilitates the activation of calmodulin/calmodulin dependent kinase II (CaMKII); further phosphorylation of GluRI by CaMKII increases
the conductance of AMPA receptors and contributes to the potentiation of glutamatergic synapses (Dore et al., 2014; Petersen et al., 2003; Steiner et al., 2008).

Several other scaffolding proteins play roles in glutamatergic transmission, including the Glutamate Receptor Activating Protein (GRIP) (Dong et al., 1997; Trotman et al., 2014), synapse-activating protein of 97kDa (SAP-97) (Craven et al., 1999), protein interacting with C kinase-1 ( PICK-1) (Bertaso et al., 2008; Boudin et al., 2000), shank (Naisbitt et al., 1999), and homer (Tu et al., 1998).

1.2.4 Glutamate Clearance and Cycling

An increased level of extracellular glutamate resulting from dysregulated glutamate neurotransmission causes cellular damage (Hardingham et al., 2002; Lau and Tymianski, 2010). It is estimated that extracellular glutamate must be removed from the synaptic cleft within a millisecond time scale to avoid cellular damage (Danbolt, 2001). Unlike the case for the neurotransmitter acetylcholine, there are no extracellular enzymes to degrade glutamate. Excessive glutamate is actively transported to neurons and glia from the extracellular space against a concentration gradient via a family of sodium-dependent excitatory amino acid transporters (EAATs) (Shashidharan et al., 1994). To date, five such high affinity transporters (EAAT1-EAAP5) have been identified (Arriza et al., 1997; Arriza et al., 1994; Danbolt et al., 1990; Fairman et al., 1998; Pines et al., 1992; Shashidharan et al., 1994). EAAT1 is selectively expressed in astrocytes throughout the CNS (Lehre et al., 1995; Takayasu et al., 2009). EAAT2, the chief glutamate transporter, is predominantly expressed in astrocytes (Danbolt et al., 1992;
EAAT3 is a neuronal transporter, expressed in the majority of neurons throughout CNS. EAAT3 is present in neuronal soma and dendrites, devoid from axons (Cheng et al., 2002; Holmseth et al., 2012; Shashidharan et al., 1997). EAAT4 is another neuronal transporter, predominantly found in dendrites of cerebellar purkinje neurons, with weak expression in a subset of forebrain neurons, vestibular hair cells and calyx endings (Dalet et al., 2012; de Vivo et al., 2010; Dehnes et al., 1998; Fairman et al., 1995). Finally, EAAT5 is expressed in the retina, while the levels in the brain are low (Arriza et al., 1997; Dalet et al., 2012; Eliasof et al., 1998). EAAT5 expression is also found in vestibular hair cells and calyx endings (Dalet et al., 2012).

EAATs also conduct chloride, but the chloride conductance is not coupled to the uptake of glutamate. EAAT4 and EAAT5 are efficient chloride channels with the largest chloride conductance, but with low glutamate transport activities, suggesting that some neuronal EAATs may function as inhibitory glutamate receptors, instead of glutamate transporters (Dehnes et al., 1998; Gameiro et al., 2011; Machtens et al., 2015; Mim et al., 2005; Schneider et al., 2014; Veruki et al., 2006; Zhou and Danbolt, 2014).

Regardless, EAATs enhance the signal-to-noise ratio of glutamate signaling, and maintain low levels of glutamate at the synaptic cleft and return glutamate to presynaptic neurons for recycling and release.

### 1.2.5 Glutamate Signalling in Non-neuronal Tissues
There is growing evidence for a function of glutamate signalling in several non-neuronal tissues in the body including pancreas, heart, kidney, skin, bone, lung, liver, stomach and intestine (Danbolt, 2001; Julio-Pieper et al., 2011; Skerry and Genever, 2001). Mechanisms of these signaling events, however, remain to be explored.

1.2.5.1 Pancreas

Several components of glutamate signalling have been found to be present in the pancreatic islets of Langerhans, consisting of insulin secreting β-cells and glucagon secreting α-cells (Hayashi et al., 2003; Inagaki et al., 1995; Weaver et al., 1996). The endocrine islets contain the hormone-loaded secretory granules (SGs) and synaptic-like micro vesicles (SLMV). SLMVs resemble synaptic vesicles (Anhert-Hilger et al., 1996; Thomas-Reetz and De Camilli, 1994); both SGs and SLMVs undergo Ca\(^{2+}\) and SNARE-dependent exocytosis, similar to synaptic vesicle release (MacDonald and Wheeler, 2003; Proks and Lippiat, 2006). Metabolic enzymes for the synthesis of glutamate (GOT, PAG, GDH) and glutamine (glutamine synthetase, GS) are also present in islets (Li et al., 2004). In α-cells, VGLUT1 and VGLUT2 co-package glutamate and glucagon into secretory granules, which co-release glutamate and glucagon (Hayashi et al., 2003). In β-cells, VGLUT3 and EAAT2 are co-expressed in insulin releasing secretory granules. However, β-cells do not release glutamate (Hayashi et al., 2003; Hoy et al., 2002; Maechler and Wollheim, 1999). A recent study suggests that glutamate acts as a signalling molecule to promote insulin maturation and release from secretory granules (Gammelsaeter et al., 2011). The conversion of pro-insulin to mature insulin, and its subsequent release requires the acidification of secretory granules. Intracellular influx
and efflux of glutamate from secretary granules, mediated by VGLUT3 and EAAT2, respectively, facilitates acidification (Gammelsaeter et al., 2011).

Both ionotropic and metabotropic glutamate receptors are also expressed by islet cells, and they stimulate $\text{Ca}^{2+}$ influx that potentiates glucagon and insulin secretion from islet cells (Braun et al., 2004; Brice et al., 2002; Cabrera et al., 2008; Gonoï et al., 1994; Jenstad and Chaudhry, 2013; Morley et al., 2000).

1.2.5.2 Placenta

Glutamate metabolism is important for the development of the human fetus. Glutamate levels, which can be neurotoxic at high levels, must be strictly regulated in the fetal circulation (Noorlander et al., 2004). Exchange of nutrients, gases, metabolic waste and biologically active substances between the maternal and fetal systems is performed by the placenta (Hay, 1995). The placenta supplies the fetus with a number of amino acids including glutamine, but removes glutamate from the fetal circulation (Christensen, 1992; Hoeltzli et al., 1990; Moe and Smith, 1989; Schneider et al., 1979; Vaughn et al., 1995). A number of amino acid transporters have been identified in the placenta (Moe, 1995), including EAAT1, EAAT2, and EAAT3 (Noorlander et al., 2004) that take up glutamate from the fetal circulation. The fetal liver removes glutamine from the circulation and releases glutamate. A recent study demonstrated that fetal blood has higher concentrations of glutamate compared to the maternal blood (Zlotnik et al., 2012); high glutamate levels in the fetal blood might be essential for normal brain development,
as excitatory amino acids are known to be crucial in the development of brain plasticity in animals and humans. Fetal blood also has elevated GOT activity levels relative to maternal blood (Zlotnik et al., 2012); elevated levels of GOT might play a role in maintaining the balance between positive and negative effects of glutamate on the developing brain of the fetus (Khanna et al., 2014).

1.2.5.3 Megakaryocytes

Megakaryocytes are progenitors for platelets, the blood cells necessary for thrombosis, or blood clotting. Thrombosis is a major pathophysiological cause for stroke and acute coronary syndromes. Several studies demonstrated a critical role of glutamate signalling in megakaryocytopoiesis, a process by which bone marrow progenitor cells develop into mature megakaryocytes. This makes it a suitable target for the treatment of thrombosis and other hematological disorders (Berk et al., 2000; Mahaut-Smith, 2012; Skerry and Genever, 2001). In megakaryocytes vesicular glutamate transporters VGLUT1 and VGLUT2 load glutamate into vesicles, and glutamate is released in a SNARE-dependent exocytosis process similar to the CNS (Kasatkina and Borisova, 2013; Thompson et al., 2010).

Glutamate transporters EAAT1 and EAAT2 (Ferrarese et al., 2001; Hoogland et al., 2005; Zoia et al., 2004) and multiple glutamate receptors have also been identified in platelets. NMDA receptor activation inhibits platelet activation and aggregation (Franconi et al., 1998; Franconi et al., 1996; Genever et al., 1999; Hitchcock et al., 2003).
By contrast, activation of AMPA and kainate receptors promotes platelet activation and aggregation (Morrell et al., 2008).

1.2.5.4 Heart

Amino acids, in particular glutamate, aspartate, glutamine and alanine, play important roles in the metabolism of heart myocytes (Dinkelborg et al., 1996). The human heart extracts glutamate from the circulation in larger quantities than any other amino acids, and this uptake is further enhanced during hypoxic or ischemic conditions (Kimose et al., 1996; Mudge et al., 1976). Glutamate signalling plays an important role in heart function, which strictly depends on balanced intracellular Ca^{2+} concentrations (Bozic and Valdivielso, 2014). The glutamate transporters EAAT1 and EAAT3 (Nakayama et al., 1996), as well as ionotrophic and metabotropic glutamate receptors (Gill et al., 2007), have been identified in the heart. Glutamate transporters are localized at the inner membrane of cardiac mitochondria, and function as glutamate carriers for the malate/aspartate shuttle (Ralph et al., 2004). Malate-aspartate shuttle translocates electrons produced during glycolysis across the semipermeable inner membrane of the mitochondria for oxidative phosphorylation and regulates cellular redox and bioenergetics states (Digerness and Reddy, 1976; Nielsen et al., 2011).

Dysregulated activation of heart NMDA receptors, which affects the electrical activity of cardiac myocytes, contributes to cardiac arrhythmogenesis (Bozic and Valdivielso, 2014; D'Amico et al., 1999). Moreover, the glutamate metabolic enzyme
GOT, has emerged as a new therapeutic target for treatment of ischemic stroke injury: a recent study demonstrated that lower blood glutamate levels and higher levels of GOT are associated with a better neurological outcome in patients after ischemic stroke (Khanna et al., 2014).

1.2.5.5 Bone

Bone maintenance is regulated by bone-forming osteoblasts and bone-resorbing osteoclasts. An imbalance between the two cell populations leads to pathogenesis of metabolic bone diseases including osteoporosis. The balance between bone formation and reabsorption is subject to endocrine (estrogen and parathyroid hormone) and para/autocrine (insulin-like growth factor, interleukin and fibroblast growth factor) control (Ducy et al., 2000; Teitelbaum, 2000). Several studies proposed that glutamate functions as an autocrine factor for intercellular communication between bone cells (Bozic and Valdivielso, 2014; Chenu et al., 1998; Mason et al., 1997).

Functional components required for glutamate release, including VGLUT1 and basic machineries required for vesicle targeting and exocytosis, have been identified in osteoblasts (Bhangu et al., 2001)(Bhangu, 2003). Both ionotropic and metabotropic glutamate receptors are expressed in, and regulate the activities of both osteoblasts and osteoclasts (Chenu et al., 1998; Gu and Publicover, 2000; Patton et al., 1998). The glutamate transporter EAAT1 is expressed by osteoblasts, but not in osteoclasts (Huggett et al., 2000). Electrophysiological recordings of NMDA receptors from patch-clamped clonal human osteoblast-like cells exhibited similar although not identical gating
properties to the same receptors in the CNS (Laketic-Ljubojevic et al., 1999). Similar to neurons, bone cells also exhibit an elevation in intracellular Ca\(^{2+}\) following receptor activation (Skerry and Genever, 2001). It has been proposed that the opening of stretch sensitive Ca\(^{2+}\) channels by mechanical load results in spontaneous release of glutamate from osteoclasts, which auto-activates osteoblast glutamate receptors (Mason, 2004). At high concentrations, glutamate inhibits pre-osteoblast differentiation by inhibiting cystine/glutamate antiporter activity and reducing glutathione levels, whereas the activation of specific glutamate receptors potentiates the differentiation and bone forming activity of osteoblasts (Brakspear and Mason, 2012; Takarada-Iemata et al., 2011; Uno et al., 2007). During osteoblast differentiation, glutamate concentrations are tightly regulated by glutamine synthetase (GS), which converts glutamate to glutamine (Olkku and Mahonen, 2008).

### 1.2.5.6 Kidney

Glutamate and aspartate along with other amino acids are filtered by the kidney glomeruli and reabsorbed in the tubuli. Glutamate transporters (EAATs) mediate reabsorption of amino acids (Hediger, 1999; Sacktor et al., 1981; Schneider et al., 1980). The kidney renal tubular cells have high levels of glutamate dehydrogenase (GDH), an enzyme that catalyzes the reversible conversion between glutamate, \(\alpha\)-ketoglutarate and ammonia. In the kidney, glutamate deamination by GDH produces ammonia to control acidosis (Schoolwerth et al., 1983; Spanaki and Plaitakis, 2012). Glutamate transporters EAAT2 and EAAT3 are expressed in the kidney (Kanai and Hediger, 1992; Peghini et
al., 1997; Shayakul et al., 1997), and are proposed to take part in regulating cell volume under osmotic stress (Hediger, 1999). Both ionotropic (Deng et al., 2002) and metabotropic glutamate receptors are also expressed in the kidney (Gill et al., 2000; Julio-Pieper et al., 2011). Basal activation of NMDA receptors is necessary for the maintenance of normal tubular and kidney function, while excess activation has pathophysiological consequences due to disturbances in kidney homeostasis (Bozic and Valdivielso, 2014). Metabotropic receptors were proposed to regulate water and electrolyte transport (Gill et al., 2000; Julio-Pieper et al., 2011).

1.2.5.7 Liver

In the liver, glutamate is the most abundant amino acid and regulates several metabolic pathways including ureagenesis, gluconeogenesis and glutathione synthesis (Bachmann et al., 1982; Jungas et al., 1992; Lu, 1998). As the central organ of ammonia metabolism, enzymes that are involved in glutamate metabolism, such as alanine aminotransferase (ALT), glutamate/oxaloacetate transaminase (GOT), glutamate dehydrogenase (GDH), glutamine synthetase (GS) and glutaminase, are found in abundance in the liver (Brosnan and Brosnan, 2009; Spanaki and Plaitakis, 2012). The glutamate transporter EAAT2 is expressed in the liver and EAAT2-mediated glutamate transport is involved in liver cholestasis (a condition where bile cannot flow from the liver to the duodenum) (Kim et al., 2003; Najimi et al., 2014; Utsunomiya-Tate et al., 1997). Both ionotropic and metabotropic glutamate receptors are expressed in liver with poorly understood functions (Do et al., 2007; Jesse et al., 2009; Lin et al., 1996; Storto et
Glutamate signaling has also been implicated in benign and malignant tumors (Willard and Koochekpour, 2013). Increased expression of mGluR1 was found in human prostate tumors; further, serum glutamate levels correlated with primary prostate cancer aggressiveness (Koochekpour, 2013; Koochekpour et al., 2012). Inhibition of extracellular glutamate release or glutamate receptor activation has been shown to decrease growth, migration and invasion and induce apoptosis in breast cancer, melanoma, glioma and prostate cancer cells (Cavalheiro and Olney, 2001; Kalariti et al., 2005; Prickett and Samuels, 2012; Teh and Chen, 2012; Willard and Koochekpour, 2013).

1.2.6 Regulation of Glutamate Signalling

Glutamate acts as a widespread cytokine and influences cellular activity in a range of tissue types. Glutamate acts on a number of different receptors, and dysregulated glutamate signalling causes glutamate excitotoxicity (Lau and Tymianski, 2010). Aberrant glutamate signalling is implicated in several neurodegenerative and psychiatric disorders such as autism spectrum disorders, schizophrenia, Amyotrophic Lateral Sclerosis (ALS), stroke, traumatic brain injury (TBI), and major depression (Lau and Tymianski, 2010; Soni et al., 2014; Willard and Koochekpour, 2013). Therapeutic intervention for glutamate excitotoxicity is likely to have broad disease implications.
Several therapeutic strategies have attempted to reduce glutamate excitotoxicity. Several glutamate receptor antagonists have been developed to attenuate neuronal damage, however their clinical outcome has been poor and associated with severe side effects (Lau and Tymianski, 2010; Willard and Koochekpour, 2013). In a different approach to decrease glutamate release, drugs that block sodium/calcium channels and inhibit/decrease glutamate release have been developed. Riluzole, a P/Q-type calcium channel blocker (Weller et al., 2008), is approved for ALS treatment (Louvel et al., 1997), but it affects normal brain function and produces severe adverse effects such as decreased lung function and pancreatitis (Soni et al., 2014).

Another strategy was to accelerate glutamate clearance by EAATs. Increasing the expression of several EAATs was found to provide neuronal protection for some neurodegenerative models (Soni et al., 2014). There are, however, caveats associated with EAAT overexpression. EAAT activities have been implicated in glutamate excitotoxicity by the reverse transport of glutamate during ischemic stroke; therefore overexpression of EAATs may worsen brain injury (Khanna et al., 2014; Weller et al., 2008).

An emerging alternative strategy is to provide neuronal protection by increasing blood glutamate scavenging. The glutamate metabolic enzyme, GOT, has been shown to exert protective effects after traumatic brain injury in rats (Ruban et al., 2014; Zlotnik et al., 2008) and after ischemic stroke in humans (Khanna et al., 2014). GOT has emerged as a new therapeutic target to protect against ischemic stroke injury (Khanna et al., 2014).
GOT mediated neuronal protection is achieved by converting glutamate with oxaloacetate into α-ketoglutarate and aspartate. In fact, in human placenta, GOT plays a critical protective role in the fetal brain, by clearing glutamate (Zlotnik et al., 2012). More research needs to be focused on mechanisms regulating glutamate synthesis. As glutamate signalling has important functions in both neuronal and non-neuronal tissues, identifying the tissue-specific factors that regulate glutamate synthesis will be useful to develop successful therapeutics for several systemic diseases.
1.3 The Physiological Role of GABA Signalling

The activity state of the central nervous system (CNS) is established by balanced physiologic inputs from excitatory and inhibitory neurotransmission. Dicarboxylic amino acid glutamate mediates excitatory neurotransmission, whereas mono carboxylic amino acids GABA and glycine mediate inhibitory neurotransmission. Glutamate and GABA are the main excitatory and inhibitory neurotransmitters in the mature nervous system, respectively. Like glutamate, in addition to the CNS, GABA is present in the peripheral nervous system (PNS), and endocrine and non-neuronal tissues, where it may serve diverse physiological roles. A reduced level of GABA, or an imbalanced excitatory/inhibitory input contributes to multiple neurodevelopmental disorders including Parkinson's disease, epilepsy, and mood disorders including major depression and anxiety.

1.3.1 GABA Biosynthesis and Release

GABA is synthesized from glutamate by glutamic acid decarboxylase (GAD) (Miller et al., 1978). GAD undergoes pyridoxal 5’ phosphate dependent inter-conversion between active (holo-GAD) and inactive (apo-GAD) forms; GAD activation is the rate-limiting step in GABA synthesis (Martin, 1993; Martin and Rimvall, 1993). GABA, ATP, and aspartate inhibit GAD activity by promoting the apo-GAD state and stabilizing it. Pyridoxal phosphate, on the other hand, converts apo-GAD to holo-GAD. GAD is also activated by changes in energy state—depolarization, acidosis, increased carbon dioxide, low bicarbonate, low phosphocreatine, increased magnesium, increased ADP, and
decreased ATP (Petroff, 2002).

Mammalian GADs exist in two forms, GAD\textsubscript{65} and GAD\textsubscript{67}. Products of two different genes, GAD\textsubscript{65} and GAD\textsubscript{67} differ not only by molecular weights, but also by catalytic and kinetic properties, subcellular localization, and physiological functions (Bu et al., 1992; Kaufman et al., 1991). GAD\textsubscript{65} is concentrated in nerve terminals (Martin and Rimvall, 1993) and the vesicular GABA at synapses is primarily synthesized by GAD\textsubscript{65} (Soghomonian and Martin, 1998). GAD\textsubscript{67}, by contrast, is distributed evenly throughout the neuronal cytoplasm, including soma, axons, dendrites and synapses (Erlander and Tobin, 1991). GABA generated by GAD67 mainly functions as a trophic factor for synaptogenesis during early development and neuronal protection after injury, as energy source via the GABA shunt, and as a regulator of redox potential under oxidative stress (Lamigeon et al., 2001; Pinal and Tobin, 1998; Waagepetersen et al., 1999). In nerve terminals, GAD\textsubscript{65} associates with the synaptic vesicle (SV) membrane and with mitochondria. Upon sustained neuronal stimulation, the SV membrane-bound GAD\textsubscript{65} increases in level, and forms a complex with the heat shock cognate 70 (HSC70), cysteine string protein (CSP) and the vesicular GABA transporter (VGAT) (Jin et al., 2003). It has been proposed that GABA generated by GAD\textsubscript{65} is taken up preferentially over that generated by GAD\textsubscript{67}, because GAD\textsubscript{65} and VGAT are functionally coupled at the SV membrane (Buddhala et al., 2009).

GABA synthesized at the SV membrane is uploaded into vesicles by the vesicular GABA transporter (VGAT) (Erlander and Tobin, 1991). VGAT recognizes both
GABA and glycine; therefore the expression of GAD determines GABAergic neuronal identity (Kish et al., 1989). VGAT uses a proton gradient generated by the vacuolar ATPase as a secondary energy source to transport GABA against its concentration gradient. In addition to classic GABA release via calcium-dependent exocytosis; GABA was shown to be released from glial cells through reversed operation of GABA transporters or through openings of the bestrophin 1 (Best1) anion channel (Glykys and Mody, 2007). Best1 encodes a functional $\text{Ca}^{2+}$-activated anion channel (CAAC) in non-neuronal tissues and peripheral neurons (Hartzell et al., 2008). Best1 is directly activated by intracellular $\text{Ca}^{2+}$ concentrations and shows unique permeability to large anions such as GABA and glutamate (Lee et al., 2010b; Woo et al., 2012).

### 1.3.2 GABA as an Inhibitory Neurotransmitter

GABA signalling plays key roles in modulating the development, plasticity and function of neuronal networks. GABA released at nerve terminals exerts its effects through two classes of receptors: ionotropic GABA$_A$ and metabotropic GABA$_B$ receptors (Glykys and Mody, 2007). GABA$_A$ are the fast responding GABA receptors and are permeable to chloride and bicarbonate (Schofield et al., 1987). In the mammalian CNS, 20 GABA$_A$ subunits have been identified. Activation of GABA$_A$ receptors by binding of GABA triggers opening of a chloride ion-selective pore, and the increased chloride conductance generates a membrane hyperpolarization and a reduction of action potential firing (Olsen and Sieghart, 2009; Olsen and Tobin, 1990).
GABA_B receptors mediate the slower response of GABA and are members of the G protein coupled receptor family that operate through G_i and G_o proteins (Bowery, 1993; Couve et al., 2000). Two different subunits of GABA_B receptors have been identified; GABA_B1 and GABA_B2, and fully functional GABA_B receptors require co-assembly of both subunits (Bowery and Brown, 1997; Schwarz et al., 2000). GABA_B receptors are localized on both pre- and post-synaptic membranes. At the presynaptic termini, GABA_B receptors inhibit P/Q- and N-type voltage gated Ca^{2+} channels via G_i and G_o proteins to reduce neurotransmitter release (Amico et al., 1995; Chen and van den Pol, 1998; Mintz and Bean, 1993; Poncer et al., 1997). At post-synaptic termini, GABA_B receptors increase K^+ conductance by activating potassium channels GIRK or KIR3, leading to neuronal hyperpolarization, resulting in slow IPSPs (Inhibitory Post Synaptic Potentials) (Ben-Ari et al., 2007; Dutar and Nicoll, 1988; Luscher et al., 1997).

### 1.3.3 GABA as an Excitatory Neurotransmitter

Although GABA is inhibitory in mature neurons and the adult mammalian brain, it can act as an excitatory neurotransmitter for some developing neurons, neuroblasts and neural stem/progenitor cells (NSCs/NPCs) (Pontes et al., 2013). A recent study suggests that GABA may be the principle excitatory neurotransmitter during early development (Ben-Ari et al., 2007).

