INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA

UMI®
800-521-0600

RICE UNIVERSITY

THE DROSOPHILA *inebriated/rosA* TRANSPORTER: DUAL ROLES IN THE CONTROL OF NEURONAL EXCITABILITY AND OSMOTIC STRESS RESPONSE

by
XI HUANG

A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE
DOCTOR OF PHILOSOPHY

APPROVED, THESIS COMMITTEE

Michael Stern, Associate Professor
Biochemistry and Cell Biology

Bonnie Bartel, Assistant Professor
Biochemistry and Cell Biology

Kathleen M. Beckingham, Professor
Biochemistry and Cell Biology

George N. Bennett, Professor
Biochemistry and Cell Biology

Joan E. Strassmann, Professor
Ecology and Evolutionary Biology

Houston, Texas
January, 2000

ABSTRACT

The Drosophila *inebriated/rosA* Transporter: Dual Roles in the Control of Neuronal Excitability and Osmotic Stress Response

by

Xi Huang

Members of the Na^+/Cl^- dependent neurotransmitter transporter family perform the re-uptake of neurotransmitter released into synapses and thus control the magnitude and duration of synaptic transmission. The importance of these molecules in the nervous system is further demonstrated by the fact that Na^+/Cl^- dependent neurotransmitter transporters are targets of psychoactive drugs such as cocaine and Prozac, and are linked to diseases such as Autism. In addition, studies in cultured kidney cells have shown that these molecules also accumulate osmolytes and maintain normal cellular structure and function under hypertonic stress. Inhibition of neurotransmitter transporters in the kidney can cause kidney failure.

In this thesis is described the positional cloning of the Drosophila *inebriated(ine)/rosA* gene that encodes a putative Na^+/Cl^- neurotransmitter transporter. Mutations in this gene cause increased neuronal excitability at the neuromuscular junction and photoreceptor cells, as well as increased sensitivity to a hypertonic environment. The *ine/rosA* gene produces two proteins that differ only in their N-terminal intracellular domain. One form, *ine/rosA*-l, contains an additional unique sequence of 313 amino acids at the N-terminus compared to the second form, *ine/rosA*-s. The two transcripts of this gene have very similar distribution patterns in Drosophila embryos. Functional studies on the *ine/rosA*

gene include various attempts to identify the substrate(s) for the *ine/rosA* transporter and the localization of the *ine/rosA* products. Overexpression of *the ine/rosA*-s cDNA in the Malpighian tubules, the Drosophila analog of mammalian kidney, enhanced Drosophila salt resistance.

These studies have established that the *ine/rosA* gene plays a vital role in the regulation of both neuronal excitability and water and salt metabolism, and have laid a foundation for further elucidation of neurotransmitter transporter function that may lead to new targets and clues for the treatment of disorders in the nervous system and the kidney.

Acknowledgments

I would like to thank my advisor Dr. Michael Stern whose lectures inspired me, who has provided me with opportunities for learning and training and who has been supportive in many aspects of my research and my life. I am very appreciative of members of my committee, who have made valuable suggestions on the direction and technicalities of my research project.

Many thanks go to the postdoctoral fellows, technicians, graduate students and undergraduate students in the Stern lab and Beckingham lab whose names may or may not appear in my thesis. They have been extremely helpful in my research effort.

I would also like to thank my wife, Yan Jin, who has become very understanding on both the long hours I put into research and the subject of neurotransmitter transporters.

Finally, a little pat on the back to myself as well.

Table of Contents

List of Figures

List of Tables

Abbreviations

3': The end of nucleic acid where the 3' position of the sugar residue is not connected to another one.

5': The end of nucleic acid where the 5' position of the sugar residue is not connected to another one.

cDNA: complementary DNA containing mRNA sequences.

DAT: dopamine transporter

GABA: γ-aminobutyric acid

GAT: GABA transporter

kb: kilo base pairs of nucleic acid

kDa: kilo Dalton

LB: Luria-Bartani medium for bacteria

MAPK: mitogen-activated protein kinase

NET: norepinephrine transporter

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PKA: protein kinase A

PKC: protein kinase C

RFLP: restriction fragment length polymorphism

RT-PCR: reverse transcriptase PCR. (Use PCR to amplify reverse transcription products from mRNA templates)

SDS: sodium dodecyl sulfate

SERT: serotonin transporter

UTR: untranslated region in mRNA

Chapter 1. Introduction

1. Two major roles of neurotransmitter transporters

i. Neurotransmitter transporters

A. Neurotransmitters and their transporters

Chemical synaptic transmission is a process used by excitable cells, such as neurons and muscle cells, to communicate with each other. In this process, neurotransmitters, such as GABA, dopamine, norepinephrine and glutamate, are released from the presynaptic cell, traverse the synapses and bind to their receptors on the postsynaptic cell to trigger a postsynaptic response. Synaptic transmission is usually terminated by the neurotransmitter transporters that are located in neuronal and glial plasma membranes and clear the released neurotransmitters from the synapse (Amara and Kuhar, 1993; Beckman and Quick, 1998; Nelson, 1998; Saier, 1994).

Plasma membrane neurotransmitter transporters have structural features and preferences of electrochemical gradients which are distinct from those of vesicular transporters, which pack neurotransmitters into synaptic vesicles (Hediger, 1994). Several amino acids, such as glycine, glutamate and proline, are used as neurotransmitters in the nervous system. Plasma membrane transporters for these transmitter amino acids have very strict substrate specificity, in contrast to general amino acid transporters (Nelson, 1998). Due to the large number of neurotransmitters, their diverse functions and the specificity of the transporters, it is estimated that there are at least 30 plasma membrane neurotransmitter transporters in mammalian brain (Liu *et al.*, 1992b).

Neurotransmitter transporters can carry their substrates using energy stored in Na^+ electrochemical gradient established by primary ion pumps such as Na^+-K^+ exchange ATPase (Kanner, 1983). Depending on their co-transporting ions, the 12

transmembrane domain neurotransmitter transporters can be divided into Na^+/Cl^- dependent transporters such as GABA, norepinephrine and serotonin transporters, and Na^+/K^+ dependent transporters such as the glutamate transporter. These two groups of transporters have distinct primary and putative secondary structures.

B. Structure-function relationships among neurotransmitter transporters

From hydropathy analysis (Guastella *et al.*, 1990) and analogy to structurally similar sugar transporters (Kaback *et al.*, 1997), neurotransmitter transporters are proposed to contain 12 transmembrane domains (figure 1.1). This 12 transmembrane domain topology was supported by experimental results from glycosylation scanning mutageneses on a GABA transporter (Bennett and Kanner, 1997), as well as cysteine-modification assays on a human serotonin transporter (Chen *et al.*, 1997). The N-terminus and the C-terminus of human norepinephrine and dopamine transporters were localized to the cytosolic side the membrane using fluorescent and electron microscopic immunostudies (Bruss *et al.*, 1995; Melikian *et al.*, 1994; Nirenberg *et al.*, 1996).

Even though there are certain hallmarks identified, such as the LXNWRFPXXXYXNGGGXF sequence in the first transmembrane domain and the following loop, there is no common motif for substrate transport detected among neurotransmitter transporters (Liu *et al.*, 1992b; Soehnge *et al.*, 1996). Studies have identified sequences and structures in some or most neurotransmitter transporters that are important for substrate binding and translocation, as well as inhibitor and drug effects (Nelson, 1998).

Site-directed mutageneses and chimeric protein studies have shown that almost every transmembrane domain is involved in the binding of one or more known inhibitors or drugs (Nelson, 1998). The Y 140 of the GABA transporter is

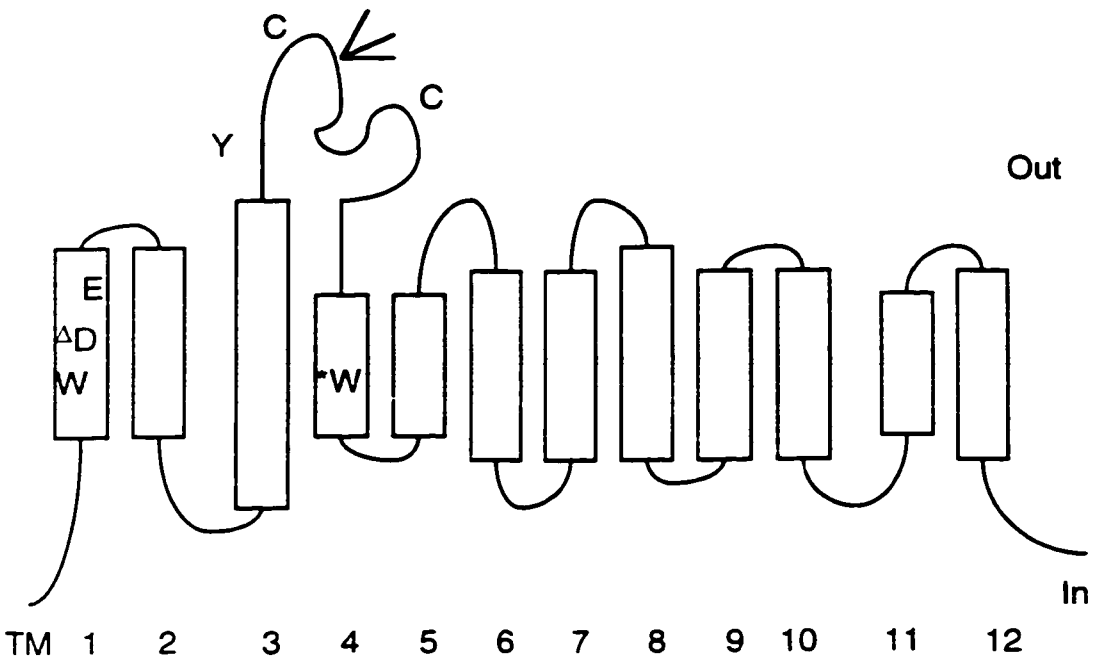


Figure 1.1 The putative structure of Na^+/Cl^- dependent neurotransmitter transporters. There are 12 putative transmembrane domains with internal N and C-termini. Amino acids important in activity such as substrate and ion binding are marked. Amino acids superscripted with "Δ" are specific for monoamine transporters such as dopamine, norepinephrine and serotonin transporters. Those superscripted with "*" are specific for GABA and taurine transporters. For more details see text. Modified from Nelson, 1998.

important for the binding of the amino group in GABA or other substrates (Bismuth *et al.*, 1997), W68 in the first transmembrane domain facilitates the Na^+ ion transport (Mager *et al.*, 1996), E101 is essential for transport activity(Keshet *et al.*, 1995), the tryptophan in the fourth transmembrane domain is important for GABA binding (Kleinberger-Doron and Kanner, 1994) and external loops 4, 5 and 6 are important for GABA and β-alanine binding (Tamura *et al.*, 1995), possibly by forming an external pocket. D79 is implicated in the dopamine affinity of the dopamine transporter (Kitayama *et al.*, 1992), whereas the seventh and eleventh transmembrane domains are important for dopamine translocation (Kitayama *et al.*, 1993). The second extracellular loop of the serotonin transporter, containing two cysteine residues (C200 and C209) that may form a disulfide bond, is crucial to the transport activity (Chen *et al.*, 1997; Stephan *et al.*, 1997). Glycosylation in the external loop between the third and the fourth transmembrane domains is important in the activities of certain neurotransmitter transporters (Liu *et al.*, 1998).

C. Kinetics of neurotransmitter transporters

a. Transport of neurotransmitters

The classical model of neurotransmitter transporter substrate uptake is the stoichiometric model, in which transmitter substrates are cotransported across the membrane with a certain number of Na^+, Cl^- or K^+ ions (figure 1.2 A). For example, the binding of Na^+ or/and Cl^- to the GABA transporter causes conformational changes that allow the binding of substrate GABA (Mager *et al.*, 1993). After the translocation of substrates and ions, neurotransmitter transporters return to their initial state spontaneously to be charged with ions and substrates again (figure 1.2 A). The serotonin transporter returns to the initial state through a proton or K^+ gradient (Rudnick and Clark, 1993). Through Hill co-efficiency estimates, the

stoichiometry of some neurotransmitter transport is as follows: both GABA and dopamine are cotransported with two Na^+ and one Cl^-, one norepinephrine is cotransported with one Na^+ and one Cl^- (Rudnick and Clark, 1993). Mammalian serotonin transport is electroneutral (Galli *et al.*, 1997), and invertebrate serotonin transporter is electrogenic (Bruns *et al.*, 1993; Galli *et al.*, 1997).

Because of the low turnover rate of neurotransmitter transporters, usually about 10/s, they are more effective in catalyzing the uptake of slow acting modulatory neurotransmitters that exert postsynaptic effects through second messenger pathways. For example, pharmacological blockade of GABA transporters affects both the amplitude and decay phases of G-protein coupled $GABA_B$ receptor mediated response, but has little effect on the response of the $GABA_A$ receptor, which is a fast ligand-gated ion channel (Isaacson *et al.*, 1993). However, evidence has emerged showing that the glutamate transporter can act as a diffusion sink and quickly sequester glutamate (Diamond and Jahr, 1997). This method of clearing neurotransmitters allows transporters to function in fast ligand-gated ion channel receptor mediated synapses as well.

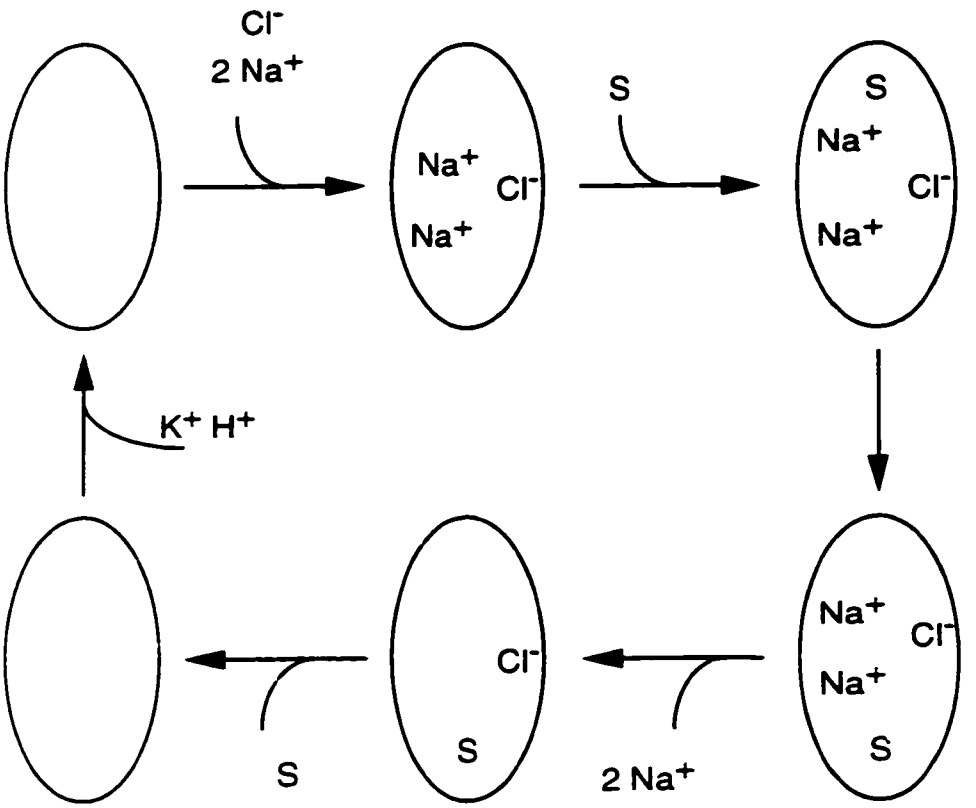
Neurotransmitter transporters can also catalyze the efflux of neurotransmitters from neurons (Attwell *et al.*, 1993; Schwartz, 1987). Reverse uptake (release) of GABA, glutamate and glycine has been reported (Attwell *et al.*, 1993). The Ca^{++}-independent release of GABA was reported to be a major mode of synaptic transmission in toad and catfish horizontal cells (Schwartz, 1987).

b. Ion conductance

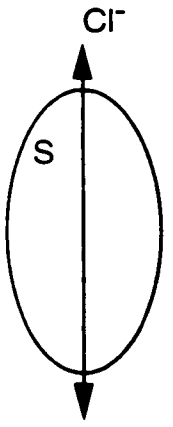
The ion conductance properties of neurotransmitter transporters can not be fully explained by the stoichiometric model derived from kinetic studies. Electrophysiological studies have shown that these transporters are also able to cause non-stoichiometric ion fluxes in a substrate dependent or independent manner.

Figure 1.2 Neurotransmitter transport and ion conductances of neurotransmitter transporter using the GABA transporter as a model. A. The classical stoichiometric model in which two Na^+, one Cl^- and the substrate (S) are cotransported. B. Substrate-dependent non-stoichiometric mode of ion conductance. C. Substrate-independent leak channel mode that may or may not require another protein component (shaded vertical bar on the left). Modified from Nelson, 1998.

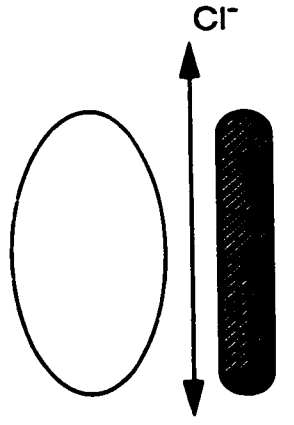
A



B



C



The substrate dependent ion conductances of neurotransmitter transporters resembles that of ligand-gated ion channels (figure 1.2 B), and the conductances thus created are larger than stoichiometric conductances but smaller than the conductances of ligand-gated ion channels (Mager *et al.*, 1994). Like ligand-gated ion channels, the current triggered by ligand binding is voltage dependent and the direction of the current can be reversed at particular voltages. This ligand-gated ion channel mode of ion conductance is detected in the rat GABA transporter (Cammack *et al.*, 1994), human dopamine transporter (Sonders *et al.*, 1997), norepinephrine transporters (Galli *et al.*, 1996), and serotonin transporters (Galli *et al.*, 1997; Mager *et al.*, 1994).

The substrate independent mode of neurotransmitter transporter ion conductance is typically inhibited by the presence of the substrate and called the "leak channel" mode (figure 1.2 C). The leak channel currents are much larger than those of the ligand-gated ion channel and carried by different ions in different transporters. The human norepinephrine transporter (Galli *et al.*, 1995), rat GABA transporter (Cammack *et al.*, 1994) and rat serotonin transporter (Lin *et al.*, 1996) exhibit leak channel currents.

D. Regulation of neurotransmitter transporter function

The PKC (protein kinase C) pathway is the most well-established regulatory mechanism of neurotransmitter transporters. Activation of PKC down-regulates activities of the GABA transporter (Quick *et al.*, 1997), dopamine transporter (Huff *et al.*, 1997)and the glutamate transporter (Casado *et al.*, 1993; Conradt and Stoffel, 1997). PKC can either directly phosphorylate the transporter protein (Conradt and Stoffel, 1997; Huff *et al.*, 1997) or indirectly regulate a transporter by controlling its subcellular localization (Quick *et al.*, 1997).

Other kinase pathways are also involved in the regulation of neurotransmitter transporter function. An increase in cAMP level increases activities of serotonin transporters (Ramamoorthy *et al.*, 1993) and glial glutamate transporter (Gegelashvili *et al.*, 1996). Calmodulin can upregulate serotonin transport (Jayanthi *et al.*, 1994). Activation of the arachidonic acid pathway inhibits glutamate and dopamine transporter activity (Barbour *et al.*, 1989; Trotti *et al.*, 1995; Zhang and Reith, 1996).

Extracellular acidity affects the functional activity of certain neurotransmitter transporters. Lower extracellular pH caused increased serotonin-induced currents from *Xenopus* oocytes expressing serotonin transporter (Cao *et al.*, 1997) and suppressed both forward and reverse glutamate transport by glial glutamate transporter (Billups and Attwell, 1996).

Receptor activity is also a factor regulating neurotransmitter transporter activity. Activation of AMPA/kainate receptors drastically increases glutamate transporter activity (Gegelashvili *et al.*, 1996), whereas the activation of NMDA receptors inhibits the glutamate transporter via the arachidonic acid pathway (Barbour *et al.*, 1989) as a feedback loop for potentiation. The binding of antagonists to certain histamine receptors increases serotonin uptake in human platelets (Launay *et al.*, 1994).

Finally, neurotransmitter transporter activity can be regulated at the transcriptional level as well. Long-term activation of AMPA/kainate receptors causes increased glutamate transporter gene transcription (Gegelashvili *et al.*, 1996); the transcription of a serotonin transporter gene can be upregulated through the cAMP pathway in human placental cells (Ramamoorthy *et al.*, 1993); the transcription of the GABA/betaine transporter gene can be induced by extracellular hypertonicity (see chapter 1, 1, iii, B, d).

E. Versatility of neurotransmitter transporters

Neurotransmitter transporters participate in a wide array of important physiological processes, largely because of the important biological functions of their substrates. The regulation of neuronal excitability in the central nervous system and the involvement in the osmotic stress response in the kidney are discussed in the following sections.

Creatine transporters in various tissues help to move creatine from the bloodstream, where its concentration is high, into tissue cells as an energy source. In platelets, serotonin transporters control the blood serotonin concentration, which regulates blood vessel constriction. Norepinephrine transporters regulate noradrenergic transmission in the heart (Galli *et al.*, 1996). Monoamine transporters are also present in the placenta where they transport placental amines (Galli *et al.*, 1996).

In the nervous system, neurotransmitter transporters can regulate intracellular pH. This ability was first identified in the glutamate transporter, which may co-transport H^+ or OH^- and cause intracellular acidification (Bouvier *et al.*, 1992). Other transporters such as the GABA transporter were also reported to be able to carry inward H^+ currents (Cao *et al.*, 1997).

ii. Regulation of neuronal excitability

A. Control of the magnitude and duration of neurotransmitter effects

The fundamental function of neurotransmitter transporters is to control neurotransmitter levels in the synapse and thereby regulate synaptic transmission (Zorumski *et al.*, 1996). The effect of a neurotransmitter transporter is not only felt in the synapse where the transporter is present, but also in areas to which the neurotransmitter substrate can diffuse (Isaacson *et al.*, 1993). Both short-term blockage of neurotransmitter transporters by inhibitors and long-term reduction of

neurotransmitter transporter functions by genetic approaches exert huge impacts on neuronal excitability.

In rat, blocking the serotonin transporter with inhibitors such as cocaine prolongs both the peak and the decay of the postsynaptic potentials (Pan and Williams, 1989). The inhibition of the glutamate transporter increases both the amplitude and duration of NMDA glutamate receptor response (Hestrin *et al.*, 1990). The effect of GABA transporter inhibitors on GABA mediated synaptic transmission is discussed in section 1, i, C, a of this chapter.

The long-term effect of the disruption of neurotransmitter function is manifested in the observation that dopamine knockout mice have lower dopamine receptor D1 and D2 gene expression (Giros *et al.*, 1996), and in neurological defects caused by aberrations on neurotransmitter transporter genes discussed below.

B. Drugs targeting neurotransmitter transporters and disorders caused by aberrations on these molecules

Monoamine transporters, such as dopamine, norepinephrine and serotonin are involved in many neurological phenomena such as mood, depression, panic disorders, attention deficit hyperactive disorder (Gainetdinov *et al.*, 1999), and drug abuse. They are the targets of drugs such as cocaine, amphetamines and various antidepressants such as Prozac (Giros *et al.*, 1996; Pan and Williams, 1989; Ramamoorthy and Blakely, 1999). Serotonin transporters in Drosophila also are affected by cocaine (Corey *et al.*, 1994; Demchyshyn *et al.*, 1994).

A truncated version of the serotonin transporter is found in some Autism patients (Holden, 1997). Reduction on hippocampal reverse GABA transport was implicated in epileptic seizures (During *et al.*, 1995). Dopamine transporter knockout mice are insensitive to cocaine and amphetamine and are

hyperlocomotive (Giros *et al.*, 1996). Serotonin transporter knockout mice are less sensitive to 3, 4- methylenedioxymethamphetamine ("Ecstasy") (Bengel *et al.*, 1998).

The drugs that target these molecules and neurological disorders caused by alterations in neurotransmitter transporter genes further demonstrate the importance of this family of neurotransmitter transporters in the nervous system.

iii. Overview of water and salt metabolism and osmotic stress response

A. Water reabsorption in animals

It is critical for animals to reabsorb water released into the urine before excretion in order to maintain water and salt homeostasis. The kidney is the organ where urine is formed and concentrated in mammals. Primary urine is ultrafiltrated from the blood in the renal cortex. The primary urine has the same composition as the blood with the exception that all of the proteins are absent and is almost isotonic to the blood. The primary urine then flows into tubular loops in the renal medulla where water is drawn from the urine across the collecting duct epithelium. This water transport occurs because of the high interstitium osmolarity (figure 1.3). This high interstitium osmolarity, essential in urine concentration, is established by active transport and accumulation of Cl^- and its counterions in the interstitium of the medulla (Schmidt-Nielsen, 1975).

The maintenance of a constant level of tonicity in the animal body lies in the kidney's ability to produce urine of varying osmolarities according to the hydration status of the organism. When water intake is abundant, solute concentrations in the medulla interstitium drop. Therefore, cells in the kidney medulla have to live in a hypertonic environment with widely fluctuating osmolarity.