The physiologic basis for a membrane depolarizing, instead of hyperpolarizing effect of GABA relies on the fact that newborn neurons have a much higher intracellular
concentration of chloride ions. Such a chloride gradient is the result of high expression of the chloride loader (NKCC1) and low expression of the chloride extruder (KCC2) in immature neurons (Blaesse et al., 2009; Dzhala et al., 2005; Rivera et al., 1999; Yamada et al., 2004). In this case, GABA-mediated activation of GABA_A receptors leads to an outward flow of Cl\(^{-}\), which causes membrane depolarization (Cserep et al., 2012; Owens and Kriegstein, 2002). Increased expression of chloride extruder (KCC2) in the mature neurons drains out intracellular chloride, and decreased chloride concentration in mature neurons allows GABA to become an inhibitory neurotransmitter (Achilles et al., 2007; Dzhala et al., 2005; Valeeva et al., 2013).

The depolarizing action of GABA during early neurogenesis is important for activity- and experience-induced regulation of NSC quiescence, NPC proliferation, neuroblast migration and the maturation and functional integration of newborn neurons (Pontes et al., 2013). Inhibitory GABA signaling plays a principle role in reducing neuronal excitability and is proposed to allow sparse and static functional networking essential for learning/memory development and maintenance (Ben-Ari et al., 2007; Pontes et al., 2013). Developmental regulation of transporter expression requires the disrupted-in-schizophrenia 1 (DISC1) and brain-derived neurotrophic factor (BDNF) signaling (Aguado et al., 2003; Rivera et al., 2002). Importantly, dysfunctional DISC1 and BDNF signaling have both been strongly associated with a spectrum of neurological disorders (Boxall et al., 2011; Zuccato and Cattaneo, 2009; Zuccato et al., 2011).
1.3.4 GABA Uptake and Metabolism

The postsynaptic clearance of GABA and the maintenance of extracellular GABA levels is carried out by high affinity GABA transporters in the plasma membrane (Iversen and Kelly, 1975). GABA transporters are expressed in neurons and glia; in neurons, they are present not only at the plasma membrane but also in synaptic vesicles (Kish et al., 1989). Na$^+$ and Cl$^-$ gradients drive GABA uptake (Radian et al., 1986). To date, four mammalian GABA transporters, GAT1, GAT2, GAT3 and BGT, have been identified. These transporters move GABA against its concentration gradient by utilizing the inward sodium gradient. The stoichiometry for transport is two to three sodium, one chloride, and one GABA (Kanner, 1994). Expression of GAT1 and GAT3 is restricted to the brain, whereas BGT1 and GAT2 are found in the brain and also in other tissues. GAT1 is expressed throughout the brain, primarily in presynaptic GABAergic neurons (Conti et al., 1998; Durkin et al., 1995). In contrast to GAT1, GAT3 is selectively expressed in astrocytes (Durkin et al., 1995; Minelli et al., 1996). GAT2 and BGT1 are predominantly expressed in hepatocytes of the liver and kidney. In the adult brain, GAT2 shows strong expression in leptomeninges and ependyma, while BGT1 is expressed in leptomeninges. Hence, GAT2 and BGT1 may play a more important role in GABA signalling in peripheral tissues (Evans et al., 1996; Takanaga et al., 2001). It should be noted that these transporters are not restrictive to GABA: GAT2 and GAT3 also transport β-alanine and taurine, and BGT1 also transports betaine (Liu et al., 1993).

The uptaken GABA can be either repackaged in synaptic vesicles for synaptic transmission or enter the metabolic pathway (Tillakaratne et al., 1995). The first step of
GABA catabolism, or GABA shunt, is catalyzed by the GABA transaminase (GABA-T), which converts GABA to succinic semialdehyde through a stoichiometric conversion between α-KG and glutamate (Gibson et al., 1985; Medina-Kauwe et al., 1994; White and Sato, 1978). Succinic semialdehyde is then oxidized by the NAD$^+$ dependent enzyme succinic semialdehyde dehydrogenase (SSADH) in mitochondria to produce succinic acid (Cash et al., 1979). Both succinic acid and α-KG enter the Krebs cycle (Tillakaratne et al., 1995) (Figure 4).

Figure 4: A Diagram of the GABA Shunt.
GAD, glutamate decarboxylase; GABA-T, 4-aminobutyrate aminotransferase; SSADH, succinic semialdehyde dehydrogenase.
1.3.5 GABA Signaling in Peripheral Tissues

Components of GABA signaling, including GABA metabolic enzymes (GAD, GABA-T, SSADH), transporters, and receptors are expressed in several non-neuronal tissues: liver, kidney, pancreas, testis, ova, oviduct, adrenal, sympathetic ganglia, gastrointestinal tract and circulating erythrocytes (Du et al., 2013; Erdo, 1984; Erdo and Wolff, 1990; Tillakaratne et al., 1995). In pancreas, GABA released within the islets modulates the release of insulin and glucagon, an activity that involves activating both GABA<sub>A</sub> and GABA<sub>B</sub> receptors (Taneera et al., 2012). GABA plays an important role in maintaining homeostasis of spermatogenesis in the testes (Du et al., 2013), stimulation of testosterone production, and the regulation of uterotubal and gut motility (Ritta et al., 1987; Tillakaratne et al., 1995). GABA has also been implicated in the control of hormone release in the adrenal medulla and the gastrointestinal tract (Castro et al., 1989; Harty and Franklin, 1986; Kataoka et al., 1984). In several human cancer cells and tissues, increased GABA<sub>B</sub> receptor expression has been detected and in the rat models, GABA<sub>B</sub> receptor agonist baclofen inhibited the tumor growth (Jiang et al., 2012; Neman et al., 2014). The exact mechanism by which GABA regulates these various processes is not known. As GABA metabolites succinic acid and α-KG support oxidative metabolism through the TCA cycle, it is speculated that GABA may serve as an alternative energy source in peripheral tissues (Neman et al., 2014; Tillakaratne et al., 1995).
1.4 Co-transmission/Co-release of Multiple Neurotransmitters at a Synapse

The information that neurons convey is defined by neurotransmitter identity, and neurons are often classified by the neurotransmitter they contain. For many years, it has been assumed that neurons release a single neurotransmitter, known as Dale’s principle (Dale, 1935). However, several lines of emerging evidence to suggest that many, if not all neurons are capable of releasing two or more neurotransmitters. The co-release of ATP and acetylcholine (ACh) in the electric organ of *Torpedo californica* was the first example noted for dual-transmitter neurons (Silinsky, 1975; Whittaker et al., 1972), and later studies showed that neurons can co-transmit multiple primary neurotransmitters (Hnasko and Edwards, 2012; Vaaga et al., 2014).

Neurons can either co-transmit or co-release neurotransmitters: co-transmission refers to the release of multiple neurotransmitters from non-overlapping pools of synaptic vesicles; co-release, on the other hand, refers to two or more neurotransmitters packaged into a single set of synaptic vesicles to be co-released (Vaaga et al., 2014).

1.4.1 Functional Implications of Dual Neurotransmission

The vesicular uptake of one neurotransmitter can regulate the loading of the other by altering the $\mathrm{H}^+$ electrochemical driving force (Gras et al., 2008). The $\mathrm{H}^+$ electrochemical gradient ($\Delta \mu_{\mathrm{H}^+}$) produced by the vacuolar-type H-ATPase comprises a
chemical gradient ($\Delta p\text{H}$) and a membrane potential ($\Delta \Psi$) (Hnasko and Edwards, 2012). Different neurotransmitters exhibit differential reliance on components of $\Delta \mu_{\text{H}^+}$ for vesicle loading. For example, the vesicular loading of monoamines and acetylcholine (ACh) depend more on $\Delta p\text{H}$ than on $\Delta \Psi$, loading of GABA and glycine depends equally on $\Delta p\text{H}$ and $\Delta \Psi$ (Hell et al., 1990; Kish et al., 1989), whereas glutamate loading depends primarily on $\Delta \Psi$ (Tabb et al., 1992). The entry of anions - glutamate, ATP, and $\text{Cl}^-$ - promotes $\Delta p\text{H}$ by consuming $\Delta \Psi$ (Maycox et al., 1988). Consistent with this mechanism, co-release of glutamate can increase packaging of monoamines (Hnasko et al., 2010) and Ach into synaptic vesicles by consuming $\Delta \Psi$, which results in increased $\Delta p\text{H}$ (Gras et al., 2008).

Neurons that release more than one neurotransmitter can be divided into three classes: neurons that release two fast acting neurotransmitters; neurons that release a fast acting neurotransmitter and a slow acting monoamine; and neurons that release a fast acting neurotransmitter and a neuromodulator such as neuropeptides, neurotrophins, ATP and $\text{Zn}^{2+}$ (Vaaga et al., 2014). For neurons that co-release multiple fast acting neurotransmitters (such as glutamate/GABA and GABA/glycine), net synaptic transmission is determined by the combinatorial receptors present at pre- and post-neuronal pairs and their synaptic versus extra synaptic distribution (Apostolides and Trussell, 2013; Dugue et al., 2005). When a neuron co-releases a fast acting neurotransmitter and a slow acting monoamine, additional functional outputs include intrinsically slower currents, the effect of the second messenger cascade and modulation of presynaptic receptors (Vaaga et al., 2014). In the case of dual transmission of
neuromodulators with fast acting neurotransmitters, neuromodulators may alter presynaptic activity and gene expression, and provide trophic support to post-synaptic membrane. For example, in the hippocampus, insulin release modulates activity-dependent synaptic plasticity that involves activation of NMDA receptors and the downstream PI3K pathway (van der Heide et al., 2005). When Zn$^{2+}$ is co-released with glutamate, it can bind NMDA receptors and prolong NMDA receptor-mediated EPSC (Tovar and Westbrook, 2012).

It should be noted that despite the potential widespread occurrence of co-release of multiple neurotransmitters and neuromodulators, their physiological significance remains largely unexplored.

1.4.2 Co-release of GABA and Glycine

Co-release of GABA and glycine was detected in the spinal cord, in the auditory brain stem and in the neonatal respiratory network, where miniature IPSCs contained both GABAergic and glycnergic components (Apostolides and Trussell, 2013; Ishibashi et al., 2013; Jonas et al., 1998; Rahman et al., 2013). Vesicular GABA transporter (VGAT) recognizes GABA as well as glycine (Chaudhry et al., 1998; McIntire et al., 1997), providing a potential explanation for the co-release of GABA and glycine from the same set of vesicles. However, because of the low affinity of VGAT for its substrates (Wojcik et al., 2006), it remains unclear what factors control differential loading and the subsequent release of GABA and glycine. A recent study suggests that cytoplasmic concentration of GABA and glycine may regulate their ratio in synaptic
vesicles, because acute changes in the ratio between cytosolic GABA and glycine modulate the IPSC amplitude independent of vesicle turnover (Apostolides and Trussell, 2013).

1.4.3 Co-transmission of GABA and Acetylcholine

In the vertebrate retina, starburst amacrine cells (SACs) process complex visual signals by direction selective motion sensing. Radially symmetric dendrites of SACs overlap with dendrites from neighboring SACs as well as direction-selection ganglion cells (DSGCs) in the inner plexiform layer. In response to light SACs release two classic fast neurotransmitters of opposite excitability, GABA and Ach, onto the DSGCs (Duarte et al., 1999).

The DS ganglion cells respond to their preferred direction (direction in which a set of neurons respond most strongly) with a large excitatory postsynaptic potential as a result of Ach release from SACs (Lee et al., 2010a). GABA release by SACs is selective for movement in the opposite or null direction (Lee et al., 2010a) (Fried et al., 2002). In SACs, both ACh and GABA release depends on external Ca\(^{2+}\), but with a different sensitivity to membrane depolarization, suggesting that different vesicle populations may mediate the release of the two transmitters (Duarte et al., 1999). The proposed model is that SACs encode the direction selectivity through GABA release and motion sensitivity through the release of Ach, thereby reducing the number of retinal circuits and circuit components required for integration of these two visual cues (Lee et al., 2010a).
1.4.4 Dual Transmission of GABA and Dopamine

In the olfactory bulb, a subset of periglomerular cells express both tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine (DA) synthesis, and GAD, the rate-limiting enzyme for GABA synthesis (Gall et al., 1987; Maher and Westbrook, 2008). These two neurotransmitters are loaded into different vesicle pools and are differentially released: a single action potential results in a fast GABAergic IPSC, followed by slow dopamine release over a period of seconds (Borisovska et al., 2013; Liu et al., 2013).

1.4.5 Co-release of Glutamate and Ach

Co-expression of the vesicular glutamate transporter (VGLUT) in a striatal cholinergic neuron population improves vesicular Ach uptake and storage. Co-released glutamate further activates ionotropic glutamate receptors on medium spiny neurons (Gras et al., 2008; Higley et al., 2011). Co-release of glutamate and ACh in mammalian spinal cord motor neurons excites Renshaw cells and other motor neurons (Nishimaru et al., 2005). Some cholinergic neurons in the medial habenula release glutamate and Ach from different vesicle populations, with differential release properties (Ren et al., 2011).

1.4.6 Dual Neurotransmission of Glutamate and GABA

Perhaps the most striking and unexpected case is the dual transmission of glutamate and GABA. Tightly regulated vesicular transport and release of glutamate and
GABA allows precise segregation of excitatory and the inhibitory neurotransmission in a neural network. However, multiple studies have shown co-release of glutamate and GABA as neurotransmitters in the same neuronal pool, although the physiological function of co-release is not well understood.

The co-presence of glutamate transporter VGLUT and GABA transporter VGAT has been reported at nerve terminals of hippocampal CA3 mossy fibers (glutamatergic), cerebellum mossy fibers (glutamatergic), cerebellum GABAergic basket cells, and hypothalamic proopiomelanocortin (POMC) neurons (peptidergic) (Beltran and Gutierrez, 2012; Jarvie and Hentges, 2012; Safiulina et al., 2006; Somogyi et al., 2004; Walker et al., 2001; Zander et al., 2010). In hippocampal mossy fibers that are glutamatergic neurons, both VGAT and GAD are transiently expressed in the first three weeks after birth, implying a period of transient GABA release (Safiulina et al., 2006). Interestingly, in adult glutamatergic granule cells, epileptic activity can revive GABAergic phenotypes, suggesting that even adult neuronal identity could be altered (Gutierrez, 2000; Nadler, 2003). The potential developmental transition from GABA/glutamate co-release to a dominant neurotransmitter type has led to the speculation that neurotransmitter co-release may play a role in synapse stabilization and circuit formation (Beltran and Gutierrez, 2012; Walker et al., 2001).

In the lateral habenula, a brain region that lacks GABAergic interneurons, glutamate and GABA are co-released by glutamatergic synapses. GABA and glutamate co-exist in the same vesicle pool. Consistent with the co-release of excitatory and
inhibitory neurotransmitters, activation of habenular neurons leads to inhibition followed by excitation or excitation followed by inhibition, and GABA exerts a greater inhibitory effect on habenular neuron firing under high frequency stimulation (Root et al., 2014; Shabel et al., 2014). Interestingly, a rat depression model exhibits a reduced GABA- to glutamate-mediated synaptic response ratio, and an increased GABA signalling ratio after treatment using a serotonin-based antidepressant in the lateral habenula. These studies suggest that depression associates with imbalanced GABA/glutamate signalling, and manipulation of GABA/glutamate co-release may provide a potential approach for treatment of mood disorders (Shabel et al., 2014).

Injury or disease affects the co-release of different neurotransmitters. In patients with Parkinson’s disease, olfactory bulb interneurons, which co-transmit GABA and dopamine, exhibit increased expression of dopamine-synthesizing enzyme HT (Huisman et al., 2004; Mundinano et al., 2011; Ross et al., 2008). The portion of VTA neurons that co-transmit glutamate and dopamine increases rapidly after lesion induced by a neurotoxin 6-OHDA (Berube-Carriere et al., 2009; Dal Bo et al., 2008). Given the dynamic modulation of neurotransmitter phenotype, it is critical to understand the molecular mechanisms that regulate the balance between different neurotransmitters at synapses or neurons. These provide the key to uncovering new therapeutic strategies for several neurodevelopmental disorders.
1.5 Objectives

My Ph. D study started with identifying genes that regulate the motor output of *C. elegans*. I identified loss-of-function mutants for *ubr-1*, the sole *C. elegans* homologue of the UBR1 family E3 ligase, to regulate two different motor phenotypes. Through genetic, metabolic and functional imaging analyses of the *ubr-1* mutants, I address the cellular function and molecular mechanisms through which the UBR-1 E3 ligase regulates the *C. elegans* motor output. In the first data chapter, I present studies that reveal a previously unknown role of UBR-1 in negative regulation of glutamate signaling, which functions through a small network of the *C. elegans* premotor interneurons to establish the phase lag of motor neurons to enable body bending. In the second data chapter, I present studies that reveal a role of UBR-1 in regulating the glutamate/GABA homeostasis in a GABAergic motor circuit that regulates the defecation motor program.

These studies establish the *C. elegans* motor circuit, which utilizes glutamatergic and GABAergic signaling to control two distinct motor outputs, as new genetic models to address the cellular functions and mechanisms of UBR-1. These studies reveal an intriguing hypothesis that dysfunctional glutamate metabolism and/or signaling may be the common cause that underlies the Johanson Blizzard Syndrome (JBS).
The Johanson-Blizzard Syndrome Ubiquitin Ligase

UBR-1 Regulates Glutamate Metabolism and Signaling

2.1 Abstract

The Johanson-Blizzard Syndrome (JBS) is a congenital disorder characterized by pancreatic insufficiency, facial dysmorphism, and cognitive impairments. JBS results from the functional loss of an E3 ubiquitin-ligase UBR1, but mechanisms that underlie pathogenesis remain elusive. We report here that the functional loss of its sole Caenorhabditis elegans ortholog UBR-1 reduces the animal’s ability to bend during backward locomotion, and the reduced bending results from dysregulated glutamate signaling in a small motor circuit. Specifically, we show that 1) UBR-1 functions through premotor interneurons, AVE, RIM and AVA, to regulate the phase-lag between the A-type motor neurons that promotes bending; 2) the bending defect of ubr-1 mutants is rescued by removing either a glutamate synthesizing transaminase (GOT) or a vesicular glutamate transporter (VGLUT3) from AVE and RIM, or, by removing a glutamate receptor (GluCl) from their postsynaptic partner AVA; 3) ubr-1 mutants exhibit increased glutamate. Hence UBR-1 regulates glutamate signaling between premotor interneurons to affect the motor output pattern. These results raise an intriguing possibility that dysregulated glutamate metabolism and signaling may underlie systemic or neurodevelopmental defects of JBS.
2.2 Introduction

Targeted proteolysis eliminates misfolded proteins, generates antigens, and controls cell signaling by restricting the concentration, localization, or subunit composition of its regulators. In eukaryotes, the ubiquitin-proteasome system mediates selective degradation of intracellular proteins (Pickart and Eddins, 2004; Schwarz and Patrick, 2012). In this system, a cascade of enzymatic reactions by the ubiquitin-activating enzyme, conjugating enzyme, and ligase (E3) leads to the covalent attachment of a single, or a chain of ubiquitin(s) to a lysine residue of a substrate, often targeting the substrate for proteasomal degradation. E3s confer substrate specificity via direct interaction with degradation signals in substrates (Ciechanover and Iwai, 2004; Ciechanover et al., 2000; Hershko and Ciechanover, 1998; Varshavsky, 1996).

UBR1 is an E3 ligase of the N-end rule, through which a protein’s metabolic stability is regulated by the identity and post-translational status of its N-terminal amino acid (Sriram et al., 2011; Varshavsky, 1996). In yeast and mice, the UBR family proteins have been implicated in a variety cellular functions, such as chromosomal stability (Rao et al., 2001), apoptosis (Piatkov et al., 2012), neurogenesis and cardiovascular development (An et al., 2006). Loss-of-function mutations in UBR1 cause JBS, a multisystemic, neurodevelopmental disorder (Zenker et al., 2005). It remains unclear whether UBR function is conserved between yeast, mice and humans, and how mutation of UBR1 contributes to JBS pathophysiology.
Glutamate is the primary excitatory neurotransmitter in the vertebrate central nervous system. Glutamate signaling plays a crucial role in animal development, motor functions, and learning and memory (Brockie and Maricq, 2003; DiAntonio, 2006; Mattson, 2008; Ottersen and Storm-Mathisen, 1984). Glutamate acts on many receptors, including the excitatory ionotropic NMDA, AMPA and kainate receptors, and the metabotropic mGluRs. Invertebrates also have glutamate-gated inhibitory chloride channels (GluCls), closely related to mammalian glycine receptors (Wolstenholme, 2012; Wolstenholme and Rogers, 2005). Aberrant glutamate signaling may lead to excitotoxicity (Dong et al., 2009) and has been implicated in neurological disorders (Siegel and Sanacora, 2012). In addition to being the main excitatory neurotransmitter (Ottersen and Storm-Mathisen, 1984), glutamate also functions as a key metabolite (Kelly and Stanley, 2001) and the precursor of the inhibitory neurotransmitter GABA (Costa et al., 1979).

*C. elegans* has a single UBR family protein, UBR-1. Here we demonstrate that UBR-1 regulates glutamate metabolism and signaling, which, through a small motor circuit, modulates body bending during backward locomotion. We present genetic, neurophysiological and metabolic evidence that UBR-1 affects the motor output through glutamate signaling between the AVE, RIM, and AVA premotor interneurons. This involves a molecular pathway that consists of a glutamate synthesizing transaminase GOT-1 (GOT), a vesicular glutamate transporter EAT-4 (VGLUT3), and a glutamate-gated chloride channel AVR-15 (GluCl). These findings reveal a novel role for UBR-1, as well as a previously unknown circuit mechanism for locomotion. It raises the
possibility that dysregulated glutamate metabolism and signaling may be relevant to the pathophysiology of JBS.

2.3 Materials and Methods

2.3.1 EMS Mutagenesis and Forward Genetic Screen

L4 stage C. elegans were harvested from 5-6 plates with M9 buffer, washed three times, and placed into 47mM ethane methyl sulfonate (EMS) in M9 for 4 hours at room temperatures (20-25C). These animals were washed thoroughly with M9 buffer after mutagenesis, and allowed to recover for 2 hours on the OP50-seeded NGM plates. Young adult (P0) animals were picked onto fresh plates (3-4 P0s per plate) and allowed to lay eggs for 3 days. Progenies (F1s) were transferred to fresh plates (1 per plate) and allowed to propagate. Their progenies (F2s) were screened for body curvature defects. Animals with body curvature defects were isolated and propagated for one more generation (F3s) to verify the penetrance of phenotypes.

2.3.2 UBR-1 and GOT-1 Cloning

Single Nucleotide Polymorphism Mapping

All isolated mutants were backcrossed against N2 background 3-4 times before mapping. Isolated mutations were first rough-mapped to a region on a specific chromosome using single nucleotide polymorphism (SNP) mapping (Davis and Hammarlund, 2006; Doitsidou et al., 2010). A specific mutant in the N2 background was
crossed into the Hawaiian mapping strain CB4856, which differs from N2 by a number of SNPs distributed over all six chromosomes. These SNPs introduce sites for various restriction enzymes such that when short regions around these SNPs were amplified by PCR and digested with an appropriate enzyme, the N2 and Hawaiian digestion pattern looked different. 50 mutant animals from the F2 generation of these crosses were isolated and propagated as separate lines that have the N2/CB recombination events at different positions on the chromosomes. The closer the mutation was to a particular SNP, the less recombination with the Hawaiian strain occurred, and the more it was enriched for the N2 digest pattern.

**Whole Genome Sequencing**

In parallel with standard SNP mapping, WGS of two isolated mutants (*hp684* and *hp731*) was performed. WGS was performed using genomic DNAs prepared from the same 50 N2/CB hybrid mutant lines that were used for SNP mapping. The F3 and F4 populations for all 50 lines were pooled and the genomic DNA was isolated. Sequencing was done on the HiSeq 2000 platform, at the Genome Sciences Centre; BC Cancer Agency, Vancouver, BC and data analysis was done using the MAQGene software by Stephane Flibotte and Donald Moerman at University of British Columbia. N2 differs from CB4856 by roughly 100,000 SNPS. Based on the ratio of N2 to CB SNP variants, a region enriched for the N2 pattern was identified and mutations that cause amino acid and splicing changes in this region were called for further validation.
After the re-sequencing of the original hp684 and hp731 mutant strains to verify the called mutations, overlapping fosmids that cover the identified genomic region were injected and tested for rescue ability of behavioral phenotypes to select for causative mutations. An A-to-T mutation in Exon 15 of C32E8.11 (UBR-1) was identified by WGS and re-verified with sequencing. A fosmid WRM0617cA07 alone rescued the behavioral phenotypes exhibited by hp684. From WRM0617cA07, a 15kb fragment containing a containing a single open reading frame C32E8.11 (pJH2913) fully rescued the behavioral phenotypes of hp684. Deletion alleles for C32E8.11 were subsequently generated by CRISPR/Cas9 genomic editing; they showed identical phenotypes as hp684 that were fully rescued by the same 15kb fragment. We conclude that hp684 represents a loss of function allele of UBR-1.

A G-to-A mutation in the 2nd Exon of T01C8.5 (GOT-1.2) was identified by WGS and re-verified with sequencing. A fosmid WRM067cF08 rescued the behavioral rescue exhibited by hp731. From WRM067cF08, a 8.4kb fragment containing a single open reading frame T01C8.5 (pJH2781) fully rescued the behavioral phenotypes of hp731. We conclude that hp731 represents a loss of function allele for GOT-1.2.

2.3.3 C. elegans Strains and Transgenic Lines

All strains were cultured using standard conditions on Nematode Growth Medium (NGM) plates seeded with OP50 E. coli bacteria and maintained at 22°C (Brenner, 1974). ubr-1(hp684), and got-1.2(hp731) mutant strains were isolated from Ethyl Methane Sulfate (EMS) mutagenesis screens. Mutations were mapped using a combination of SNP
mapping and whole genome sequencing (Davis and Hammarlund, 2006; Doitsidou et al., 2010) and rescued using fosmid reporters and genetic deficiency crosses. \textit{ubr-1}(hp820, hp821, and \textit{hp}821 \textit{hp}833) mutant strains were created using genetic editing approaches (Friedland, 2013; Tzur et al., 2013) as described in the results. Several strains were acquired from the National Bioresource Project (Japan) or the \textit{Caenorhabditis} Genetics Center (CGC) (USA). All mutant strains were outcrossed several times against the N2 Bristol strain prior to study and confirmed by PCR genotyping. Transgenic animals carrying extra-chromosomal arrays (\textit{hpEx}) were created by co-injecting the DNA plasmid of interest and a co-injection marker at 5-30 ng/μL. When appropriate, extra-chromosomal arrays were integrated into the genome using UV irradiation to create stable, transgenic lines (\textit{hpIs}) (Mello et al., 1991). Since GOT-1 overexpression for tissue-specific rescue experiments rendered very sick and at times, non-viable, animals, \textit{got-1} tissue-specific rescue lines were generated using Mos1 single copy insertion, as previously described (Frokjaer-Jensen et al., 2008). Integrated strains were outcrossed several times prior to study. For a complete list of constructs as well as strains used, including strains used for genetic interactions, imaging, microscopy and transgenic lines, refer to Table S1-4.

\textbf{Strains for calcium imaging and optogenetic ablation: ZM8426 \textit{hpIs460}, ZM8749 \textit{ubr-1} (hp684); \textit{hpIs460}, ZM8750 \textit{ubr-1} (hp684); \textit{got-1.2} (hp731); \textit{hpIs460}, ZM8839 \textit{ubr-1} (hp684); \textit{eat-4} (ky5); \textit{hpIs460}, ZM8913 \textit{ubr-1} (hp684); \textit{avr-15} (ad1051) \textit{hpIs460}, ZM7108 \textit{hpIs327}, ZM8737 \textit{ubr-1} (hp684); \textit{hpIs327}, ZM8844 \textit{ubr-1} (hp684); \textit{got-1.2} (hp731); \textit{hpIs327}, ZM8892 \textit{ubr-1} (hp684); \textit{eat-4} (ky5); \textit{hpIs327}, ZM9043 \textit{hpIs579},
ZM9044 ubr-1 (hp684); hplS579, ZM9045 avr-15 (ad1051); hplS579, ZM9046 ubr-1 (hp684); avr-15 (ad1051); hplS579.

Strains for VGlut3/EAT-4 and glutamate receptor expression: ZM6902 ubr-1 (hp684) nulS25, ZM7884 ubr-1 (hp684) nulS25; got-1.2 (hp731), ZM9015 ubr-1 (hp684) nulS25; eat-4 (ky5), ZM9016 ubr-1 (hp684) nulS25; avr-15 (ad1051), ZM8863 ubr-1 (hp684); hplS516, ZM8864 ubr-1 (hp684); got-1.2 (hp731); hplS516, ZM8866 ubr-1 (hp684); eat-4 (ky5); hplS516, ZM9017 ubr-1 (hp684); avr-15 (ad1051); hplS516, ZM8490 unc-119(ed3) III; ddIs263 (EAT-4::GFP), ZM8492 unc-104; ddIs263, ZM8892 unc-104; ddIs263; hplS321.

Table 1: Strains for genetic interactions

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<td>ubr-1; asns-2 (ok3108)</td>
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<td>ubr-1; eat-4 (ky5)</td>
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Table 2: Primers for Genotyping *C. elegans* Strains Used in Chapter 2

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<th>Wild type</th>
<th>Mutant</th>
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OZM3451  agctgcggccgcatgattgtcgatttaat  A forward primer to amplify the N-terminal of UBR-1 minigene (ATG)
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OZM3582  cccatttaaagagagaatgcgatg  To amplify a fragment of UBR-1 cDNA
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OZM3584  ggatg/gac/tac/aaa/gac/cat/gac/ggt/ gat/tat/aaa/gat/cat/gat/atgc  A 5' primer to amplify the ubr-1 cDNA at ATG
OZM3585  ggcgcggatcactgcatcattttataatcaccgtcatgctgtttgtga  To construct the FLAG-tagged UBR-1 construct
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OZM3681  ttagtgcacccatattaaagacgcagatgcatg  A 3' primer for ubr-1 cDNA
OZM3682  gggatccgggagaagctctgtcacaatgaaa  A primer to amplify the ubr-1 cDNA (near bp2412)
OZM3683  tttcgcagacgtgtatatgtgccaccatg  A 3' primer for ubr-1 cDNA. (used with OZM3682)
OZM4002  ttgg/gga/tcc/att/gtc/gat/tta/ata/caa/tca/gct  A 5' primer for ubr-1 cDNA with an
added BamHI site at ATG, GGA reading frame.

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Table 4: A list of constructs and transgenes generated for Chapter 2
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| PJH3390 | Pglr-1::UBR-1 minigene | hp684 | hpEx3541 | ZM8404 |
| PJH3436 | Pnmr-1::UBR-1 minigene | hp684 | hpEx3564 | ZM8457 |
| PJH3469 | Prig-3::UBR-1 minigene | hp684 | hpEx3595 | ZM8551 |
| PJH3476 | Popt-3::UBR-1 minigene | hp684 | hpEx3596 | ZM8550 |
| PJH3485 | Plgc-55::UBR-1 minigene | hp684 | hpEx3597 | ZM8582 |
| PJH3502 | Pgcy-13::UBR-1 minigene | hp684 | hpEx3622 | ZM8627 |
| PJH3487 | Pttx-3::UBR-1 minigene | hp684 | hpEx3598 | ZM8581 |
| PJH3476 | Popt-3::UBR-1 minigene | hp684 nls25 | hpEx3731 | ZM9014 |

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| PJH2998 | Pgot-1.2::GOT-1.2-GFP MosCl | unc-104; TT51605 | hpSi10 | ZM7289 |
| PJH3396 | Ppan::GOT-1 cDNA MosCl | unc-104; TT51605 | hpSi16 | ZM8458 |
| PJH3397 | Pglr-1::GOT-1 cDNA MosCl | unc-104; TT51605 | hpSi18 | ZM8460 |
| PJH3399 | Punc-47::GOT-1 cDNA MosCl | unc-104; TT51605 | hpSi20 | ZM8462 |
| PJH3471 | Prig-3::GOT-1 cDNA MosCl | unc-104; TT51605 | hpSi22 | ZM8497 |
| PJH3531 | Popt-3::GOT-1 cDNA | hp684; hp731 | hpEx3661 | ZM8760 |
| PJH3501 | Pgcy-13::GOT-1 cDNA | hp684; hp731 | hpEx3621 | ZM8759 |

**EAT-4**

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| PJH3314 | Pglr-1::EAT-4 minigene::SL2::wCherry | hp684; ky-5 | hpEx3502 | ZM8311 |
| PJH3336 | Pacr-2::EAT-4 minigene::SL2::wCherry | hp684; ky-5 | hpEx3552 | ZM8443 |
| PJH3344 | Punc-47::EAT-4 minigene::SL2::wCherry | hp684; ky-5 | hpEx3501 | ZM8314 |
| PJH3454 | Pnmr-1::EAT-4 minigene::SL2::wCherry | hp684; ky-5 | hpEx3574 | ZM8494 |
| PJH3455 | Prig-3::EAT-4 minigene::SL2::wCherry | hp684; ky-5 | hpEx3575 | ZM8495 |
| PJH3482 | Popt-3::EAT-4 minigene::SL2::wCherry | hp684; ky-5 | hpEx3612 | ZM8598 |
| PJH3509 | Pgcy-13-EAT-4 minigene::SL2::wCherry | hp684; ky-5 | hpEx3636 | ZM8652 |

**AVR-15**

| PJH3615 | Pdpy-30::AVR-15 minigene | hp684; avr-15 | hpEx3698 | ZM8938 |
| PJH3612 | Ppan::AVR-15 minigene | hp684; avr-15 | hpEx3697 | ZM8939 |
| PJH3617 | Prig-3::AVR-15 minigene | hp684; avr-15 | hpEx3699 | ZM8937 |
| PJH3618 | Punc-4::AVR-15 minigene | hp684; avr-15 | hpEx3700 | ZM8940 |

**Calcium Imaging**

<p>| PJH3137 | Punc-4-GcamP6::w-cherry | lin-15 | hpIs460 | ZM8426 |
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**Generation of GOT-1::GFP using CRISPR-mediated Homologous Recombination**

GOT-1::GFP animals (*hpSi26*) were generated as previously described (Tzur et al., 2013) with some modifications. The CRISPR template for GOT-1::GFP replacement (pJH3629) was generated by including 1 Kb of the sequence upstream to the *got-1* coding sequence (promoter region), the entire *got-1* coding sequence, and 1.5 kb sequence downstream of *got-1* (UTR region). GFP was inserted in-frame with the last amino acid of GOT-1. LoxP-Prps-27-Neo resistant gene-loxP cassette was insert between stop codon and 3’ UTR. sgRNA targeting site in *got-1* was mutated. The resulting replacement vector was co-injected into wild-type N2 animals with RFP marker, Peft-3-driven Cas9 expressing vector and P U6-driven sgRNA expressing vector as well Phsp16.1-driven peel-1. After injection, animals were placed on NGM plates and allowed to lay eggs overnight at 25°C before applying G418 to select between animals containing the replacement and extra-chromosomal arrays. G418-resistant progeny were allowed to grow for an extra seven days and was subjected to heat shock at 34°C for 2 hours to activate expression of PEEL-1. Animals with extra-chromosomal arrays died after heat shock treatment. Animals that survived and did not possess any RFP markers were candidates for replacement. Candidates were genotyped by PCR and GOT-1::GFP
expression was tested. The resulting strain with GOT-1::GFP replacement (hpSi26) was crossed into \textit{ubr-1 (hp684)} mutant.

2.3.4 Locomotion Analysis

**Behavior Acquisition for \textit{ubr-1, got-1, and \textit{avr-15} Mutants}**

\textit{C. elegans} typically spend 90\% of the time moving forward, with brief interruptions of backward movement. We quantified body curvature, initiation frequency, and duration on one-day old young adult worms using two different experimental conditions to increase the frequency of backing. In a similar manner, as previously described (Gao et al., 2015), 35 mm NGM plates with limited food (lightly seeded OP50 bacteria) were used for all behavior experiments.

For all experiments that did not involve the \textit{eat-4 (ky5)} mutation, we used the chemical copper chloride (CuCl\textsubscript{2}), an aversive stimulus for \textit{C. elegans} (Hilliard et al., 2002). Immediately before transferring worms onto NGM plates, a small amount (~200 uL) of 100 mM CuCl\textsubscript{2} was placed on the surface of the NGM plate, leaving a small circle (~10 mm in diameter) in the middle for the animal to roam. The CuCl\textsubscript{2} “ring” was allowed to dry for few minutes before transferring the worms onto the plate. For each genotype, an animal was placed in the center of the circle and allowed to habituate for one minute prior to recording. Behavioral recordings lasted for three minutes.

**Behavior Acquisition for \textit{eat-4} Mutants**

When exposed to 100 mM CuCl\textsubscript{2} or other aversive chemicals tested, \textit{eat-4(ky5)}
mutants exhibit reduced behavioral responses, consistent with previous reports that *eat-4* mutants possess various sensory defects (Clark et al., 2007; Lee et al., 1999; Rankin and Wicks, 2000). Therefore, we utilized another assay to induce and quantify backward locomotion in animals containing the *eat-4* mutation. Similar to wild-type animals, *eat-4* mutants will initiate backward locomotion in response to mechanical stimulation to the head region (i.e., head tap). To simulate mechanical stimulation to the head region, we utilized a 0.001 inch-thick “spacer” made of polycarbonate (Catalog # 9513K12 from McMaster-Carr, kind gift from Dr. Aravithan Samuel, Harvard University) to serve as a “barrier.” When in contact with this “spacer,” both wild-type and *eat-4* animals respond by moving backwards. For all *eat-4* locomotion analysis, “spacers” were placed on the surface of the lightly seeded NGM plate, leaving a small (approximately 9 x 9 mm) square in the middle for the animal to roam. Animals were placed in the center of the square and allowed one minute to habituate before recordings. Behavior recordings lasted for three minutes.

**Behavior Tracking and Data Analysis**

Behavior was recorded using a Zeiss Axioskop 2 Plus equipped with an ASI MS-40000 motorized stage and a CCD camera (Hamamatsu Orca-R2). Tracking acquisition and analysis were performed using Micromanager and Image J software plug-ins developed in-house (courtesy of Dr. Taizo Kawano). Image sequences were sampled at 100-msec exposure (10 frames per second). For the post-imaging analysis, an Image J plugin was used to skeletonize the worm and extract its centerline. The centerline was divided into 29 equal segments, and the angle between each segment and its tangent line...
was calculated to quantify the curvature of the animal. These segments were binned into four groups in most cases and into six groups in the case of the eat-4 mutants to capture the best curvature trends across the whole worm. To calculate directionality of movement, the midline point for each worm was determined based on its position in the field-of-view and the stage coordinates. The directionality of movement (forward vs. backward) was determined by comparing the changes in the anterior-posterior axis of the “head” and “tail” points manually defined at the first two frames and verified throughout the recording. Image sequences wherein animals touched the edge of the recording field and crossed over on themselves were not processed.

Quantification and statistical analyses of the output data were carried out using an R program-based code developed in-house (courtesy of Dr. Michelle Po). From a group of recordings (N≥10 for each genotype per experiment), we quantified the following parameters, among other behavioral parameters analyzed by our in-house program: 1) Body curvature, the average curvature of each animal at each segment; 2) Initiation (defined as the frequency of directional change for each animal; 3) Duration (defined as the time spent moving in the same direction for >3 frames or 300msec, calculated for each bout of forward or backward initiation). Body curvature, initiations, and durations were calculated for forward and backward locomotion separately.

2.3.5 Optogenetic cell ablation

MiniSOG is a light-activated flavoprotein miniSOG, which generates singlet oxygen upon blue-light activation (Shu et al., 2011). Cells that express mitochondrially-
targeted miniSOG can be effectively killed without damaging surrounding tissues in a light-inducible manner (Qi et al., 2012). Animals that express mitochondrially targeted miniSOG under a RIM neuron specific promoter (Pcex-1-tomm20-miniSOG-UrSL-wCherry) were subjected to a blue LED light exposure for 45 minutes, and animals were allowed to recover overnight in darkness. The RFP expressed under the same promoter as the miniSOG served as a marker to examine the morphology of the neurons before and after ablation. Animals with ablated RIM typically showed a dead RIM neuron with unstructured soma (lacking the RFP-negative nucleus) without neurites, and with scattered RFP signals that were taken into the peripheral tissues, most prominently in muscles. For behavioral analyses after RIM ablation, all animals were visually examined to confirm the successful ablation before subjecting them for locomotion analyses.

2.3.6 Calcium Imaging and Data Analysis

Animals were crossed into calcium imaging reporter worms hpIs579 (Prig-3-GCaMP6::w-cherry) and hpIs460 (Punc-4-GCaMP6::w-Cherry) for AVA and A-motor neuron imaging, respectively. To record calcium transients from AVA and A-type motor neurons, larval stage 4 (L4) C. elegans were placed on top of a 2.5% agar pad with M9 buffer to allow for movement. Each recording lasted for 3mins (duration). Images were captured using a 40x objective on a Zeiss Axioskop 2 Plus equipped with an ASI MS-40000 motorized stage, a dual-view beam splitter (Photometrics) and a CCD camera (Hamamatsu Orca-R2). The fluorescence excitation light source from X-CITE (EXFO Photonic Solution Inc.) was reduced to prevent saturation of imaging field. The fluorescent images were split by Dual-View with a GFP/RFP filter set onto the CCD
camera operated by Micromanager. The 4x-binned images were obtained at 50-msec exposure time (10 frames per second). As previously described, regions of interest (ROIs) containing the neuron of interest was defined, and the GFP and RFP fluorescence intensities from the of the neuron(s) was measured using Image J plug-ins developed in-house (Kawano et al., 2011). For AVA data analysis, the amplitude of calcium transients is defined as the percent change of peak GFP/RFP fluorescence intensity compared to baseline averaged for each worm during a 3min (duration) recording. The rise and decay were calculated using the linear slope (change in amplitude divided by the change in time). For A-motor neuron data analysis, cross-correlation analysis on MATLAB (MathWorks) was used to calculate the phase-lag between DA7, VA10, and VA11 motor neurons. An $N \geq 10$ was used for each genotype per experiment.

2.3.7 Whole Animal Metabolomics Analyses by HPLC and LC−MS/MS

Preparing the Samples:

The *C. elegans* metabolomics were performed using gram quantity of animals of the following genotypes: (a) wild type *C. elegans* (N2), (b) *ubr-1* (hp684), (c) *got-1* (hp731), (d) *ubr-1* (hp684); *got-1* (hp731), and (e) *ubr-1* (hp684); *got-2* (gk109644) animals.

Grow 10-15 large NGM agar plates for each genotype. When the most worms grow to adult, wash worms off plate into a 50 ml tube using sterile M9. Spin the tube at 4000rpm for 2mins, let the worms settle down and discard the supernatant. Repeat the washing step two more times. Add 3x volume bleaching solution (formula for bleaching
solution: 8ml water, 1ml concentrated bleach, and 1ml 10N NAOH) to the tube, vortex continuously for 6-8mins or as soon the solution goes clear (It is recommended to check the samples by placing a drop on a slide and checking under the dissecting scope). As soon the solution goes clear, immediately (very important to do it as quick as you can) fill the tube with sterile M9 solution (this step helps to dilute the bleach and reduces its effect on eggs), spin down and discard the supernatant. Repeat the washing step four more times. Transfer the eggs to a 100ml flask with M9 and gently shake at RT for about 20 hrs. When all the eggs hatch, spin again (it is better to spin the L1s in 15ml tubes or even in small Eppendorf tubes) and transfer them to several seeded plates.

When the worms grow to L4 stage, wash the samples thoroughly several times using the M9 solution to get rid of bacterial contamination, and last wash before extraction should be with water to minimize salt reactions with extraction buffer. After the washes, Snap freeze packed washed worms (in sterile screw-cap microfuge tube) in liquid N2.

Keeping frozen, trim the bottom of the tube with a razor blade, trying to not cut into the pellet. Holding the tube upside down, pierce the bottom of the tube with a push-pin, to force the pellet loose from the tube. Transfer pellet to the pre-chilled (in liquid N2) cell-crusher. Powder frozen worm pellet using the "Cell crusher". Transfer the pellet to pre-weighed and dry-ice chilled fresh sterile screw-cap microfuge tube.
I transfer the pellet into tube by lightly taping a cone of weigh paper that has a small hole cut into the bottom. Keep the entire tube, and part of the weigh paper cone, within the dry ice, to prevent any thawing of the sample. Gently push the powder into the tube with an ice chilled spatula. Remove taped cone carefully, so that no tape remains adhered to the weighed microfuge tube. Back on dry ice, and then very quickly weigh the tube with the sample, to get sample weight.

**Metabolite Extraction:**

Add 1 mL dry ice chilled extraction solvent (40% Acetonitrile (HPLC grade), 40% Methanol, 20% Water) to the frozen worm powder (Add 200ul extraction solvent first and vortex it, leave them on dry ice until you finish adding to all the samples, then add remaining 800ul and vortex it. **Should be very careful when adding extraction buffer, as sometimes pellet pops up.** Vortex the tubes to suspend the worm powder in the solvent and let sit on dry ice for 1 hour with occasional thawing and vortexing. The extraction solvent mix will eventually freeze if kept on dry ice.

Shake the samples in a thermo mixer (Eppendorf, Germany) for 1 h at 1400 rpm at 4°C. Centrifuge samples at 4°C at 14 Krpm for 10 mins and harvest the supernatant (first extraction). Supernatant transferred to fresh tubes and lyophilized in a CentreVap concentrator at 40 °C (Labconco, MO).

Re-suspend the pellet in 1 mL extraction solvent. Use a flame sealed 200uL pipette tip to mash the pellet around before and after addition of solvent because the
pellet is difficult to re-suspend. Shake the samples in a thermo mixer for 1 h at 1400 rpm at 4°C. Centrifuge samples at 4°C at 14Krpm for 10mins and harvest the supernatant (second extraction). Transfer the supernatant into the same tube with the lyophilized sample from first extraction and lyophilize (harvest supernatants from both the extractions into the same tube. Start drying the supernatant after the 1st extraction and then add the supernatant from the 2nd extraction into the same tube and dry). The extract samples were stored at −80 °C and used for either HPLC or LC–MS analysis.

**Metabolite Quantitation**

Amino acid quantitation was performed using the Waters Pico-Tag System. After the hydrolysis and pre-column derivatization of the sample by PITC, samples were analyzed by reverse phase HPLC (Amino acid facility, SPARC BioCentre, Sick Kids, Toronto, CA). Metabolite analysis by LC–MS/MS was performed as previously described (Abdel Rahman et al., 2013). Raw values were normalized against the total protein concentration as determined based on the Bradford dye-binding method.

**Quantitation of protein in metabolite pellets**

**CHAPS/Urea Lysis Buffer for extraction:**

4 M urea (2.4 g)

1% triton X-100 (1 mL 10%)

4% CHAPS (4 mL 10%)

10 mM Tris pH 8 (100 ul 1 M)

Add water to 10 mL, mix to dissolve. Store at room temperature.
**Procedure**

1. Add 50-500ul lysis buffer to pellet. For typical extraction sizes, 100ul-150ul is recommended, although this can be adjusted up or down for very small or very large pellet sizes.

2. Vortex pellet and lysis buffer for 5 minutes at room temperature

3. Heat pellet 5 minutes at 95 degrees.

4. For protein quantitation, use Bio-Rad protein assay (BIO-RAD), exactly as per kit directions. Measure on spectrophotometer as directed.

**2.3.8 Measurement of AST/GOT Activity**

The Aspartate Aminotransferase Activity Assay kit is based on the transfer of an amino group from aspartate to α-ketoglutarate resulting in the generation of glutamate, which further results in the production of a colorimetric (450 nm) product proportional to the AST enzymatic activity. One unit of AST is the amount of enzyme that will generate 1.0 m mole of glutamate per minute at pH 8.0 at 37 °C.