In insects, the Malpighian tubules and the hindgut are the functional analogs of the mammalian kidney. In Drosophila, the primary urine is secreted into the

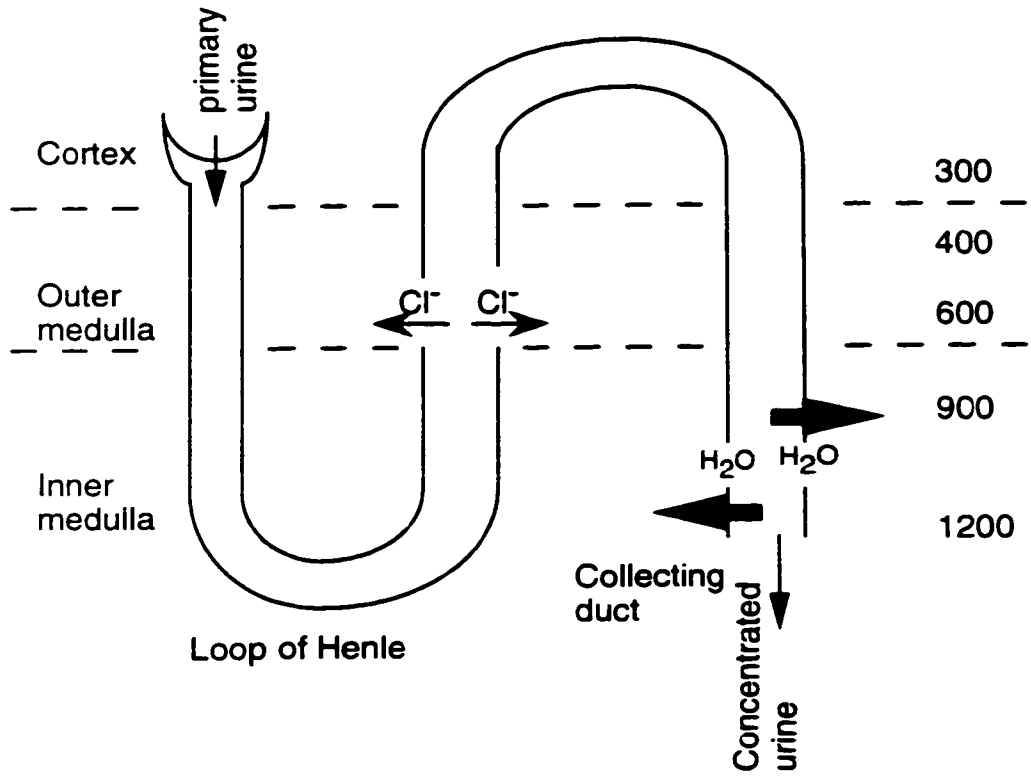


Figure 1.3 Different layers of the mammalian kidney, and mechanisms of urine concentration. The osmolarity in the cortex and medulla are labeled on the right (units are milliosmolar). The descending loop of Henle is water permeable and Na^+ impermeable; thus the primary urine is concentrated in the medulla. The ascending limb of the loop of Henle is less permeable to water. In this limb, Cl^- is actively pumped out of the urine into the interstitial space. The diluted urine then flows to the collecting duct which is permeable to water so that water is absorbed again when passing the hypertonic inner medulla. Modified from Schmidt-Nielsen, 1997.

upper Malpighian tubules. Active K^+ transport is the driving force in this location (Randall, 1997). The primary urine flows into other parts of the Malpighian tubules and the hindgut where water, salts and nutrients are reabsorbed before excretion (Bradley, 1989; O'Donnell and Maddrell, 1995).

The model commonly used to explain water transfer, sometimes against the osmotic gradient, in biological systems is the Curran three compartment model. This model is derived from the experiment illustrated in figure 1.4 using a cylinder divided into three compartments, A, B, and C. These compartments contain 0.1 M sucrose, 0.5 M sucrose and water respectively. The cellophane membrane between A and B is not permeable to sucrose, whereas the porous glass between B and C allows the flow of both water and sucrose. Left alone, compartment B will draw water from A and C and expand. However, when the volume of compartment B is fixed, water will go from A to B but sucrose can't go from B to A. The resultant pressure will move both sucrose and water from B to C. Thus the net movement of water is from A to C. The sucrose concentration in A rises due to loss of water to B and the sucrose concentration in B drops due to the movement of water and sucrose from B to C. The flow will stop when sucrose level in compartment B drops to that in compartment A. If the high sucrose concentration in B is maintained by adding sucrose, the flow will continue.

The concentration of urine in the insect hindgut is illustrated in figure 1.5, where hindgut lumen, the hindgut wall and the hemolymph are analogous to compartment A, B and C of the Curran model. The hindgut wall is very hypertonic in order to move water from the hindgut lumen to the hemolymph. Like the mammalian kidney, the primary driving force of water reabsorption is Cl^- transport, which establishes the hypertonicity in the hindgut wall (Phillips, 1998). In support of this model, small patches from the cockroach hindgut exhibit higher osmolarity than the hemolymph (Wall, 1970).

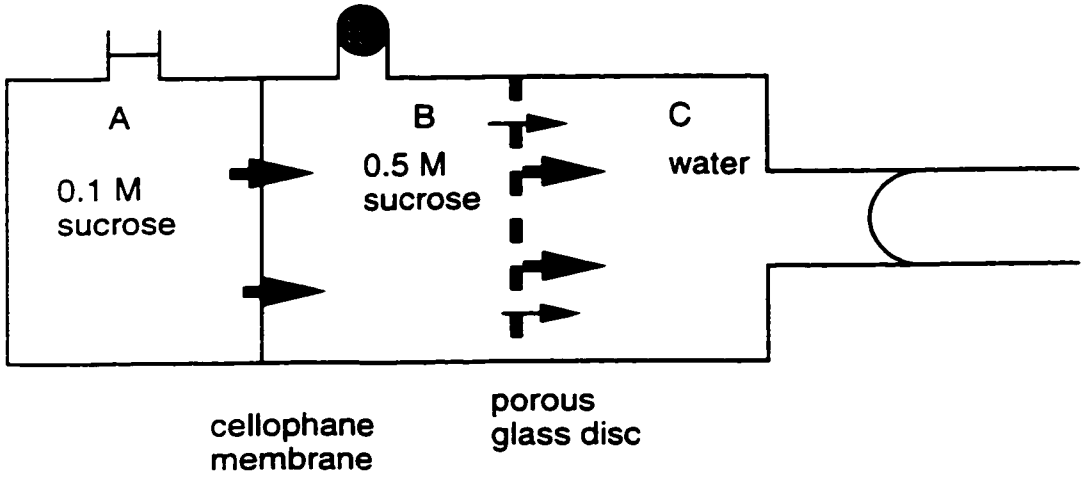


Figure 1.4 The three compartment experiment performed by Curran and MacLintosh in 1962. In this system, water is transferred from compartment A to C against the sucrose gradient. The cellophane membrane is permeable to water but not to sucrose. The porous glass disc is permeable to both water and sucrose. Thick arrows indicate water movement. Thin arrows indicate sucrose movement. For more explanation see text. Modified from Schmidt-Nielsen, 1975.

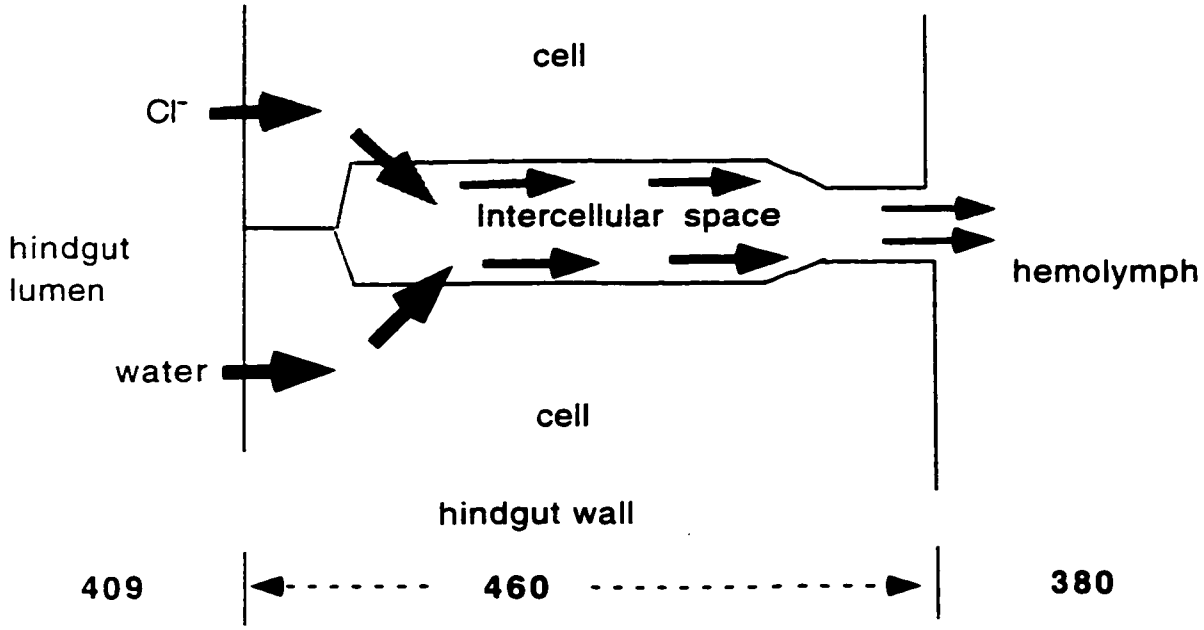


Figure 1.5 Water absorption in the insect hindgut. Cl^- and its counterions are actively transported into the deep, convoluted intercellular space, creating a high osmolarity. This high osmolarity in the intercellular space draws water from the hindgut lumen across the hindgut wall cells through osmosis. Numbers in bold font indicate the osmolarity in different compartments in milliosmolar. Gray arrows indicate the movement of Cl^-, black ones that of water. Ions and water are not spatially separated even though they appear to be so here in this figure for clarity. Modified from Wall et al., 1970.

Cells in Malpighian tubules and the hindgut walls, like the kidney medulla cells, must be capable of enduring the hypertonic environment that is required for fluid reabsorption.

B. Strategies adopted by cells in different organisms to handle hypertonic stress

Due to the permeability of membranes to water, changes in extracellular solute concentrations lead to changes in cell volume, intracellular ionic strength and transmembrane chemoelectrical gradient, all of which could negatively impact normal cellular structure and functions. The initial response of cells to hypertonic stress is to restore cell volume by actively accumulating ions followed by water (Sone *et al.*, 1995). However, high concentrations of ions such as Na^+ and K^+ have deleterious effects on macromolecules such as enzymes and DNA (Yancey *et al.*, 1982).

Cells in most organisms maintain a constant level of intracellular salt content through the extrusion or sequestration of ions, while staying isotonic to a hypertonic environment through the gradual accumulation of a group of small, nonperturbing molecules called osmolytes. These osmolytes, unlike ions, can be accumulated at a high concentration inside the cell without drastically affecting activities of macromolecules such as enzymes (Yancey *et al.*, 1982). Osmolyte accumulation is also important to maintain normal transmembrane ion gradients necessary for transport systems dependent upon these gradients such as Na^+/H^+ antiport and $Cl^-/HCO3^{--}$ antiport (Beck *et al.*, 1998).

The mechanisms of the osmotic stress response differ from one organism to another, even between different organs of the same organism. However, ion transport and the subsequent accumulation of osmolytes is the common theme across species.

a. Bacteria

Increases in environmental tonicity cause rapid water loss and cell volume reduction. The resultant low turgor activates the *E. coli* Kdp ATPase K^+ transport protein (Epstein, 1992). Kdp, together with another K^+ transport system Trk (Bakker, 1992; Schlosser *et al.*, 1995), enables rapid accumulation of K^+ and draws in water to recover the cell volume (Poolman and Glaasker, 1998). K^+ and other ions in *E. coli* are then replaced by osmolytes such as betaine and proline via the constitutively expressed ProP and inducible ProU transport systems (Lucht and Bremer, 1994). Synthesis *de novo* of trehalose is also induced by hypertonic stress to stabilize the bacterial membrane (Welsh and Herbert, 1999).

b. Yeast

The initial response to hypertonic stress in yeast is the up-regulation of a cation transport system to transport ions and adjust cell volume (Nishikawa *et al.*, 1999). The *de novo* synthesis of the main osmolyte in yeast, glycerol, is induced to replace ions. After the accumulation of osmolytes, yeast reduces intracellular ion concentrations by sequestering Na^+ in intracellular compartments such as pre-vacuoles and vacuoles. This sequestration is accomplished with the Na^+/H^+ antiport. Intracellular ion content is further reduced by actively pumping ions out of the plasma membrane with cation pumps (Nass and Rao, 1998). Under severe dehydrating condition, trehalose is also accumulated through *de novo* synthesis to protect yeast membranes.

c. Plants

Two principal means to handle hypertonic stress were identified in plants. In Arabidopsis, Na^+ can be sequestered into vacuoles to reduce intracellular salt level via a Na^+/H^+ antiport (Apse *et al.*, 1999), which utilizes the proton gradient established by proton pumps across the vacuolar membrane (Nelson, 1994). Osmolytes such as proline are accumulated by plant cells to enhance salt tolerance and maintain cellular function (Nanjo *et al.*, 1999).

d. Mammals

Under hypertonic stress, cultured renal medulla cells shrink due to water loss. The medulla cell initially responds to changes in cell volume by the import of ions via hypertonicity inducible Na^+/H^+ exchange (Bookstein, 1994). The influx of NaCl brings in water and restores the medulla cell volume and the Na^+ ion is later replaced by K^+ (Beck *et al.*, 1998). The increase in intracellular ion concentrations triggers the induction of the expression of various genes encoding transporters and enzymes that accumulate osmolytes and replace intracellular ions (Burg *et al.*, 1996). As osmolytes gradually accumulate, intracellular ion concentrations return to a normal level (Sone *et al.*, 1995).

Major osmolytes in the kidney include trimethylamines such as betaine and glycerophosphorylcholine (GPC), polyols such as sorbitol and inositol, and amino acid derivatives such as taurine (Garcia-Perez and Burg, 1991a; Garcia-Perez and Burg, 1991b). These osmolytes are accumulated through different mechanisms.

Increases in the osmolarity of the medulla interstitial fluid inhibit GPC:choline phosphodiesterase, which degrades GPC in medulla cells (Garcia-Perez and Burg, 1991b). The tonicity-responsive enhancer element (TonE) upstream of the open reading frame of the Na^+/Cl^- dependent GABA/betaine transporter (BGT1) drives the expression of this transporter and enables betaine accumulation in cultured kidney medulla cells under hypertonic stress (Takenaka *et al.*, 1994). Also in cultured kidney medulla cells, the transcription of the gene encoding aldose reductase (*AR*), the enzyme that synthesizes sorbitol, is upregulated by hyperosmolarity (Smardo *et al.*, 1992), whereas the gene for sorbitol dehydrogenase, which degrades sorbitol, is downregulated (Grunewald *et al.*, 1995; Sands and Schrader, 1990). The *AR* gene is regulated by an osmolarity response element (ORE) very similar to the ToneE of *BGT1* (Ferraris *et al.*, 1996;

Ko *et al.*, 1997). These two *cis* elements may be regulated by the same transcription factors. Under a given extracellular osmolarity, the sum of the four osmolyte concentrations in renal medulla cells remains at a stable level, whereas the concentration of each varies a great deal according to changes in urea levels or the availability of certain osmolyte(s) (Peterson *et al.*, 1992).

Taurine transporter gene transcription is also induced by hypertonicity (Uchida *et al.*, 1992). Free amino acids are less important in quantity, but they can be accumulated faster than the trimethylamines and polyols (Law, 1991a).

Transcription of the genes encoding certain heatshock proteins such as HSP 72 and OSP 94 (osmotic stress protein) are also induced by hypertonicity (Kojima *et al.*, 1996). The concentrations of heatshock proteins such as HSP 25 and HSP 72 correlate well with extracellular osmolarity in the kidney (Muller *et al.*, 1996).

C. Signal transduction pathways involved in the regulation of the osmotic stress response

In eukaryotes, two major groups in the mitogen-activated protein kinase (MAPK) superfamilies, extracellular-signal-regulated protein kinases (ERKs) and stress-activated protein kinases (SAPKs), are at the center of osmotic signal transduction. The SAPK subfamily includes SAPK1s such as Jun-NH2 terminal kinase (JNK), SAPK2s such as p38, and the HOG1 (high osmolarity glycerol) MAPK of yeast (Kutz and Burg, 1998).

a. Bacteria

Bacteria, such as *E. coli*, both sense osmotic stress and respond to it through a two-component system that consists of a primary sensor, EnvZ, and a response regulator OmpR. The latter is an inducible transcription factor that binds to the *cis*-elements and regulates the expression of genes, such as *OmpF* and *OmpC*, that modify membrane permeability (Kutz and Burg, 1998), and perhaps other genes

in the osmotic stress response. Two-component systems have also been discovered in fungi and plants.

b. Yeast

A two-component system similar to that of *E. coli* was found to be the primary sensor of osmolarity in yeast (Maeda *et al.*, 1994; Wurgler-Murphy and Saito, 1997). Unlike *E. coli*, the yeast two-component system can't directly regulate gene expression of downstream targets. Both an alternative osmosensor, SHO1 (Posas and Saito, 1997), and the two-component system exert their effect through the MAPK cascade, including different MAPKKKs, MAPKK(PBS2) and HOG1 (MAPK). The MAPK pathway in turn induces the expression of genes encoding enzymes that accumulate glycerol (GPD1) (Brewster *et al.*, 1993).

c. Plants

All of the plant MAPKs identified so far belong to the ERK family and are called PERKs. PERKs are strongly induced by hypertonic stress in Arabidopsis (Mizoguchi *et al.*, 1996) and *Pisum sativum* (Popping *et al.*, 1996). The PERK gene from the latter was able to rescue yeast *hog1Δ* mutants under hypertonic stress (Popping *et al.*, 1996).

d. Mammals

In the mammalian kidney, three types of kinases, ERK, SAPK2 (p38) and SAPK1 (JNK) are activated by hypertonic stress (Berl, 1997). Direct correlation between p38 activity and *BGT1* induction has been established in kidney cell culture (Sheikh-Hamad *et al.*, 1998). Some mammalian MAPK genes are also able to rescue yeast *hog1Δ* mutants under hypertonic stress (Galcheva-Gargova *et al.*, 1994). Transcription factors such as ATF2, MEF2C, c-Jun and Elk1 are targets of SAPK1 and SAPK2, although not all of them are induced by hypertonicity. The SAPK2 pathway also induce growth-arrest and DNA damage inducible genes such as GADD 45 and

GADD 153 in response to hypertonic stress in kidney medulla primary cell cultures (Kultz *et al.*, 1998).

Post-translational regulation is an important regulatory mechanism in mammals. PKC (protein kinase C) inhibits betaine and inositol uptake in cultured kidney medulla cells and cultured retinal pigment epithelial cells (Karihaloo *et al.*, 1997; Preston *et al.*, 1995). PKA (protein kinase A) also exerts effects on betaine and inositol uptake in these cell lines (Karihaloo *et al.*, 1997; Preston *et al.*, 1995).

e. Insects

A p38 MAPK was shown to be induced by hypertonic stress in Drosophila cell lines (Han *et al.*, 1998). The expression of this p38 MAPK can rescue the osmosensitive phenotype of yeast *hog1Δ* mutants.

iv. Summary of the two major roles of neurotransmitter transporters

Neurotransmitter transporters regulate neuronal excitability by controlling the extracellular neurotransmitter levels thus affecting the duration and magnitude of synaptic transmission. The transporters also accumulate osmolytes to maintain cellular structure and volume, macromolecule functions and transmembrane gradients.

2. Characteristics of the *inebriated/rosA* mutants

A powerful way to identify genes of interest in Drosophila is through mutagenesis. After identifying mutants with features of interest and isolating the mutated gene, we can interpret the functions of the gene or the protein it encodes by sequence analysis and by the physiological defects, or phenotypes, caused by the mutations.

i. Morphological and electrophysiological phenotypes of *inebriated* mutants

Our lab is interested in using Drosophila as a model system to study the function of the nervous system. Mutagenesis was used to identify genes whose mutations cause increased neuronal excitability, which is ultimately effected by changes in ion channel activities. Due to the redundancy of ion channel function in Drosophila, sometimes it is necessary to either chemically or genetically block one type of ion channel in order to accentuate the effect of a mutation affecting another.

The *Shaker* (*Sh*) gene is on the X chromosome encoding a potassium channel. Mutations in *Sh* cause leg-shaking under ether and prolonged depolarization in the motor neuron (Kamb *et al.*, 1987). In a *Sh* background, some mutations that cause increased neuronal excitability such as *ether a go-go* (*eag*), another K^+ channel mutation (Stern *et al.*, 1990; Warmke *et al.*, 1991), and *Dp para*$^+$, a duplication of a Na^+ channel gene (Stern *et al.*, 1990), exhibit the morphological phenotype of indented thorax and down-turned wings.

Dr. Stern et al. performed EMS (ethylmethane sulfonate) mutagenesis on Drosophila chromosome 2 in *Sh* mutant background to isolate mutations on novel genes that cause the visible morphological changes, hence possibly increased neuronal excitability. Two mutant alleles, *ine*^{1} and *ine*^{2} *that* fail to complement each other, display the phenotype of indented thorax and down-turned wings (Stern and Ganetzky, 1992). The *ine; Sh* doubt mutants walk with an uncoordinated gait and flip over frequently, thus the name "inebriated".

Electrophysiology studies on the neuromuscular junction were performed by Dr. Stern et al. on *ine* mutant flies. In the presence of quinidine, a drug that blocks certain type of potassium channel, the *ine*^{1} mutation causes an increased rate of onset of facilitation over wildtype flies. Facilitation is a drastic increase (about 10 fold) in the amplitude of action potential after repetitive stimulation on the neuron. In flies carrying mutations that cause increased neuronal excitability such as *Dp para*$^+$, *frequenin*, a mutation in a gene encoding a Ca^{++} binding protein that

reduces K^+ channel activity (Rivosecchi *et al.*, 1994), and *Hyperkinetic*, a K^+ channel mutation (Stern and Ganetzky, 1989), onset of facilitation is more rapid than in wild type flies.

Both the morphological and electrophysiological phenotypes suggest that the *ine* gene encodes either an ion channel or a protein regulating ion channel activities.

ii. Defects in Drosophila photoreceptor

Mutations in the Drosophila receptor oscillation A (*rosA*) gene cause an oscillation superimposed on the electroretinogram (ERG), an extracellular recording of the response of the eye to light stimuli that indicates photoreceptor activity, as well as a reduction on the amplitude of ERG (Burg, 1996). The *rosA* mutations are localized to the 24F/25A region of chromosome 2, which is the same location as the *ine* mutations (see next section on the localization of *ine* mutations), via deficiency mapping (Burg, 1996). In a *Sh* background, *rosA* mutations fail to complement *ine* morphologically (Dr. Stern, personal communication). The *inebriated* mutant flies also show defects in Drosophila photoreceptor similar to those of *rosA* flies (Dr. Burg, personal communication). These results indicate that *ine* and *rosA* are allelic.

iii. Preliminary localization of the *inebriated*^{1} mutation

Through recombination mapping using visible genetic markers on the second chromosome, *ine*^{1} was localized between *echinoid* and *Sternopleural*, roughly position 24 - 25 on the left arm of the Drosophila second chromosome. Two deficiency lines carrying deletions in 24E1 - 25A2 and 24C2 - 25A2 failed to complement the *ine*^{1} mutation, thus positioning *ine* to the 24F/25A region (Stern and Ganetzky, 1992).

In order to clone the *ine* gene, genomic DNA fragments from the 24F/25A region are required to localize *ine* through RFLP (restriction fragment length polymorphism) analysis (for more details on the RFLP scheme see chapter 2) and to identify genomic fragments that are likely to carry the *ine* gene. These genomic fragments were obtained by chromosome walking.

Holly Soehnge used a YAC (yeast artificial chromosome) containing about 100 kb DNA from the 24 EF region to initiate chromosome walking, which was conducted by screening cosmid library and later genomic phage library. Positions of genomic phage DNA fragments, either isolated by Holly Soehnge or provided by Marie Becker, on the chromosome were verified via *in situ* polytene chromosome hybridization and their distances from the *ine* locus gauged by RFLP analysis. Genomic phages, such as 13A-1 and 5A-3, that are important for the RFLP mapping effort will be discussed further in chapter 3.

3. Studies presented in this thesis

The goal of the project described in this thesis was to isolate the *ine* gene and conduct functional studies on this gene. The localization of the *ine* mutations by recombination and RFLP mapping, followed by the isolation of one of the *ine* cDNAs and the tissue distribution of the *ine/rosA* transcripts using RNA probes , is presented in chapter 3. Attempts to identify substrate(s) for proteins encoded by the *ine/rosA* gene are described in chapter 4. Efforts to generate antibodies against proteins encoded by the *ine/rosA* gene are presented in chapter 5. Another important phenotype of *ine* mutants and evidence supporting the importance of the *ine/rosA* gene in Drosophila osmotic stress response are presented in chapter 6. Chapter 7 provides possible mechanisms to explain the various phenotypes caused by *ine/rosA* mutations and speculations on future directions of this project.