**Preparing the Samples:**

Synchronized larval stage 4 (L4) worms were grown on 100 mm NGM agar plates seeded with OP50 bacteria. Worms were collected using M9 buffer and were washed thoroughly to remove bacterial contamination. Samples were homogenized (using sonicator) in 100-200ul ice-cold AST buffer (provided with the kit). Samples were centrifuged at 13,000Xg for 10mins to remove insoluble materials. The supernatant was used for the AST assay and pellets were used to quantify the total protein in the
samples (as described above).

The AST/GOT activity was measured using a colorimetric assay according to the manufacturer’s instructions (Sigma-Aldrich). Readings were measured using spectrophotometric multiwell reader in Jeff Wrana’s lab. Data were normalized against the total protein content of the whole worm lysate as determined by the Bio-Rad protein assay (BIO RAD).

2.3.9 Fluorescence Microscopy and Confocal Imaging

For quantification of GLR-1::GFP, animals were crossed into the integrated fluorescent reporter line *nuIs25 (GLR-1::GFP)* (Rongo et al., 1998). Images of the *C. elegans* dorsal nerve cord were captured using a 63x or 100x objective on a Zeiss Axioplan 2 connected to a Hamamatsu ORCA-ER digital camera and processed using Improvision Open Lab software. Images were processed using minimal deconvolution levels to remove background fluorescence. Confocal images of transgenic strains carrying either *Pubr-1-UBR-1::GFP, Pnmr-1-wCherry,* or *EAT-4^{FOS}::GFP* were acquired on a Nikon Eclipse 90i confocal microscope. Image processing was conducted using Adobe Photoshop. ROIs were defined for the neuron(s) of interest and GFP or RFP fluorescence intensities in the cell body or along the axons were calculated using Image J. The GFP fluorescence intensity was normalized to the total area defined by the ROI.
2.3.10 *C. elegans* Biochemistry

Mixed stage *C. elegans* were grown on 100 mm NGM plates seeded with OP50 and collected using M9 buffer. Lysates were prepared as described previously (Liao et al., 2004; Hung et al., 2007). For western blotting, protein concentrations were determined using a Bradford Protein Assay (Bio-Rad), equivalent amounts ran on an SDS PAGE gel. An anti-GFP antibody (Roche) was used to probe for GOT-1::GFP and an anti-alpha tubulin antibody was used for a loading control.

2.3.11 Statistical Analysis

For curvature analyses, statistical significance was determined using two-way repeated measures (RM) ANOVA and subsequent post-hoc analysis. For metabolite analyses, two-tailed Student’s t-tests were applied to determine statistical differences. For calcium imaging analyses, statistical significance was determined using Kruskal-Wallis tests. P< 0.05 were considered to be statically significant. All statistics were performed using Prism software (GraphPad).
2.4 Results

2.4.1 *ubr-1* Mutants Exhibit Reduced Bending During Backward Movement

Wild-type *C. elegans* generate sinusoidal locomotion where a body bend propagates along the animal to generate movement. In a forward genetic screen for mutants with altered motor behaviors, we isolated a recessive mutation, *hp684* (*Figure 5A*) that led to reduced bending during backing. *hp684* mutants were fully capable of backward movement, but did so with limited flexes hence appear stiff (*Figure 5B*). The stiffness, visible by eye, was progressive as animals developed from larvae to adults.

We quantified the motor phenotypes of the wild type and *hp684* one-day adults (Methods). We compared several parameters: the initiation frequency, duration of backward movements and the curvature across the body during backing (*Figure 5C*). Compared to wild-type, *hp684* mutants exhibited significantly decreased mean curvature value throughout the body (p<0.001, N=12; Figure 1D), significantly longer duration (p<0.001, N=12; Figure 1F), and reduced initiation frequency for backing (p<0.01; N=12; *Figure 5G*). These parameters for forward movements were only mildly, or not changed in *hp684* mutants (data not shown).
Figure 5: ubr-1 Mutants Exhibit Reduced Body Curvature
**Figure 5: ubr-1 Mutants Exhibit Reduced Body Curvature**

A) A diagram depicting the structure of the UBR1 family protein. The UBR zinc finger motif, a region rich in basic amino acids (BRR) and a RING-type zinc finger domain are found is shared by the UBR family proteins. The position and amino acid substitutions in alleles of ubr-1- hp684, hp821hp833 are denoted in the upper panel. Nonsense mutations (red dots), frame shift mutations (blue triangles), and small in-frame deletions (green diamonds) in human UBR1 that lead to JBS are shown in the lower panel.

B) Consecutive snapshots of wild-type and ubr-1 animals during backing (left to right panels). The wild-type animal generated sinusoidal body bend, whereas ubr-1 (hp684, hp821hp833) animals exhibited almost no bending. This ‘stiffness’ in movement was rescued by restoring the expression of UBR-1. Black, white and red dots indicate position of tail in animals of various genetic backgrounds.

C) A schematic of the curvature analysis. The body of the animal is divided into 32 segments, and relative curvature is calculated between each segment from anterior to posterior. They were grouped into 4 sections (1, 2, 3, 4) with each containing an average curvature of 8 segments.

D, E) In ubr-1 mutants (grey line), the body curvature is reduced throughout head to tail when compared to wild type (black line); this phenotype was rescued by restoring expression of UBR-1 under its endogenous promoter (+ UBR-1). D- ubr-1(hp684); E- ubr-1(hp821hp833).

F, H) ubr-1 mutants have longer backing durations. This phenotype was rescued by restoring UBR-1. F- ubr-1(hp684); H- ubr-1(hp821hp833).

G, I) ubr-1 mutants have fewer backing initiations. This phenotype was rescued by restoring UBR-1. G- ubr-1(hp684); I- ubr-1(hp821hp833).

H) UBR-1 translational reporter starts expression during early embryogenesis and expresses in the nerve ring (NR), motor neurons (MN) and body wall muscles (M).

D, E) ***p<0.001; **p<0.01; *p< 0.05 by Two-way RM ANOVA. Error bars, SEM. F, G) ***p< 0.001; **p<0.01; *p< 0.05 by Kruskal-Wallis test. Error bars, SEM.

**Credit** – Raw images used to prepare for Panel J were taken by Dr. Wesley Hung.
I mapped and cloned \( hp684 \), a nonsense mutation that truncates the last 194 amino acids (Q1864X) of the \( C. \) \textit{elegans} ortholog of the UBR1 E3 ligase (\textbf{Figure 5A}; Methods). The UBR family proteins have a conserved amino acid sequence and domain organization from yeast to humans. Their signature motifs include an N-terminal UBR box, and an internal module called the N motif, which consists a region enriched for basic amino acids (BRR) and a RING finger motif (Tasaki et al., 2005). The UBR box and N motif recruit the substrate and E2 of the N-end rule pathway, respectively (Xie and Varshavsky, 1999).

The nonsense mutation in \( hp684 \) is predicted to generate a small truncation near the C-terminal of UBR-1. To verify whether phenotypes of the \( hp684 \) mutant reflect the consequence of the functional loss of UBR-1, I generated three additional \( ubr-1 \) deletion alleles, \( hp820, hp821, \) and \( hp821hp833 \) (\textbf{Figure 5A}), using the \textit{C}lustered, \textit{R}egularly \textit{I}nterspaced, \textit{S}hort \textit{P}alindromic \textit{R}epeats CRISPR-Cas9 system (Friedland et al., 2013). The \( hp820 \) allele harbors a twenty-four base in-frame deletion near N-terminal (ΔR18-W25). The \( hp821 \) allele harbors a four base pair deletion near N-terminal that leads to frame-shift and a premature stop codon (E34X). The \( hp821hp833 \) allele harbors, in addition to \( hp821 \), a second seven base pair internal deletion that leads to frame shift and premature stop codon (E1315X) (\textbf{Figure 5A}). Each of these mutants exhibited motor defects similar to \( hp684 \), including reduced curvature, decreased initiation frequency, and increased duration of backward movements (\textbf{Figure 5B, E, H, I}; \textbf{Figure 6}).
Figure 6: All *ubr-1* Alleles Exhibit Similar Motor Defects to *hp684*
Figure 6: All *ubr-1* Alleles Exhibit Similar Phenotypes

A) Images of wild-type and *ubr-1* animals during backing (left to right panels). The wild-type animal generated sinusoidal body bend, whereas *ubr-1*(*hp820, hp821*) animals exhibit no bending, which was rescued by restoring the expression of UBR-1. Dots denote position of tail.

B, E) In *ubr-1* mutants (grey line), the body curvature is reduced throughout head to tail when compared to wild type (black line), and this was rescued by restoring expression of UBR-1 (Red line). B- *ubr-1*(*hp820*), E- *ubr-1*(*hp821*). *ubr-1* mutants have fewer initiations (C, F) and longer backing durations (D, G).

B, E) ***p<0.001; **p<0.01; *p< 0.05 by Two-way RM ANOVA. Error bars, SEM. C, D, F, G) ***p< 0.001; **p<0.01; *p< 0.05 by Kruskal-Wallis test. Error bars, SEM.
Importantly, all *ubr-1* alleles are recessive, with their motor defects rescued by UBR-1 expression driven by the endogenous *ubr-1* promoter (*Figure 5B-I; Figure 6*).

The comparable phenotypic severity among *hp684*, *hp820*, *hp821* and *hp821hp833* suggests that these motor defects likely represent the consequence of a near complete functional loss of UBR-1. This complements findings from JBS patient cohorts, where individual UBR1 mutations, whether resulting in early or late stop codons, reading frame shifts, or even small internal in-frame deletions, all exert similar pathologic effects (*Figure 5A, lower panel*). For the rest of this study, we only present results from the *hp684* allele unless specified, and refer to it as the *ubr-1* mutant. For behavioral analyses, we only present data on curvature during backing unless specified, and refer to it as the motor phenotype.

2.4.2 UBR-1 is Functions through AVE/RIM Premotor Interneurons to Regulate Bending

Our functional UBR-1 translational reporter showed that UBR-1 was broadly expressed in neurons and muscles (*Figure 5J*). To pinpoint the cellular origin of the motor phenotypes of the *ubr-1* mutants, we examined the effect of restoring UBR-1 expression using exogenous cell-type specific promoters. When UBR-1 expression was restored by a panneuronal promoter (*Prgef-1*), the motor defect in *ubr-1* mutants was fully rescued, whereas restoring UBR-1 expression in muscles did not rescue (*Table 5*; data not shown), indicating its neuronal requirement for proper locomotion. Using promoters for neuronal subgroups, we further identified that UBR-1 function is required
and sufficient in neurons that express the glutamate receptors (*Pglr-1* or *Pnmr-1*).

Restoring UBR-1 expression in the cholinergic or GABAergic motor neurons did not rescue the motor pattern of *ubr-1* mutants (Table 5; data not shown).

*Pglr-1* and *Pnmr-1* express in an overlapping set of interneurons (Brockie et al., 2001a; Brockie et al., 2001b; Maricq et al., 1995); most of them, the AVA, AVD, AVE and RIM premotor interneurons, have been implicated in regulating backward movement (Alkema et al., 2005; Ben Arous et al., 2010; Chalfie et al., 1985; Chronis et al., 2007; Faumont et al., 2011; Gray et al., 2005; Kawano et al., 2011; Piggott et al., 2011; White et al., 1976; Wicks et al., 1996; Zheng et al., 1999). To further narrow down UBR-1’s requirement, we restored its expression with promoters that are active in neurons partially overlapping with the *Pglr-1* and *Pnmr-1* positive premotor interneurons. The restoration of UBR-1 in the AVA and non-overlapping neurons (*Prig-3*) led to a weak rescue of bending and backing durations (Figures 8A-C). By contrast, the restoration of UBR-1 in the AVE, RIM and non-overlapping neurons (*Popt-3*) fully restored the *ubr-1* mutants’ defects for all parameters during backing (Figures 7A, B; Figures 8B, C). Restoring the UBR-1 expression in RIM alone (*Pgcy-13*) was insufficient to rescue *ubr-1*’s motor defects (Figures 7A, B; Figures 8B, C). We could not test the effect of restoring UBR-1 in AVE alone due to the lack of a suitable promoter.

Taken together, UBR-1 is expressed and functions through the AVE and RIM premotor interneurons to affect body bending, initiation frequency and duration of backward movement.
### Table 5: A List of UBR-1 and GOT-1 Tissue specific Rescue Transgenic Lines

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Tissue</th>
<th>Rescue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pubr-1::UBR-1</td>
<td>Neurons and muscle</td>
<td>++++</td>
</tr>
<tr>
<td>Prgef-1::UBR-1</td>
<td>All neurons</td>
<td>++++</td>
</tr>
<tr>
<td>Pmyo-3::UBR-1</td>
<td>Muscle</td>
<td>−</td>
</tr>
<tr>
<td>Pglr-1::UBR-1</td>
<td>AVA, AVE, RIM, AVD, PVC, AVB, others</td>
<td>++++</td>
</tr>
<tr>
<td>Pnmr-1::UBR-1</td>
<td>AVA, AVE, RIM, AVD, PVC</td>
<td>++++</td>
</tr>
<tr>
<td>Popt-3::UBR-1</td>
<td>AVE, RIM, DVB, AVL, CAN</td>
<td>++++</td>
</tr>
<tr>
<td>Prig-3::UBR-1</td>
<td>AVA</td>
<td>+</td>
</tr>
<tr>
<td>Pgcy-13::UBR-1</td>
<td>RIM</td>
<td>−</td>
</tr>
<tr>
<td>Punc-47::UBR-1</td>
<td>GABAergic neurons</td>
<td>−</td>
</tr>
<tr>
<td>Pacr-2::UBR-1</td>
<td>Ach neurons</td>
<td>−</td>
</tr>
<tr>
<td>Plgc-55::UBR-1</td>
<td>AVB</td>
<td>−</td>
</tr>
<tr>
<td>Ptx-3::UBR-1</td>
<td>AIY</td>
<td>−</td>
</tr>
<tr>
<td>Pinx-1::UBR-1</td>
<td>AIB</td>
<td>−</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Tissue</th>
<th>Rescue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prgef-1::GOT-1</td>
<td>All neurons</td>
<td>++++</td>
</tr>
<tr>
<td>Pglr-1::GOT-1</td>
<td>AVA, AVE, RIM, AVD, PVC, AVB, others</td>
<td>++++</td>
</tr>
<tr>
<td>Popt-3::GOT-1</td>
<td>AVE, RIM, DVB, AVL, CAN</td>
<td>++++</td>
</tr>
<tr>
<td>Prig-3::GOT-1</td>
<td>AVA</td>
<td>+</td>
</tr>
<tr>
<td>Pgcy-13::GOT-1</td>
<td>RIM</td>
<td>−</td>
</tr>
<tr>
<td>Punc-47::GOT-1</td>
<td>GABAergic neurons</td>
<td>−</td>
</tr>
</tbody>
</table>
Figure 7: *ubr-1* and its suppressor *got-1.2* function primarily through the AVE/RIM premotor interneurons to regulate backward locomotion.
Figure 7. *ubr-1* and its suppressor *got-1.2* function primarily through the AVE/RIM premotor interneurons to regulate backward locomotion.

A) Snapshots of animals during backing movement of different genetic backwards (left to right panels). Top to bottom: wild-type, *ubr-1*, *ubr-1* transgenic animals (+AVE/RIM UBR-1, +RIM UBR-1). Black, white, red, blue dots denote the position of tail in motion.

B) The reduced bending curvature in *ubr-1* mutants (grey line) was rescued by restoring UBR-1 expression in AVE/RIM premotor interneurons (red line). Expression of UBR-1 in RIM neuron alone did not rescue (blue line).

C) Snapshots of *ubr-1;got-1.2* and transgenic animals during backing: *ubr-1;got-1.2* double mutants (red dots), *ubr-1;got-1.2* with restored GOT-1.2 expression in AVE/RIM (blue dots), RIM alone (Green dots). Color dots indicate position of tail.

D) Loss of function mutations in *got-1.2* suppresses *ubr-1*’s inability to bend (red line). Restoring GOT-1.2 in AVE/RIM in *ubr-1;got-1* reverted curvature to that of *ubr-1* (blue line), whereas restoring the expression of GOT-1.2 in RIM alone did not have any effect (green line).

E) A diagram depicting the structure of GOT-1. The *hp731* allele contains a cysteine to tyrosine change in its pyridoxal phosphate-binding domain.

F) GOT catalyzes reversible transfer of amino group (NH2) from glutamate to oxaloacetate to form α-ketoglutarate and aspartate.

G) Functional GOT-1 translational reporter is ubiquitously expressed in somatic tissues, including the nervous system, gut, seam cells, excretory canal, hypodermis, and body wall muscles. N=10 animals (B, C, E, F).

***p<0.001; **p<0.01; *p<0.05 by Two-way RM ANOVA. Error bars, SEM.
Figure 8: *ubr-1* and its suppressor *got-1* are required in AVE/RIM to regulate backward movement
Figure 8. *ubr-1* and its Suppressor *got-1* are Required in the AVE to Regulate Backward Movement

A. In *ubr-1* mutants (grey line), the body curvature is reduced throughout head to tail when compared to wild type (black line); this phenotype was weakly rescued by restoring expression of UBR-1 in AVA neuron.

B) The frequency of backing initiations (number of events per 5 minutes). In *ubr-1* mutants, the backing initiation frequency is decreased, which is rescued by restoring the expression of UBR-1 in AVE/RIM. Restoring the expression of UBR-1 in RIM neuron alone or in AVA has no effect.

C) Average duration of backward movement in seconds. In *ubr-1* mutants, mean backing duration is increased, which is rescued by restoring the expression of UBR-1 in the AVE/RIM. Restored expression of UBR-1 in RIM neuron alone or in AVA did not rescue.

D) *got-1* suppresses *ubr-1*'s inability to bend (red line). *got-1* mutants also have slightly deeper body curvature compared to wild type (blue line). Restoring GOT-1 in AVA in *ubr-1; got-1* double mutants showed weak reversion to that of *ubr-1* (green line).

E) The loss of function mutation in *got-1* suppresses the decreased initiations of *ubr-1* mutants.

F) *got-1* suppresses *ubr-1*'s longer backing duration. Restoring GOT-1 expression in AVE/RIM reverted the phenotype of *ubr-1; got-1* back to that of *ubr-1*, whereas restored expression of GOT-1 in RIM alone had no effect. Expression of GOT-1 in AVA showed weak reversion.

A, D) **p<0.01 by Two-way RM ANOVA. Error bars, SEM.**
B, C, E, F) ***p< 0.001; **p<0.01; *p< 0.05 by Kruskal-Wallis test. Error bars, SEM.
2.4.3 Removing a glutamate metabolic enzyme GOT rescues *ubr-1’s* motor defects

If UBR-1 regulates neuronal function through negative regulation of components of an unknown signaling pathway, the motor defects of *ubr-1* mutants should be fully or partially restored by a simultaneous decrease of its signaling activity. Accordingly, we isolated *ubr-1* suppressors that could restore *ubr-1*’s motor defects, screened by the rescue of the bending pattern during backward locomotion.

The *hp731* genetic suppressor restored bending in *ubr-1* mutants ([Figures 7C, D]), similar to that of the *hp731* mutant animals, which exhibited a slightly deeper bending compared to wild-type animals during backing ([Figure 8D]). *hp731* also rescued other parameters of backing in *ubr-1* mutants ([Figures 8E-F]). The *hp731* allele harbors a C184Y missense mutation at the pyridoxal phosphate-binding domain in *got-1.2* ([Figure 7E]), a gene that encodes one of the four predicted glutamate-oxaloacetate transaminases (GOT), also referred to as the aspartate transaminase (AST). GOT/AST catalyzes the reversible transfer of an α-amino group between aspartate (Asp) and α-ketoglutarate (α-KG), which converts them to oxaloacetate (OAA) and glutamate (Glu), respectively (Hayashi et al., 1990; Hirotsu et al., 2005) ([Figure 7F]). Consistent with *hp731* representing a loss of function allele of *got-1.2*, placing *hp731* over a deficiency *mnDf1* fully recapitulated the rescuing effect in *ubr-1* mutant background (data not shown). Furthermore, the genetic interaction between *ubr-1* and *got-1.2* is specific: loss-of-function mutations in the other three GOTs did not rescue the bending defect ([Table 6]). From here on, we refer to *got-1.2* as *got-1*. 

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Our functional GOT-1 translational reporter exhibited ubiquitous, cytoplasmic expression in all somatic tissues, including the nervous system (Figure 7G). Restoring GOT-1 expression by a panneuronal promoter (Prgef-1) fully reverted the motor pattern of the ubr-1; got-1 mutants to that of ubr-1 mutants (Table 5; data not show). We further restored GOT-1 expression in subgroups of neurons in ubr-1; got-1 double mutants (Table 5; data not shown). Similar to the case for UBR-1, restored expression of GOT-1 in both AVE and RIM led to an almost full reversion of ubr-1; got-1’s motor patterns to that of ubr-1 mutant animals (Figures 7C, D; 8E, F). Identical to the case for UBR-1, restoring GOT-1 in RIM alone did not cause reversion (Figures 7C, D; 8E, F), and restoring it in AVA had a weak reversion in bending, but no other backing parameters (Figures 8D-F).

Taken together, removing a metabolic enzyme GOT-1 in ubr-1 mutants rescues their motor defects during backward movement. In this regard, the function of both UBR-1 and GOT-1 are critically required in the AVE and RIM premotor interneurons.
2.4.4 Modulating glutamate signaling between the AVE/RIM and AVA premotor interneurons rescues \textit{ubr-1}'s motor defects

The predicted enzymatic activity of GOT-1 affects amino acid and metabolite homeostasis, impacting several metabolic pathways that could affect nervous system development and function: glutamate metabolism, the TCA cycle (LaNoue et al., 1973), the urea cycle (Brosnan, 2000) and mTOR signaling (Zepeda et al., 2009). Glutamate is also the precursor for GABA (Costa et al., 1979; Miller et al., 1978). To determine the pathway through which the \textit{got-1} mutation mediates the suppression of the motor defects of \textit{ubr-1} mutants, we systematically examined genetic interactions between \textit{ubr-1} and viable loss-of-function mutations in components of both metabolic and neuronal signaling pathways (Table 6). Among 32 candidates, we identified two additional \textit{ubr-1} suppressors: loss-of-function mutations in VGLUT3, EAT-4 (Figures 9A, B) and GluCl, AVR-15 (Figures 9D, E). None of the other tested mutants suppressed \textit{ubr-1}'s bending defects (Table 6).
Table 6: A List of Metabolic Enzymes/Components Tested for Genetic Interaction with *ubr-1*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Allele</th>
<th>Curvature Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate/oxaloacetate transaminase</td>
<td><em>got-1.2</em></td>
<td><em>hp731</em></td>
</tr>
<tr>
<td>Glutamate/oxaloacetate transaminase</td>
<td><em>got-1.1</em></td>
<td><em>tm2311</em></td>
</tr>
<tr>
<td>Glutamate/oxaloacetate transaminase</td>
<td><em>got-1.3</em></td>
<td><em>tm3424</em></td>
</tr>
<tr>
<td>Glutamate/oxaloacetate transaminase</td>
<td><em>got-2.1</em></td>
<td><em>gk109644</em></td>
</tr>
<tr>
<td>Glutamic acid decarboxylase</td>
<td><em>unc-25</em></td>
<td><em>e156</em></td>
</tr>
<tr>
<td>Glutamine Synthetase</td>
<td><em>gln-1</em></td>
<td><em>gk329791</em></td>
</tr>
<tr>
<td>Glutaminase</td>
<td><em>glna-2</em></td>
<td><em>gk536170</em></td>
</tr>
<tr>
<td>Alanine aminotransferase</td>
<td><em>C32F10.8</em></td>
<td><em>tm2997</em></td>
</tr>
<tr>
<td><em>nkat-1</em></td>
<td><em>F28H6.3</em></td>
<td><em>ok566</em></td>
</tr>
<tr>
<td>Asparagine Synthetase</td>
<td><em>asns-2</em></td>
<td><em>ok3108</em></td>
</tr>
<tr>
<td>GABA transaminase</td>
<td><em>gta-1</em></td>
<td><em>ok517</em></td>
</tr>
<tr>
<td>Vesicular glutamate transporter</td>
<td><em>eat-4</em></td>
<td><em>ky5</em></td>
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<tr>
<td>Vesicular glutamate transporter</td>
<td><em>vglu-3</em></td>
<td><em>gk765782</em></td>
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<tr>
<td>Vesicular glutamate transporter</td>
<td><em>vglu-1</em></td>
<td><em>ok2356</em></td>
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<tr>
<td>Vesicular glutamate/aspartate transporter</td>
<td><em>C38C10.2</em></td>
<td><em>gk821665</em></td>
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<tr>
<td>Glutamate transporter</td>
<td><em>glt-4</em></td>
<td><em>bz69</em></td>
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<td>Glutamate transporter</td>
<td><em>glt-5</em></td>
<td><em>bz70</em></td>
</tr>
<tr>
<td>GABA transporter</td>
<td><em>snf-11</em></td>
<td><em>ok156</em></td>
</tr>
<tr>
<td>GABA vesicular transporter</td>
<td><em>unc-47</em></td>
<td><em>e307</em></td>
</tr>
<tr>
<td>Isocitrate dehydrogenase</td>
<td><em>idhb-1</em></td>
<td><em>ok2368</em></td>
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<tr>
<td>Pyruvate dehydrogenase</td>
<td><em>pdhk-2</em></td>
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<td>Pyruvate carboxylase</td>
<td><em>pyc-1</em></td>
<td><em>tm3788</em></td>
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<td>Pyruvate kinase</td>
<td><em>pyk-1</em></td>
<td><em>ok1754</em></td>
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<tr>
<td>Carbamoyl phosphate synthetase1</td>
<td><em>T28F3.5</em></td>
<td><em>tm4283</em></td>
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<tr>
<td>S6K</td>
<td><em>rsks-1</em></td>
<td><em>ok1255</em></td>
</tr>
<tr>
<td>Caspase</td>
<td><em>ced-3</em></td>
<td><em>n717</em></td>
</tr>
<tr>
<td>Caspase</td>
<td><em>ced-4</em></td>
<td><em>n1162</em></td>
</tr>
<tr>
<td>AMPA-type/Ionotropic</td>
<td>glr-1</td>
<td>tm5549</td>
</tr>
<tr>
<td>----------------------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td></td>
<td>glr-2</td>
<td>ok2342</td>
</tr>
<tr>
<td></td>
<td>glr-5</td>
<td>tm3506</td>
</tr>
<tr>
<td>NMDA-type/Ionotropic</td>
<td>nmr-1</td>
<td>ak4</td>
</tr>
<tr>
<td>Metabotropic</td>
<td>mgl-1</td>
<td>tm1811</td>
</tr>
<tr>
<td>Glutamate-gated chloride channels</td>
<td><em>avr-15</em></td>
<td><em>ad1051</em></td>
</tr>
<tr>
<td></td>
<td>glc-3</td>
<td>ok321</td>
</tr>
<tr>
<td></td>
<td>glc-4</td>
<td>ok212</td>
</tr>
</tbody>
</table>
Both EAT-4 and AVR-15 are components of glutamate signaling. As reported, \textit{eat-4} mutants exhibited a drastic reduction of spontaneous backing (Chalasani et al., 2007; Lee et al., 1999). During induced backward movements, \textit{eat-4} mutants exhibited a slightly deeper curvature than wild-type animals (Figure 10B). \textit{ubr-1; eat-4} mutants exhibited fully restored bending and backing durations, similar to that of \textit{eat-4} mutants (Figures 9A, B; 10D). The suppression was fully reverted by an EAT-4::GFP fosmid translational reporter (Sarov et al., 2012) (Figure 10A). We further examined the critical neuronal subgroups required for \textit{eat-4}-mediated suppression (Table 7).

Among all tested subgroups, restored expression of EAT-4 in the AVE and RIM exhibited the strongest partial reversion of \textit{ubr-1; eat-4} motor phenotypes, with reduced bending, identical to that of \textit{ubr-1} mutants in posterior segments (Figure 9A, B).

We examined the expression pattern of the functional EAT-4::GFP fosmid reporter. To facilitate cell identification, we examined this functional reporter in a \textit{kinesin/unc-104} mutant background, where the EAT-4::GFP signal was retained at the soma of many neurons. Consistent with a previous study (Serrano-Saiz et al., 2013), we identified robust EAT-4::GFP expression in RIM, and no expression in AVA and AVD, in all transgenic animals (Figure 9C). In addition, we observed strong expression in AVE in \textasciitilde70\% of transgenic animals (Figure 9C). These results are consistent with the role of EAT-4 in AVE and RIM for exerting its genetic interaction with UBR-1.
Figure 9: Modulating glutamate signalling between AVE/RIM and AVA restores *ubr-1* body curvature.
Figure 9: Modulating glutamate signalling between AVE/RIM and AVA restores ubr-1 body curvature.

A) Snapshots of ubr-1; eat-4 with suppressed bending (red dots), expression of EAT-4 in AVE/RIM (blue dots), RIM alone (green dots). Color dots indicate position of tail.

B) Loss of function mutations in eat-4 suppress ubr-1 reduced curvature (red line), restoring EAT-4 in AVE/RIM reverted curvature back to ubr-1 (blue line), whereas expression of EAT-4 in RIM alone did not rescue (green line).

C) Expression pattern of EAT-4 using a fosmid reporter crossed into unc-104 mutants to identify the cell bodies. EAT-4 fosmid reporter (green) co-localizes with Pnmr-1::RFP in AVE and RIM premotor interneurons.

D) Snapshots of ubr-1; avr-15 animals with suppressed bending (red dots), expression of AVR-15 in AVA premotor interneuron (blue dots), A-type motor neurons (green dots). Colored dots indicate position of tail.

E) Loss of function mutations in avr-15 suppress ubr-1 reduced curvature (red line), restoring AVR-15 in AVA premotor interneuron alone reverted curvature back to ubr-1 (blue line), whereas expression of AVR-15 in A-type motor neurons didn’t rescue (green line).

F) A model for how UBR-1 regulates body curvature. UBR-1 maintains the synaptic glutamate levels. Glutamate synthesized by GOT-1 at the synapse is loaded into synaptic vesicles by EAT-4. Glutamate released from AVE/RIM neurons regulate AVR-15 activity in the post-synaptic AVA neuron and regulates body curvature.

***p<0.001; **p<0.01 by Two-way RM ANOVA. Error bars, SEM.
Figure 10: *eat-4* and *avr-15* reduce longer backing durations in *ubr-1* Mutants
Figure 10: *eat-4* and *avr-15* reduce longer backing durations in *ubr-1* Mutants

A) Snapshots of *ubr-1;eat-4* with suppressed bending (red dots), expression of EAT-4 under its own promoter +EAT-4 (green dots) reverts the suppression back to *ubr-1*. Color dots indicate position of tail.

B) In *eat-4* mutants (blue line), the body curvature is deeper similar to *ubr-1;eat-4* double mutants (red line).

C) Quantification of backing initiations frequency. The loss of function mutation in *eat-4* didn’t rescue decreased initiation in *ubr-1* mutants’ backward movements.

D) Quantification of mean backing durations. The loss of function mutation in *eat-4* suppressed longer duration in *ubr-1* mutants’ backward movements. Restoring EAT-4 expression in AVE/RIM reverted the phenotype of *ubr-1;eat-4* back to that of *ubr-1*. Expression of EAT-4 in RIM alone showed partial reversion.

E) In *avr-15* mutants (blue line), the body curvature is deeper similar to *ubr-1;avr-15* double mutants (red line).

F) Quantification of backing initiations frequency. The loss of function mutation in *avr-15* suppressed the decreased initiation in *ubr-1* mutants’ backward movements. Expression of AVR-15 in AVA but not in A-type motor neurons reverted back the phenotype.

G) Quantification of mean backing durations. The loss of function mutation in *avr-15* suppressed longer duration in *ubr-1* mutants’ backward movements. Restoring AVR-15 expression in AVA, but not in the A-type motor neurons reverted the phenotype of *ubr-1;avr-15* back to that of *ubr-1*.

C, D, F, G) ***p< 0.001; **p<0.01; *p< 0.05* by Kruskal-Wallis test. Error bars, SEM.
Table 7: A List of EAT-4 and AVR-15 Tissue specific Rescue Transgenic Lines

<table>
<thead>
<tr>
<th>EAT-4</th>
<th>All neurons</th>
<th>+++</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prgef-1::EAT-4</td>
<td>AVA, AVE, RIM, AVD, PVC, AVB, others</td>
<td>+++</td>
</tr>
<tr>
<td>Pglr-1::EAT-4</td>
<td>AVA, AVE, RIM, AVD, PVC</td>
<td>+++</td>
</tr>
<tr>
<td>Pnmr-1::EAT-4</td>
<td>AVA, AVE, RIM, AVD, PVC</td>
<td>+++</td>
</tr>
<tr>
<td>Popt-3::EAT-4</td>
<td>AVE, RIM, DVB, AVL, CAN</td>
<td>++</td>
</tr>
<tr>
<td>Prig-3::EAT-4</td>
<td>AVA</td>
<td>+</td>
</tr>
<tr>
<td>Pgcy-13::EAT-4</td>
<td>RIM</td>
<td>−</td>
</tr>
<tr>
<td>Punc-47::EAT-4</td>
<td>GABAergic neurons</td>
<td>−</td>
</tr>
<tr>
<td>Pacr-2::EAT-4</td>
<td>Ach neurons</td>
<td>−</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>AVR-15</th>
<th>All Tissues</th>
<th>+++</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pdpy-30::AVR-15</td>
<td>All Tissues</td>
<td>+++</td>
</tr>
<tr>
<td>Prgef-1::EAT-4</td>
<td>All neurons</td>
<td>+++</td>
</tr>
<tr>
<td>Prig-3::EAT-4</td>
<td>AVA</td>
<td>+++</td>
</tr>
<tr>
<td>Punc-4::EAT-4</td>
<td>A-class motor neurons</td>
<td>−</td>
</tr>
</tbody>
</table>
GluCls, the invertebrate-specific glutamate-gated chloride channels closely related to mammalian glycine receptors, have been implicated in feeding and sensorimotor functions (Wolstenholme, 2012; Wolstenholme and Rogers, 2005). ubr-1; avr-15 mutants also exhibited fully restored bending and other parameters for backward movements (Figures 9D, E; 10F, G). avr-15 mutants, similar to got-1 and eat-4, exhibited deeper bending than wild-type animals (Figure 10E).

AVR-15 expression has been observed in pharyngeal muscles and some motor neurons using a transcriptional reporter (Dent et al., 1997). We first determined that AVR-15 functions through neurons to regulate bending. Driven by a panneuronal promoter (Prgef-1), the restored expression of avr-15 in neurons fully reverted ubr-1; avr-15 mutants to that of ubr-1 (Table 7; data not shown). We further tested the postsynaptic neurons of AVE and RIM as candidates through which AVR-15 regulate ubr-1’s motor defects. AVE makes chemical synapses to the A-type motor neurons that drive backward movement, and both AVE and RIM make chemical synapses to AVA, which provides the majority of inputs to the A-type motor neurons (White et al., 1976). Restoration of AVR-15 in AVA and other neurons (Prig-3), but not in the A-type motor neurons (Punc-4), fully reverted the motor patterns of ubr-1; avr-15 to that of ubr-1, with even slightly reduced bending (Figures 9D, E; 10F, G).

In summary, the activities of UBR-1, GOT-1 and EAT-4 are critical in the AVE and RIM premotor interneurons, whereas the activity of AVR-15 is critical in their postsynaptic partner AVA to regulate backing. These results suggest that decreased UBR-
1 activity leads to aberrant glutamate signaling from AVE/RIM to AVA (Figure 9F).

2.4.6 UBR-1 regulates calcium dynamics in the AVA premotor interneuron

To further test this hypothesis, we examined AVA activity in ubr-1 and one of the
ubr-1 suppressor avr-15 by in vivo calcium imaging (Figure 11A-D). A GCaMP6::w-Cherry calcium sensor, driven by Prig-3, exhibited similar expression levels in AVA
between wild-type, ubr-1, avr-15, and ubr-1; avr-15 animals. Consistent with previous
reports (Ben Arous et al., 2010; Chronis et al., 2007; Faumont et al., 2011; Kawano et al.,
2011), the initiation of reversals temporally correlated with an increase in the intracellular
calcium levels in AVA, and the decrease with the animal’s transition from backing to
forward locomotion; such a correlation was observed for all genotypes (dotted vertical
lines in Figure 11A-D).

The Ca\(^{2+}\) transient change (ΔF/F) had no significant difference across genotypes,
although ubr-1 and avr-15 mutants showed a trend towards a slight decrease and
increase, respectively, compared to wild-type animals (Figure 11G; Figure 12).
However, we observed significant changes in the kinetics of the calcium transients in
AVA in ubr-1 mutants. Both the calcium transient rise and decay were significantly faster
in ubr-1 mutants (P<0.001 against wild-type animals; Figure 11E-F). avr-15 mutants
showed wild-type calcium transient rise and decay (Figure 11E-F). In ubr-1; avr-15
mutants, the decay rate was rescued (P<0.01 against ubr-1, Figure 11F), but not rate of
rise (Figure 11E).
Altered kinetics of AVA calcium transients in *ubr-1* mutants as indicated by increased rate of rise and decay is consistent with UBR-1 regulating glutamatergic inputs to AVA. These results also indicate a functional contribution of the GluCl AVR-15 in modulating AVA’s activity decay.
Figure 11: Altered AVA premotor interneuron activation pattern in \textit{ubr-1} mutants
Figure 11: Altered AVA premotor interneuron activation pattern in *ubr-1* mutants

A-D) Imaging of the premotor interneuron AVA in moving animals. Calcium transient traces shown as the GFP/RFP ratio over time (bottom panels) and correlated with the initiation of backward movement (top panels). The periodic rise of calcium transients in AVA correlated with the initiation of backward movement (dotted vertical line).

E) Quantification of slope values for AVA activity rise. The rate of calcium transients rise in AVA in *ubr-1* mutants is faster (indicated by increased slope values) compared to wild-type. Loss of function mutations in *avr-15* fails to suppress *ubr-1*’s faster AVA activity rise.

F) Quantification of slope values for AVA activity decay. The rate of calcium transients decay in AVA in *ubr-1* mutants is faster (indicated by increased slope values) compared to wild-type. Mutations in the glutamate-gated chloride channel *avr-15*, causes slower decay compared to wild-type. Loss of function mutations in *avr-15* restores *ubr-1*’s faster AVA activity decay.

G) Quantification of the mean amplitude of Ca$^{2+}$ spikes in AVA neuron in wild-type, *ubr-1*, *avr-15* and *ubr-1; avr-15*. There is no significant change in the mean amplitude between different genotypes.

N=10-15, ***p<0.001, **p<0.01, *p<0.05 by Kruskal-Wallis test.
Figure 12: *ubr-1* mutants and faster AVA activity rise and decay constants
Figure 12. *ubr-1* mutants and faster AVA activity rise and decay constants

Raster plots of all calcium traces of AVA activity in wild type, *ubr-1*, *avr-15* and *ubr-1; avr-15* animals. Each horizontal line corresponds to the GFP/RFP ratio of one animal of the respective genotypes during the 3-minute recording.
2.4.8 UBR-1 is required for the phase lag between A-type motor neurons to allow bending

How does altered glutamate signaling between these premotor interneurons lead to reduced bending in *ubr-1* mutants? The AVE and AVA premotor interneurons make chemical synapses and AVA makes gap junctions onto the cholinergic A-type motor neurons that coordinate backward movements (Figure 13A). The ventral A-type (VA) and dorsal A-type (DA) motor neurons are excitatory and innervate the ventral and dorsal body wall muscles, respectively (Figure 13B) (Chalfie et al., 1985; White et al., 1976; Wicks et al., 1996). The VA and DA motor neurons also exhibit rhythmic calcium patterns (Haspel et al., 2010; Kawano et al., 2011). Their activities have to be spatially and temporally regulated to alternate dorsal and ventral bends that propagate along the body. We examined the temporal activation pattern of a subset of A-type motor neurons that innervate the ventral (VA10, VA11) and dorsal (DA7) body wall muscles by simultaneous in vivo calcium imaging.

Across all genotypes examined, the rhythmic calcium transients in these motor neurons corresponded with backward movements (dotted boxes in Figure 13C-H). VA10 and VA11 innervate adjacent ventral muscle groups (Figure 13B) (Haspel and O'Donovan, 2011). In wild-type animals, the calcium transient change was out-of-phase, as expected from the sequential firing of adjacent ventral muscles (red and blue traces in Figures 13C, Figures 13I and 14A). The activation of DA7 was out-of-phase with that of VA10 (green and red traces in Figure 13C, Figures 13K and 14A); this is consistent with the notion that DA7 innervates more dorsal muscles that directly appose
VA10’s targets (Figure 13B) (Haspel and O'Donovan, 2011). By contrast, the activation of VA11 and DA7 was more in-phase (green and blue traces in Figures 13C, Figures 13J and 14A), reflecting an out-of-phase propagation of the ventral and dorsal muscle contraction sequence.

*ubr-1* mutants exhibited striking difference in their temporal activation pattern: the change of calcium transients for all three A-type motor neurons (VA11, VA10 and DA7) were in-phase (blue, red and green traces in Figures 13D; I-K and 14B). These results suggest that the reduced bending in *ubr-1* mutants results from a failure to establish phasic relationship between the A-type motor neurons, instead of a lack of neuronal activity. Further, when UBR-1 expression was restored in the AVE and RIM neurons, transgenic animals exhibited restored A-type motor neuron phasic relationship similar to that of wild-type animals (Figures 13E, I-K; 14C), coinciding with the restoration of bending. In accordance with the behavioral suppression, *ubr-1; got-1, ubr-1; eat-4*, or *ubr-1;avr-15* all exhibited full or partial restoration of the phase relationship between these motor neurons (Figures 13F-K; 14D-F).

These results indicate that glutamate signaling in a small premotor interneuron network regulates the phase-lag of the A-type motor neurons. In *ubr-1* mutants, dysregulated glutamate signaling in this network disrupts this phasic relationship, resulting in backing with reduced body curvature.
Figure 13: ubr-1 regulates bending through AVE-dependent phase lag of A-type motor neurons
**Figure 13. ubr-1 regulates bending through AVE-dependent phase lag of A-type motor neurons**

A) A schematic diagram of connectivity of the *C. elegans* backward motor circuit. Hexagons represent premotor interneurons, circle indicates A-type motor neurons, arrow is for chemical synapses and line represents gap junctions.

B) The anatomic organization of A-type motor neurons (VA11, DA7, VA10) that innervates ventral and dorsal muscles.

C-H) Simultaneous imaging of multiple A-type motor neurons in free moving animals. Calcium transient traces shown as the GFP/RFP ratio over time. Backward movement is indicated in dotted box.

C) In wild-type animals, during backward movement, VA11 and VA10 motor neurons that innervate different muscles often show an out-of phase change. VA11 and DA7 activate together and exhibit in-phase changes, whereas VA10 and DA7 exhibit out of phase changes.

D) In *ubr-1* mutants, VA11, VA10 and DA7 activate together and exhibit in-phase changes.

E) UBR-1 expression in AVE/RIM premotor interneurons restored in-phase activity changes of A-type motor neurons in *ubr-1* mutants back to wild-type.

F-H) Loss of function mutations in *ubr-1* suppressors got-1, eat-4 and avr-15 suppress *ubr-1*’s in-phase activity changes of A-type motor neurons.

I-K) Quantification of real-time cross correlation of activity changes between different A-type motor neurons. I) Cross correlation between VA11 and VA10 activity changes.

J) Cross correlation between VA11 and DA7 activity changes. K) Cross correlation between DA7 and VA10 activity changes.

N=10-15, ***p<0.001; **p<0.01 by Kruskal-Wallis test.
Figure 14: *ubr-1* mutants exhibit in-phase changes in A-type motor neuron activity pattern.
Figure 14: *ubr-1* mutants exhibit in-phase changes in A-type motor neuron activity pattern

A-E) Cross correlation images of different A-type motor neurons In wild type, *ubr-1*, +AVE/RIM UBR-1, *ubr-1; got-1*, *ubr-1; eat-4*; *ubr-1; avr-15* animals.

A) In wild type cross correlation of VA11 to VA10, DA7 to VA10 shows negative correlation or a phase lag, whereas there is no lag between VA11 and DA7 A-type motor neurons.

B) All pairs of motor neurons exhibit in-phase activity changes with no lag.

C) Expression of UBR-1 in AVE/RIM neurons restores in-phase activity changes in *ubr-1* mutants for VA11 to VA10, DA7 to VA10 motor neurons.

D-E) *ubr-1* suppressors *got-1*, *eat-4* and *avr-15* suppress in-phase activity changes in *ubr-1* mutants for VA11 to VA10, DA7 to VA10 motor neurons.
2.4.9  *ubr-1* mutants have elevated glutamate

How does UBR-1 regulate glutamate signaling? The predicted bi-directional transaminase activity of GOT-1, which could suppress *ubr-1*’s bending defects when mutated, suggests a potential involvement of metabolite homeostasis, primarily between aspartate, glutamate, α-ketoglutarate (α-KG), and oxaloacetate (OAA) (*left panel, Figure 15A*). However, the activity of the GOT enzymes has not been determined *in vivo*. To determine the metabolic changes associated with loss of UBR-1 and GOT-1, we profiled free amino acids from synchronized L4 stage *C. elegans* by high profile liquid chromatography (HPLC). Compared to wild-type animals, *ubr-1* mutants exhibited a moderate, but consistent and significant increase in glutamate (*N=5, p<0.05*). *ubr-1; got-1* mutants, on the other hand, exhibited a moderate, but consistent and significant reduction in glutamate (*N=4, p<0.05*) when compared to either *ubr-1* or wild-type animals (*Figure 15B*). A similar degree of glutamate reduction was observed in *got-1* mutants. Importantly, the loss of GOT-2, another predicted *C. elegans* GOT, did not reduce *ubr-1*’s glutamate level (*Figure 15B*).

*ubr-1* mutants exhibited a slight, but consistent increase of aspartate (*N=5, p<0.05* against wild-type animals). *got-1* and *ubr-1; got-1* double mutants, on the other hand, accumulated massive amount of aspartate (*Figure 15C*). These results suggest that GOT-1 preferentially synthesizes glutamate from aspartate (*Figure 15A, right panel*). The observation that glutamate was decreased in *ubr-1*, and eliminating GOT-1 alone was sufficient to reduce the glutamate level in both wild-type animals and *ubr-1* mutants highlights it’s key contribution to glutamate synthesis *in vivo* (see Discussion).
In addition to amino acid homeostasis, GOT-1’s activity also impacts the synthesis of OAA and α-KG, which may further affect other metabolites and metabolic pathways. To determine how the loss of GOT-1 affects other metabolites, we performed liquid chromatography-mass spectrometry (LC-MS) using the whole-animal extract from synchronized L4 larvae (Methods). In both ubr-1 and ubr-1; got-1 mutants, OAA was slightly decreased when compared to wild-type animals (Figure 15D). We did not observe a consistent trend for α-KG (Figure 15D), or for other components of the TCA cycle across all genotypes (Figure 16B). In got-1 and ubr-1; got-1 mutants, we did observe an increased AMP/ATP ratio (Figure 16C), as well a decreased ratio between reduced and oxidized glutathione (GSH/GSSG) (Figure 15E); both indicative of increased metabolic stress and cellular toxicity (Son et al., 2013), which could result from the massive accumulation of aspartate.

Together, these results suggest that GOT-1 is a key synthesizing enzyme for glutamate. The loss of UBR-1 leads to an elevated glutamate, and the subsequent cellular defect could be compensated through reducing its synthesis by GOT-1.
Figure 15: *u*br-*I* mutants have increased glutamate level
Figure 15. *ubr-1* mutants have increased glutamate level

A) GOT catalyzes reversible transfer of amino group (NH2) from glutamate to oxaloacetate to form α-ketoglutarate and aspartate (left panel). Our amino acid profiling indicates that GOT-1.2 preferentially catalyzes synthesis of glutamate from aspartate (right panel).