Chapter 2 Materials and Methods

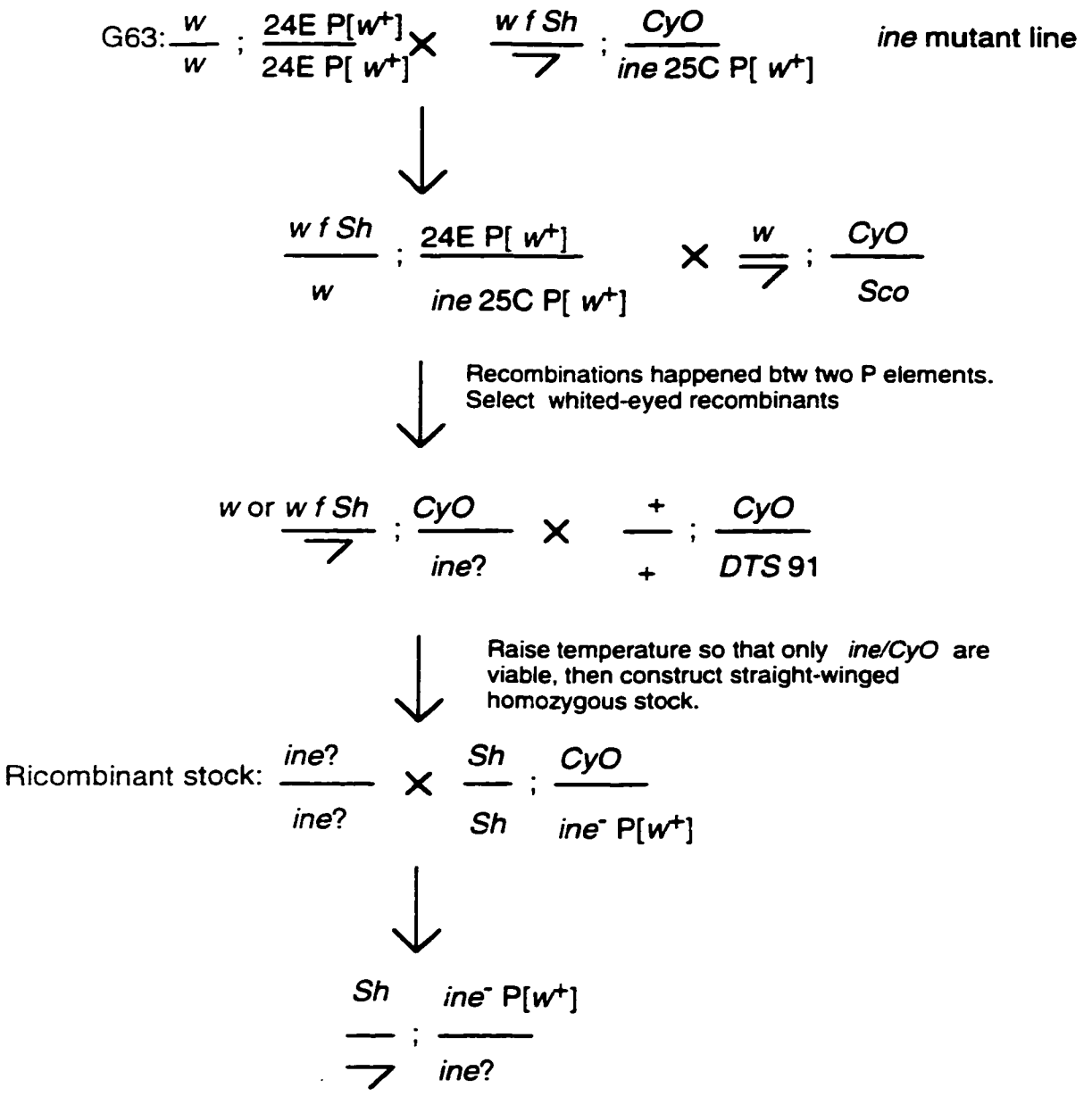
1. Recombination mapping and RFLP mapping of *inebriated* mutations on the Drosophila second chromosome

i Fly stocks and genetic markers used in recombination mapping

Two fly lines with different second chromosomes were used in the recombination mapping of *ine* mutations. G63 is a strain that has a white-eyed background and a P-element carrying a white gene (w^+) at 24E region of the second chromosome. This w^+ can render G63 line red eye color. The second line carries the ine^1 or ine^2 mutation, a P-element carrying w^+ at 25C of the second chromosome and a dominant mutation in the *Shaker* (*Sh*) gene on the X chromosome. Both ine^1 and ine^2 flies are created from *iso brown* parental line via EMS mutagenesis. For this reason, the second chromosome of ine^1 and ine^2 mutants are often designated as *iso brown*, or "I" in later chapters. Two additional *ine* mutant alleles, ine^3 and ine^4, were created from γ radiation mutagenesis. A fly stock that has a balancer second chromosome *CyO* and DTS^{91} on the sister second chromosome was used to cross to recombinants and generate homozygous recombinant stocks. DTS^{91} is a dominant temperature sensitive mutation that causes lethality under heatshock. *CyO* is a balancer second chromosome characterized by curly wings and is homozygous lethal (Figure 2.1).

ii. Generation of recombinants for RFLP mapping

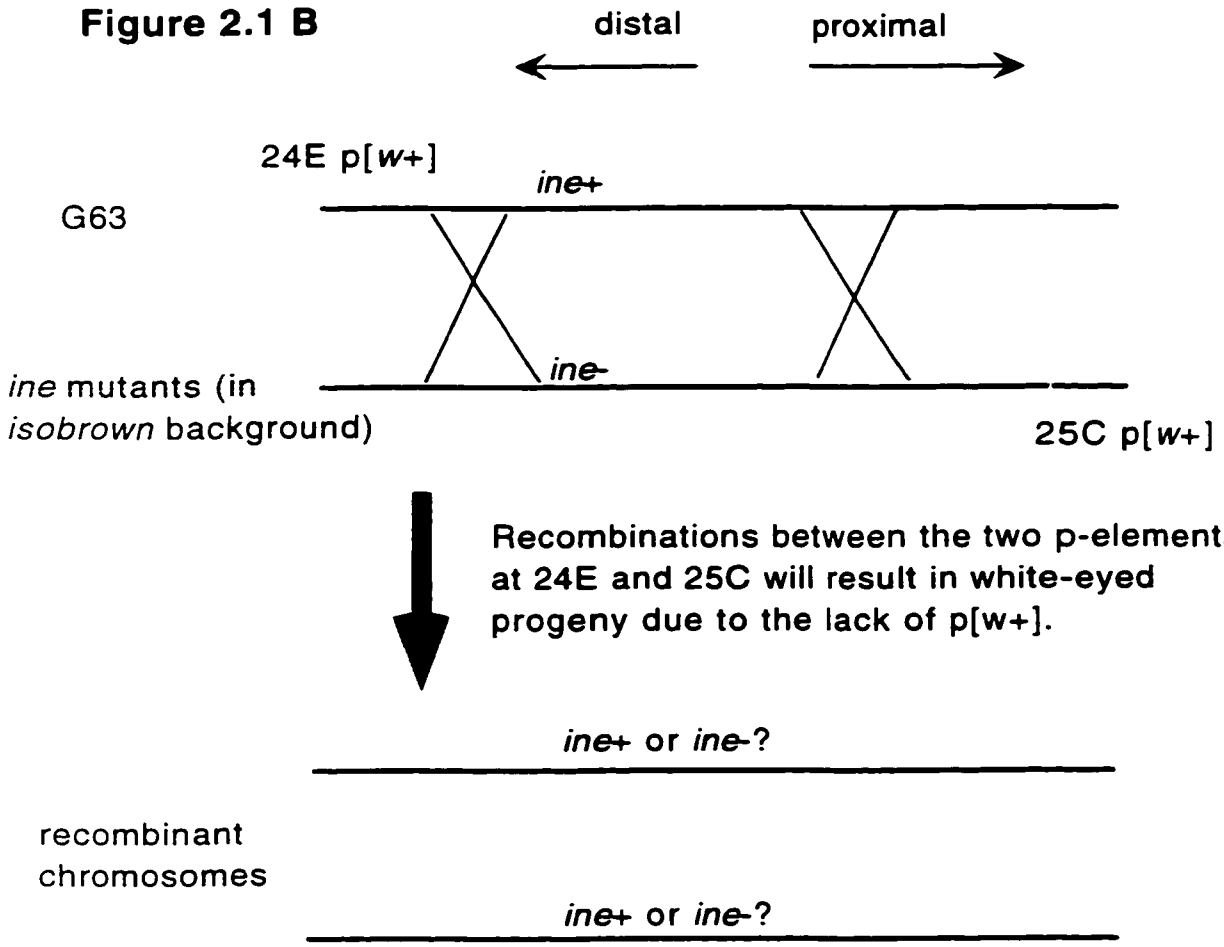
The scheme used to generate recombinants carrying recombination events between the P-element at 24 E and the *ine* locus is shown in figure 2.1. We first collected recombination events between the two P-elements by crossing ine^1 and ine^2 mutant lines to G63 line and choosing white-eyed progeny. These progeny have the two P-elements crossed out. Each white-eyed recombinant was then



If more than 20 straight- winged male progenies don't display downturned wings, a recombinant stock is considered *ine*$^{+}$; otherwise, it is *ine*$^{-}$

Figure 2.1 A

Figure 2.1 B



The *ine+* flies carry recombination events between 24 E and *ine* locus, whereas the ine- flies carry those between *ine* locus and 25C. The *ine-* genotype is indicated by the downturned wings and indented thorax in *Sh* background.

Figure 2.1 Generation of recombinant stocks for RFLP (restriction fragment length polymorphism) mapping. A. Crossing scheme used to generate recombinations between the P[w+] element on 24E and the inebriated locus. B. A schematic picture using chromosome DNAs to show the same scheme described in A. Distal: toward the end of the second chromosome. Proximal: toward the centromere. For more explanation see text.

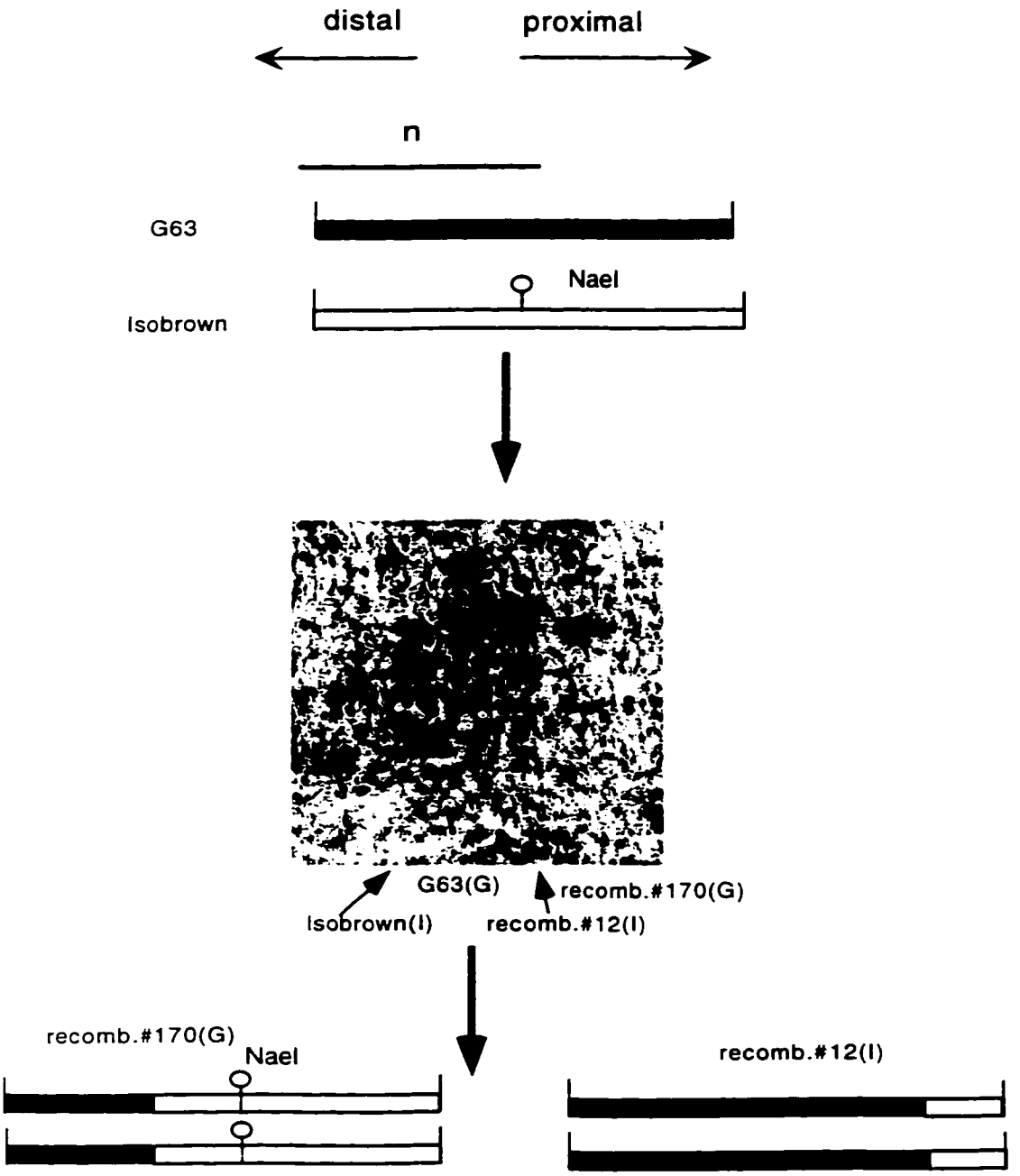
crossed to *CyO/DTS*^{91} stock to generate a homologous recombinant stock. To test if a homozygous recombinant stock was *ine*$^{+}$ or *ine*$^{-}$, we crossed it to a *Sh; CyO/ine1 mutant* stock. If we detected downturned wings among *Sh/ine* double mutant male progeny, then the recombinant is *ine*$^{-}$, otherwise it is *ine*$^{+}$. We collected *ine+* recombinant stocks carrying recombinations that occurred between 24E and *ine* to conduct RFLP mapping

iii. RFLP mapping of *inebriated* mutations

The idea behind RFLP (restricted fragment length polymorphism) mapping is to use restriction enzyme sites that exist in one parental chromosome of recombinants but not the other (polymorphisms) as genetic markers for mapping. Through restriction digestion of genomic DNA from a homologous recombinant fly line and Southern blots using a genomic DNA fragment covering the restriction polymorphism as probe, we could tell if a polymorphism existed in the fly line and if the recombination event happened to the left or right of the restriction fragment polymorphism. The ratio between recombination evens occurred to the left and those to the right indicates the distance between the *ine* locus and the polymorphism because the distance between the P- element in 24 E and *ine* locus was roughly known. Further, we could determine which genomic DNA fragment will be the most likely to carry the *ine* gene.

I used EcoRI and NaeI restriction polymorphisms to localize the *ine*^{2} mutation. An example of RFLP mapping is shown in figure 2.2 using a NaeI polymorphism. This NaeI site exists in *iso brown*, the parental line of *ine* flies, but not G63. In Southern blots of genomic DNA digested with NaeI, the second chromosome from *iso brown* should give a band shorter than that from G63. If a recombination happened to the right of (distal to) the NaeI polymorphism, the NaeI

Figure 2.2 The RFLP (restriction fragment length polymorphism) mapping process. Thick horizontal bars indicate *Drosophila* chromosome, with the black one being form G63, white one from *iso brown*, mixed ones from recombinants #170 and #12. The borders between black and white indicate sites of recombination. The thin horizontal bar on the top is the genomic DNA fragment 'n' used as probe in the Southern blot shown in the center. Vertical bars are NaeI sites existing in both G63 and *iso brown* chromosome 2. The lollipops indicate the NaeI sites that exist in the *iso brown* line but not in G63. For more explanation see text.


distal
proximal
n
G63
NaeI
Isobrown
G63(G)
recomb.#170(G)
Isobrown(I)
recomb.#12(I)
recomb.#170(G)
NaeI
recomb.#12(I)

site should be in the recombinant chromosome and a lower band shows in the Southern blot as in *iso brown* ("I"). Otherwise, a recombinant will give a higher band as in G63 ("G"). Two recombinants between 24E and *ine*^{2}, #12 and #170, were tested here. The #12 line showed "I" polymorphism on the Southern blot and thus carried a recombination event to the right of NaeI. The #170 line showed "G" polymorphism and thus carried a recombination to the left of NaeI.

In figure 2.2, the existence of recombinations to the right of NaeI site indicates that *ine* is further right than the NaeI site. By performing RFLP analysis on a larger number of recombinants, the ratio between the numbers of "I"s and "G"s will reflect the physical distance between a polymorphism and *ine*, assuming an even distribution of recombination events.

iv. Isolation of Drosophila genomic DNA and Southern blotting

Total Drosophila genomic DNA was isolated from recombinant flies using the method described by McGinnis & Beckendorf, (1983). This DNA was digested with restriction enzymes and separated by electrophoresis on 1% agarose gel. The transfer of DNA species from agarose gel to nylon membranes (Schleicher & Schuell), the preparation of ^{32}P labeled probes and hybridization were performed according to Sambrook, (1989).

2. Polymerase chain reaction (PCR), subcloning and bacterial transformation

i. PCR

PCR amplifications of DNA were usually done as following (for 100 µl reaction)

10 µl 10 x buffer

2 µl both primers (10 pmole/µl)

2 µl 10 mM dNTP

2 µl template (about 100 µg/ml)

1 µl Taq or Vent (New England Biolabs)

Add water to 100 µl

This mixture was subjected to denaturation at 94 °C for 5 minutes followed by 35 PCR cycles. Each cycle consists of 30 seconds at 94 °C, 45 seconds at 56 °C, and 1 minute/1 kb of template at 72 °C. After 35 cycles, PCR reactions were incubated at 72 °C for 10 minutes.

If the Hi-Fidelity PCR system (Boehringer Mannheim) was used, instructions for the product were followed to amplify DNA.

ii. Subcloning

Vector DNAs were digested with the restriction enzymes overnight, treated with SAP (Shrimp Alkaline Phosphatase) for 1 hour at 37 °C to dephosphorylate the vector and prevent self-ligation, and run on a 1 % agarose gel to separate the DNA fragments. The desired fragment was excised from the gel with a razor blade, put in a microfuge tube with a hole and glass wool at its bottom, and then collected by spinning at 5000 rpm in a microcentrifuge. The collected DNA was then recovered by phenol/chloroform extraction and ethanol precipitation as described in Sambrook, (1989).

Some insert DNAs were PCR products that are purified using the Qiagen PCR purification kit, digested with restriction enzymes and purified again with phenol/chloroform extraction and ethanol precipitation (Sambrook, 1989). Others were excised from other vectors and gel purified as described in the previous paragraph without the dephosphorylation. For blunt end ligations, 3 unites of T4 DNA polymerase were added and dNTPs were added to 0.5 mM, after the restriction enzyme digestion to fill the overhangs before gel purification.

T4 DNA ligase (Promega, New England Biolabs if blunt end ligation was desired) was used to carry out the ligation at 14 °C or room temperature for blunt end ligation.

iii. Bacterial transformation

Frozen $CaCl_2$ competent cells were prepared as described (Sambrook, 1989). For each transformation, 50 µl of competent cells (XL-1 blue or BL21 DE3 LysS) and 75 µl 0.1 M $CaCl_2$ were mixed in an microfuge tube. An aliquot of 1 µl plasmid or 5 µl ligation reaction was then added to the tube. The mixture was incubated on ice for 30 minutes, heat shocked for 1 minute at 42 °C and then plated on LB/Amp plates that had been prewarmed at 37 °C for 30 minutes. Plates were incubated overnight to grow transformant colonies. The ampicillin concentration of LB/Amp plate was 100 µg/ml.

3. Isolation of Drosophila mRNAs and Northern blotting

Isolation of Drosophila mRNAs and Northern blotting was performed as described (Soehnge *et al.*, 1996), with the exception of using Qiagen Oligotex mRNA miniprep kit to select mRNAs.

4. Whole-mount embryo *in situ* hybridization of *inebriated/rosA* transcripts.

Hybridizations *in situ* were performed as described in (Soehnge *et al.*, 1996). In order to study the localization of different *ine/rosA* transcripts, DNA sequences that are specific to *ine/rosA*-s and *ine/rosA*-l were amplified by PCR and subcloned into pcDNA1/Amp vector (Invitrogen), which has T7 and Sp6 promoters flanking the multiple cloning site. T7 and Sp6 RNA polymerases were used to synthesize digoxigenin labeled anti-sense and sense RNA probes. In some experiments, the

probes were examined for digoxigenin labeling with Boehringer Mannheim hybridization strips.

5. Uptake assays on *Xenopus* oocytes and S2 cells expressing the *inebriated/rosA* transporter

i. Expression of neurotransmitter transporters on *Xenopus* oocytes.

A. Isolation of *Xenopus* oocytes

Stage V *Xenopus* oocytes were obtained as described (Krieg *et al.*, 1984). Collagenase (A, or D from Boehringer Mannheim) solution was made by adding 13 mg collagenase into 20 ml Ca^{++} free Barth's buffer (Krieg *et al.*, 1984).

B. Expression of neurotransmitter transporters

The *inebriated/rosA*-s cDNA was subcloned into an eukaryotic expression vector (pcDNA1 Amp, Invitrogen), which was kindly provided by Dr. Jim Patrick at Baylor College of Medicine (figure 2.3). The Drosophila serotonin transporter (dSERT) cDNA (Corey *et al.*, 1994), provided by Dr. Michael Quick at University of Alabama, Birmingham, was also subcloned into pcDNA1 Amp vector to be expressed in oocytes as a positive control.

Each oocyte was injected with 13.8 nl of DNA constructs at 2 mg/ml or water. After injection, oocytes were incubated at 18^{o}C for 3 days before uptake assays.

ii. Maintenance and transfection of cultured Drosophila S2 cells.

A. Maintenance of the Drosophila S2 cell culture

The Drosophila S2 cell culture was derived from a hematopoietic precursor in the Drosophila embryo (Cherbas *et al.*, 1994). The average dividing time of S2 cells is about 24 hours. S2 cells were purchased from Invitrogen and were passaged every 2 to 3 days as described (Cherbas *et al.*, 1994). The medium for untransfected S2 cells

was Drosophila Schneider cell medium with L-glutamine (Gibco), 10 % Fetal Bovine Serum (heat inactivated, Gibco), 50 u/ml of penicillin G and 50 µg/ml of streptomycin. To the above medium, hygromycin B was added to 300 µg/ml to maintain stably transfected S2 cell lines.

B. Vectors and constructs used in transfection

The plasmid pMTA (Invitrogen) is a S2 cell expression vector that harbors a metallothionine promoter inducible by heavy metals such as Cu^{++}. Initially, *ine/rosA*-l and *ine/rosA*-s cDNAs were cloned without codons for their last five amino acids and stop codons and were in frame with the hexohistidine and V5 tags. Later, in order to express native Ine/RosA proteins without tags, the *ine/rosA* -s and *ine/rosA*-l open reading frames, from start to stop codons, were subcloned into pMTA.

The pMT-lacZ construct (Invitrogen) was used to inducibly express β-galactosidase as a control in some experiments. The pCoHYGRO vector was used to co-transfect S2 cells with other constructs and to render hygromycin resistance.

C. Transient and stable transfection

S2 cells were transfected with long or short forms of *ine/rosA* cDNA or both in pMTA, using a calcium phosphate transfection kit from Invitrogen. Calcium phosphate precipitate was removed the second day. For transient expression, Cu^{++} was added to 600 µM 48 hours after the transfection. For stable transfection, the cDNAs were co-transfected with a hygromycin resistance vector (pCoHYGRO) at a 19:1 ratio. To select stably transfected cells, hygromycin was added 72 hours after transfection to the culture to 300 µg/ml. Stable lines inducibly expressing Ine/RosA-l or Ine/RosA-s or both were established 3 to 4 weeks after transfection, as well as a line expressing β-galactosidase as controls used in uptake assays.

D. Detection of *inebriated/rosA* expression in the S2 cell culture

The expression of *ine/rosA* was detected at the transcriptional level by Northern blotting, and at the translational level by Western blotting. Anti-V5 monoclonal antibodies (Invitrogen) were used to detect *ine/rosA* recombinant proteins with the V5 epitope tag. Antisera to the N-terminus of *ine/rosA*-l protein (ROSA-N) were used to detect the *ine/rosA*-l protein without the V5 epitope tag.

iii. Uptake assays using tritium labeled substrates.

A. The *Xenopus* oocyte system

The uptake assay on *Xenopus* oocytes was performed as described (Liu *et al.*, 1992a), except that concentrations of tritium labeled neurotransmitters or osmolytes may vary as specified in the result sections of chapter 4.

B. The S2 cell system

About a million S2 cells transiently or stably transfected with cDNAs were induced with 600 μM Cu^{++} for two days. Cells were spun down, washed with PBS once and pelleted again. Cells were then resuspended and incubated in Na+ free buffer (150 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Tris.HCl pH 7.5) for 15 minutes. Cells were then transferred to uptake buffer (150 mM NaCl, 1 mM $CaCl_2$, 2 mM KCl, 1 mM $MgCl_2$, 10 mM Tris.HCl pH 7.5) containing 0.1 μM ^3H (tritium) labeled neurotransmitters or osmolytes.

After 30 minutes of incubation in uptake buffer, cells were washed with uptake buffer without neurotransmitters and then spun down. These cells were lysed by 200 μl of 10% SDS and transferred to scintillation fluid. Radioactivity was measured by scintillation counting

6. Generation of antisera against *inebriated/rosA* proteins

i. Expression and purification of recombinant antigen proteins from *E. coli*

Fragments of *ine/rosA*-s cDNA that encode the N-terminus and the C-terminus of Ine/RosA-s protein was amplified with PCR. I subcloned these PCR

products into pGEX-KT vector (Hakes and Dixon, 1992) behind the coding region of the glutathione S transferase (GST) protein, as well as the pET15-b and pET23-a vectors (Novagen, 1996). A cDNA fragment encoding the N terminus 300 amino acids of Ine/RosA-l was amplified and subcloned into the pET23-a vector with a T7 promoter and a C-terminus hexohistidine tag. This cDNA was subcloned into the pGEX-KT vector as well.

After sequencing the constructs to ensure that the *inebriated/rosA*-s cDNA fragments were in frame with the GST protein and histidine tag, I transformed them into an *E. coli* strain called BL21 DE3 pLysS (Novagen, 1996). Protein expression was induced by the addition of IPTG to 1 mM to cells grown to O.D. 0.5 at 37 ^{o}C. After IPTG induction for 3 hours at 37 ^{0}C, bacteria were harvested by centrifugation, lysed with electrophoresis loading buffer containing 2% SDS, and analyzed by SDS PAGE and Western blotting as described in Molecular Cloning, 18.47 (Sambrook, 1989).