B-D) Free amino acids were measured from whole worm lysates using HPLC, normalized to total protein in the lysate and then normalized to wild-type.

B) Glutamate levels are significantly increased in *ubr-1* and *ubr-1got-2* mutants; whereas *got-1* and *ubr-1; got-1* mutants have reduced levels of glutamate.

C) Aspartate levels are increased in *ubr-1*, and *ubr-1got-2* mutants, whereas *got-1* and *ubr-1; got-1* mutants have 20-fold higher levels of Asp compared to wild-type.

D-E) Amount of metabolites measured using mass spectrometry normalized to total protein in the lysate and then normalized to wild-type

D) OAA is decreased in all the mutants, whereas the levels of α-KG are unchanged.

E) Ratio of NADP/NADPH is increased in the *got-1* and *ubr-1; got-1* mutants, no change in *ubr-1* mutants (left panel). Glutathione (GSH) to glutathione disulfide (GSSG) ratio is decreased in the *got-1* and *ubr-1; got-1* mutants, no change in *ubr-1* mutants (right panel).

**p<0.01; *p<0.05 by Student T test.**
Figure 16: Metabolic profiling in \textit{ubr-1} and its suppressor

A) Alanine levels are decreased in \textit{got-1} and \textit{ubr-1} mutants compared to wild-type and \textit{ubr-1} respectively.

B-C) Amount of metabolites measured using mass spectrometry normalized to total protein in the lysate and then normalized to wild type.

B) Other metabolite components of the TCA cycle didn’t show any coordinated changes in mutants.

C) Ratio of AMP/ATP is increased in the \textit{got-1} and \textit{ubr-1; got-1} mutants, no change in \textit{ubr-1} mutants.

D) Amount of amino acids in wild-type normalized to total protein in the lysate. In \textit{C. elegans} alanine is the most abundant amino acid, followed by glutamate. Aspartate and GABA are maintained at low abundance.

\*\*p <0.01; \*p < 0.05 by student T test.
2.4.10 AVA has reduced excitatory ionotropic glutamate receptor in *ubr-1* mutants

How does elevated glutamate in *ubr-1* mutants alter signaling between the premotor interneurons? Glutamate induces synaptic plasticity by altering the distribution and expression of ionotropic glutamate receptors (Featherstone et al., 2002; Grunwald et al., 2004; Lissin et al., 1999; Wang et al., 2010). AVA receives extensive inputs from other premotor interneurons, including AVE and RIM. Increased glutamate in *ubr-1* mutants may elevate presynaptic glutamate inputs to AVA, resulting in a compensatory change in their ionotopic glutamate receptors.

We addressed this possibility by examining two excitatory glutamate receptors expressed in AVA - the NMDA-type receptor glutamate receptor 1 (NMR-1) and the AMPA-type glutamate receptor 1 (GLR-1). A functional translational GLR-1::GFP reporter displayed clustered fluorescent puncta along the ventral nerve cord, where AVA was nearly exclusively postsynaptic to other premotor interneurons (Grunwald et al., 2004). In *ubr-1* mutants, these puncta were significantly decreased in intensity (N=15-20, p<0.0001, Figures 17A-A’). The intensity of GLR-1::GFP puncta was fully rescued by restoring the expression of UBR-1 in AVE and RIM (N=15-20, p<0.0001, Figures 17A-A’). Consistent with the notion that reduced intensity of the GLR-1::GFP puncta results from increased presynaptic glutamate inputs to AVA, GLR-1::GFP puncta intensity was restored in *ubr-1; got-1* and *ubr-1; eat-4*, but not in *ubr-1; avr-15* mutants (Figures 17A-A’), despite the similar rescue of motor pattern in all three mutants.
Figure 17: Abnormal glutamate signalling due to increased activity of GOT in \textit{ubr-1} mutants leads to reduced expression of ionotrophic glutamate receptors.
Figure 17. Abnormal glutamate signalling due to increased activity of GOT in *ubr-1* mutants leads to reduced expression of ionotropic glutamate receptors

A) Representative images of wild-type, *ubr-1*, *ubr-1+AVE/RIM UBR-1*, *ubr-1; got-1*, *ubr-1; eat-4* and *ubr-1;avr-15* carrying the GLR-1::GFP transgene. A’) Quantification of GLR-1::GFP intensity. GLR-1::GFP intensity is reduced in *ubr-1* mutants compared to wild-type and can be restored by expressing UBR-1 in AVE/RIM neurons. The reduced GLR-1::GFP intensity in *ubr-1* mutants is suppressed by loss of function mutations in *got-1* and *eat-4*, but not by *avr-15*.

B) GOT activity is measured in whole worm lysates, normalized to total protein in the lysate and then normalized to wild-type. *ubr-1*, *ubr-1;got-2* mutants have increased activity of GOT, and GOT activity is decreased in both *got-1* and *ubr-1;got-1* mutants compared to wild type.

C) Western blot analyses on *C. elegans* lysates from strains carrying the same integrated transgenic arrays expressing GOT-1::GFP (left panel) or panneuronal GOT-1::GFP (right panel)

D) UBR-1 regulates the activity of GOT-1, thereby maintains the synaptic glutamate levels. Glutamate synthesized at the synapse is loaded into synaptic vesicles by EAT-4. Glutamate released from AVE neuron regulates AVR-15 activity in the postsynaptic AVA neuron.

D’) Glutamate-mediated activity changes in AVA neuron regulates phase lag of A-type motor neurons, which is necessary for proper body curvature.

N=10-15, ***p<0.001; **p<0.01 by Kruskal-Wallis test.
Activity also induces glutamate-dependent transcriptional regulation of glutamate receptors (Myers et al., 1999). We tested this possibility using a transcriptional reporter for NMR-1 (*Pnmr-1-wcherry*). In wild-type animals, a similar level of fluorescent signals was observed at the soma of the AVA, AVE, RIM, and AVD premotor interneurons. *ubr-1* mutants exhibited a pronounced decrease of fluorescent signal intensity in AVA. Such a decrease was unique for AVA: the ratio between AVA and AVE fluorescence intensity was significantly decreased in *ubr-1* (N=10, p<0.0001, Figure 18), whereas there was no difference between the ratio between AVE and RIM fluorescence intensity between wild-type and *ubr-1* (Figure 18). Restoring the expression of UBR-1 in AVE and RIM was sufficient to restore the intensity of *Pnmr-1* signals in AVA in *ubr-1* mutants. Also similar to that of the GLR-1::GFP reporter, the *Pnmr-1* fluorescence intensity in AVA was rescued in *ubr-1;got-1* and *ubr-1;eat-4* (N=10, p <0.001 against *ubr-1*, Figure 18), but not in *ubr-1;avr-15* (Figure 18).

These results support the notion that in *ubr-1* mutants, increased glutamate signaling results in the down-regulation of excitatory ionotropic glutamate receptors (*Figure 17D-D’*), altering glutamate signaling between the AVE/RIM and the AVA interneurons.
Figure 18: Reduced expression of the ionotrophic glutamate receptors in premotor interneuron AVA in ubr-1 mutants

A) Representative images of wild type, ubr-1, ubr-1 animals with UBR-1 expression in AVE/RIM neurons, ubr-1; got-1, ubr-1; eat-4 and ubr-1; avr-15 carrying the Pnmr-1::w-cherry transgene. Pnmr-1 is expressed in AVA, AVE, AVD and RIM premotor interneurons.

B) Quantification of relative intensity ratio of Pnmr-1::w-cherry between AVA/AVE neurons- AVA/AVE ratio is decreased in ubr-1 mutants compared to wild type and rescued by UBR-1 expression in AVE/RIM. ubr-1 suppressors got-1 and eat-4 suppress pnmr-1::w-cherry intensity in AVA neuron, whereas avr-15 has no effect.

C) There is no change in the relative intensity ratio of Pnmr-1::w-cherry between AVE/RIM.

N=10-15, **p < 0.01 by Kruskal-Wallis test.
2.4.11  *ubr-1* mutants exhibit increased transaminase activity but maintain similar levels of GOT-1

Our genetic analyses attributes the motor defects of the *ubr-1* mutants to increased glutamate level and/or release from premotor interneurons. How does UBR-1 negatively regulate glutamate levels? We assayed total glutamate oxaloacetate transaminase activity in *C. elegans* lysates, and observed a mild but significant increase in *ubr-1* mutants compared to wild-type animals (N=4, p<0.05). This increase was attenuated in *ubr-1; got-1* mutants (N=4, p<0.01, **Figure 17B**). The attenuation was specific to GOT-1: the functional loss of GOT-2, a homolog of GOT-1, did not suppress the increased transaminase activity in *ubr-1* mutants (**Figure 17B**). As expected, the transaminase activity was drastically reduced, but not completely lost in *got-1 or ubr-1; got-1* mutants (N=4, p<0.01, **Figure 17B**). Thus, increased GOT activity can increase glutamate synthesis and elevate the glutamate level in *ubr-1* mutants.

Despite increased GOT activity, glutamate level and glutamate loading, we did not observe a change in the GOT-1 protein level in *ubr-1* mutants (**Figure 17C**). This conclusion was verified by comparing the level of GOT-1::GFP expressed either from an exogenous panneuronal promoter, or by its endogenous genomic locus (Figure 7C). Therefore, UBR-1 is unlikely to target GOT-1 for degradation. We propose that UBR-1-mediated regulation of glutamate metabolism and signaling involves targeting other components of the pathway, including kinases or phosphatases that may modify protein activity in a tissue-specific manner, for degradation.
2.5 Discussion

Dysregulated E3 ligase activities have been implicated in the pathological development of several neurological disorders, including JBS. Using the C. elegans model, we demonstrate a new role for UBR1 in glutamate metabolism and signaling. Through dissecting a small circuit that affects C. elegans body curvature, we uncover a glutamate signaling pathway, comprised of a glutamate synthesizing transaminase, a vesicular glutamate transporter, and a glutamate-gated chloride channel, that is negatively regulated by UBR-1. This study brings forth a hypothesis that defects in glutamate homeostasis may be a common cellular defect that underlies JBS pathology.

2.5.1 Glutamate is the primary metabolic change that underlies the genetic interaction between ubr-1 and got-1

Our metabolomics analysis on ubr-1 mutants revealed an increased free glutamate level, coinciding with an elevated glutamate oxaloacetate transaminase activity. Removing GOT-1.2 reduces glutamate level in wild-type, as well as in the ubr-1 mutant background. This effect was not recapitulated by removing another predicted GOT, GOT-2. The differential modifying effect on the metabolite profile by GOTs parallels their effects on the genetic modification of ubr-1’s motor defects: removing GOT-1.2, but not any other GOT, restored curvature during backing.
As previously reported (Falk et al., 2008), *C. elegans* is unusual in that the most abundant amino acid is alanine, followed by glutamate, whereas aspartate is maintained at low abundance (Figure S6D). The massive aspartate accumulation and partial glutamate reduction in *got-1.2* mutants establishes GOT-1.2 as the key transaminase that synthesizes glutamate from aspartate in *C. elegans* and also establishes aspartate as a key amino group donor. The reduced, but not abolished glutamate level in *got-l* mutants may reflect compensatory increase of transaminase activities that utilize other amino acids as the amino donor to maintain glutamate abundance; this notion was corroborated by a consistent and significant decrease of alanine in *got-l* and *ubr-l; got-l* mutants (Figure 16A).

In *ubr-l* mutants aspartate levels were also slightly increased and these levels were further increased in the background of *got-l*. Activation of other metabolic pathways in response to glutamate accumulation may account for a slight increase in aspartate, to maintain glutamate homeostasis. With the exception of glutamate and aspartate, we did not observe consistent changes or related trends for other amino acids in *ubr-l, got-l* and *ubr-l; got-l* mutants.

Genetic mutations in specific metabolic enzymes are unlikely to restrict the metabolic consequence to their primary substrates or immediate metabolic pathways. In addition, GOT-1 not only does it convert specific amino acids, but it also affects two metabolites OAA and α-KG critical for energy production, lipid synthesis and multiple other cellular pathways. A global survey of metabolic consequences of *ubr-l and got-l*
mutants by our LC-MS analyses further provided a glimpse on the complexity of compensatory or adaptive changes in response to metabolic dysfunction induced by single mutations. In both ubr-1 and got-1 mutants other than decreased OAA levels, we didn’t observe any significant or consistent changes with other TCA cycle metabolites including α-KG (Figures 15C; 16B). Such an in vivo deviation from the expected metabolic enzymatic reactivity could be attributed to functional contribution of additional regulators for these metabolites. However, we favour an alternative possibility that these results reflect the ability of cells to activate different metabolic pathways to compensate or adapt for the perturbation of a major branch of glutamate synthesis. In parallel, we failed to identify any genetic suppressors from regulators of other metabolic pathways partaking by α-KG or OAA (Table 6).

Taken together, these results argue that the suppression of the bending phenotype in the ubr-1 background by got-1 is due to reduced glutamate synthesis, rather than indirect metabolic effects.

2.5.2 Increased glutamate signaling in premotor interneurons underlies ubr-1’s defects

Glutamate is not only an essential amino acid, but also an excitatory neurotransmitter and the sole precursor for GABA. Through glutamate, UBR-1 and GOT-1 can impact metabolism as well as neuronal signaling. In contrast to the failure to identify additional ubr-1 suppressors from metabolic enzymes, we uncovered them from glutamate transporters and receptors. Removing either the VGLUT3 (EAT-4) or a GluCl
(AVR-15) restored bending in \textit{ubr-1} mutants; hence GOT-1’s interaction with UBR-1 is likely through affecting synaptic glutamate metabolism and signaling.

Results from our tissue specific rescue experiments solidify this notion, and further highlight where such a glutamate signaling takes place. To restore or affect bending, UBR-1, GOT-1 and EAT-4 are critically required in the AVE and RIM, and AVR-15 in AVA. The premotor interneuron AVA receives synaptic inputs from all other premotor interneurons including AVE and RIM (White et al., 1976), and provides the majority of inputs to all motor neurons that coordinate backward locomotion (Chalfie et al., 1985; White et al., 1976). Therefore, glutamate signaling within the premotor interneuron network, specifically the glutamatergic inputs to AVA, holds the key to modify \textit{ubr-1}’s bending pattern.

How does the glutamate signaling change? Removing GOT-1 or EAT-4 predicts reduced glutamatergic inputs to AVA, and removing AVR-15 increased AVA’s response to glutamatergic inputs. Synaptic plasticity, where the presynaptic glutamate inputs induce compensatory changes in postsynaptic ionotropic glutamate receptors provides a potential reconciliation for the seemingly contradictory effect of glutamate signaling by \textit{ubr-1} suppressors. Blocking presynaptic activity, or decreasing presynaptic glutamate release results in an increased expression of postsynaptic glutamate receptors in cultured neurons, \textit{C. elegans} and \textit{Drosophila} (Featherstone et al., 2002; Grunwald et al., 2004; Lissin et al., 1999). Indeed, we observed pronounced reduction of both AMPA and NMDA-type receptor markers in \textit{ubr-1} mutants, and importantly, such a reduction was uniquely profound in AVA, but not in other premotor interneurons including AVE and
RIM. These results indicate that in \textit{ubr-1} mutants, increased glutamate signaling inputs to AVA induces the down regulation of excitatory postsynaptic glutamate receptors; this results in a reduced postsynaptic response that is compensated by removing a negative regulator of AVA activity. This notion is strongly supported by the observation that restoring UBR-1 in non-AVA premotor interneurons was sufficient to restore the level of excitatory ionotropic receptors in AVA. Taken together, increased glutamate signaling to AVA in \textit{ubr-1} mutants affects motor output, suggesting that UBR-1 negatively regulates glutamate signaling.

These results also implicate a role for GOT-1 in mediating the synthesis of synaptic glutamate. Previous studies suggest that synaptic glutamate is synthesized from glutamine that is secreted by astrocytes and taken up by neurons (McKenna, 2007; Palmada and Centelles, 1998). However, the glutaminase required for this synthesis may not be expressed in all glutamatergic neurons (Laake et al., 1999; Ottersen et al., 1998). Other studies suggest that glutamine taken up by neurons may instead be utilized for ATP production (Bradford et al., 1978; Hassel and Sonnewald, 1995; Zielke et al., 1998). Therefore other enzymes may be present at the synapse to synthesize synaptic glutamate. The notion that \textit{C. elegans} GOT-1 plays a role in synaptic glutamate signaling is consistent with a previous finding that GOTs synthesize synaptic glutamate in cultured mammalian neurons (Takeda et al., 2012).
2.5.3 AVA calcium kinetics regulates the phase lag between A-type motor neurons

A byproduct of the behavioral and functional imaging analyses of *ubr-1* mutants and their genetic suppressors is a regulatory mechanism for the *C. elegans* motor circuit. Specifically, these results determine a role of glutamate signaling within the premotor interneuron network to modify the bending pattern during backward locomotion.

How the *C. elegans* motor circuit generates locomotion has been extensively examined since the 80s. The decoding of the *C. elegans* wiring diagram (Chen et al., 2006; White et al., 1986), which guided and was corroborated by ablation studies (Chalfie et al., 1985; Kawano et al., 2011; Wicks et al., 1996) led to the discovery of the functional components of a small circuit that drive directional locomotion. Follow-up studies, most notably the functional imaging analyses on the AVA in moving animals, established AVA as the critical component of the motor circuit (Ben Arous et al., 2010; Chronis et al., 2007; Faumont et al., 2011; Kawano et al., 2011). AVA forms both electrical and chemical synapses with the A-type motor neurons that execute backward locomotion, and whose activation coincides with and sustains backing (Chalfie et al., 1985; Kawano et al., 2011; White et al., 1976). Signaling mechanisms through which AVA regulates backward locomotion however remained to be solved, and appeared more complicated.

We found that in *ubr-1* mutants, an increased glutamatergic input to AVA is associated with faster rise and decay of the calcium transients. Activation of glutamate-
gated chloride channels leads to membrane hyperpolarization (Mellem et al., 2002; Wolstenholme, 2012). Consistently, removing AVR-15 specifically reduced the decay kinetics of AVA calcium transients. The rise phase of calcium transients, unaffected by *avr-15*, is hence regulated by other signaling components.

How AVA regulates the A-type motor neuron activity remains a mystery. AVA, being the shared postsynaptic partner of all other premotor interneurons, does not express VGLUT (Serrano-Saiz et al., 2013). The activation of AVA is regulated by, but dispensable of glutamatergic inputs (Brockie et al., 2001a; Marvin et al., 2013). A surprising finding of our A-type motor neuron imaging is that our original hypothesis that the bending defect of *ubr-1* mutants would be due to reduced A-type motor neuron activity was incorrect. In fact, the bending defect was due to a disrupted phase lag between A-type motor neurons innervating adjacent regions of muscle. The glutamate signaling to AVA seems to be key to control this phasic relationship. The change in AVA kinetics in *ubr-1* mutants appear may provide a clue to how such a modulation is achieved. How altered AVA kinetics leads to phasic activity pattern change between A-type motor neurons is unknown. Below we provide hypotheses that are speculative and mean to serve as possible starting points for future endeavors by us and others to explore this matter further.

AVA forms both gap junctions and chemical synapses to all A-type motor neurons. Gap junctions serve dual roles: at rest, they function as current shunts to reduce AVA’s excitability and self-inhibit the activation of backing motor circuit to bias forward motor circuit output and foraging behaviors (Kawano et al., 2011). Upon activation, AVA
effectively transitions animals from forward to backward movement. Such an effect may be transduced through both gap junctions and chemical synapses.

Altering the decay kinetics of presynaptic activity can alter the activity pattern of its postsynaptic partners (Choe and Miikkulainen, 2003). In \textit{ubr-1} mutants, the faster decay phase of calcium transients in the AVA premotor interneuron may therefore result in more synchronized activation of all the A-type motor neurons, resulting in the “stiff” backing phenotype as opposed to a smooth, sinusoidal pattern. Our results suggest that the decay phase of AVA activation is critical for its inputs to the A-type motor neurons to be spatially and temporally organized. One simple hypothesis, given that gap junctions to all A-type motor neuron somas make it a difficult to achieve asynchrony, and chemical synapses are more likely to achieve this role, inhibitory glutamatergic input to AVA may be in place to allow AVA’s chemical synaptic output to outweigh that of the gap junctions. Hence, glutamate signaling to AVA plays a role to adjust phasic relationship to modify the bending pattern during backward movement.

2.5.4 \textbf{The C. elegans motor phenotype to investigate mechanisms of UBR1 and JBS}

UBR family proteins have been extensively studied in yeast and mice. Their activities were addressed in the context of the E3 ligases for the N-end rule substrates (Sriram et al., 2011; Varshavsky, 1996). However, questions remain as whether the N-end rule substrates underlie all UBR-dependent cellular functions, and whether they are physiologically relevant to \textit{JBS} (Lee et al., 2005; Rao et al., 2001). For example, pro-apoptotic fragments are UBR1’s N-end rule substrates in mammalian cells (Piatkov et al.,
2012), but we found that removing *C. elegans* caspase-encoding genes did not rescue the bending defects of *ubr-1* mutants (Table 6). Several reported UBR1 substrates are targeted through uncharacterized internal degrons (Kitamura et al., 2001; Sasaki et al., 2006; Takeda and Yanagida, 2005; Xia et al., 2008a). Among multiple human UBRs that degrade the N-end rule substrates, mutations in UBR1 alone cause *JBS* (Zenker et al., 2005).

*C. elegans* possesses a single ortholog of UBR1, bypassing functional redundancy. Our data suggest that it is unlikely that *C. elegans* UBR-1 directly interacts with the components of glutamate signaling identified as genetic suppressors in this study, including GOT-1. Our results underscore an important function for UBR-1 in glutamate metabolism and signaling, which may be more relevant when investigating mammalian UBR and UBR1-mediated phenotypes.

Dysregulated glutamate metabolism and signaling may underlie defects in *JBS*. Among the multiple human UBR1 homologues (UBR1, UBR2, UBR4, and UBR5), only mutations in UBR1 are associated with *JBS* (Zenker et al., 2005). In *JBS* patients, pathogenic sequence changes in UBR1 include nonsense and frame shift mutations that lead to premature stop codons, splice site mutations, missense mutations, and small in-frame deletions that affect conserved amino acid residues and are found throughout the protein (Sukalo et al., 2014). Regardless of the nature and location of the pathological mutation, all JBS patients exhibit severe exocrine pancreatic insufficiency, nose wing hypo/aplasia, and dental defects (Sukalo et al., 2014).
In our study, we isolated four mutant alleles of *C. elegans* UBR-1 (*hp684*, *hp820*, *hp821*, and *hp821 hp833*) that lead to truncation of different parts of the protein. All these mutant alleles exhibited a similar degree of motor impairment, which was fully rescued by expression of endogenous UBR-1. This is reminiscent of what is observed in *JBS* patients: pathogenic mutations in UBR1, regardless of its location or type, may result in general protein instability, thereby causing similar disease phenotypes.

How may dysfunctional glutamate metabolism and signaling manifest in *JBS* patients? A case study has reported increased GOT activity in a patient with severe cognitive impairments (Fallahi et al., 2011), suggesting that abnormal glutamate metabolism may be pathologically relevant to disease. Furthermore, a growing body of evidence reveals the importance of glutamate signaling in non-neuronal tissues, including the pancreas, in addition to its well-characterized role in the nervous system (Gammelsaeter et al., 2011; Hayashi et al., 2003; Jenstad and Chaudhry, 2013). Since pancreatic exocrine insufficiency is a severe and common symptom present in all JBS patients, it may be the case that UBR1-mediated abnormal glutamate signaling underlies this symptom.

Aberrant glutamate signaling has also been implicated in other diseases (Siegel and Sanacora, 2012). Most therapies have focused on the postsynaptic regulation of glutamate signaling (Lau and Tymianski, 2010; Willard and Koochekpour, 2013), or strategies to improve glutamate clearance by EAATs (Soni et al., 2014). Targeting glutamate dysfunction at the level of glutamate synthesis, such as GOT, and its regulator
UBR1 may provide alternative strategies to treat patients wherein glutamate signaling is dysregulated.

In summary, our studies elucidate a previously unknown role for UBR-1/UBR1 in glutamate metabolism and signaling using *C. elegans*. The motor output of the *ubr-1* model may provide mechanistic insights into the cellular function, as well as to the underlying pathophysiology in other systems, including *JBS* patients.

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Chapter 3
3.1 Abstract

Defects in the establishment and maintenance of Excitatory-Inhibitory (E-I) balance has emerged as a potential unifying pathogenesis of multiple neurodevelopmental disorders, but the molecular mechanisms that regulate the E-I balance are poorly understood. We report here that in *C. elegans*, an E3 ubiquitin ligase UBR-1 regulates the synaptic balance of GABA and glutamate in some GABAergic neurons. The functional loss of UBR-1 leads to reduced GABA signaling, which could be compensated by removing vesicular loading of glutamate in the same neurons. I propose that an increased glutamate in *ubr-1* mutants is responsible for reduced activity and GABAergic signaling observed in these neurons. Mutations in the human UBR1 are associated with the Johanson-Blizzard Syndrome (JBS). An intriguing implication of these findings is that UBR-1 is a potential regulator of the E-I balance whose disruption contributes to the pathology of this disorder.
3.2 Introduction

Overall excitability of the central nervous system (CNS) is determined by the physiological activity of excitatory and inhibitory neurotransmission. In mammals, the primary excitatory neurotransmitter in the brain is glutamate, whereas inhibitory neurotransmission involves GABAergic and glycinergic signaling (Binzegger et al., 2004; DeFelipe, 2002; Eichler and Meier, 2008). In response to alterations in neuronal activity, a balance between excitation and inhibition in neural circuits is maintained to prevent hyperactivity or quiescence (Zhang and Sun, 2011). An E-I imbalance in neural circuits has been proposed to underlie or contribute to several neurological diseases, ranging from neuropsychiatric conditions such as schizophrenia and Tourette’s syndrome, to neurodegenerative conditions like the Parkinson’s and Huntington’s diseases (Gatto and Broadie, 2010; Marin, 2012). A large number of neurodevelopmental diseases including autism, epilepsy, Rett syndrome, and Fragile X syndrome are proposed to result from an E/I shift (Gatto and Broadie, 2010; Marin, 2012). Despite the emerging importance of the functional E-I balance, little is known about the molecular players involved in maintaining this homeostasis.

An E/I balance in neural circuits is influenced by the activity of neurotransmitters, a process that is regulated developmentally and activity-dependent. For example, GABA signaling shifts from being excitatory in immature neurons and developing circuit, to being inhibitory in the mature nervous system (Ben-Ari et al., 2007; Blaesse et al., 2009; Dzhala et al., 2005; Rivera et al., 1999; Yamada et al., 2004).
Moreover, further complexity arises from emerging evidence for the co-release of excitatory and inhibitory neurotransmitters in several regions of brain, which is critical for proper circuit refinement. Vesicular glutamate transporters (VGLUT) and vesicular GABA transporters (VGAT), which selectively uptake glutamate and GABA neurotransmitters, respectively, co-exist in the same synaptic terminal of the hippocampal (CA3) mossy fiber, cerebellar mossy fiber, cerebellar GABAergic basket cells, the hypothalamic proopiомelanocortin (POMC) neurons, as well as the lateral habenula (Beltran and Gutierrez, 2012; Jarvie and Hentges, 2012; Safiulina et al., 2006; Somogyi et al., 2004; Walker et al., 2001; Zander et al., 2010). These observations imply the possibility of the same synapse to engage in both excitatory and inhibitory signaling, but both mechanisms and physiological functions of these synapses are poorly understood (Beltran and Gutierrez, 2012; Jarvie and Hentges, 2012; Safiulina et al., 2006; Somogyi et al., 2004; Walker et al., 2001; Zander et al., 2010). Addressing mechanisms that regulate E/I homeostasis helps to uncover therapeutic strategies for neurodevelopmental disorders.

In the previous chapter, I show that UBR-1 is a regulator of synaptic glutamate homeostasis in the nematode Caenorhabditis elegans (C. elegans). In a simple motor circuit, UBR-1 maintains glutamate-signaling homeostasis between the AVE and AVA premotor interneurons. I identified a glutamate metabolic enzyme GOT-1, a vesicular glutamate transporter EAT-4 (VGLUT3), and a glutamate-gated chloride channel AVR-15 as components of the signaling pathway negatively regulated by UBR-1. ubr-1 mutants exhibit an increased level of glutamate as a consequence of increased GOT
activity. Loss-of-function mutations in got-1 suppress ubr-1’s bending defects by reducing glutamate levels. Further reducing synaptic glutamate release through genetic ablation of eat-4 or regulating glutamate activity through mutations in the glutamate-gated chloride channel avr-15 also suppressed ubr-1 behavioral phenotypes. Our results suggest that the absence of UBR-1 leads to an increased glutamate release from AVE, which leads to altered AVA activity dynamics.

Here, we report that both GABA and glutamate are likely released by some C. elegans GABAergic neurons and UBR-1 regulates the balance between these two neurotransmitters through glutamate homeostasis. We show that ubr-1 mutants exhibit reduced GABA signaling at a simple motor circuit that controls the defecation motor program and reducing synaptic glutamate uptake in these motor neurons can restore the motor defects. Using in vivo calcium imaging, we further show that UBR-1 is required for maintaining the GABAergic neuronal activity; the reduced GABAergic neuronal activity is suppressed by removing the metabolic enzyme GOT-1. Together our findings implicate a novel role for UBR-1-mediated glutamate homeostasis in GABAergic neurons and GABA signaling.
3.3 Materials and Methods

3.3.1 Strains, Constructs, and Transgenic lines

All strains were maintained on NGM plates at 22°C using standard methods (Brenner, 1974). The wild-type strain was Bristol (N2). \textit{ubr-1 (hp684)} was identified in a forward genetic screen looking for mutants with behavioral deficits. \textit{ubr-1 (hp821)}, and \textit{ubr-1 (hp821hp833)} alleles were generated using the Clustered, Regularly Interspaced, Short Palindromic Repeats (CRISPR)-Cas9 system (Farboud and Meyer, 2015; Friedland, 2013; Lo et al., 2013; Schwartz and Sternberg, 2014; Tzur et al., 2013).

Strains for EAT-4 Expression Pattern:

\textbf{ZM8490} - TH463- \textit{unc-119(ed3)} III; \textit{ddIs263 (EAT-4}^{FOS}\textit{::GFP)}
\textbf{ZM8492} - \textit{unc-104}; \textit{ddIs263 (EAT-4}^{FOS}\textit{::GFP)}
\textbf{ZM8722} - \textit{otIs388 (EAT-4}^{FOS}\textit{-SL2-YFP)}

Strains for Calcium Imaging:

\textbf{ZM8519} - \textit{ubr-1 (hp684)}; \textit{hpIs468}
\textbf{ZM8633} - \textit{eat-4 (ky5)}; \textit{hpIs468}
\textbf{ZM8623} - \textit{ubr-1 (hp684)}; \textit{eat-4 (ky5)}; \textit{hpIs468}
\textbf{ZM8871} - \textit{ubr-1 (hp684)}; \textit{hpIs556}
\textbf{ZM9018} - \textit{got-1.2 (hp731)}; \textit{hpIs556}
\textbf{ZM8872} - \textit{ubr-1 (hp684)}; \textit{got-1.2 (hp731)}; \textit{hpIs556}

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Table 8: A list of constructs and transgenes generated for Chapter 3

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Host strain</th>
<th>Transgene</th>
<th>Strain Name</th>
</tr>
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<tbody>
<tr>
<td>PJH2913</td>
<td>Pubr-1::UBR-1</td>
<td>hp684</td>
<td>hpEx3161</td>
<td>ZM7440</td>
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<tr>
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<td>hpEx3481</td>
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<td>hp684</td>
<td>hpEx3535</td>
<td>ZM8389</td>
</tr>
<tr>
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<td>hp684</td>
<td>hpEx3536</td>
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<tr>
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<td>hp684</td>
<td>hpEx3537</td>
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<tr>
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<td>hpEx3562</td>
<td>ZM8455</td>
</tr>
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<td>hpEx3538</td>
<td>ZM8391</td>
</tr>
<tr>
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<td>hp684</td>
<td>hpEx3541</td>
<td>ZM8404</td>
</tr>
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<td>hpEx3500</td>
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<td>lin-15</td>
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<td>hp684; hpSi468</td>
<td>hpEx3610</td>
<td>ZM8589</td>
</tr>
<tr>
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<td>Punc-47-w-cherry</td>
<td>unc-104; TH463</td>
<td>hpEx3501</td>
<td>ZM8585</td>
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<tr>
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<td>lin-15</td>
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<td>hpEx3637</td>
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<tr>
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<td>otIs388</td>
<td>hpEx3659</td>
<td>ZM8722</td>
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</table>
3.3.2 Behavioral Assay for Defecation Motor Program

The defecation phenotype was scored as described previously (Thomas, 1990). Briefly, L4 stage hermaphrodites were transferred to a new NGM plate. After about 20–24 hours, each animal was scored for total number defecation cycles for 10 minutes. Ten animals were examined for each genotype. Results were presented as mean (cycle per minute) ± SEM for each genotype. Two-tailed Mann-Whitney U test was performed to determine the statistic significant difference.

3.3.3 Confocal Imaging

Confocal imaging of UBR-1::GFP, EAT-4fos::GFP and EAT-4-SL2-YFP strains were acquired on a Nikon Eclipse 90i confocal microscope. Image processing was carried out in the Adobe Photoshop. Intensity of GFP or RFP in the cell body or along the axons was calculated using Image J.

3.3.4 Calcium Imaging

DVB neuron imaging was performed under conditions that allow animals with relatively free movement. We used two independent integrated lines, *hpIs468* and *hpIs556*, for DVB motor neuron calcium imaging. Animals were placed on freshly made 2% wet agarose pads, mounted with a few microliters of M9 buffer, covered by a coverslip, and imaged with a 40x objective equipped with the automated tracking system. Periods of DVB activity in each recording were isolated; frequency of the periodic DVB
calcium spike was determined for each animal; differences of the GFP/RFP ratio between the base and the peak of the transient during each period were normalized against the baseline (ΔF/F). The two-tailed Mann-Whitney U test was used to determine the statistic significance. Statistical difference at p < 0.05 was considered to be significant.

3.4 Results

3.4.1  *ubr-1* Mutants have Reduced Frequency of Expulsions During the Defecation Motor Program

*ubr-1* mutants, in addition to locomotion defects, exhibit morphological phenotypes including a distended intestine, which often results from constipation (Wang et al., 2013b). I thus examined the defecation motor program (DMP), a well characterized, ~50 second-cycle rhythmic behavior. The DMP consists three sequential motor steps: posterior body wall muscle contraction (pBoc), anterior body wall muscle contraction (aBoc), and enteric muscle contraction (EMC); the last step is accompanied by the expulsion of the gut content (Exp) (Thomas, 1990) (Figure 19A). The last step of the DMP, enteric muscle contraction and expulsion is controlled by the GABAergic neurons AVL and DVB (McIntire et al., 1993b) (Figure 19B). GABA, which is an inhibitory neurotransmitter for locomotion (McIntire et al., 1993a), acts as an excitatory neurotransmitter by activating an excitatory GABA-gated cation channel EXP-1 on enteric muscles (Beg and Jorgensen, 2003).
A. The defecation motor program in *C. elegans* consists of three sequential muscle contractions that are activated approximately every 50 seconds. Initiation of the cycle is characterized by posterior body contraction (pBoc), followed by a brief relaxation phase. Anterior body contraction (aBoc) happens ~2 seconds after the relaxation phase and is followed by enteric muscle contraction, leading to expulsion of the gut contents (Exp).

B. A schematic of the enteric muscles. The enteric muscles consist of four cells: the anal depressor, sphincter, and two bilaterally symmetric intestinal muscle cells. GABAergic neurons AVL and DVB innervate enteric muscles through activating the excitatory GABA receptor EXP-1 expressed by the enteric muscle. GABA release from AVL and DVB causes the anal depressor and intestinal muscles to contract, coordinating the opening of anus and explosion of the gut content.
Figure 20: ubr-1 Mutants Exhibit Reduced GABA Signaling
Figure 20. ubr-1 Mutants Exhibit Reduced GABA Signaling

A) A diagram depicting the structure of the UBR1 family protein. The UBR zinc finger motif, a region rich in basic amino acids (BRR) and a RING-type zinc finger domain are found in all UBR homologues. Position and amino acid changes in two alleles of ubr-1, hp684 and hp821hp833 are denoted.

B) A UBR-1 translational reporter exhibits expression in the AVL and DVB neurons.

C, D) Quantification of the frequency of aBoc and pBoc events in wild type animals and ubr-1 mutants. ubr-1 mutants exhibit normal aBoc and pBoc frequency.

E) Quantification of the frequency of expulsions (Exp). ubr-1 (hp684) mutants exhibit decreased expulsion frequency. The reduction in expulsion frequency could be rescued by restoring UBR-1 expression in neurons, but not in the muscle. Restricted restoration of UBR-1 in all GABAergic motor neurons, including Ds, AVL and DVB (Punc-47) fully rescued the Exp phenotype, whereas a restored expression of UBR-1 in only either the D-type GABA motor neurons, cholinergic motor neurons or GLR-positive interneurons did not rescue the defecation defect.

F) Other alleles of ubr-1, hp821 and hp821hp833, also exhibit a reduced expulsion; this phenotype was rescued by restoring the expression of UBR-1 under its endogenous promoter.

N=10-15 animals. ***p < 0.0001; by Mann Whitney test. Error bars, SEM.

Credit – Dr. Wesley Hung: Panel B
To identify whether *ubr-1* mutants possess defects in the DMP, I quantified the number of defecation cycles within a 10-minute period. Compared to wild-type, *ubr-1 (hp684)* mutants exhibited normal numbers of the aBOC and pBOC events (Figure 20C and 20D), but a ~60% reduction in the Exp frequency (N=15, p < 0.0001) (Figure 20E). Similar to *hp684*, two other deletion alleles of *ubr-1*, *hp821* and *hp821hp833*, exhibited a similar degree of reduction in expulsion events (N=10, p < 0.0001) (Figure 20F). For all three alleles, the expulsion phenotype was rescued by restoring UBR-1 expression under its endogenous promoter (N=10, p < 0.0001) (Figure 20F). For subsequent studies, we presented data obtained from the *hp684* allele and refer it as the *ubr-1* mutant.

To address where UBR-1 is required to regulate expulsions, I examined the effect of restoring UBR-1 expression using tissue-specific promoters in *ubr-1* mutants. I found that UBR-1 was strictly required in neurons, and not in muscles to rescue reduced expulsions (Figure 20E). I further dissected the functional requirement of UBR-1 using neuronal subtype promoters. I found that a restoration of UBR-1 specifically in GABAergic motor neurons, including the Ds, AVL, DVB and others (*Punc*-47) was sufficient to rescue the defecation phenotype of *ubr-1* mutants (Figure 20E). I further restricted the functional requirement to the AVL and DVB neurons, because restoring UBR-1 expression in all GABAergic motor neurons except AVL and DVB using a fragment of *Punc*-25; (Jin et al., 1999) failed to rescue the defecation defects (Figure 20E). This is consistent with the previous study that the expulsion step in DMP is regulated by a simple circuit that includes only AVL and DVB (McIntire et al., 1993b). Lastly, using a functional UBR-1 translational reporter, we found that UBR-1 is indeed
expressed in both AVL and DVB neurons (Figure 20B). These results indicate that UBR-1 functions in the AVL and DVB GABAergic motor neurons to regulate enteric muscle contraction and expulsion. Reduced defecation in ubr-1 mutants indicates reduced GABA signaling in this small motor circuit.

3.4.2 ubr-1 Mutants Exhibit Increased GABA Level

Loss-of-function mutants for UNC-25, the C. elegans homolog of glutamic acid decarboxylase (GAD), GABA synthesizing enzyme, exhibit a near complete abolishment of expulsion events, confirming a specific role of GABA in the regulation of Expulsions (McIntire et al., 1993a; Thomas, 1990). The observation that expulsion events were reduced in ubr-1 mutants is consistent with an attenuation of GABAergic signaling in these mutants. To identify precise changes in GABA levels in the absence of UBR-1, I profiled the free amino acid content in lysates from synchronized larval stage 4 (L4) stage animals using High Profile Liquid Chromatography (HPLC). Surprisingly, ubr-1 mutants exhibited a ~40% increase in GABA (compared to wild type controls, N=5, p<0.05) (Figure 21B). This observation, however, was fully consistent with the finding that glutamate, the sole precursor of GABA synthesis, was increased in ubr-1 mutants.

3.4.3. Removing the Glutamate Metabolic Enzyme GOT-1 does not Rescue the Expulsion Defects of ubr-1 Mutants

Loss of function mutations in glutamate metabolic enzyme GOT-1 leads to an effective suppression of the locomotion defects of ubr-1 mutants (Figure 6D-F).
However, got-1 did not suppress the expulsion defects of ubr-1 mutants: ubr-1; got-1 double mutants exhibited a further (~15%) reduction in expulsion frequency when compared to ubr-1 mutants (N=15, p < 0.001) (Figure 21A). This may reflect a simple additive effect because got-1 mutants also exhibited a ~20% reduction in expulsion frequency (compared to wild type animals, N=15, p < 0.0001) (Figure 21A).

As expected, the increased GABA level in ubr-1 mutants was fully attenuated by removing the glutamate metabolic enzyme GOT-1 (N=4, p<0.05) (Figure 21B). There was no significant difference between ubr-1; got-1 and got-1 mutants, both of which exhibited a significant reduction (~33%) in the GABA level (compared to wild-type animals, N=4, p<0.05) (Figure 21B). These observations were consistent with the observation that removing GOT-1 further enhances the defecation defects by decreasing GABA levels.
Figure 21: Loss of Function Mutations in *ubr-1*’s Suppressors Modify Reduced GABA Signaling Phenotype.
Figure 21. Loss of Function Mutations in *ubr-1*’s Suppressors Modify Reduced GABA Signaling Phenotype.

A) Quantification of the frequency of expulsions (Exp) in wild type, *ubr-1*, *got-1* and *ubr-1; got-1* animals. Loss of function mutations in *got-1* enhances *ubr-1* reduced Exp phenotype. *got-1* mutants also have reduced Expulsions compared to wild type. B) Free amino acids were measured from whole worm lysates using HPLC, normalized to total protein in the lysate and then normalized to wild type. GABA levels are significantly increased in *ubr-1* mutants; whereas *got-1* and *ubr-1; got-1* mutants have reduced levels of GABA. C) Quantification of the frequency of expulsions (Exp). Loss of function mutations in *eat-4* suppress *ubr-1* reduced Exp phenotype; further restoring EAT-4 in GABA neurons +AVL/DVB neurons reverted expulsions back to *ubr-1*, whereas expression of EAT-4 in other classes neurons did not rescue. E) Expression pattern of EAT-4 using two different fosmid reporters. EAT-4::GFP is crossed into *unc-104* mutants to identify the cell bodies. Both EAT-4::GFP (left panel) and EAT-4-SL2-YFP (right panel) fosmid reporters co-localizes with *Punc-47::RFP* in DVB neurons, EAT-4::GFP expression is also seen in the GABAergic motor neurons (bottom panel). N=10-15 animals (A, C). ***p < 0.0001; **p <0.001, *p <0.01 ; by Mann Whitney test. Error bars, SEM. B) *p < 0.05 by student T test.

Credit – Jyothsna Chitturi and Dr. Maria Lim: Panel C; Dr. Wesley Hung- Panel D
3.4.4 Reducing Vesicular Glutamate uptake in AVL and DVB Neurons Restores Expulsion defects in ubr-1 Mutants

How do we reconcile the counter-intuitive finding that the ubr-1 mutants exhibit a GABA level increase but decreased expulsions, a phenotype caused by decreased GABA signaling? (McIntire et al., 1993a; Thomas, 1990) The simplest potential explanation is that the AVL/DVB motor neurons are defective in the release of GABA. Previously, we found that removing the vesicular glutamate transporter EAT-4 led to a suppression of ubr-1 mutants locomotion defects (Figure 10). Remarkably, we found that placing ubr-1 in the background of eat-4 also rescued its defecation phenotype. Specifically, ubr-1; eat-4 double mutants exhibited a fully restored expulsion frequency (N=10, p < 0.0001) (Figure 21C). There was no significant difference in expulsion frequency between eat-4 single mutants and wild type animals (Figure 21C). The observation that eat-4 genetically interacts with ubr-1 to suppress its defecation phenotype suggests that abnormal synaptic glutamate may underlie the GABA signaling defects found in ubr-1.

To determine whether EAT-4 functions directly or indirectly to affect the activity of GABAAergic motor neurons that regulate defecation, we first investigated the expression pattern of EAT-4, using two different EAT-4 fosmid-based reporters (Sarov et al., 2012; Serrano-Saiz et al., 2013). We found weak EAT-4 expression in the DVB motor neuron in both reporters, and a subset of D-type motor neurons in one fosmid reporter (Figure 21D). We did not detect EAT-4 expression in AVL using either reporter.
We next restored the expression of EAT-4 using different neuronal subtype promoters in *ubr-1; eat-4* double mutants, and quantified the expulsion frequency in respective transgenic strains. We found that restoring the expression of EAT-4 in AVL, DVB and D-type GABAergic motor neurons in *ubr-1; eat-4* mutants fully reverted expulsion frequency back to that of the *ubr-1* mutants (N=10, p < 0.0001) (Figure 21C). Expression of EAT-4 in D-type GABAergic motor neurons alone did not revert the Exp frequency back to that of *ubr-1* mutants (Figure 21C).

Together, these observations suggest potential co-transmission of GABA and glutamate in the DVB motor neuron. Moreover, aberrantly increased synaptic glutamate synthesis and/or vesicular loading in the DVB GABAergic motor neuron may underlie defective GABA signaling in *ubr-1* mutants.
3.4.5 *ubr-1* Mutants Exhibit Reduced DVB Neuronal Activity

Because of the functional requirement of UBR-1 in the DVB motor neuron, we investigated how *ubr-1* regulates DVB motor neuron activity? To address this question, I performed in vivo calcium imaging of the DVB neuron in animals during the defecation motor program, and compared the frequency of DVB activation and amplitude of DVB-Ca\(^{2+}\) transients.

In wild-type animals, the DVB neuron exhibited periodic large intracellular Ca\(^{2+}\) spikes that tightly correlate with the defecation motor program. The rise of each Ca\(^{2+}\) spike immediately followed each pBoc step, peaked before the onset of Exp step, and returned to the baseline after the Exp step (Figures 22A, 22E and 22F). Hence, the DVB motor neuron exhibits correlative rhythmic active pattern with Exp motor set. *ubr-1* mutants exhibited significant reduction in the amplitude (Figures 22B and 22E) and frequency (Figures 22B and 22F) of the Ca\(^{2+}\) transients in DVB (N=14, p < 0.0001). These results correlated with reduced expulsion frequency in *ubr-1* mutants (Figure 20E). Importantly, restoring UBR-1 expression in the AVL, DVB and D-type GABAergic motor neurons rescued the Ca\(^{2+}\) transient profile to that of wild-type animals (N=14, p < 0.0001) (Figures 22C, 22E and 22F), whereas a restored expression of UBR-1 in the D-type GABAergic motor neurons alone did not show any rescue (Figures 22D, 22E and 22F). These results imply that UBR-1 regulates activity of the GABAergic DVB neuron.
Figure 22: *ubr-1* Mutants Exhibit Reduced DVB Neuronal Activity
Figure 22: *ubr-1* Mutants Exhibit Reduced DVB Neuronal Activity

A-D) By calcium imaging analyses, *ubr-1* mutants exhibited reduced DVB activity, which was rescued to wild-type level by restoring UBR-1 expression in AVL, DVB, and D-type GABA motor neurons (*Punc-47::UBR-1*), whereas expression in the D-type GABA motor neurons alone (*Punc-25::UBR-1*) did not rescue. Left panels: Representative calcium traces in DVB neuron, shown as the GFP/RFP ration over time. Right panels: Raster plots of all calcium traces; each horizontal line corresponds to the GFP/RFP ratio of one animal of the respective genotypes during the 3 minute recording. E) Quantification of the frequency of DVB activation during 3 minute recording. *ubr-1* mutants exhibited reduced frequency of DVB activation; this defect was rescued by restoring UBR-1 in AVL and DVB motor neurons. F) Quantification of the mean amplitude of Ca\(^{2+}\) spikes in DVB neurons. In *ubr-1* mutants, the amplitude was reduced, and such a defect was rescued by restoring UBR-1 in expression in AVL and DVB motor neurons.