If overexpression of desired recombinant proteins was detected, GST fusion proteins were purified as described in (PharmaciaBiotech, 1994). After the detection of its overexpression, the ROSA-N was found in the inclusion bodies that was then purified as described in (Novagen, 1996).

ii. Immobilization of antigens and affinity purification of antibodies

Various recombinant protein and peptide antigens were immobilized onto the cyanogen bromide activated agarose (Sigma, instructions for cyanogen bromide). In order to affinity purify antibodies against Ine/RosA peptides, a bleed was first subjected to columns with immobilized GST protein to eliminate the anti-GST antibodies, and then to columns with immobilized GST fusion proteins to affinity purify anti-Ine/RosA antibodies. Antibodies bound to the immobilized antigens were eluted with 3M NaSCN and then dialyzed against PBS buffer.

iii. Preparation of Ine/RosA-s expressed in S2 cells

Cells stably transfected with *ine/rosA*-s cDNA were induced with 600 μM Cu^{++} for two days, harvested and washed with PBS once. These cells were resuspended with buffer A (50 mM Tris pH 8.0, 20% glycerol, 1% Triton X-100) and lysed by sonication. After extraction in Buffer A for about 30 minutes, the sonicate was centrifuged at 30,000 x g to remove debris. Ni-NTA resin (Qiagen) was then added to the lysate and incubated at 4 °C overnight.

Ni-NTA resins were then packed onto a column and washed with buffer A, wash buffer (buffer A containing 10 mM imidazole), and finally eluted with 1M imidazole in buffer A. Proteins remaining on the Ni-NTA resin were stripped by boiling the resin in 2% SDS solution.

iv. SDS-PAGE and Western blotting

SDS-polyacrylamide gels were made and run according to (Sambrook, 1989). Proteins were transferred from SDS-polyacrylamide gel onto nitrocellulose membrane (PROTRAN BA85, Schleicher & Schuell) using semi-dry transfer system as described (Harlow, 1988), page 488. After the transfer, the nitrocellulose membrane was blocked in blotto (1xTBS (Sambrook, 1989), 3% non-fat powdered milk and 0.2% tween 20) for 1 hour, incubated at 4 °C in blotto containing primary antibodies overnight, and washed three times for 10 minutes with blotto. If the primary antibody was already conjugated with peroxidase, the membrane would be visualized with either colorimetric substrates (Vector Laboratories) or chemiluminescent substrates (PIERCE) according to the instructions for the respective products. If the primary antibody was not conjugated with peroxidase, the membrane was then incubated with peroxidase conjugated secondary antibodies for 1 hour, washed three time for 5 minutes in blotto and once for 5 minutes in PBS,

and then subjected to colorimetric and chemiluminescent detection as described above.

v. Chemical modifications of protein antigens

A. Performic acid and hydrogen peroxide oxidation

1 mg protein was dissolved into 0.5 ml formic acid, added 0.1 ml methanol and chilled on ice for 30 minutes. 1 ml performic reagent (prepared by mixing 9.5 ml formic acid and 0.5 ml 30% H_2O_2 and incubating for 2 hours at room temperature and 30 minutes on ice) was added and mixed, followed by incubation on ice for 150 minutes. Protein was then precipitated by dialyzing against PBS solution overnight, resuspended by sonication, and sent out for antibody generation.

B. Dinitrophenol coupling

The procedure was performed as described in Antibodies, a laboratory manual, page 125 (Harlow, 1988), except that 6 M urea was present in the 0.5 M sodium carbonate solution (pH 9.5) to keep ROSA-N solublized.

vi. Separation of proteins by DEAE-cellulose and P-cellulose chromatography

Both DEAE-cellulose and P-cellulose resins were equilibrated in 10 mM KCl solution overnight and then mixed with GST and GST fusion proteins in 50 mM Tris.HCl pH 7.5 for 1 hour. The resins were then eluted with buffers containing different amount of KCl, from 10 mM to 500 mM.

7. Drosophila osmotic stress response assays and eating behavior assays

i. Osmolarity sensitivity assays

Fly food used in these assays was made by mixing equal volumes of instant Drosophila food (Carolina Biological Supply Company) and water or solutions.

Freshly eclosed flies with different genetic backgrounds were maintained on food containing various concentrations of NaCl. Four to six days later, the number of surviving flies was counted.

ii. Observation of Drosophila food intake

To observe the eating behaviors of flies with various genetic backgrounds, phenol red and bromophenol blue were added to fly food to 5 mg/ml and 2.5 mg/ml respectively. The mixture of these two dyes gives the Drosophila midgut a purple color if ingested. After 24 hours of maintenance on food with dyes, flies were dissected and the color of their midguts recorded with a camera.

Chapter 3. Cloning, structure and expression pattern of the *inebriated/rosA* gene

1. Introduction

The approach adopted in our lab is to use mutagenesis to identify novel genes that regulate neuronal excitability. Increased neuronal excitability is characterized by electrophysiological and morphological examinations. In the *Sh* mutant background, *ine* mutants exhibited indented thorax and downturned wings morphological phenotype and increased the rate of onset of augmentation in neuromuscular junction in the presence of quinidine (Stern and Ganetzky, 1992).

The general strategy to clone a gene after mutagenesis is first to localize its mutations on the Drosophila chromosome and then to use genomic DNA fragments from the mutation locus to screen cDNA libraries and isolate potential cDNA(s) of the gene. Various tests, such as sequencing of the mutated gene, studies of transcriptional levels and rescue assays, are conducted in order to prove that the cDNA thus isolated is the cDNA of the gene of interest. Northern blots can be used to see if the cloned cDNA represents the whole length of the transcript.

The distribution of the transcripts of a newly identified gene is usually studied after the cloning. Expected distribution patterns will corroborate the characterization of the gene from its phenotype. Unexpected distribution patterns will lead to previously unsuspected functions of the gene.

2. Results

i. Mapping of the ine^2 mutation on chromosome 2 of Drosophila

The ine^1 mutation had been localized to 24 F/25 A region of the second chromosome (Stern and Ganetzky, 1992). Previous recombination tests had already shown that the distance between a P-element at 24E and *ine* locus is about 100 kb

(figure 2.1 B). RFLP mapping was employed to more precisely localize the *ine* mutations and to identify the genomic DNA fragments that are the most likely to carry the *ine* gene. The RFLP mapping scheme is explained in detail in the Materials and Methods section.

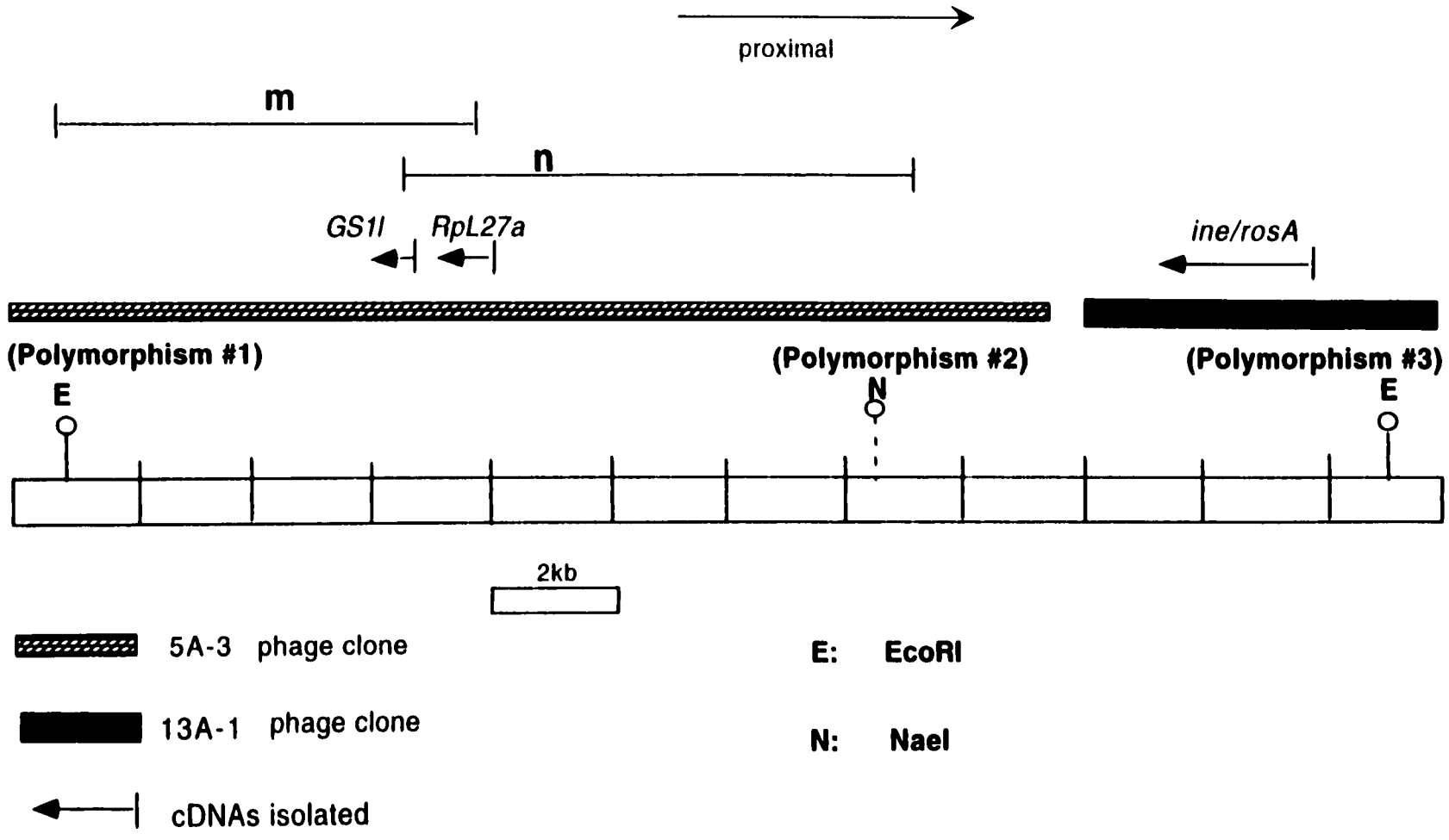
Genomic DNA fragments from phage clones at the vicinity of the *ine* locus, such as 5A-3 and 13A-1 (Figure 3.1), were either given by Marie Becker or isolated by Holly Soehnge through chromosome walking (Soehnge *et al.*, 1996). The G63 line, "G" and *iso brown*, "I" are the parental lines of the recombinants between 24E and *ine* locus (figure 3.1). An EcoRI site (polymorphism #1) on the 'm' DNA of the 5A-3 chromosome walk exists only in "G" but not in "I", so does another EcoRI site (polymorphism #3) on the 13A-1 chromosome walk. In contrast, an NaeI site (polymorphism #2) on the 'n' DNA of the 5A-3 chromosome walk exists only in "G" but not in "I". The 'm' DNA was used as probe for polymorphism #1, 'n' DNA was used for polymorphism #2 and 13A-1 DNA was used for polymorphism #3 on Southern blots. A "G" type recombinant, the existence of either EcoRI sites in the two locations or the absence of the NaeI site detected in Southern blots using corresponding probes, carries a recombination that occurred to the left of a certain polymorphism. An "I" type recombinant carries a recombination that occurred to the right. The ratio of the number of "G"s versus that of "I"s tells how far *ine* is from the polymorphism.

Using these polymorphisms, Diana Conover performed RFLP mapping on ine^1 mutation, whereas I performed the RFLP analysis on ine^2. Table 3.1 shows the results of our experiments.

Results from RFLP mapping using polymorphism #1 and the "m" DNA as the probe on ine^1 recombinants showed that out of 43 recombinations between the 24E and *ine* loci, 2 occurred to the right of polymorphism #1 (Diana Conover and Holly Soehnge). Assuming that recombinations are evenly distributed along

Figure 3.1 Restriction polymorphism sites and genomic DNA fragments used in RFLP mapping. The distance between a P-element at 24E position of the second chromosome and the *ine* locus is estimated to be about 100 kb. Lollipops indicate polymorphisms and arrows indicate cDNAs isolated by screening cDNA libraries using overlapping genomic DNA fragments as probes. These fragments include the 'm' and 'n' DNAs from the 5A-3 phage clone, and the 13A-1 phage DNA clone.

RFLP Mapping Scheme



probe used in Southern blots	'm' DNA	'n' DNA	13A-1 phage clone
polymorphism restriction site	#1 EcoR1	#2 NaeI	#3 EcoRI
I:G ratio of RFLP on *ine*1	2 : 41	0 : 43	0 : 43
I:G ratio of RFLP on *ine*2	6 : 40	2 : 44	0 : 46

Table 3.1 Tabulation of RFLP results. We have used 44 ine^{+} recombinant stocks carrying recombination events between 24E and ine^{1}, 46 ine^{+} recombinant stocks with recombination events between 24E and ine^{2}. Positions of probe DNAs and polymorphism sites are labeled in figure 3.1. "I" indicates a recombination happened to the right of a polymorphism; "G" indicates otherwise.
See text for explanation

chromosome, the *ine* locus should be about 4.6 kb right of polymorphism #1, at the 3′ end of the "m" DNA. Results from RFLP mapping using polymorphism #1 on ine^2 recombinants showed that out of 46 recombinations between the 24 E and *ine* loci, 6 happened to the right of polymorphism #1. This puts ine^2 to be 13.2 kb to the right of polymorphism #1, at the 3′ end of 5A-3 DNA. Further, using polymorphism #2 and the "n" DNA as the probe, 2 in the 46 ine^2 recombinations occurred to the right of polymorphism #2, setting ine^2 to be in the 13A-1 region, 4.4 kb from polymorphism #2.

ii. Transcriptional studies on two genes, *GS1l* and *RpL27a*, located in the *inebriated/rosA* vicinity.

Based on the location of ine^1 mutation from RFLP mapping data, Holly Soehnge and Penn Whitley screened cDNA libraries using probes made from the DNA fragments around the calculated position of ine^1. The 'm' DNA probe was utilized to screen a 0-24 hour embryonic library in the λEXLX vector. A 1.0 kb cDNA called 2I was isolated from this library. The "n" DNA probe was employed to screen an eye disc library in the λgt10 vector. A cDNA of 0.6 kb called 12B was uncovered from this library. Both cDNAs were subcloned into pBluescript(SK+) vector and sequenced.

Through sequence analysis using databases in the Genbank, 2I has 47.2% amino acid sequence identity to a human gene GS1, whose function remains unknown. The gene 2I cDNA represents was named *GS1l* (GS1 like), it has open reading frame (ORF) that encodes for a 231 amino acid protein. The 12B sequence was found to have 98% amino acid sequence identity to a Drosophila ribosome protein L27a, as well as homology to human and mouse L27a genes. The gene represented by the 12B cDNA was named *RpL27a* and it encodes a 149 amino acid protein (Soehnge *et al.*, 1997). I performed Northern blotting using *GS1l* and *RpL27a*

cDNA probes to study their transcription in wild type flies. The *GS1l* and *RpL27a* probes have recognized transcripts of 3.2 kb and 0.75 kb respectively (figure 3.2). The *RpL27a* transcript is much more abundant than that of *GS1l* and was later used as loading controls in many experiments, because of the house-keeping nature of ribosomal genes.

The *RpL27a* cDNA was ruled out as the *ine* cDNA because it is very unlikely that a mutation in a ribosomal protein gene causes neuronal defects. Also mutations on ribosomal genes usually have distinct phenotypes, such as *Minute* phenotypes, that are not exhibited in *ine* mutants (Kay, 1987; Qian *et al.*, 1987). In order to decide if the *GS1l* cDNA is the *ine* cDNA, RT-PCR was performed on *ine* mutant flies to amplify the *GS1l* transcript. Sequencing of the resulting RT-PCR products failed to show any changes in *GS1l* mRNA sequence in *ine* mutant flies, indicating that *GS1l* gene was not likely to be *ine* either.

iii. Sequencing and verification of an *inebriated/rosA* cDNA.

Data from RFLP mapping on ine^2 mutations suggested that ine^2 might be in the 13A region of the chromosome. Penn Whitley used 13A genomic DNA as a probe to screen another embryonic library and isolated a cDNA named 2A. Holly Soehnge PCR amplified the 2A cDNA and sequenced the PCR products.

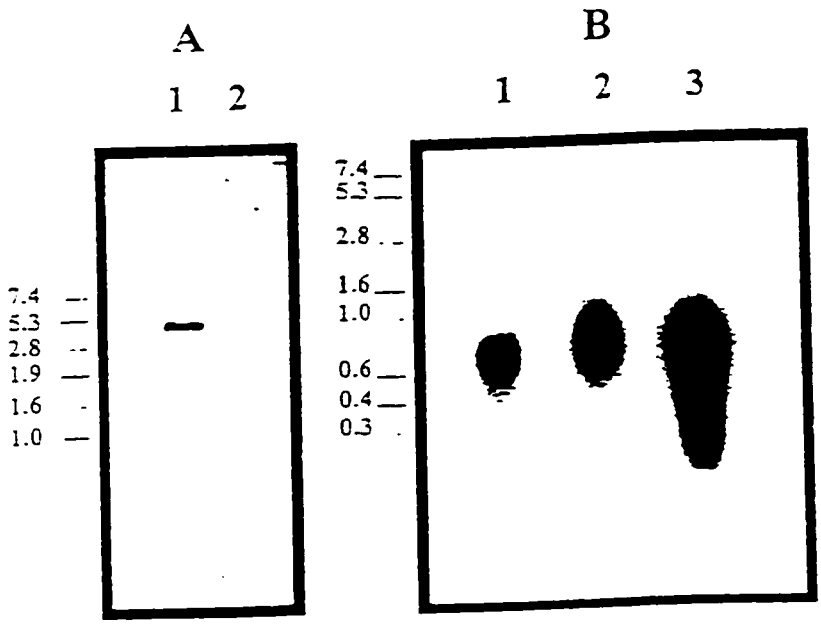
In order to confirm the cDNA sequence and correct possible PCR errors, I subcloned this cDNA from a phage clone into pBluescript(SK+). I then sequenced both strands using primers in the 2A sequence designed by Holly Soehnge and Penn Whitley, aligned them and compared the sequences with that of the PCR product sequence of 2A cDNA. The final cDNA sequence and the deduced amino acid sequence are shown in figure 3.3.

The 2A cDNA has an ORF that encodes for a protein of 658 amino acids with a molecular weight of 74.6 kDa. Hydropathy analysis using an Internet software

Figure 3.2 Northern results using *GS1l* and *RpL27a* probes

A. The *GS1l* cDNA (2I) was ^{32}P labeled and used as probe. Lane 1 is mRNA (5 μg) from whole flies; lane 2 is total RNA (40 μg) from whole flies. An RNA molecular weight ladder is labeled to the left. The size of *GS1l* transcript is about 3.2 kb.

B. The *RpL27a* cDNA (12B) was used as probe. Lane 1, 2, and 3 are total RNA from whole flies (40 μg), total RNA from fly heads (40 μg) and mRNA from whole flies (5 μg) respectively. An RNA molecular weight ladder is labeled to the left. The size of *RpL27a* transcript is 0.75 kb.


A
1 2
7.4
5.3
2.8
1.9
1.6
1.0
B
1 2 3
7.4
5.3
2.8
1.6
1.0
0.6
0.4
0.3

PROSITE predicted that the 2A protein would contain 12 transmembrane domains. By searching the Genbank database, we determined that the amino acid sequence of the protein encoded by the 2A cDNA sequence has about 40% sequence identity to members of the Na^+/Cl^- dependent neurotransmitter transporter family. These sequences are most conserved in the first transmembrane domain and more divergent at the C terminus. Both N and C termini are on the cytoplasmic side. Proteins in this family include GABA, dopamine, norepinephrine, serotonin, glycine and proline transporters. The putative protein encoded by 2A has the highest amino acid sequence identity with GABA/betaine transporters. It also shares similarity with creatine and taurine transporters which also function outside of the nervous system. An alignment of neurotransmitter transporter amino acid sequences was created using the GCG software package and SeqVu (figure 3.4).

I studied the transcription of the 2A gene by means of Northern blotting (Figure 3.5). Due to the low abundance of 2A transcripts, they can hardly be detected on Northern blots using total RNA preparations. Two major bands, the 2.2 kb and the 3.7 kb ones, were detectable on Northern blots on mRNA preparations, with the latter being more abundant. The 4.8 kb band was hard to see in some experiments. The 2.2 kb band is roughly the size of the 2A cDNA. The possible identities of the 3.7 kb and 4.8 kb bands will be discussed later.

Through sequencing of RT-PCR and genomic PCR products and Southern analysis, Penn Whitley found that ine^3 is a large deletion and ine^4 is a nonsense mutation at codon #177 of the 2A cDNA (figure 3.3). The ine^1 mutant flies have a very reduced level of 2A transcript (figure 3.5). The ine^2 mutation has not been defined yet. No mutation was found in the 2A cDNA through RT-PCR sequence analysis and the 2A transcript level is normal in the ine^2 mutants. These results proved that the 2A cDNA is a cDNA of the *ine* gene. Rescue results shown in later chapters further confirmed this conclusion.

Figure 3.3 Nucleotide sequence and deduced amino acid sequence of the *ine* cDNA. Putative transmembrane domains are underlined and labeled with Roman numerals. Consensus sequences for CK2 (filled bar), PKC (filled circle) and glycosylation (asterisk) sites are shown. The introns are indicated with bars and numbered 1-7. The nonsense ine^4 mutation (G to A531) is indicated by a #. The deletion in ine^3 flies is indicated by an arrow starting from G879 to the 3' end of the ine gene.

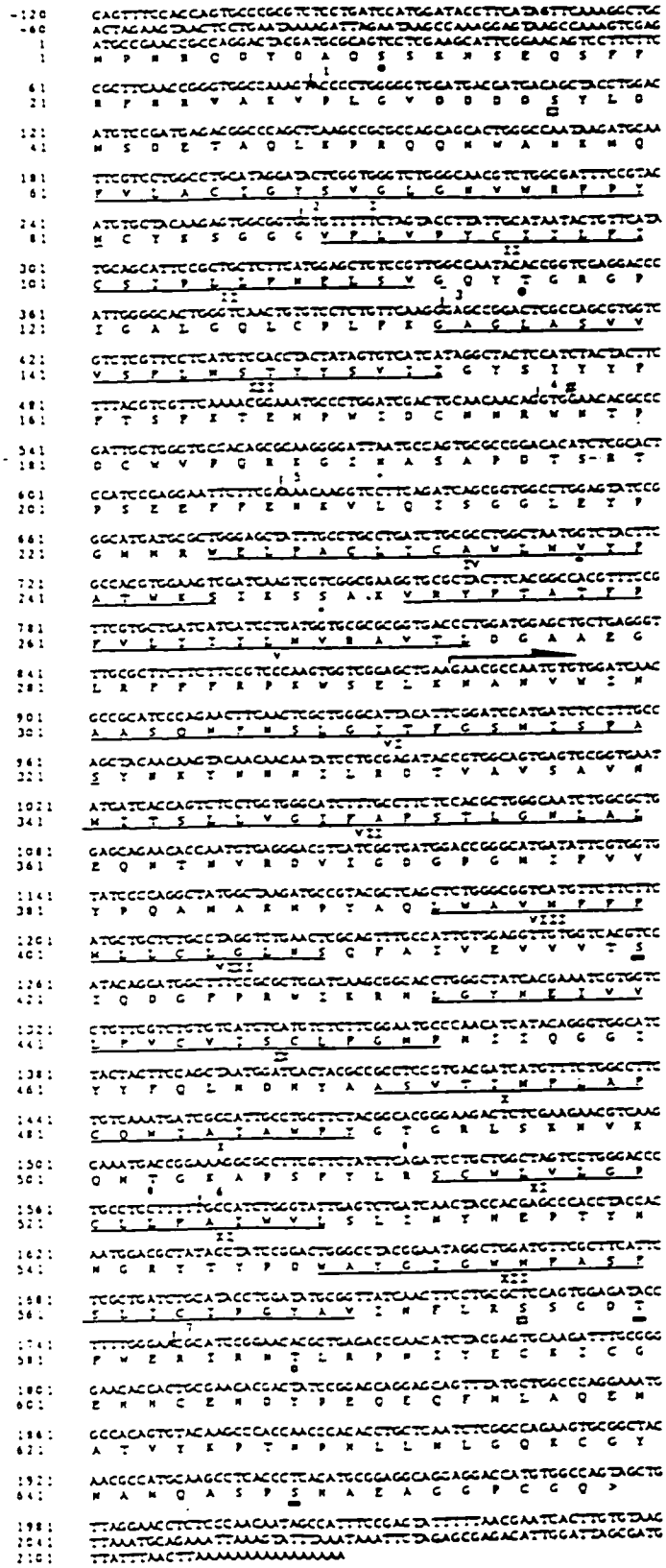
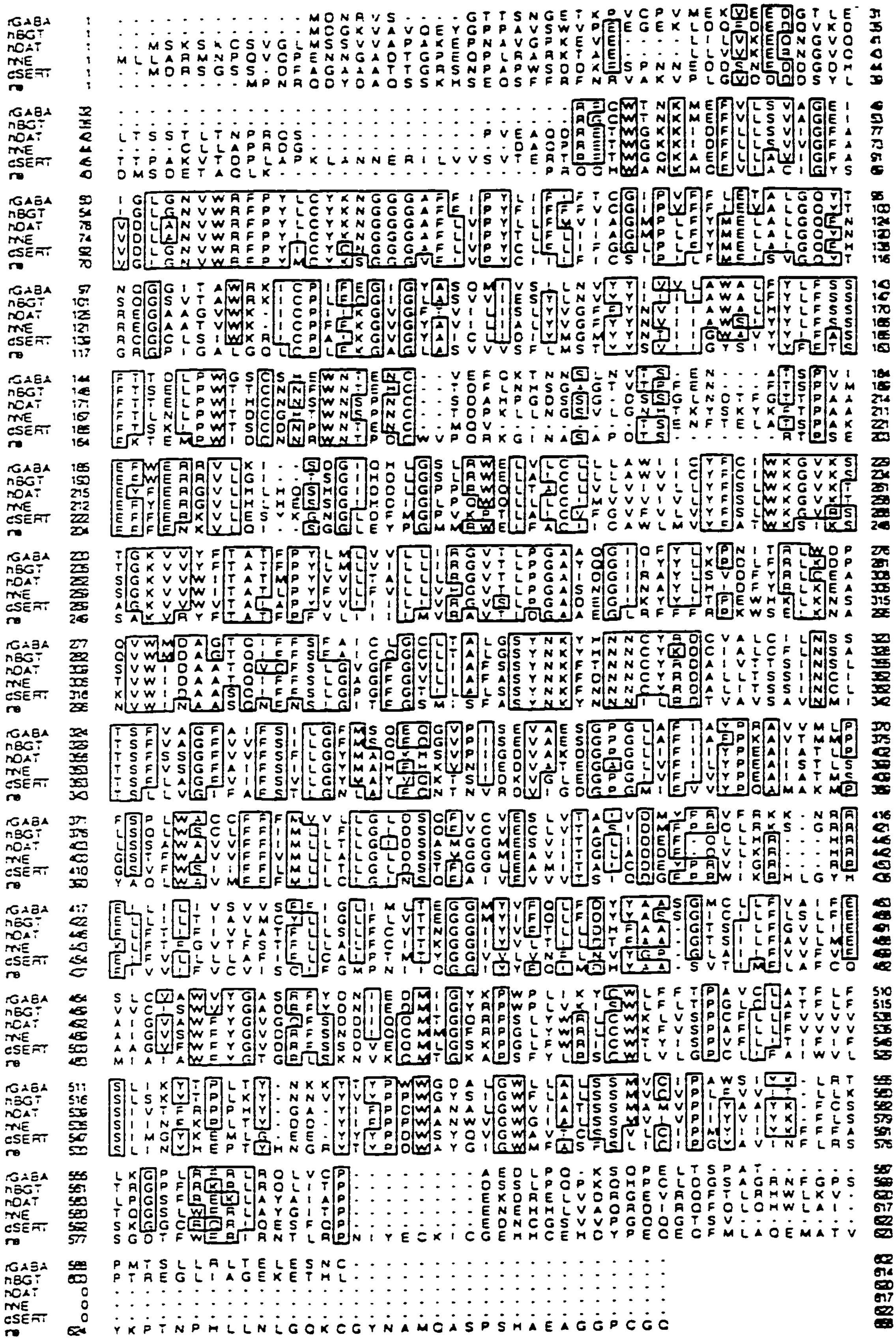


Figure 3.4 Sequence alignment of the amino acid sequence of the *ine* transporter with some members of the Na+/Cl- dependent neurotransmitter transporter family. Sequence sources are as follows: human betaine/GABA transporter (hBGT) (Borden *et al.*, 1995), rat GABA transporter GAT (rGABA) (Borden *et al.*, 1992), human dopamine transporter (hDAT) (Vandenbergh *et al.*, 1992), human norepinephrine transporter (hNE) (Pacholczyk *et al.*, 1991), and Drosophila serotonin transporter (dSERT) (Corey *et al.*, 1994). Boxed areas indicate identical amino acids among sequences. Alignments are based on a Dayhoff matrix using the PILEUP program of the Genetics Computer Group sequence analysis software package and SeqVu. The gap creation penalty is 12 and the gap extension penalty is 4.



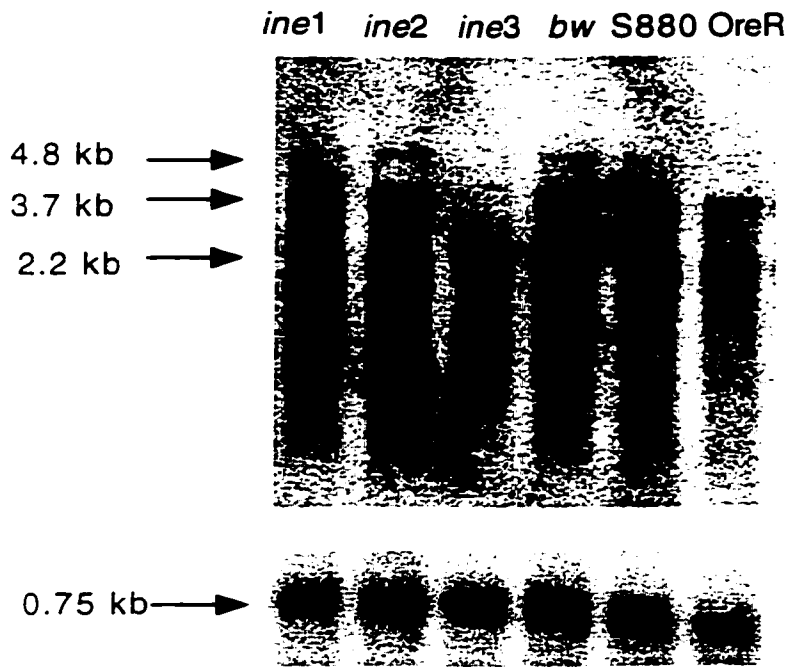


Figure 3.5 Northern analysis of transcriptional levels of the 2A cDNA sequence in wildtype and *ine* mutant flies. In each lane, about 5 µg of mRNA prepared from adult flies was loaded. Three wild type strains are shown here, labeled as OreR, S880 and *bw*, with the last one being the parental stock of *ine* mutants *iso brown*. The upper panel is probed with the 2A cDNA. The lower panel is the loading control using *RpL27a* cDNA as probe.

iv. Two forms of *inebriated/rosA* cDNA correspond to two transcripts detected on Northern blots.

Burg et. al. discovered the Drosophila *rosA* gene whose mutations cause an oscillation superimposed on the photoreceptor potential in Drosophila, as well as a reduction of the electroretinogram (ERG) amplitude and off- and on- transient currents (Burg, 1996). The *rosA* gene was localized to the same position on the Drosophila second chromosome as *ine*. The *rosA*P^{219} mutation failed to complement *ine*^{1} for the morphological phenotypes (Dr. Michael Stern, personal communication). Moreover, the *ine*^{1} mutants also showed photoreceptor defects characteristic of *rosA* mutants (Dr. Martin Burg, personal communication). These results indicate that *ine* and *rosA* are the same genes. To clarify the nomenclature in this study, we will call the cDNA obtained by Burg et al. *ine/rosA*-l, whereas the cDNA reported in (Soehnge *et al.*, 1996) will be called *ine/rosA*-s. These cDNAs encode Ine/RosA-l and Ine/RosA-s proteins respectively. The *ine/rosA*-l cDNA is about 3.6 kb and encodes an additional 300 amino acids at the N terminus compared to the *ine/rosA*-s cDNA. The *ine/rosA*-l and *ine/rosA*-s cDNAs share the same sequence from the first intron position in *ine/rosA*-s cDNA to the 3′ end, encompassing all of the twelve putative transmembrane domains of the *ine/rosA* transporter and the poly-A site.

Northern blotting was performed to test the possibility that *ine/rosA*-s and *ine/rosA*-l cDNAs arise from different mRNA species observed in Northern blots. To make *ine/rosA*-s and *ine/rosA*-l specific probes, I amplified the cDNA sequences that are specific to *ine/rosA*-s and *ine/rosA*-l by PCR from 5′ untranslated regions (UTRs) to the first intron position in *ine/rosA*-s cDNA. The *ine/rosA* -l specific probe recognized two bands, 4.8 kb and 3.7 kb, in Northern blots of mRNA from adult flies (figure 3.6 A). The 3.7 kb band mRNA is about the size of *ine/rosA*-l cDNA, pointing to the possibility that *ine/rosA*-l is an extension from *ine/rosA*-s on

the 5′ end. The *ine/rosA*-l transcript doesn't seem to be enriched in the head as reported previously by Burg et. al (data not shown). The *ine/rosA*-s specific probe recognized a single 2.2 kb band (figure 3.6 B).

v. Organization of *inebriated/rosA* transcription on Drosophila chromosome

Penn Whitley defined the intron-exon boundaries of the *ine/rosA*-s cDNA on Drosophila chromosome by sequencing the genomic DNA. A total of seven introns were found within the *ine/rosA*-s cDNA (figure 3.3).

The *ine/rosA*-l cDNA differs from the *ine/rosA*-s from the 5′ UTR to the first intron position in the *ine/rosA*-s cDNA. From Northern results in the previous section and the position from where *ine/rosA*-l and *ine/rosA*-s sequences differ, the *ine/rosA*-l specific sequence is likely to be located 5′ of *ine/rosA*-s. Dr. Burg has reported that the *ine/rosA*-l specific sequence was not found within 50 kb 5′ of the *ine* locus (Burg, 1996), raising the possibility that the 5′ *ine/rosA*-l specific sequence is from transcript(s) of other gene(s) and fused, by chance, onto the *ine/rosA*-s transcript during cDNA library generation.

To probe this possibility, I used the published *ine/rosA*-l specific cDNA sequence to search the Drosophila genome project database. Results showed that the *ine/rosA*-l specific sequence is only about 500 base pairs away from the 5' of the *ine/rosA*-s cDNA and spread over three exons (figure 3.7).

Results from searches on the Drosophila genome project database using *ine/rosA*-s and *ine/rosA*-l specific sequence and sequences shared by both forms revealed the organization of the *ine/rosA* gene transcription unit (figure 3.7). All of the *ine/rosA* cDNA sequences were found in one Drosophila P1 phage clone DS07068. Canonical splicing sites, 5′- GU... ... AG - 3′, have been identified in 4 introns. Three introns contain half of the canonical splicing sites at either 5′ or 3′ ends, and three have atypical splicing sites. In a mammalian GABA transporter,

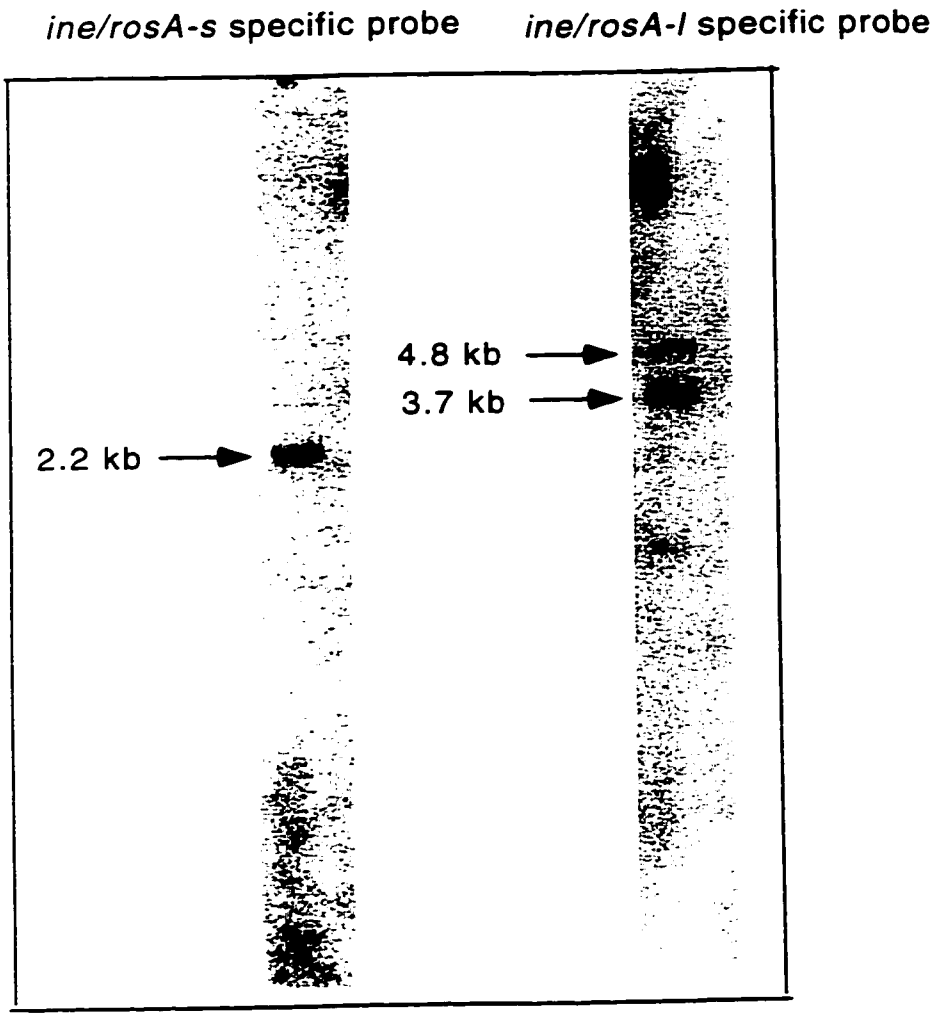


Figure 3.6 Northern blots using probes specific to *ine/rosA*-s and *ine/rosA*-l. About 5 μg of mRNA prepared from *iso brown* flies was loaded in each lane. The *ine/rosA*-s specific probe contains the *ine/rosA*-s specific exon. The *ine/rosA*-l specific probe contains all the three *ine/rosA*-l specific exons (see figure 3.7).

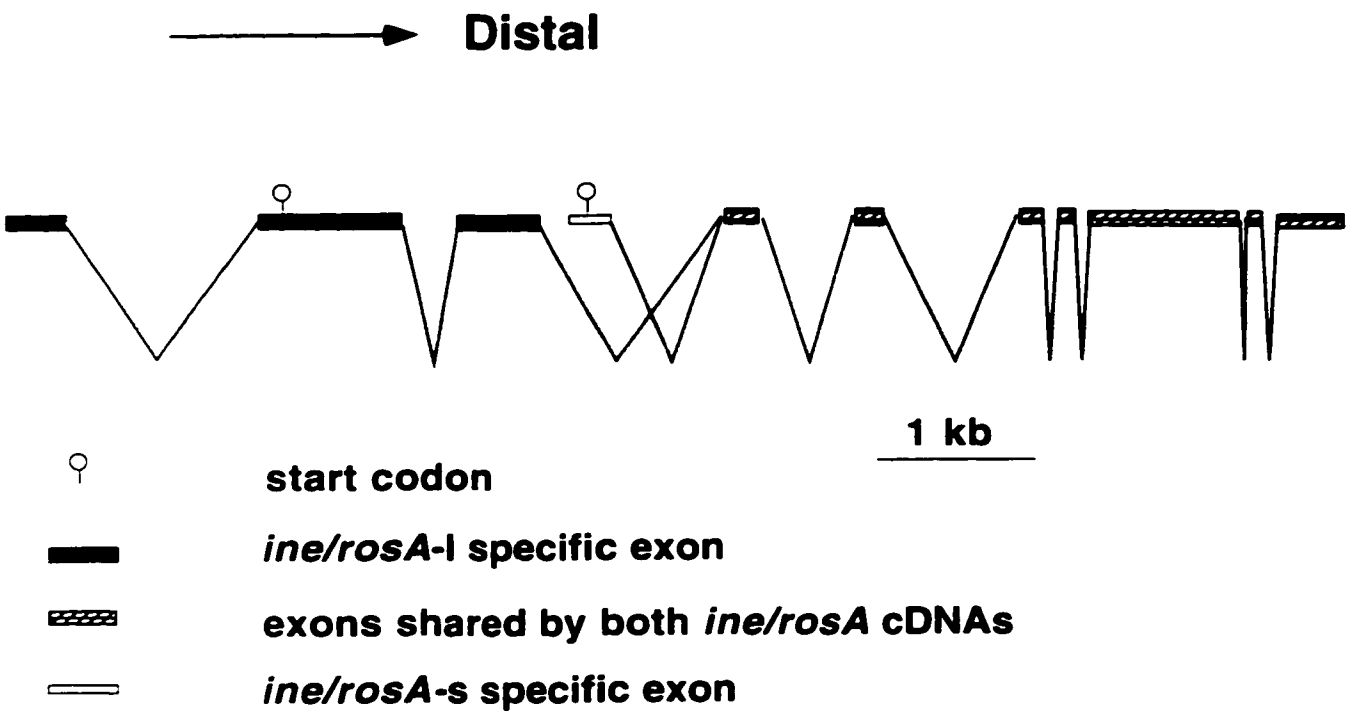


Figure 3.7 Organization of the *ine/rosA* transcription unit. There are three *ine/rosA*-l specific exons, one *ine/rosA*-s specific exon and seven exons shared by both cDNAs.

only 1 out 12 introns is not canonical (Liu *et al.*, 1992b). The significance of these non-canonical intron splicing sites is unclear.

From the aforementioned results, *ine/rosA*-l and *ine/rosA*-s have different 5' coding and UTR sequences but the same 3' sequences. This, together with the fact that both cDNAs are very close to the sizes of their transcripts on Northern blots, suggests that these transcripts are initiated from different promoters at their 5' ends. However, I can not completely exclude the possibility that *ine/rosA*-l and *ine/rosA*-s are alternatively spliced products of a gene from the same promoter. This is because I am not certain that we have the very 5' ends of the *ine/rosA* transcripts, which are usually defined by 5'-RACE PCR experiments.

The 4.8 kb transcript could be detected by *ine/rosA*-l specific sequence and whole *ine/rosA*-s cDNA probes, but not *ine/rosA*-s specific sequence (figure 3.5, 3.6). These results indicate that this 4.8 kb band could be a low-abundance transcript or partially spliced mRNA containing *ine/rosA*-l specific exon(s) and exons shared by *ine/rosA*-l and *ine/rosA*-s, but not the first exon of *ine/rosA*-s.

vi. Tissue distribution of *inebriated/rosA* transcripts

The tissue distribution of the *ine/rosA* transcripts may corroborate conclusions drawn from mutant phenotype analysis and provide new clues for gene function based on the functions of tissues. I have used whole-mount embryonic *in situ* hybridization to study the expression patterns of the *ine/rosA* gene. I used digoxygenin labeled *ine/rosA*-s antisense RNA as probe and digoxygenin labeled *ine/rosA*-s sense RNA in control experiments on 1- 16 hour developing embryos (figure 3.8). Consistent with the embryo cryo-section *in situ* results provided by Dr. Bernie Andruss, the *ine/rosA* gene is expressed in many embryonic tissues.

The posterior part of the hindgut and anal plate exhibit the strongest staining, Malpighian tubules, midgut, central nervous system (ventral ganglia), garland cells

near the proventriculus, tracheal termini, pharyngeal muscle region and small patches in the anterior region (perhaps the brain lobe), also have specific staining. It is of particular interest to notice that there is segmentally repeated staining along the ventral ganglia, with groups of cell bodies stained on either side of the midline.

For several reasons, it is possible that *ine/rosA*-l and *ine/rosA*-s are expressed in different organs of Drosophila and carry out different functions. First, from the distribution of the *ine/rosA* exons on Drosophila chromosome, it is likely that the *ine/rosA*-s and *ine/rosA*–l transcripts are initiated from different promoters. Also, as shown in later sections, *ine/rosA*-s and *ine/rosA*–l cDNAs rescue distinct sets of mutant phenotypes. For example, overexpression of *ine/rosA*-l cDNA rescues the downturned wing and indented thorax phenotype in a *Sh* mutant background, whereas overexpression of *ine/rosA*-s can't rescue these phenotypes (table 7.1). Third, members of Na^+/Cl^- neurotransmitter transporter family often have more than one transcript, probably due to alternative splicing or tissue specific promoters. Different mRNAs thus produced may have overlapping but distinct tissue specificity and functions, like in the case of GLYT-1 and GLYT-2 transporters (Borowsky *et al.*, 1993).

To address this possibility, I performed whole-mount embryo *in situ* hybridization using probes specific to *ine/rosA*-l and *ine/rosA*-s (fig 3.9). The results show that both *ine/rosA*-s and *ine/rosA*-l are expressed in the CNS, Malpighian tubules and the hindgut (figure 3.8).

3. Discussion and conclusions

From experimental results described in this chapter, we can conclude that the *ine/rosA* gene encodes a putative neurotransmitter/osmolyte transporter. Mutations in this gene cause increased neuronal excitability, possibly via altering

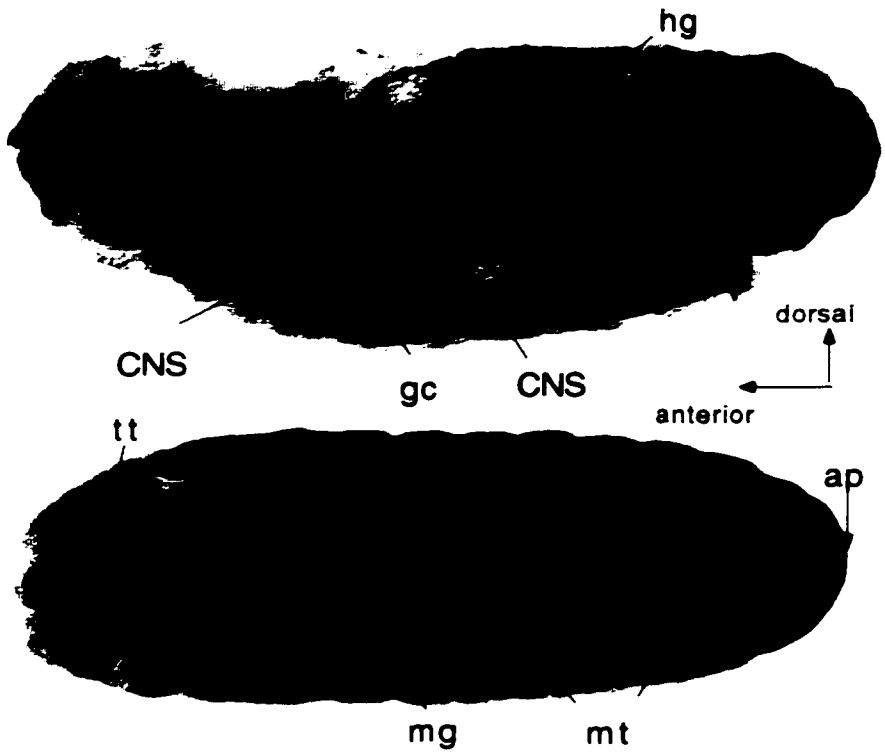


Figure 3.8 Distribution of *ine* transcript as illustrated by embryonic whole mount *in situ*. Digoxigenin labeled whole-length *ine/rosA*-s antisense RNA was used as probe. A. The lateral view of a stage 15 embryo; B. The Dorsal view of a stage 15 embryo. Abbreviations are as following. ap: anal plate; hg: hindgut; mg: midgut; mt: Malpighian tubules; tt: tracheal terminal; gc: garland cells; CNS: central nervous system. There are also small patches of staining in the anterior region that need further identification.

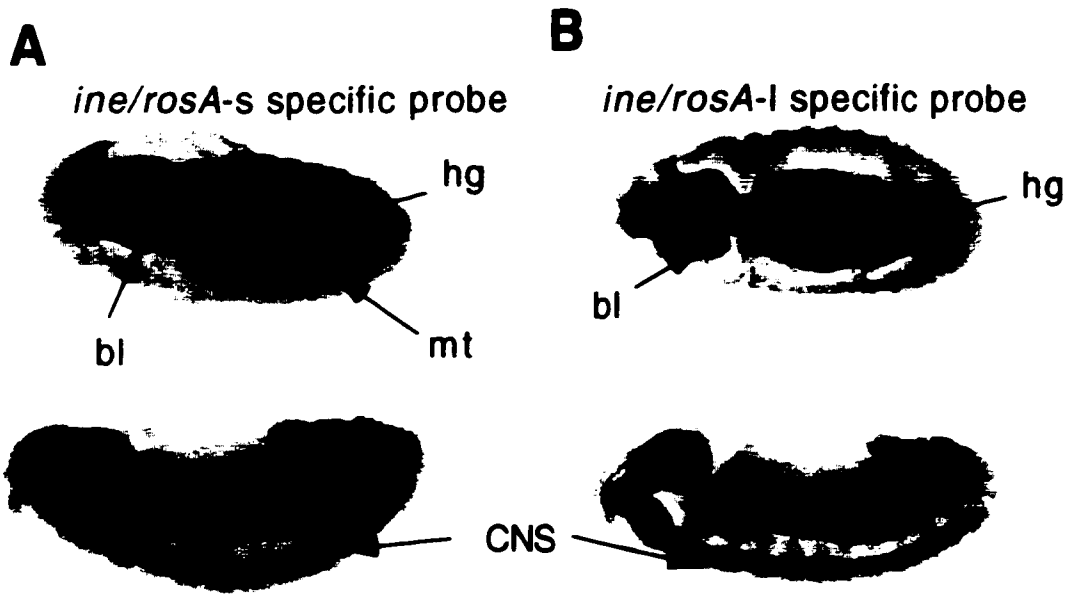


Figure 3.9 Whole-mount *in situ* hybridization using *ine/rosA*-s (A) and *ine/rosA*-l (B) specific probes. Both *ine/rosA* transcripts are found in the hindgut (hg), Malpighian tubules (mt), the central nervous system (CNS) along the ventral midline and brain lobes (bl). Lower panels: lateral view. Upper panels: dorsal view. The *ine/rosA*-s specific probe contains the *ine/rosA*-s specific exon. The *ine/rosA*-l specific probe contains all the three *ine/rosA*-l specific exons (see figure 3.7).

ion channel activities. The gene encodes two major transcripts that are localized in the same organs of Drosophila embryos.

The RFLP mapping results of *ine*^{1} and *ine*^{2} are disparate: the two mutations map about 10 kb away from each other. This discrepancy probably is due to the low number of recombination events measured. If the total number of recombinations were greater, the positions could be closer. It is also possible that recombination events are not evenly distributed on the Drosophila chromosome. It has been known that there are some "hot spots" for mutagenesis on the Drosophila chromosome. The 2A cDNA is proven to be located mainly in the 3' of the 'n' DNA fragment, and a small portion in 5' of 13-A fragment, very close to the locus positioned by the RFLP result on *ine*^{2}.

The nature of the *ine*^{2} mutation is still not clear. It could be a mutation in one of the *ine/rosA*-l specific exons that we did not examine.

Two major *ine/rosA* transcripts were detected on Northern blots that correspond to two structurally distinct transporters, Ine/RosA-l and Ine/RosA-s. It is not unprecedented for neurotransmitter transporters with different splicing products to arise from the same gene. For example, two forms of mouse glycine transporters, GLYT1-a and GLYT1-b differ only in their first exons, in which the N-terminal 10 amino acids of GLYT1-a and 15 amino acids of GLYT1-b are encoded (Liu *et al.*, 1993). It is still not known if the two transcripts are products of alternative splicing or from different promoters. These two transporters have very similar kinetics and tissue distribution pattern.