N=10-15 animals. ***p < 0.0001 by Mann Whitney test.
3.4.6 Reducing Free Glutamate, but not Vesicular Glutamate, Restores DVB Activity Patterns in ubr-1 Mutants

Since removing GOT-1 and EAT-4, components of glutamate signaling, enhance and suppress, respectively, the expulsion defects of ubr-1 mutants, I examined how they may modify the DVB motor neuron activation profile in ubr-1 mutant background.

Despite a lack of rescue of Exp frequency in ubr-1; got-1 double mutants, the DVB neuron exhibited restored frequency and amplitude of Ca\(^{2+}\) transients (N=12, p < 0.0001, compared to ubr-1 mutants) (Figures 23D, 23E and 23F). There was no significant difference in DVB Ca\(^{2+}\) transients between ubr-1; got-1, got-1 mutants and wild type animals (Figure 23C, 23E and 23F). Therefore, while the reduced DVB motor neuron activity was rescued by removing GOT-1, GABA signaling that induces defecation remains dysfunctional.

The ubr-1; eat-4 double mutants, on the other hand, did not exhibit any rescue of calcium transient amplitude (Figure 24D and 24F), but did exhibit an increased calcium transient frequency (N=15, p < 0.001, compared to ubr-1 mutants) (Figure 24E) in the DVB motor neurons. Hence, restoring DVB motor neuron activity alone was insufficient to restore functional GABA signaling at the defecation motor circuit, but removing potential vesicular glutamate loading in DVB, while not restoring DVB’s activity profile, was sufficient to restore functional GABA signaling.
Figure 23: Reducing Cytosolic Glutamate Levels Restores DVB Activity
Figure 23: Reducing Cytosolic Glutamate Levels Restores DVB Activity

A-D) By calcium imaging analyses, got-1; ubr-1 mutants exhibited restored amplitude and frequency of DVB calcium transients. Restoring GOT-1 expression in the AVL and DVB neurons in got-1;ubr-1 reverted DVB activity profile back to that of ubr-1. Left panels: Representative calcium transient traces indicated by the GFP/RFP ratio in wild type, ubr-1, got-1 and ubr-1; got-1 animals over time. Right panels: Raster plots of calcium traces; each horizontal line corresponded to the GFP/RFP ratio of an animal of the respective genotypes as in left panels during the 2 minute recording of one animal. E) Quantification of the frequency of DVB activation during 2 min recording. got-1 suppresses reduced frequency of DVB activation in ubr-1 mutants. F) got-1 rescued the DVB calcium transient changes in ubr-1 mutants. ubr-1; got-1 mutants have calcium transient level similar to that of wild-type animals.

N=10-15 animals. ***p < 0.0001 by Mann Whitney test.
Figure 24: Reducing Vesicular Glutamate Restores DVB Activity Frequency, but not Amplitude.
Figure 24: Reducing Vesicular Glutamate Restores DVB Activity Frequency, but not Amplitude.

A-D) By calcium imaging analyses, eat-4; ubr-1 mutants exhibited reduced amplitude of calcium transients in DVB. Left panels: Representative calcium transient traces in DVB neuron indicated by the GFP/RFP ration in wild-type, ubr-1, eat-4 and ubr-1; eat-4 animals over time. Right panels: Raster plots of calcium traces; each horizontal line corresponds to the GFP/RFP ratio of an animal of the respective genotypes during the 3 min recording.

E) eat-4 suppresses reduced frequency of DVB activation in ubr-1 mutants.

F) eat-4 fails to suppress reduced amplitude of DVB calcium transients in ubr-1 mutants. eat-4 mutants exhibited slightly reduced DVB calcium transient amplitude.

N=10-15 animals. **p < 0.001, *p < 0.05 by Mann Whitney test.
Based on these results, I propose a dual functional model for UBR-1 in a dual neurotransmitter neuron that may co-release opposing neurotransmitters glutamate and GABA. First, UBR-1 modulates GABA neurotransmitter release by maintaining synaptic glutamate levels (Figure 25), and second, UBR-1 also mediates GABAergic neuronal activity by affecting intracellular free glutamate levels.

Figure 25: Model: A Model for UBR-1-Mediated Glutamate Homeostasis and GABA/Glutamate Balance in GABAergic Neurons.
3.5 Discussion

Excitatory-inhibitory synaptic imbalance has been implicated in several neurodevelopmental disorders. However, molecular mechanisms that regulate the balance between E/I neurotransmitters are not well established. Previously we showed that UBR-1 maintains synaptic glutamate homeostasis by regulating the activity of metabolic enzyme GOT-1. In this study, using a simple GABAergic motor circuit that controls the defecation motor program in *C. elegans*, we demonstrate that in some GABAergic neurons, UBR-1-mediated glutamate homeostasis regulates GABA signaling, and this is likely achieved through maintaining the balance between GABA and glutamate release in these neurons. These data suggest a previously unknown mechanism by which UBR-1-mediated glutamate homeostasis is able to regulate GABA neuron activity as well as GABAergic synaptic transmission.

3.5.1 UBR-1-mediated Regulation of Neurotransmitter Balance

GABA functions as an excitatory neurotransmitter in *C. elegans* to regulate enteric muscle contraction during defecation (Beg and Jorgensen, 2003). Decreased GABA synthesis or release contributes to a near complete loss of the expulsion step in the defecation cycle (McIntire et al., 1993a). In *ubr-1* mutants, the frequency of enteric muscle contraction and expulsion were decreased, whereas other motor steps in the same program were not affected, indicating a decreased GABA signaling in the motor circuit that governs the Exp step.
Previous studies have demonstrated that defective GABA signaling by AVL and DVB motor neurons causes the specific loss of the Exp step. Our amino acid analysis showed an increased, instead of a decreased GABA level in ubr-1 mutants, ruling out the cause of ubr-1 defects in GABA signaling being the reduction of GABA synthesis. Instead, it points towards a defective GABA release being the cause.

How may GABAergic neurons that lack UBR-1 release less GABA? One hypothesis is that since vesicular glutamate and GABA transporters (VGLUT3 and VGAT) are co-expressed in GABAergic neurons, VGLUT3 may compete with GABA vesicular loading, and subsequently dampen GABA release. This model predicts a possible competition between vesicular GABA and glutamate transporters, resulting in reduced uptake of GABA in ubr-1 mutants. This is consistent with the finding that removing EAT-4 was capable of rescuing the GABA signaling defects in ubr-1 mutants.

Which mechanism could explain a potential homeostasis between the vesicular loading of two distinct neurotransmitters? Previous work has shown that GABA uptake depends equally on a chemical gradient (ΔpH) and a membrane potential (ΔΨ) (Kish et al., 1989), whereas VGLUTs that take up glutamate depend primarily on the ΔΨ (Tabb et al., 1992). Therefore I hypothesize that in ubr-1 mutants, an increased glutamate synthesis promotes increased glutamate uptake, which dissipates ΔΨ, reducing GABA uptake (Figure 25B). Therefore, regulation of glutamate homeostasis by UBR-1 also plays a regulatory role in the balance of vesicular loading of two neurotransmitters of opposing physiological properties.
3.5.2 Role of Glutamate in Maintaining GABAergic Neuronal Excitability

We show that in *ubr-1* mutants, DVB GABAergic motor neuron exhibited reduced activity, and these defects were suppressed by reducing the free glutamate level upon the removal of the glutamate-synthesizing enzyme GOT-1. Despite reducing the glutamate level and restoring DVB’s activity, *got-1* enhanced *ubr-1*’s expulsion defects. Glutamate is the sole precursor for GABA synthesis (Miller et al., 1978), and in both *got-1* and *got-1; ubr-1* mutants, GABA level was reduced. Therefore, removing GOT-1 could further reduce synaptic GABA loading and release, causing enhancement, instead of suppression of *ubr-1*’s defecation defects (*Figure 25C*). In other words, UBR-1 affects DVB motor neuron activity, and DVB-depending GABAergic signaling via two independent mechanisms.

How does glutamate homeostasis affects the DVB activity level? One possibility is that DVB activation is regulated by glutamate receptors (such as the case in the AVE-AVA circuit in locomotion). In this scenario, blocking glutamate release in *ubr-1* mutants should restore DVB activity pattern. However, *ubr-1; eat-4* mutants still exhibited aberrant DVB activity profile. These results suggest that intracellular glutamate is required to maintain DVB activity. The fact that removing GOT-1 led to a full rescue of DVB’s activity profile, suggest that increased intracellular glutamate in DVB is responsible for its reduced activity pattern. Glutamate may function as a secondary messenger, or a key metabolite to affect metabolism and function of the cell. Indeed, in pancreatic beta cells, maturation of insulin requires the acidification of secretory granule cells, a process that is affected by intracellular glutamate concentration (Gammelsaeter et
al., 2011; Maechler and Wollheim, 1999). Understanding the precise role and mechanism of glutamate as an intracellular messenger requires further investigation.

3.5.3 Functional Model for UBR-1

Based on our results and previous literature, we propose a dual functional model for UBR-1 in potentiating neuronal activity and mediating neurotransmitter balance. 

*ubr-1* mutants exhibit changes in GABAergic neuronal activity and decreased GABA release. In *ubr-1* mutants, an increased glutamate level may enhance glutamate uptake by VGLUT. The increased consumption of membrane potential gradient leads to a reduced GABA uptake, because VGAT function requires both proton and membrane potential gradients (Figure 25B). On the other hand, increased cytosolic glutamate reduces GABAergic neuronal activity through unknown mechanisms.

Decreased cytosolic glutamate levels in *ubr-1; got-1* mutants causes further reduction in GABA levels, because glutamate is the only precursor for GABA and thereby causes enhancement of GABA signaling phenotypes in *ubr-1* mutants (Figure 25C). On the other hand, by decreasing cytosolic glutamate levels, *got-1* restores GABAergic neuronal activity, without restoring the functional deficit of GABA signaling.

Upon removing *eat-4*, *ubr-1* mutants show restored expulsions but reduced GABAergic neuronal activity. Removing EAT-4 relieved the competition between the
two transporters by reducing glutamate uptake, and restoring GABA signaling in \textit{ubr-1} mutants (Figure 25D). However due to increased cytosolic glutamate levels mutations in \textit{eat-4} has no effect on GABAergic neuronal activity.

In future studies, identifying the mechanism underlying the role of glutamate as an intracellular messenger will help further dissect how excitatory-inhibitory synapses are established and maintained.

3.5.4 Synaptic Co-existence of Glutamate and GABA

In this study we provide the first line of evidence for co-existence of GABA and glutamate in \textit{C. elegans} GABAergic neurons. The fate of glutamate released from the DVB neuron requires further investigation. DVB motor neurons form neuromuscular junctions with the enteric muscles (White et al., 1986), and few glutamate gated chloride channels are known to be expressed in body wall and enteric muscles (Hunt-Newbury et al., 2007). We hypothesize that one potential role for glutamate co-release is to activate inhibitory glutamate receptors on the enteric muscles and function as an inhibitory neurotransmitter, while GABA functions as excitatory neurotransmitter by activating GABA-gated cation channel EXP-1, to fine-tune the activation pattern of the muscles.

3.5.5 Implications for Neurodevelopmental Disorders

E-I imbalance or reduced GABAergic inhibition is a common pathophysiological mechanism in several neurodevelopmental disorders including autism, epilepsy, Rett
syndrome, mood disorders and Fragile X syndrome (Eichler and Meier, 2008; Marin, 2012). Loss of function of human UBR1 causes Johanson-Blizzard Syndrome (JBS). JBS patients exhibit pancreatic insufficiency and severe cognitive impairments (Johanson and Blizzard, 1971; Zenker et al., 2005). We propose that UBR-1 mediated excitatory-inhibitory neurotransmitter balance may underlie the cognitive impairments in JBS patients. Therefore, E-I imbalance resulting from deregulated UBR-1 signaling may contribute to other neurodevelopmental disorders associated with reduced functional inhibition. UBR-1 might represent an appealing pharmacological target for maintaining the E-I balance.

3.6 Acknowledgements

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Chapter 4 Conclusions and Future Perspectives
4.1 Conclusions

Overall activity of the central nervous system (CNS) is established by the physiological activity of excitatory and inhibitory neurotransmission. Despite the importance of the functional excitatory-inhibitory (E-I) balance, mechanisms and molecular players that maintain and modulate this homeostasis remain poorly understood. An E-I imbalance or reduced GABAergic inhibition have emerged as a common pathophysiological feature of several neurodevelopmental disorders, including autism, epilepsy, Rett syndrome, mood disorders and Fragile X syndrome (Eichler and Meier, 2008; Marin, 2012). This body of work is centered UBR1, an E3 ubiquitin ligase in which mutations have been specifically associated with the neurodevelopmental disorder Johanson-Blizzard Syndrome (JBS) in human. The cellular and molecular mechanisms by which mutations in UBR1 lead to JBS remain elusive. The main goal of this research is, using the C. elegans genetic model, to 1) determine the physiological function of UBR-1 by examining the phenotypes of ubr-1 mutants, and 2) uncover the signaling pathways that are negatively regulated by UBR-1 through identifying ubr-1 genetic modifiers.

I hereby present results from these two aims and their implications. First, my studies reveal that loss-of-function mutations in the C. elegans UBR-1 lead to behavioral and physiological defects in a motor circuit that regulates body curvature during backward locomotion. Through a combination of metabolomics, behavioral genetics and functional imaging, I identified a signaling pathway that is negatively regulated during this process. Specifically, the absence of UBR-1 leads to an increased glutamate level,
and a dys-regulated glutamate signaling between the AVE and AVA premotor interneurons that disrupts the phase lag between A-type motor neurons. The inability of \textit{ubr-1} mutants to flex due to in-phase activation of ventral- and dorsal-muscle innervating motor neurons, is rescued by either reducing glutamate synthesis or synaptic vesicle glutamate loading in the AVE premotor interneurons, or, removing the glutamate-gated chloride receptors in the postsynaptic AVA premotor interneurons. Together, I propose a novel regulatory role for the E3 ligase UBR-1 in maintaining synaptic glutamate homeostasis. Next, this body of work further reveals a previously unknown mechanism by which UBR-1-mediated glutamate homeostasis regulates GABAergic synaptic transmission. I show that in another small motor circuit, where a GABAergic motor neuron controls the defecation cycle through innervating the anal muscles, the loss of UBR-1 leads to reduced GABAergic synaptic transmission and subsequently, reduced defecation. Surprisingly, these defects could be rescued by removing VGlut3/EAT-4 from these GABAergic neurons, indicating a role of glutamate homeostasis in GABAergic neurons.

Glutamate is the primary neurotransmitter at the vertebrate CNS (Ottersen and Storm-Mathisen, 1984), and many disorders such as ischemia, neurodegenerative diseases, and neurodevelopmental disorders have been associated with glutamate dysfunction. I propose that dysfunctional glutamate homeostasis mediated by UBR1 may underlie both systemic and neurodevelopmental defects in JBS patients. Therefore, E-I imbalance resulting from deregulated UBR-1 signaling may contribute to other
neurodevelopmental disorders associated with reduced functional inhibition. UBR-1 might represent an appealing pharmacological target for maintaining the E-I balance. In the next section, I summarize remaining questions, future directions of these investigations, and potential approaches to address them.

### 4.2 Future Perspectives

#### 4.2.1 Examining the Interaction Between UBR-1 and GOT-1 or EAT-4

Since UBR-1 is an E3 ubiquitin ligase, its function involves physical interaction and ubiquitination of its substrates. Results from our current studies have demonstrated elevated GOT activity in *ubr-1* mutants. A key remaining question is whether GOT-1, which was identified as a genetic modifier of UBR1, is directly regulated by UBR-1 as a substrate. If GOT-1 is a direct target of UBR-1, it should fulfill several criteria: 1) GOT-1 overexpression mimics, fully or partially, *ubr-1* defects, in a UBR-1-dosage sensitive manner; 2) GOT-1’s level, temporally or spatially is increased or becomes less restricted in *ubr-1* mutants; 3) GOT-1 physically interacts with, and is ubiquitinated by UBR-1.

To determine if GOT-1 overexpression could mimic *ubr-1*’s phenotypes in a dosage-dependent manner, GOT-1 will be overexpressed in either the premotor neuron AVE or the GABAergic neurons AVL/DVB in wild type and *ubr-1/+* animals. Body curvature and expulsion frequency will be quantified.

To investigate GOT-1’s expression level and pattern in *ubr-1* mutants, I will generate a functional tagged GOT-1 translational reporter under its endogenous promoter.
This reporter will be placed in all alleles of \textit{ubr-1} mutants. The protein level change will be quantified biochemically by western blot analyses using the tag and will be compared to appropriate controls. A caveat of the reporter analyses has been the copy number of transgenic arrays; a single copy insertion of the translational reporter, as well as endogenous genomic editing by the CRISPR/Cas9 system has become possible to mitigate this caveat.

If GOT-1 exhibits a reliable change in either expression level or pattern in \textit{ubr-1} mutants, physical interaction between UBR-1 and GOT-1 will be tested, starting with using the heterologous cell culture system. Tagged-UBR-1 and GOT-1 constructs will be co-transfected into the HEK293T cells, and reciprocal co-immunoprecipitation will be performed to determine if both interact in the same protein complex. UBR-1’s substrates likely become more unstable as a result of UBR-1 overexpression. Proteasome inhibitors (MG132) will be applied prior to harvesting transfected cells to stabilize the complex. For the ubiquitination assay, tagged UBR-1, GOT-1, and ubiquitin K63R (a Ub variant that is less efficiently recognized by the proteasome) will be co-transfected in HEK293T cells. The substrate will be immuno-precipitated and probed for the presence of ubiquitin. Pre-treating transfected cells with proteasome inhibitors will further improve the sensitivity for detecting poly-ubiquitination.

\textbf{4.2.2 Synaptic C-existence of VGAT and VGLUT3}

I have shown that vesicular glutamate transporter EAT-4 is expressed in \textit{C. elegans} GABAergic neuron DVB. Albeit a low expression level, through the defecation
assay, a physiological relevance of EAT-4 in the DVB/AVL neurons has been demonstrated.

The vesicular glutamate transporter EAT-4 and the vesicular GABA transporter UNC-47 might be co-present in the same vesicle pool, or in different pools (synapses) by the same neuron. Co-existence of VGLUT and VGAT can be tested using immunogold electron microscopy. Immunogold labeling technique in conjunction with electron microscopy provides a method for subcellular localization of endogenous and exogenous proteins. Exogenous protein tags, such as the green fluorescent protein (GFP), can be utilized as epitopes for immunogold labeling following high-pressure freezing of *C. elegans* (Rostaing et al., 2004). Transgenic strains that carry GFP tagged VGLUT3 and VGAT can be used for high-pressure freezing and immunogold labeling.

4.2.3 Dissecting the Role of Neurotransmitter Glutamate in GABAergic Neurons

In the motor circuit that regulates defecation, the fate of glutamate after its release from VGLUT-containing vesicles in DVB is unknown. One hypothesis is that it may activate glutamate receptors on either GABAergic neurons and/or enteric muscles to modulate GABAergic signaling. The fact that *eat-4* suppresses the defecation defects of *ubr-1* mutants suggests that glutamate released from DVB may function as an inhibitory neurotransmitter in the defecation motor circuit. There are six putative glutamate gated chloride channels - AVR-14, AVR-15, GLC-1, GLC-2, GLC-3, and GLC-4 - in *C. elegans*. Glutamate gated chloride channels are closely related to mammalian glycine receptors and are important for controlling locomotion, feeding and mediating sensory
inputs into behaviors in nematodes (Wolstenholme, 2012; Wolstenholme and Rogers, 2005). Indeed, some of them have been reported to be expressed in the enteric muscles.

To test this hypothesis, double mutant animals can be generated between *ubr-1* and glutamate gated chloride channel mutants. Frequency of expulsions will be quantified in all the double and single mutants. Both enteric muscle imaging (see the next section) and GABAergic motor neuron calcium imaging will be performed in these double mutants. Should any chloride channel mutants show genetic rescue of the defecation circuit, the precise tissue requirement of the respective chloride channel encoding genes will be determined.

### 4.2.4 *in vivo* Calcium Imaging of Enteric Muscle in *ubr-1* and its Suppressors

In *eat-4* single mutants and *ubr-1; eat-4* double mutants, the amplitude of DVB GABAergic motor neuron activity was reduced, but expulsion frequencies were normal. How is a neuron with reduced activity level still able to release enough GABA to maintain normal cycle of enteric muscle activation? One potential explanation is that in *eat-4* mutants, there is an increased vesicular uptake of GABA due to the reduction of competition between GABA and glutamate uptake (*Figure 25D*). The strength of GABA signaling can be estimated from the activity level of DVB’s postsynaptic enteric muscles. *in vivo* calcium imaging reporter of the enteric muscle can be generated by expressing the GCaMP6 sensor under the *Pexp-1* promoter. Quantification of the frequency and amplitude of muscle activity, and a correlation with GABAergic motor neuronal activity will provide clues to the nature of GABA signaling defects in *ubr-1* mutants, and
mechanisms through which the *ubr*-1 suppressors exert their effect.

### 4.2.5 Role of UBR-1 mediated Glutamate Homeostasis in the Pathophysiology of JBS

In a clinical study, Fallahi and colleagues collected blood plasma samples from a JBS patient harboring a nonsense mutation in UBR1 gene and display severe disease symptoms including cognitive impairments (Fallahi et al., 2011). In the patient blood samples, GOT and ALT levels, glutamate metabolic enzymes, were increased (~two fold). In another study blood test results of a patient with a missense mutation in UBR1 gene and with normal range of intellectual ability showed slight increase in the levels of GOT and ALT (Almashraki et al., 2011). In our study, I demonstrate that UBR-1 regulates the activity of GOT; therefore, it will be interesting to test more blood samples from JBS patients and to examine if there is correlation between GOT levels and specific JBS phenotypes, such as the severity of cognitive impairments.

In addition to its well-known function in the nervous system, there is growing evidence for glutamate signaling in non-neuronal tissues, including in the heart, kidney, skin, bone, lung, liver, stomach, intestine, and pancreas (Danbolt, 2001; Julio-Pieper et al., 2011; Skerry and Genever, 2001). In the pancreas, several components of glutamate signaling have been identified in the pancreatic islets of Langerhans, consisting of insulin secreting β-cells and glucagon secreting α-cells (Hayashi et al., 2003; Inagaki et al., 1995; Weaver et al., 1996). Since pancreatic exocrine insufficiency is a severe and common symptom present in all JBS patients, UBR-1-mediated abnormal glutamate...
homeostasis may underlie this symptom. The role of glutamate metabolism in the pancreatic dysfunction can be investigated by measuring glutamate levels in the pancreatic tissue of JBS patients, or, from the UBR1-/- mouse model, which also exhibits pancreatic insufficiency. Further investigation is needed to determine whether turning down glutamate signaling in neuronal or non-neuronal tissues can improve disease symptoms in both mammalian models and patients.

Glutamate dysfunction has been associated with many human conditions including ischemia, neurodegenerative diseases, and neurodevelopmental disorders. Above proposed experiments aim will not only further our understanding of UBR-1-mediated glutamate homeostasis in the pathophysiology of JBS, but may also shed light on new therapeutic pathways to address glutamate dysfunction and excitatory-inhibitory imbalance in general.
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