Localization of transcription by *in situ* hybridization has been commonly used in transporter studies. Na^+/Cl^- dependent transporters fall into two groups according to their localization patterns: the ones that are neural specific and the ones that are also seen in other tissues. Dopamine transporters belong to the former: their expression is restricted to cells in the brain that synthesize the corresponding

neurotransmitter (Ciliax *et al.*, 1999). Glycine transporters (Borowsky *et al.*, 1993) and GABA/betaine transporters (Rasola *et al.*, 1995) belong to the latter: these transporters have variants that are expressed in different tissues and locations.

Results from whole embryonic *in situ* hybridization experiments have shown that *ine/rosA* is transcribed in the CNS, as well as fluid absorption organs such as Malpighian tubules and the hindgut. Both transcripts are localized to the same organs in embryos. It is possible that Ine/RosA-l and Ine/RosA-s function together as a heterodimer. Homodimers of neurotransmitter transporters have been observed before, but its functional importance remains unclear (Rudnick, 1999). It is also possible that *ine/rosA*-l and *ine/rosA*-s are required in different parts of Drosophila at developmental stages other than embryogenesis, because all the phenotypes are observed after embryo development.

It could be imagined that in the CNS, the *ine/rosA* transporter transports neurotransmitters that modulate motor neuron excitability, whereas in the hindgut and Malpighian tubules, the *ine/rosA* proteins function to accumulate osmolytes in the cells and facilitate water absorption. There are also garland cells near the proventriculus that have strong *ine/rosA* transcription: these cells might also play a role in fluid absorption.

The *ine/rosA* transporter has the highest similarity to GABA transporters. Some GABA transporters (mouse GAT1, GAT4) have high GABA affinity and express exclusively in nervous system. These are often speculated to terminate synapse transmission. Others (mouse GAT2, GAT3) have low GABA affinity and can also transport betaine, taurine. These transporters can be present in tissues other than the nervous system, and function to transport osmolytes. These GABA transporters are reported to be expressed in diverse temporal-spatial patterns during mouse development (Nelson, 1998).

A large number of neurotransmitter transporters are being studied currently. Most of these studies start with and concentrate on isolating the cDNAs, deducing the amino acid sequences and then using probes made out of these cDNAs to isolate new transporter genes. Little has been done on the study of its structural features, even less on their physiological functions.

The study of dopamine transporter (DAT) knockout mice (Giros *et al.*, 1996) was remarkably successful in confirming the physiological role of DAT in hyperlocomotion, drug sensitivity and regulation of dopaminergic synaptic transmission. A phenomenon that is of interest is the regulatory function of DAT, shown in the downregulation of expression of dopamine receptors and dopamine synthesizing enzyme. The pursuit of the interactions between *ine/rosA* and the corresponding transmitter receptors, signal transduction components and the ultimate targets of any neurotransmission regulation, ion channels, is holding great promise for the better understanding of neurotransmission mechanism.

Chapter 4. Identification of the substrate(s) for the *inebriated/rosA* transporter

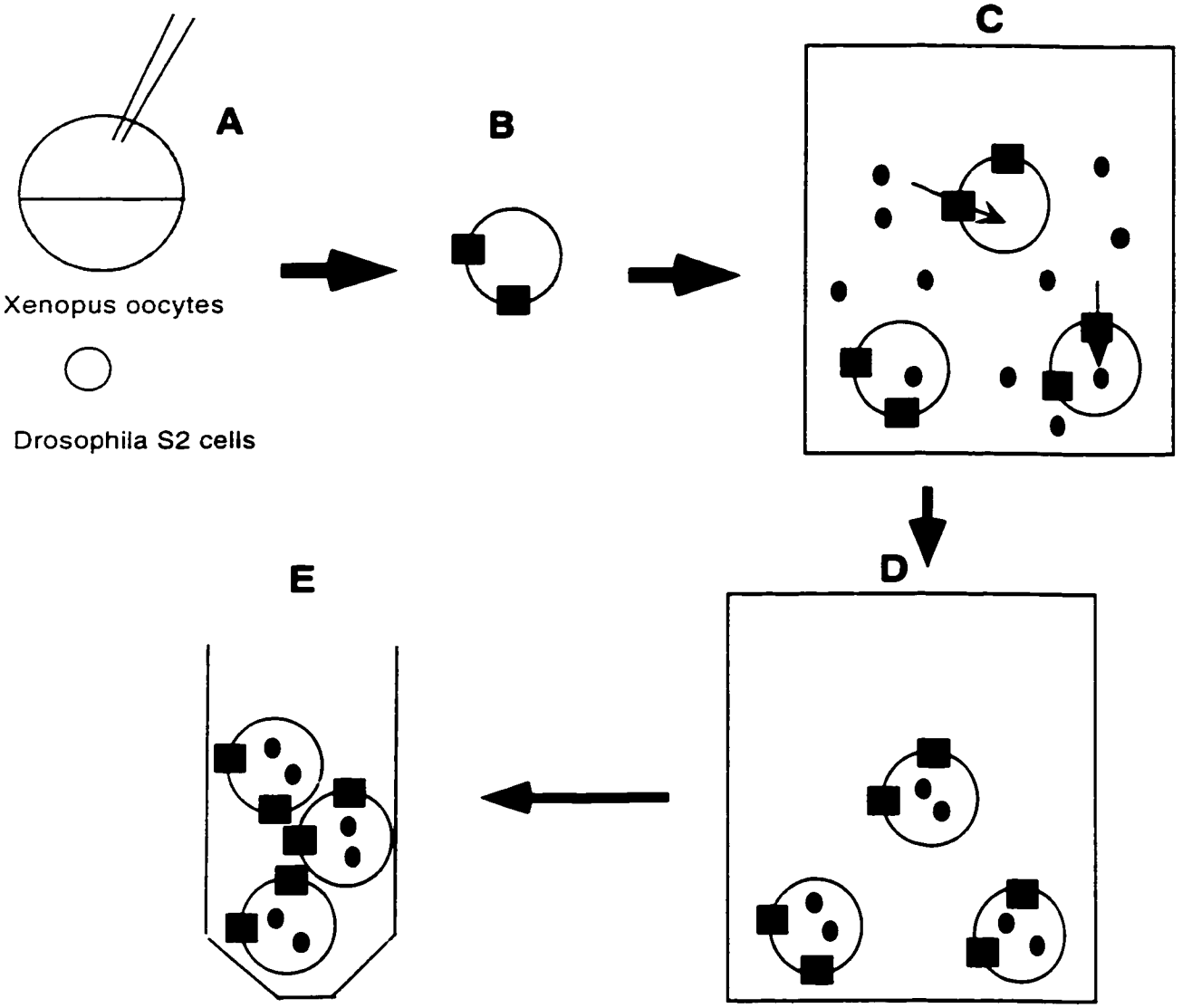
1. Introduction

The substrates of neurotransmitter transporters are usually identified through uptake assays. In this assay, neurotransmitter transporters are expressed on plasma membrane of cells. These cells are then incubated in solutions containing neurotransmitters or osmolytes labeled with ^{3}H or ^{14}C. After incubation, radioactive isotope is washed away and cells are lysed. Scintillation counting is then used to gauge how much substrate has been accumulated inside the cells (figure 4.1). Two systems are commonly used to express neurotransmitter transporters and conduct uptake assays, *Xenopus* oocytes and cultured cells.

Oocytes can be injected with cRNA (complementary RNA with mRNA sequences), mRNAs or cDNAs in expression vectors. Due to the size of *Xenopus* oocytes, this approach is suitable for electrophysiology studies and is how ion conductance of transporters and receptors are researched. The drawback is that one has to inject each individual oocyte and it is hard to examine if an oocyte is expressing the desired protein before electrophysiology or uptake assays. Commonly used cell lines include Cos-7 and Hela cell lines. Exogenous proteins are expressed using virus infection. With this system, one is examining the expression and function in a population of cells. Expression can be checked before uptake assays by taking a portion of cells and conducting Western or Northern analyses.

Knowing the substrate of a transporter will not only help interpret the function of the transporter by understanding the functions of its substrate, but also enable the further characterization of the transporter through kinetic studies. The kinetics of a neurotransmitter transporter reveals many important functional and

Figure 4.1 Schematic diagram showing the steps in uptake assays. A. Injection of *Xenopus* oocytes or transfection of cultured cells (S2 cells shown here) with transporter cDNAs. B. Expression of transporters (squares) on plasma membrane of a cell. C. Incubation of cells in solution containing ^{3}H labeled neurotransmitter or osmolyte substrates (circles). D. Cells are washed with a buffer without neurotransmitter or osmolyte substrates. E. Cells are lysed and counted for radioactivity.


A
B
C
D
E
Xenopus oocytes
Drosophila S2 cells

structural characteristics such as the affinity to the substrate(s), ion dependence, pharmacological profile, and regulatory pathways involved.

2. Results

i. Substrate uptake assays using the *Xenopus* oocyte system

A. Expression of neurotransmitter transporters

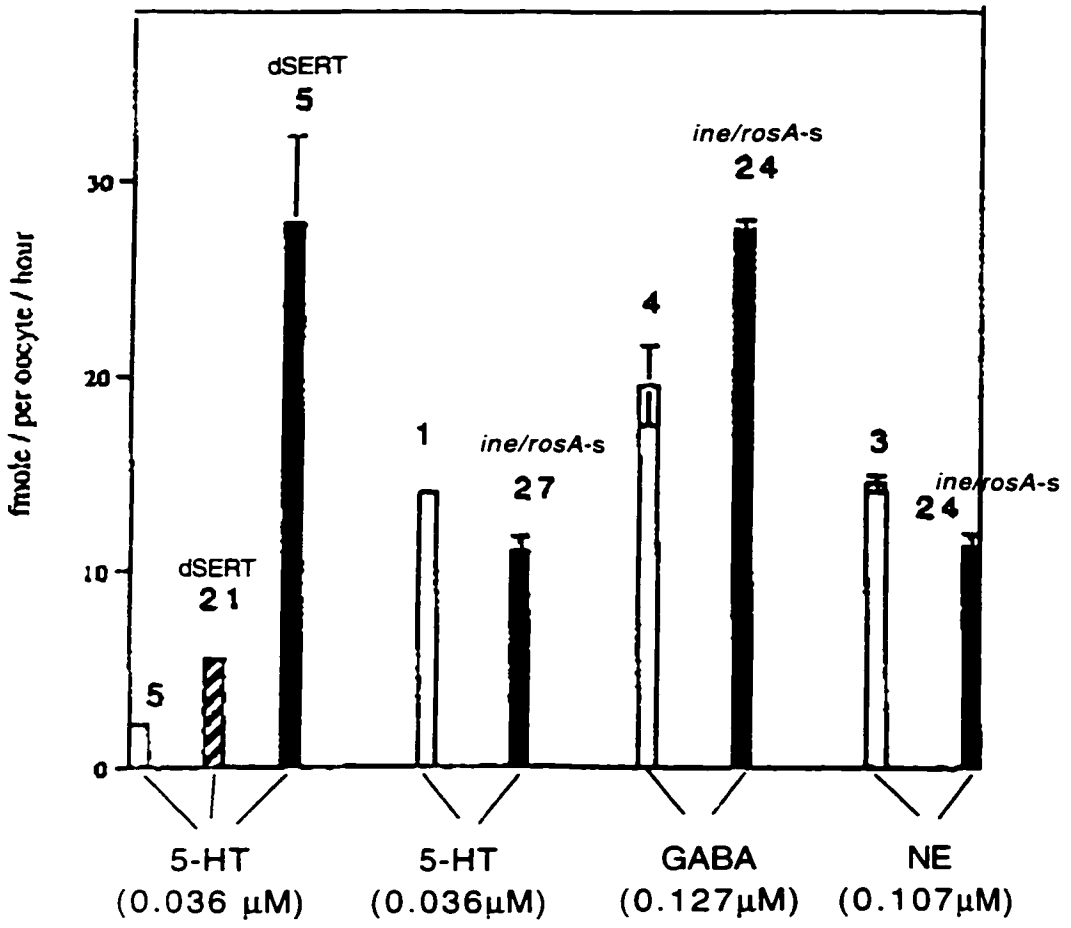
Neurotransmitter transporter cDNAs were subcloned into an expression vector (pcDNAI/Amp) driven by a cytomegalovirus (CMV) promoter (Materials and Methods). The constructs were then injected into the animal pole of stage V *Xenopus* oocytes. Due to the lack of antibodies against neurotransmitter transporters, the expression of injected cDNA was monitored by the expression of a Drosophila serotonin transporter gene (dSERT), which was analyzed by uptake assays. This Drosophila transporter was also used as a positive control to ensure that the system was working properly.

B. Potential neurotransmitter/osmolyte substrates tested

In every oocyte injection, dSERT cDNA was injected and expressed as a control to gauge the percentage of injected oocytes that can express transporter cDNAs. Usually oocytes expressing neurotransmitter transporters have uptake rates 5 to hundreds times those of uninjected oocytes. After incubation in buffer containing tritium labeled serotonin, about 20% of the oocytes injected with dSERT exhibited 5 fold or more increase in serotonin uptake than uninjected oocytes. Therefore, I estimate that about 20% of the oocytes injected with the *ine/rosA*-s cDNA may be expressing the transporter protein.

Figure 4.2 shows a representative group of data from GABA serotonin and norepinephrine (NE) uptake assays on oocytes injected with the *ine/rosA*-s cDNA.

Figure 4.2 Serotonin (5-HT), GABA and norepinephrine (NE) uptake assays on oocytes injected with transporter cDNAs. White bars are uptake by uninjected oocytes. The first black bar from the left represents serotonin uptake by oocytes injected with and expressing dSERT. Other black bars from left to right are serotonin, GABA and NE uptake by oocytes injected with *ine/rosA*-s cDNA. The first hatched bar from left indicates oocytes injected with dSERT but not taking up 5-HT. The number above each bar indicates the number of oocytes used in the test. Tritium labeled neurotransmitters and their concentrations in parentheses are at the bottom.



uninjected oocytes

oocytes injected with transporter cDNAs

oocytes injected with dSERT cDNA but have low 5-HT uptake

There are no significant increase of GABA, serotonin or NE uptake among these oocytes compared with uninjected ones.

Another set of substrates, taurine, glycine and dopamine were tested on the same system (figure 4.3). Whereas taurine and dopamine uptake did not show any significant difference between injected and uninjected oocytes, glycine uptake in 48% of the oocytes injected with *ine/rosA*-s cDNA exhibited a more than 5 fold increase compared with uninjected oocytes.

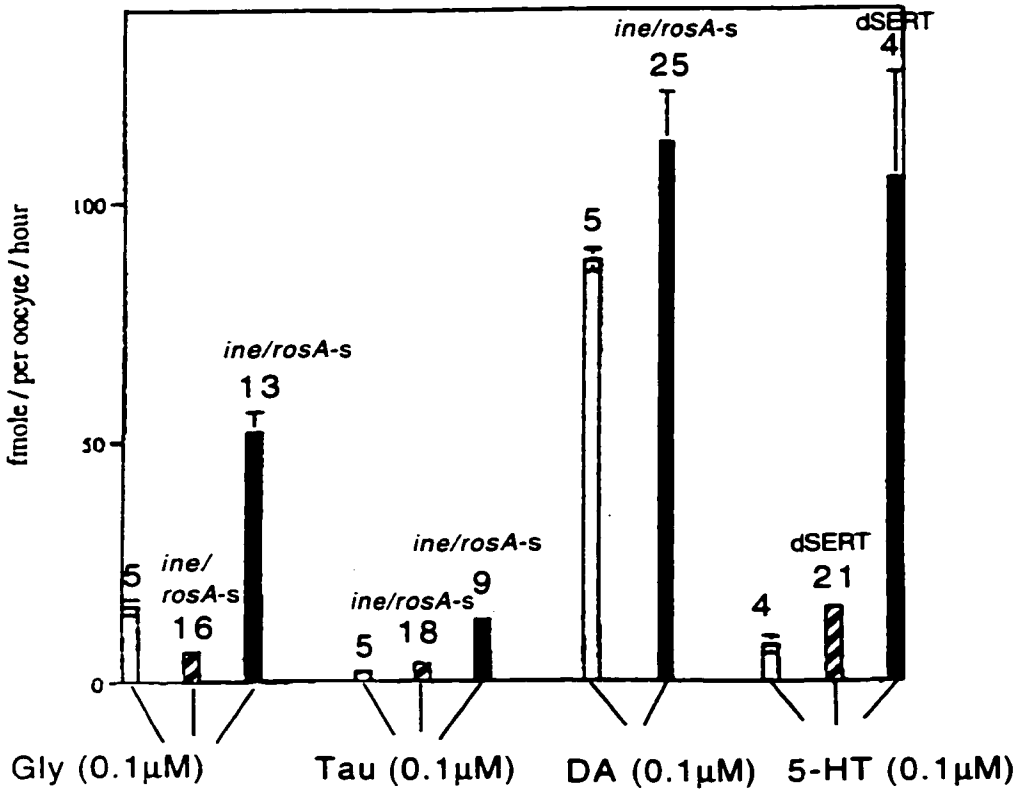
Additional uptake assays on glycine were performed to both repeat the previous result and to generate the kinetic curve as a function of glycine concentration. However, results from these experiments were sporadic and many times failed to show any difference between injected and uninjected oocytes.

These results together with the low expression rate and high variation of uptake in cDNA injection prompted me to switch to injecting oocytes with transporter mRNAs synthesized in vitro. Usually a larger fraction of oocytes injected with synthetic mRNA express transporter.

The *ine/rosA*-s and dSERT mRNA were synthesized using T7 and SP6 promoters on the pcDNAI/Amp vector. Glycine uptake assay result on oocytes injected with synthesized *ine/rosA*-s and dSERT mRNA is shown in figure 4.4. The control oocytes with dSERT failed to show serotonin uptake, which means either that the dSERT mRNA may be degraded before translation, or that too little protein is produced. In this experiment I also found that there are endogenous glycine transporters whose transport of glycine is almost completely abolished by the absence of sodium. This endogenous glycine transporter might account for the glycine uptake results I observed before (figure 4.3).

With all the problems I encountered in the *Xenopus* oocyte system, such as the great variation among individual oocytes and endogenous uptake, as well as the

Figure 4.3 Taurine, glycine (gly) and dopamine uptake assays on oocytes injected with transporter cDNAs. White bars are the uptake by uninjected oocytes. The black bar and the striped bar on the right hand side are the uptake of serotonin by control oocytes injected with dSERT, other black and striped bars are the uptake of different neurotransmitter by oocytes injected with *ine/rosA*-s cDNA. Concentrations of tritium labeled neurotransmitters are indicated in parentheses. The number above each bar indicates the number of oocytes used in the test.



uninjected oocytes

oocytes injected with transporter cDNAs

oocytes injected with transporter cDNAs but have low uptake

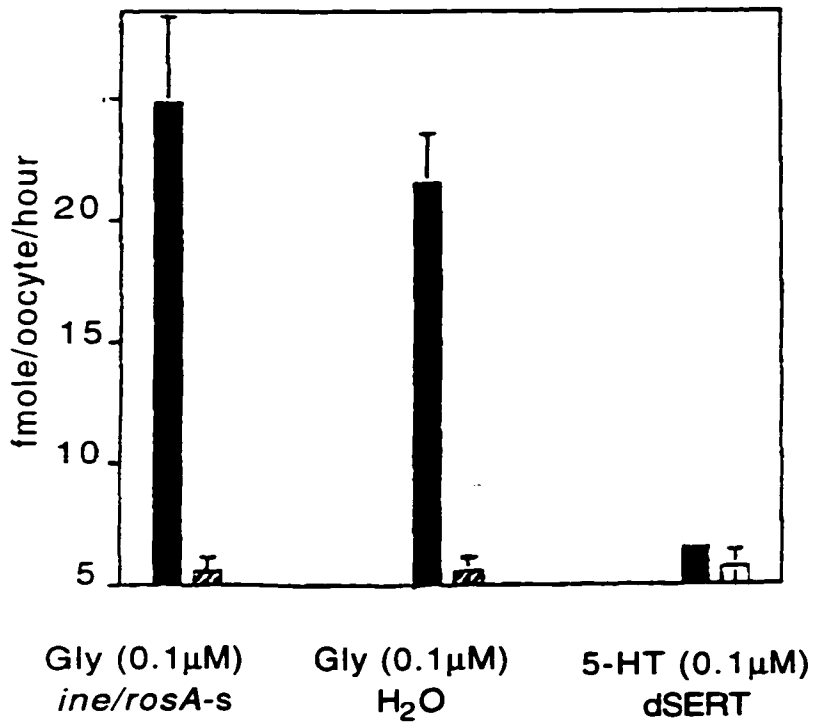


Figure 4.4 Glycine uptake assays on oocytes injected with synthetic transporter mRNAs. Black bars are glycine uptake by oocytes injected with *ine/rosA*-s synthetic RNA, H2O and dSERT synthetic mRNA, from left to right. Striped bars are glycine uptake by oocytes injected with *ine/rosA*-s and H_2O in Na^+ free buffer from left to right. Eight to ten oocytes were tested for each data point. The white bar is the uptake of serotonin by uninjected oocytes.

concern that *ine/rosA* transporter may require a Drosophila specific component to translate well or to function, I decided to switch to the S2 cell culture system.

ii. Substrate uptake assays using the Drosophila S2 cell culture

A. Expression of neurotransmitter transporters

I chose the S2 cells line to perform uptake assays because S2 cells might provide an environment similar to what Ine/RosA encounters in vivo and these cells do not produce *ine/rosA* transcripts (figure 4.5A).

Initially, *ine/rosA*-s and *ine/rosA*-l cDNAs were subcloned into the pMTA expression vector without the last few amino acids and the stop codon and were in frame with the V5 epitope tag and the hexohistidine tag. The transcription of the *ine/rosA* cDNAs was examined via Northern blotting (figure 4.5 B). Expression of protein was detected with peroxidase conjugated anti-V5 monoclonal antibody followed by chemiluminescent visualization (figure 4.6 A).

It was reported that neurotransmitter transporters can exist both in intracellular compartments, such as endoplasmic reticulum, and plasma membrane in *Xenopus* oocytes. Using anti-V5 antibody and fluorescein labeled horse anti-mouse IgG, I performed fluorescent staining on S2 cells expressing *ine/rosA*-s. I found Ine/RosA-s to be on cell surface, although it may also exist in cytosol (data not shown).

In response to concerns that the epitope tags and the removal of C-terminal amino acids in recombinant Ine/RosA expressed in S2 cells may interfere with their ability to accumulate substrate(s), I subcloned *ine/rosA* -l and *ine/rosA* -s open reading frames into the pMTA vector (Materials and Methods) to express Ine/RosA native proteins. Knowing that *ine/rosA* -l and *ine/rosA* -s are transcribed in the same organs, I have generated transient and stable lines inducibly expressing both Ine/RosA-l and Ine/RosA-s to ensure that uptake be detected if both are

Figure 4.5 The mRNA levels of *ine/rosA* in untransfected and transfected S2 cells. A. S2 cells do not express *ine/rosA*. Northern blot of mRNA (about 5 μg) extracted from wildtype flies (right lane) and from S2 cells grown in media of three different osmolarity (330, 630, and 930 milliosmolar from the second to the forth lane, normal medium for S2 cells has osmolarity of 330 Os/kg). The lower panel is the loading control using *RpL27a* probe. B. Strong *ine/rosA*-s transcription is detected in S2 cells transfected with pMTA-*ine/rosA*-s after Cu^{++} induction, shown in the left lane. The two lanes on the right are mRNAs from wild type flies. The loading is roughly equal judging from methylene blue staining (not shown here).

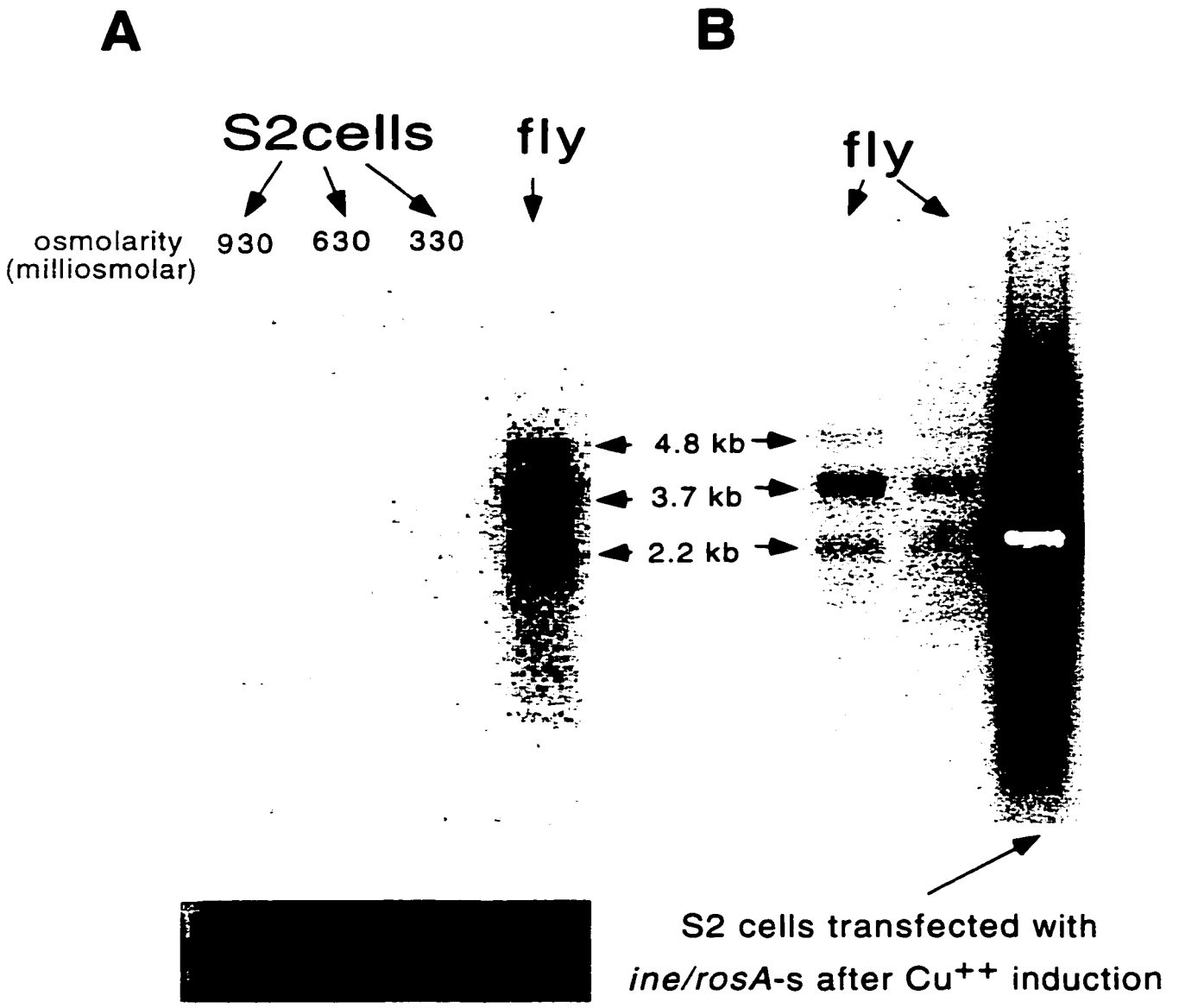
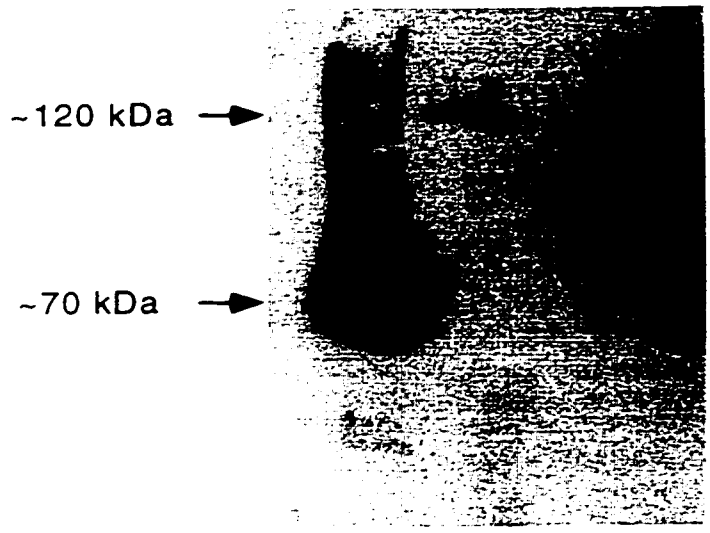

A
B
S2cells
fly
fly
osmolarity
(milliosmolar)
930
630
330
4.8 kb
3.7 kb
2.2 kb
S2 cells transfected with
ine/rosA-s after Cu++ induction

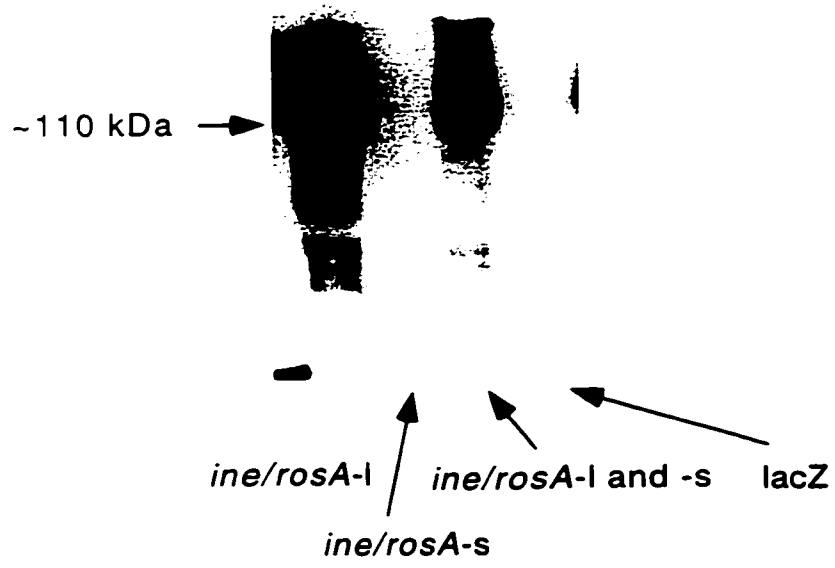
Figure 4.6 Expression of Ine/RosA in S2 cells. Proteins from about 10^5 S2 cells were loaded in each lane. A. Left lane: Western blot showing the expression of Ine/RosA-s detected by anti-V5 antibody after Cu^{++} induction of S2 cells transfected with pMT-ine. Middle lane: Expression of a lacZ control protein with V5 tag is detected. Right lane: The negative control from mock transfected S2 cells gives no signal. B. Expression of Ine/RosA-l protein in S2 cells detected by anti-serum raised against the unique N-terminus of Ine/RosA-l.

A.



ine/rosA-s lacZ untransfected

B.



required for it. Expression of transporters were verified via Western blots before each uptake assays (figure 4.6 B).

B. Potential neurotransmitter/osmolyte substrates tested

Uptake assays were performed on S2 cells expressing recombinant Ine/RosA-s protein with the V5 and hexohistidine tags. Six potential substrates were tested and none of them showed specific accumulation (figure 4.7).

Uptake assays were then conducted on 8 potential substrates (serotonin, NE, GABA, Gly, praline, choline, taurine and dopamine) using S2 cells transiently transfected with and expressing either or both whole-length *ine/rosA* cDNAs without epitope tags (figure 4.8). None of these substrates was accumulated by these S2 cells.

Considering that the transfection procedure might affect S2 cells physiologically and consequently their transport of substrates, uptake assays were also performed on S2 cells stably transfected with *ine/rosA* cDNAs (figure 4.9). In this experiment, newly established S2 stable lines expressing Ine/RosA protein were tested and a lacZ stable line established 4 months previously was used as negative control. S2 cells expressing Ine/RosA showed 4-6 fold increase in choline uptake compared to lacZ control line.

Choline uptake assays were repeated a few weeks after the establishment of S2 stable lines expressing Ine/RosA (figure 4.10). The uptake of choline by S2 cells expressing both Ine/RosA-s and Ine/RosA-l was not higher than that by the lacZ line and the uptake by both lines can be abolished by the lack of Na^+ in the uptake buffer.

It is possible that endogenous Na^+ dependent choline uptake is stimulated by transfection, because choline is a very important precursor for lipid molecules in the

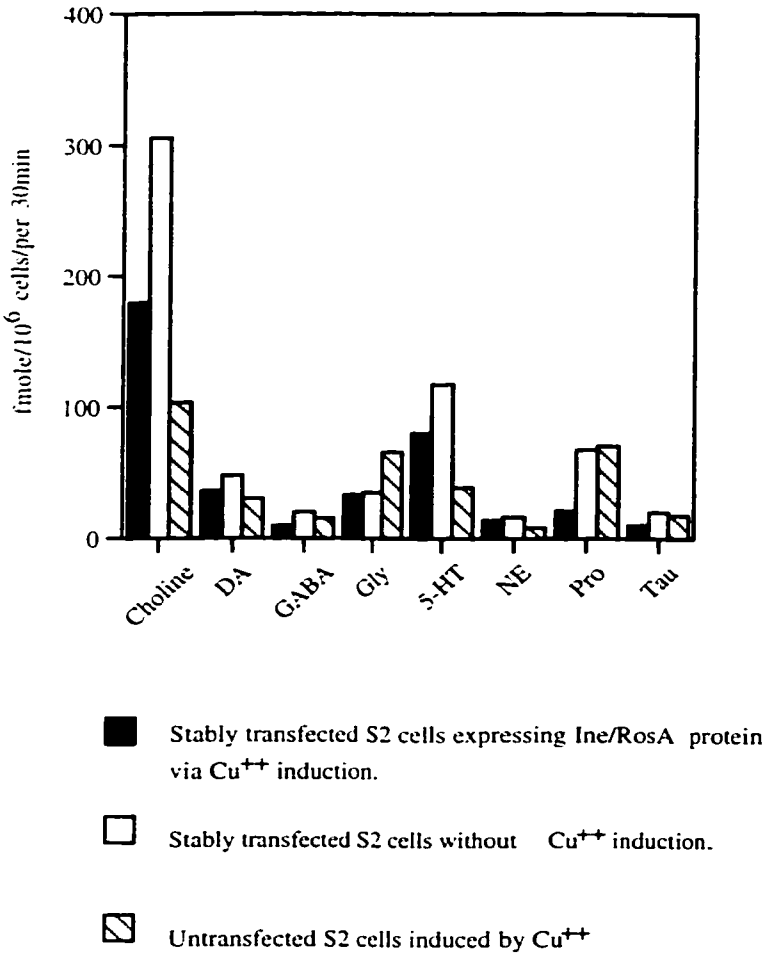


Figure 4.7 Uptake assays on S2 cells expressing Ine/RosA-s protein with hexohistidine and V5 tags. Each ^{3}H labeled substrate is used at 0.1μM. Uninduced stably transfected S2 cells and Cu^{++} induced untransfected S2 cells were used as controls.

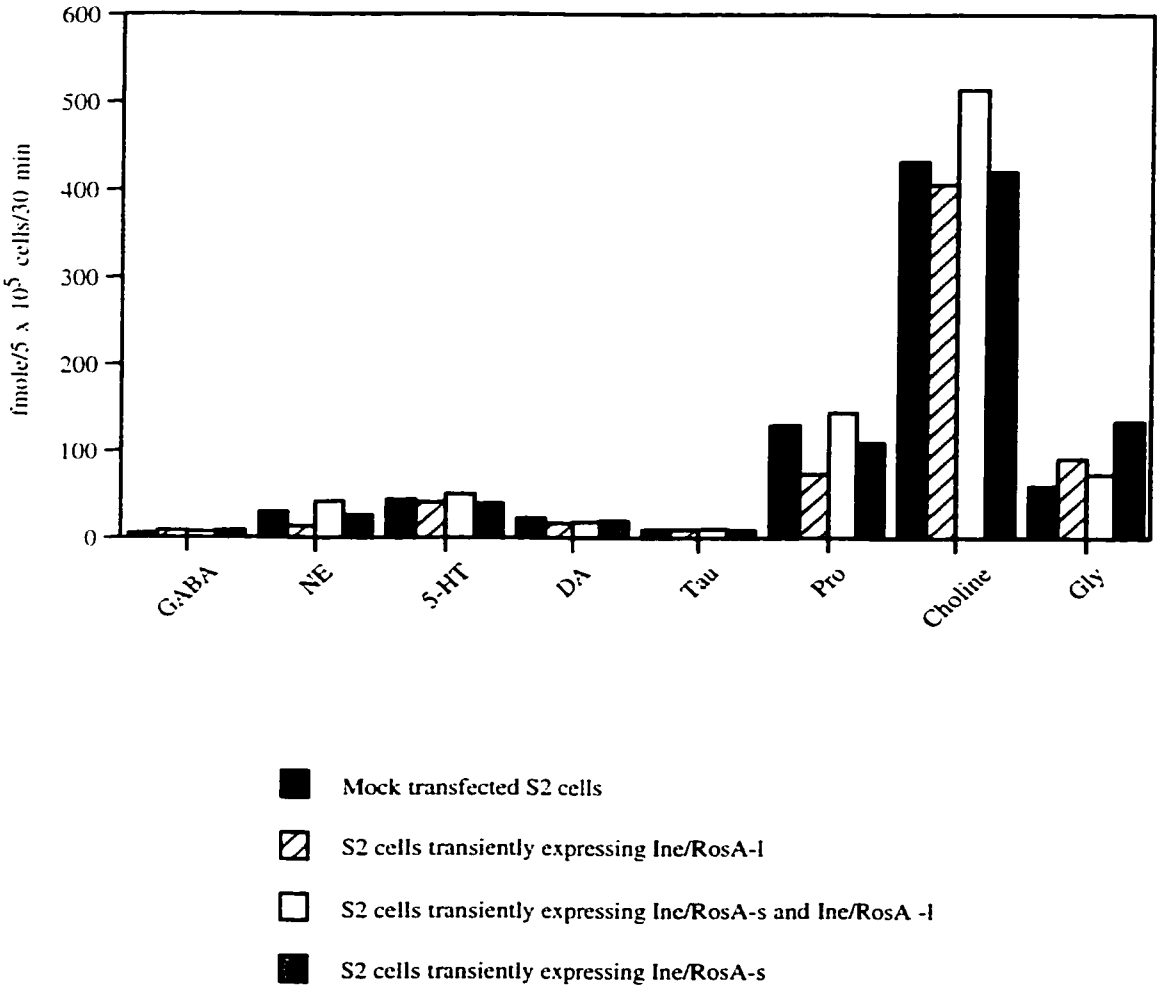


Figure 4.8 Uptake assays on S2 cells transiently expressing Ine/RosA proteins. Mock transfected S2 cells were used as negative control. Each ^{3}H labeled substrate is used at 0.1 μM.

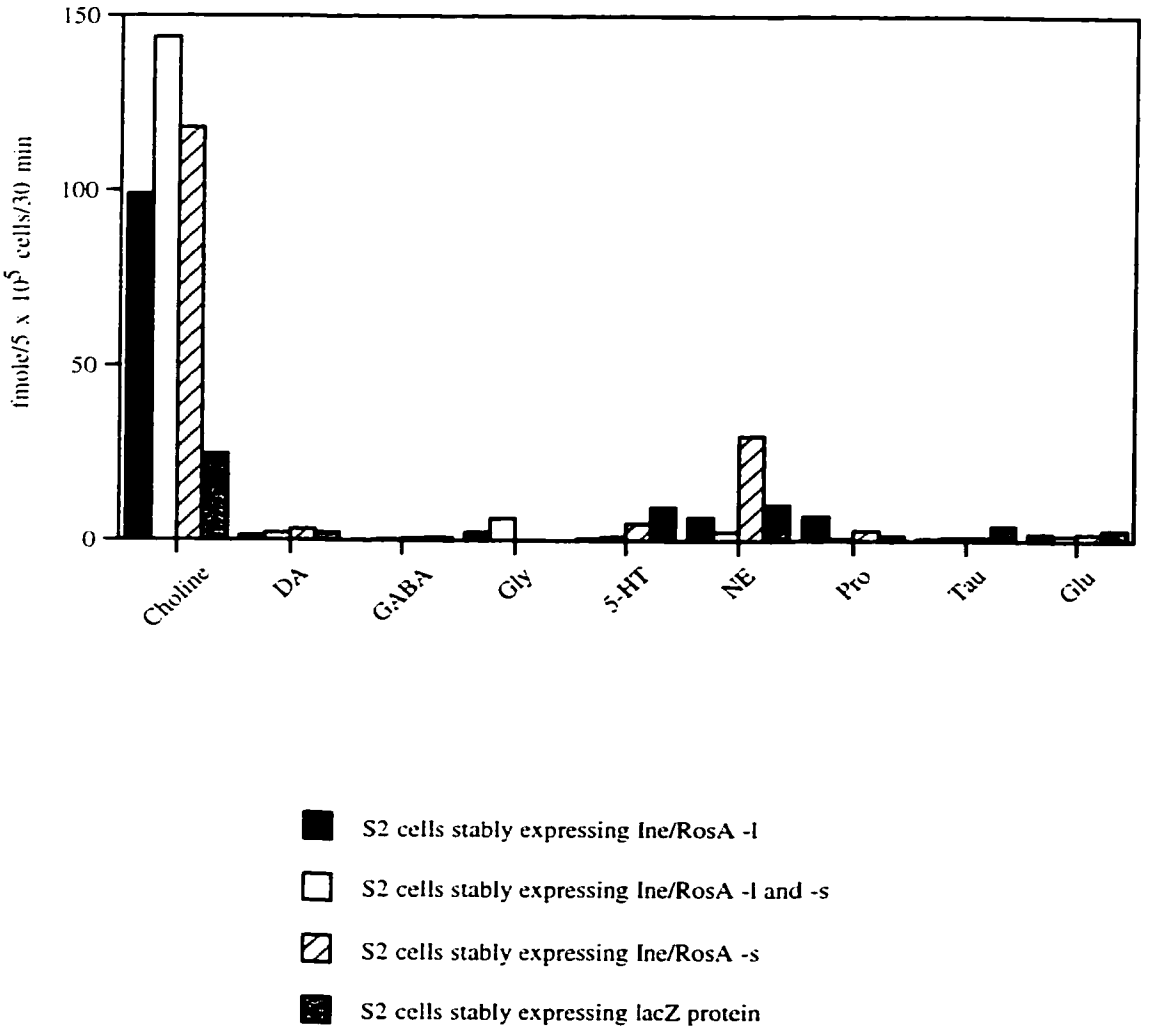


Figure 4.9 Uptake assays on newly established stable S2 cell lines. A S2 cell line stably transfected with lacZ was used as control. Each ^3H labeled substrate is used at 0.1μM.

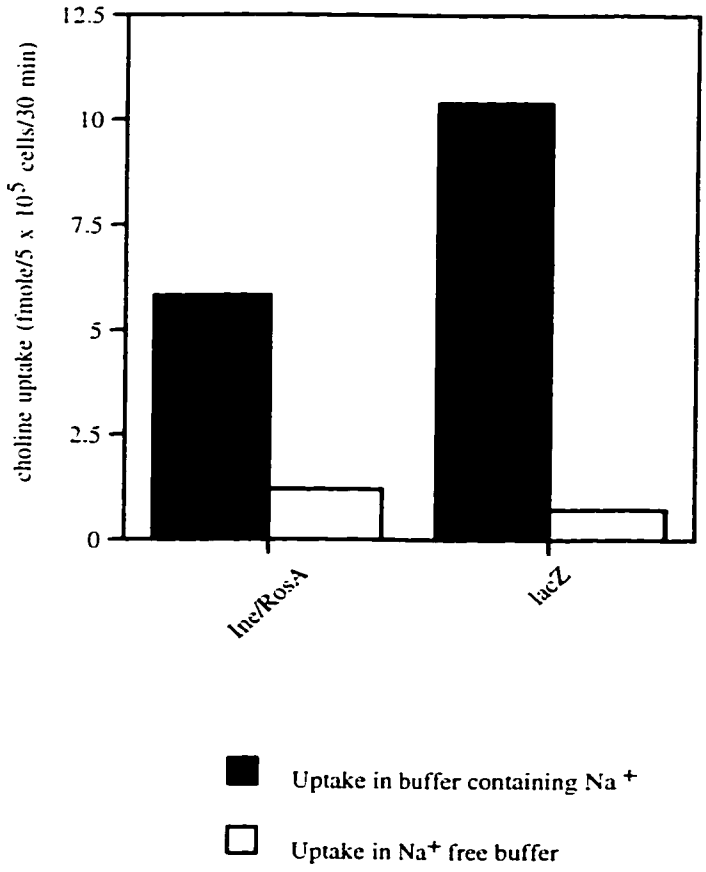


Figure 4.10 Choline uptake assays on long established stable S2 cell lines. Uptake assays were performed 3 to 4 weeks after stable transfection with both *ine/rosA*-s and *ine/rosA*-l. ^3H labeled choline is used at 0.1μM. A S2 cell line stably transfected with lacZ was used as control.

membrane that experiences drastic changes during transfection. Therefore newly transfected S2 cells may exhibit stronger choline uptake than those transfected long ago. The difference goes away as the endogenous uptake gradually subsides weeks after the transfection.

3. Discussion

There are four major subfamilies of Na^+/Cl^- dependent neurotransmitter transporters: monoamine transporters, amino acid transporters, GABA transporters and orphan transporters. The *ine/rosA* amino acid sequence shares about 40 % sequence identity to almost all of the transporters with known substrates. Identity to GABA/betaine transporter (42 %) is slightly higher than to other transporters. Monoamine transporters are usually represented by a single cDNA, whereas *ine/rosA* have multiple cDNAs like GABA and amino acid transporters.

Guessing substrate identity through global sequence comparisons has considerable limitations. Even though neurotransmitter transporters with the same substrate(s) usually share higher homology than those with different substrate(s), there are exceptions. For example, the glycine transporter GLYT2 is 48% identical to GLYT1 and is 50% homologous to a proline transporter (Liu *et al.*, 1993).

There is very limited knowledge on the primary or secondary structural features that determine substrate specificity. The *ine/rosA* transporter has the tryptophan in the fourth transmembrane domain that is characteristic of GABA, taurine and glycine transporters, but not the aspartic acid in the first transmembrane domain that is reported to be important for monoamine recognition (Chapter 1 Introduction). Despite the existence of some features common among GABA, taurine and glycine transporters, the *ine/rosA* transporter does not transport any of them, when expressed in oocytes or S2 cells.

Many neurotransmitters are found active in the Drosophila brain, including acetylcholine, serotonin, GABA, dopamine, glutamate, histamine, octopamine, and tyramine (Buchner, 1991). The major excitatory neurotransmitter at the neuromuscular junction is glutamate. There are also amines such as serotonin and octopamine in the digestive duct of insects, which facilitate ion transport (Csoknya *et al.*, 1997).

Dr. Sarjeet Gill's group in the University of California, Riverside, California is studying a Manduca neurotransmitter transporter that shows 60 - 70% amino acid sequence identity with the Drosophila Ine/RosA transporter. This Manduca *ine/rosA* homologue is expressed in axons, glia, certain muscle cells, the hindgut and Malpighian tubules. They have expressed the Manduca *ine/rosA* homologue in Xenopus oocytes, performed uptake assays on 15 potential neurotransmitter substrates, and failed to detect the accumulation of any of them.

I have tested a total of 9 neurotransmitters and osmolytes for their potential to be the substrate of the Ine/RosA transporter (listed in table 4.1). None of them seems to be the substrate of Ine/RosA transporter. Except from the limited number of neurotransmitter/osmolytes I have tested, other reasons may explain the unsuccessful search for Ine/RosA substrates. For example, Ine/RosA transporter may require an additional subunit or a protein partner for its function. There might also be cofactors or special ions needed.

Other labs also have reported that Xenopus oocytes have certain endogenous Na^+/Cl^- dependent transporters such as choline transporter, which can be activated by stimulation such as needle injection. Using two different expression systems may overcome this problem because the two systems may have different endogenous transporter portfolios.

A major issue in the uptake assays using the S2 cell culture system is the lack of proper positive control to validate this system for functional neurotransmitter

transporter expression and uptake. Several attempts to subclone dSERT and a rat GABA transporter into the pMTA S2 cell expression vector have been unsuccessful due to mutations created by PCR reactions used to amplify these cDNAs before the subcloning step.

Table 4.1 List of ^{3}H labeled neurotransmitter/osmolyte substrates tested. Specific activities and the conversion between scintillation readings and the amounts of substrates in moles are shown. 1μCi = 2.22 x 10^{6} dpm.

Substrates were purchased from DuPont.

	Specific activity	1 dpm =
Taurine	24.1 mCi/μmole	1.89x10-^{2} fmole
Dopamine	60 mCi/μmole	0.756x10-^{2} fmole
Glycine	44 mCi/μmole	1.03x10-^{2} fmole
Norepinephrine	12.56 mCi/μmole	3.12x10-^{2} fmole
GABA	100 mCi/μmole	0.45x10-^{2} fmole
Serotonin	27 mCi/μmole	1.68x10-^{2} fmole
Choline	75 mCi/μmole	0.605x10-^{2} fmole
Proline	48 mCi/μmole	0.95x10-^{2} fmole
Glutamate	46 mCi/μmole	0.99x10-^{2} fmole

Chapter 5. Generation of antibodies against the *inebriated/rosA* transporter protein

1. Introduction

Antibodies are powerful tools to study the tissue distribution and subcellular localization of proteins and to interpret their functions. Further, coimmunolocalization of a newly identified protein with other proteins with known functions can provide clues for the roles of a new protein. Antibodies can also help isolate potential interacting protein partners in co-immunoprecipitation assays.

Development of antibodies is among the first things to do in functional studies following the identification of a novel protein. The usual approach to antibody generation includes immunizing animals with either recombinant proteins expressed and purified from *E. coli*, or with synthesized peptides containing partial sequences of the protein (Bruss *et al.*, 1995; Melikian *et al.*, 1994).

Transmembrane proteins pose some difficulties to antibody development because they are insoluble and harder to overexpress in *E. coli* and purify as a whole. These difficulties can be circumvented by expressing soluble parts of a transmembrane protein, from either the cytoplasmic or the external parts. The fact that *E. coli* does not properly express exogenous peptides less than 15 kDa makes it necessary in many cases to fuse a short peptide onto another protein, such as glutathione-S transferase (GST) or maltose binding protein, in order to achieve effective expression (Krieg *et al.*, 1984).

Synthetic peptides, usually less than 18 amino acids long, from putatively soluble parts of a transmembrane protein have also been proven to be effective in generating certain antibodies. These peptides are conjugated to carrier proteins before immunizations in order to invoke an immune response (Harlow, 1988).

Many factors dictate the immunogenecity of an antigen. These factors include the size and charge of the side chains and hydropathy of amino acids, but many other unidentified factors also contribute. It is always a good strategy to start with a large protein or peptide as immunogens to maximize the chance of getting effective polyclonal antibodies. Denatured or precipitated proteins are usually better immunogens than native ones. Occasionally, antigens need to be chemically modified to become more antigenic or to overcome the presence of highly conserved protein(s) in the host (Harlow, 1988).

Affinity purification of antiserum against an antigen column may be required to increase the specificity and concentration of polyclonal antibodies. With antisera developed against fusion proteins, affinity purification is especially useful to obtain antibodies to desired amino acid sequences.

2. Results

i. Various recombinant proteins and peptides used as antigens to immunize animals

The C-terminal 450 amino acids (Ine-R1), the cytoplasmic N-terminus and C-terminus of Ine/RosA -s protein (NT and CT) were expressed in *E. coli* either as GST fusion proteins or peptides with hexohistidine tags. These proteins were purified and used as antigens. These recombinant proteins and some synthetic peptides from N-terminal and C-terminal sequences of Ine/RosA-s (Peptide-NT and Peptide-CT), together with the stages of antibody development using these antigens, are listed in table 5.1 and will be explained in more detail in this chapter.

Originally, I attempted to express large chunks of the Ine/RosA protein, regardless of their solubility, in *E. coli*. Large Ine/RosA peptides may be more antigenic. If overexpression was achieved, I could purify the protein. Otherwise, if

Table 5.1. Recombinant protein and synthetic peptide antigens with partial Ine/RosA amino acid sequences used to generate antibodies. *: hexohistidine tag. Not included in the table is the effort to purify whole length Ine/RosA-s protein from transfected S2 cells to be used as antigen (see text).

Name		Ine-R1	GST-NT	pET-NT	GST-CT	pET-CT	Peptide-NT	Peptide-CT	ROSA-N
amino acid sequence		204-658 of Ine/RosA-s	GST and 1-64 of Ine/RosA-s	6xHis* and 1-64 of Ine/RosA-s	GST and 571-658 of Ine/RosA-s	6xHis* and 571-658 of Ine/RosA-s	36-50 of Ine/RosA-s	603-617 of Ine/RosA-s	6xHis* and 1-313 of Ine/RosA-l
Expression vector		pET23-a	pGEX-KT	pET23-a pET15-b	pGEX-KT	pET23-a pET15-b	——	——	pET23-a
Overexpression in *E. coli*		no	yes	no	yes	no	——	——	yes
Purification		——	yes	——	yes	——	——	——	yes
Animals injected		——	Rabbit RC 36, RC 37	——	Guinea pig RCGP1, RCGP2 Rabbit RC 35	——	Rabbit RC 40, RC 41	Rabbit RC 38, RC 39	Rabbit RC 47, RC 48
Efficacy of antisera	On S2 cells expressing Ine/RosA	——	no	——	no	——	no	no	yes
	On fly lysate	——	no	——	no	——	no	no	no

purification was proven to be difficult, I could excise the overexpressed protein from SDS polyacrylamide gel after electrophoresis, immunize animals and then affinity purify the antibody.

I subcloned a piece of *ine/rosA*-s cDNA from an EcoRI site to the stop codon, encoding Ine-R1 that contains about 450 C-terminal amino acids with 9 transmembrane domains, into the pET-23a vector. I used the resulting construct to transform BL21 DE3 LysS (BL21) cells (Materials and Methods). This strain of bacterium is chosen because it lacks certain proteases in the cytoplasm and the membrane and reduces protein degradation after cells are broken. BL21 is especially useful for pET vectors that use T7 promoter for transcription initiation because it has the T7 polymerase gene, controlled transcriptionally by the lactose operator region, incorporated into its genome. After IPTG induction, the expressed T7 polymerase will drive the expression of cDNAs subcloned into pET vectors.

Because individual colonies containing the same expression construct may have different expression levels, in every protein expression experiment, several colonies were picked to examine their expression of proteins. I picked 9 colonies of BL21 transformed with pET23-a-Ine-R1 and none showed overexpression of proteins of predicted size on SDS-PAGE (figure 5.1). The BL21 transformants grew very slowly. It took them about 9 hours to grow to a cell density of O.D. 0.6, compared to 2-3 hours for BL21 cells with other constructs.

The cDNA fragments encoding NT and CT were cloned into the pGEX-KT vector. The resulting constructs were called pGEX-NT and pGEX-CT respectively. After IPTG induction, overexpression can be seen in BL21 bacteria transformed with both pGEX-NT and pGEX-CT. Two overexpressed proteins were seen in pGEX-NT transformants. One was about 34 kDa, the other about 41 kDa (figure 5.2). The 34 kDa protein is about the size of GST. It is possible that the fusion proteins were cleaved at

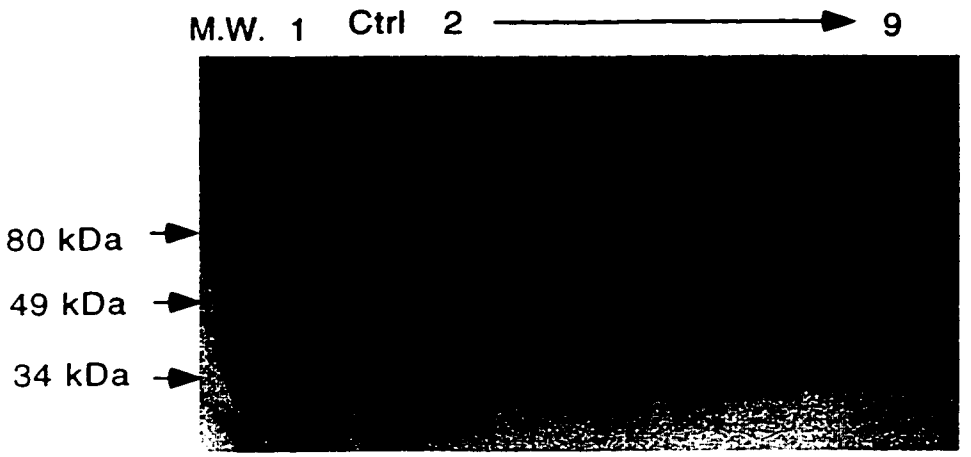


Figure 5.1 Coomassie staining of a polyacrylamide gel showing the induction of Ine-R1. M.W. : molecular weight marker. Ctrl: BL21 transformed with pET23 vector. Lane 1-9: BL21 colonies transformed with the pET23-a-Ine-R1 construct. For more details see text.

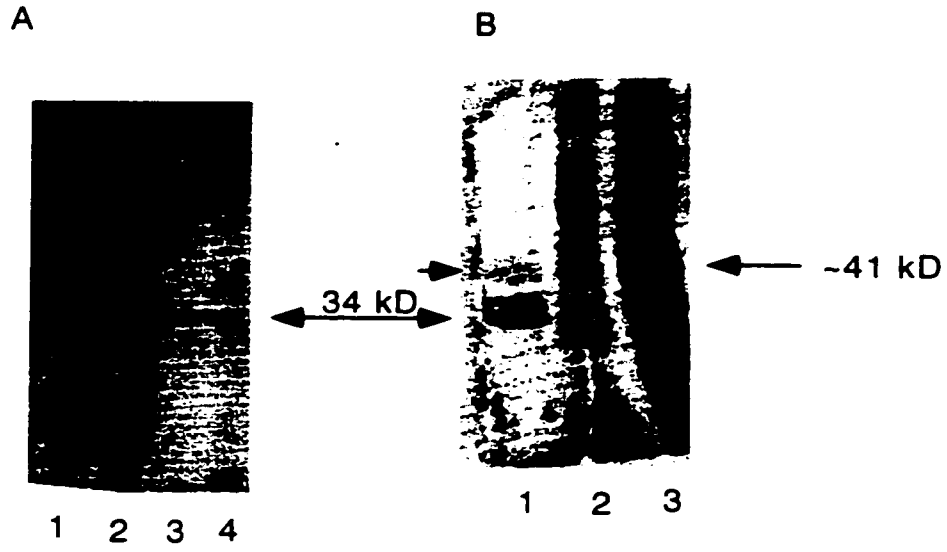


Figure 5.2 Coomassie staining results of a polyacrylamide gel showing the overexpression of GST-fusion proteins in *E. coli*. A. 1: BL21 transformed with GST-CT construct and induced by IPTG. 2: BL21 transformed with pGEX vector and induced by IPTG. 3: Purified GST protein. 4: molecular weight marker. B. 1. Purified GST-CT and GST proteins as the upper (indicated by an short arrow) and lower bands. 2. BL21 transformed with pGEX-NT before induction by IPTG. 3. BL21 transformed with GST-NT construct and induced by IPTG.

the thrombin and the "kinker" site of the fusion (Materials & Methods). The expression of GST-CT was not very strong.

After purification using glutathione columns, the purified proteins from both pGEX-NT and pGEX-CT BL21 transformants were mixtures of fusion proteins and GST (figure 5.2 and 5.3). The majority of purified proteins from pGEX-CT transformants was GST, whereas about half of purified proteins from pGEX-NT transformants was GST-NT (figure 5.3 A, B, C). More GST-CT than GST was found in the pellet after the sonication and centrifugation of bacterial lysates (figure 5.3 D), suggesting that some GST-CT was denatured and precitpitated in BL21 cells.

In order to prepare fusion antigens for immunization, I tried to separate GST from fusion proteins by DEAE-cellulose and P-cellulose chromatography (Materials and Methods). I first bound the protein mixtures to cellulose resins and then eluted them with buffers containing various concentrations of KCl, hoping to find a KCl concentration that can elute one protein but not the other. However, both GST and GST fusion proteins elute under about the same conditions.

I then decided to separate fusion proteins from GST by SDS-PAGE and use gel slices containing only GST fusion proteins to generate antibodies. The concentrations of GST fusion proteins were estimated by Coomassie blue staining against a concentration standard. About 1 mg of fusion proteins were then loaded on a large SDS polyacrylamide gel, separated from GST by electrophoresis, excised from the gel and sent to Cocalico Inc. to inject animals (figure 5.3 B).

Fifteen-amino-acid-long synthetic peptides from the N and C terminal sequences of Ine/RosA-s (Peptide-NT and Peptide-CT) were also sent to Cocalico Inc. to be coupled to a carrier protein (keyhole limpet hemacyanin) and used to immunize rabbits.

The CT and NT sequences were also subcloned into pET vectors to be expressed in BL21 and purified by Ni columns. Two pET vectors were used. The

Figure 5.3 Coomassie staining of polyacrylamide gels illustrating the purification and preparation of GST fusion proteins. A. Estimation of GST-CT concentration using Rp49 protein (from Alan Lu) as concentration standard. B. Preparation of GST-CT on a large SDS PAGE gel. The upper band, about 40 kDa in size and containing about 1 mg fusion protein, was excised, minced and sent for immunization. C. Estimation of GST-NT concentration using Rp49 protein as concentration standard. D. GST-CT is present mainly in the debris from sonication of *E. coli* expressing GST-CT.

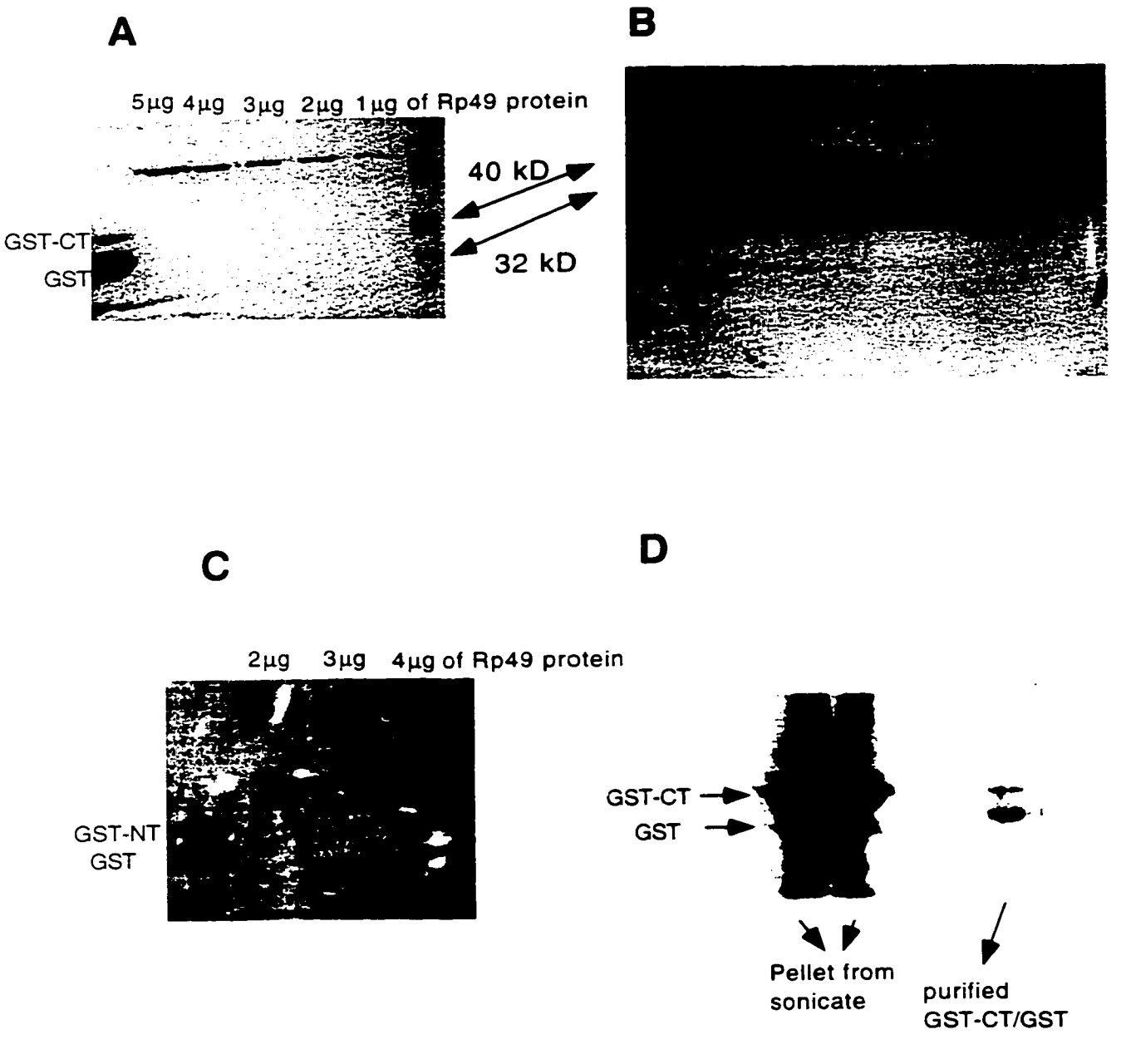
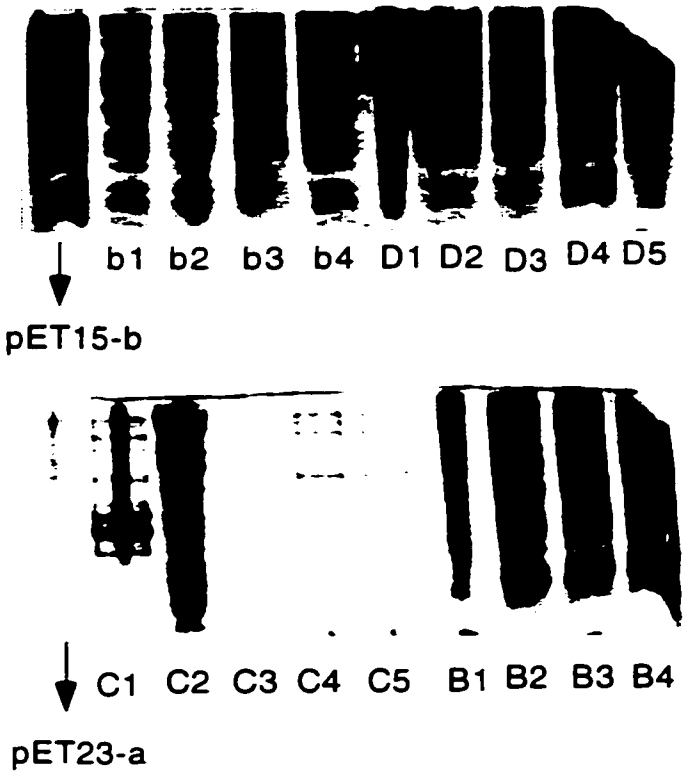
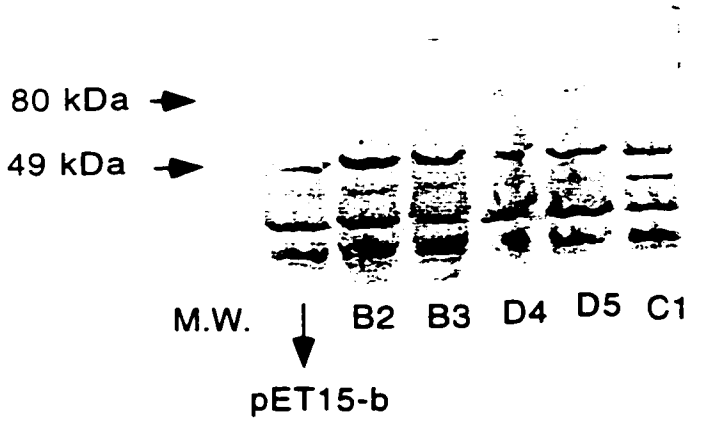

A
5µg 4µg 3µg 2µg 1µg of Rp49 protein
40 kD
32 kD
GST-CT
GST
B
C
2µg 3µg 4µg of Rp49 protein
GST-NT
GST
D
GST-CT
GST
Pellet from sonicate
purified GST-CT/GST

Figure 5.4 Expression of N and C- terminal sequences of Ine/RosA-s protein in pET15-b and pET23-a vectors. A. Coomassie blue staining of a SDS-PAGE gel showing *E. coli* clones transformed with pET15-b, CT sequence in pET15-b (b1-b4), NT sequence in pET 15-b (D1 - D5), pET23-a, CT sequence in pET23-a (C1-C5) and NT sequence in pET23-a (B1 - B4). B. Western blot using anti-His6 antibody (Invitrogen). Samples have the same nomenclature as those in A. M.W. indicates molecular weight marker.

A


b1 b2 b3 b4 D1 D2 D3 D4 D5
pET15-b
C1 C2 C3 C4 C5 B1 B2 B3 B4
pET23-a

B


80 kDa
49 kDa
M.W.
B2 B3 D4 D5 C1
pET15-b

pET23-a vector contains a C-terminal hexohistidine tag, whereas pET15-b an N-terminal hexohistidine tag. No obvious overexpression was detected in Coomassie blue stained SDS-PAGE gel after IPTG induction of all the BL21 transformants containing pET constructs (figure 5.4 A). Colonies that looked slightly promising for expression were then tested on Western blots using an antibody against histidine tags. No band was detected specific for the expression construct (figure 5.4 B). Note that many proteins were selected by antibodies to histidine tag, showing that this antibody is not very specific and may recognize many proteins that might contain runs of histidines.

The cDNA fragment of *ine/rosA*-l encoding the N-terminus 300 amino acids (ROSA-N) was subcloned into pET23-a vector to produce yet another antigen. Overexpression was detected in all the transformants after IPTG induction (figure 5.5). The BL21 culture expressing ROSA-N appeared white, which was different from the brownish yellow color typical of BL21. After cells were sonicated and centrifuged, ROSN was found to be in the white pellet. These results suggested that ROSA-N was in the inclusion bodies of BL21 cells. Lowering the fermentation temperature from 37 ^{o}C to 30 ^{o}C and the IPTG concentrations from 1 mM to 0.2 mM did not help bring ROSA-N into the solution.

The ROSA-N protein was purified using the procedure for inclusion bodies (Novagen). The purification was monitored at different stages by SDS-PAGE and Western blots (figure 5.6 A, B). After purification, a single band of about 40 kDa was obtained (figure 5.6 C). Polymerization of ROSA-N, due to formation of disulfide bonds, was detected when no DTT was added (figure 5.6 A, B). ROSN in 6 M urea and SDS polyacrylamide gel slices were used to immunize rabbits RC47 and RC48 respectively.

Jeff Tan, an undergraduate, and I tried to purify whole length recombinant Ine/RosA-s from transfected S2 cells, utilizing the hexohistidine tag at the C

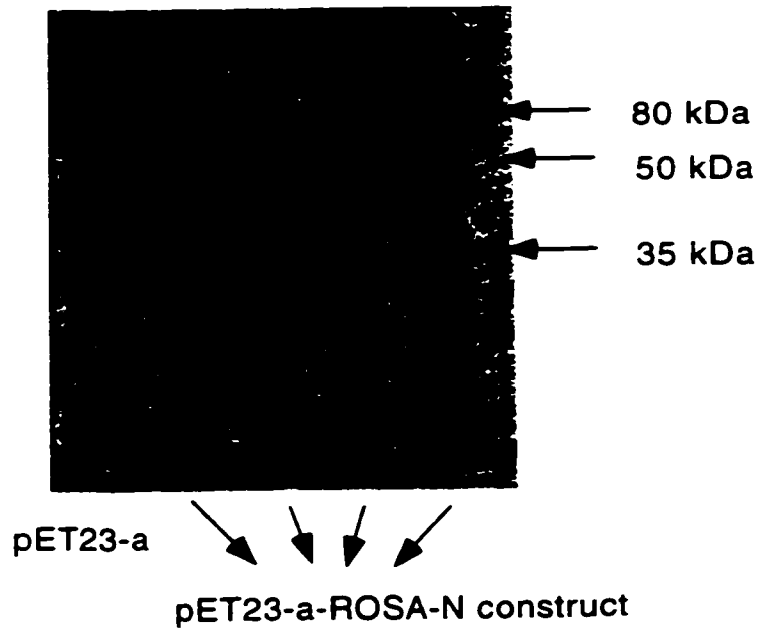
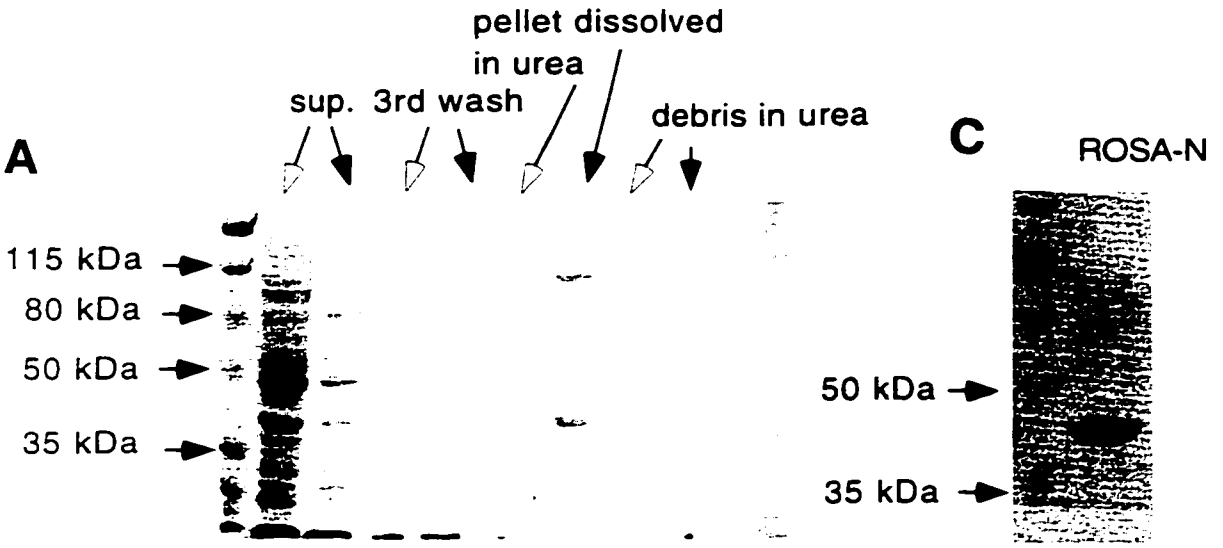
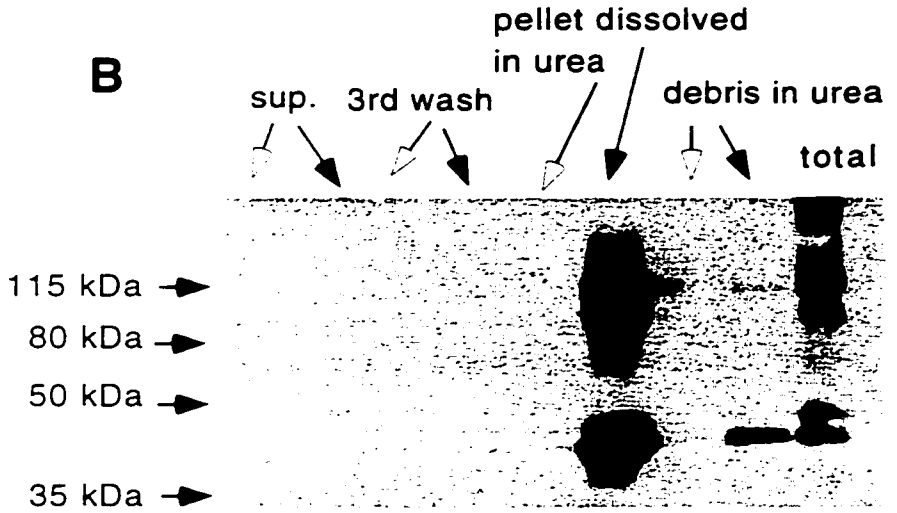


Figure 5.5 Expression of ROSA-N in BL21 DE3 pLysS A. Coomassie blue staining of a 12% SDS polyacrylamide gel showing lysates from BL21 transformed with constructs indicated at the bottom, except the right most lane that was loaded with molecular weight marker.

Figure 5.6 Purification of ROSA-N from *E. coli* inclusion bodies. "Sup." indicates the supernatant after sonication and centrifugation of BL21 cells; "3rd wash" indicates the third PBS wash of the pellet; 6 M urea was used to dissolve the pellet after three washes; "total" is the lysate from *E. coli* expressing ROSA-N. Whites arrows point to lanes loaded with sample from control, BL21 transformed with pET23-a vector. Black arrows point to lanes loaded with samples from BL21 expressing ROSA-N. A. Coomassie blue staining of a 12% SDS-PAGE gel without DTT showing different fractions of purification. B. Western blot using anti-His6 antibody (Invitrogen) to detect ROSA-N that contains hexohistidine tag. C. Addition of DTT to 0.1 M in SDS-PAGE sample buffer results in a single band with predicted size for ROSA-N after Coomassie blue staining.


A
pellet dissolved
in urea
sup.
3rd wash
debris in urea
115 kDa
80 kDa
50 kDa
35 kDa
C
ROSA-N
50 kDa
35 kDa


B
pellet dissolved
in urea
sup.
3rd wash
debris in urea
total
115 kDa
80 kDa
50 kDa
35 kDa

80 kDa

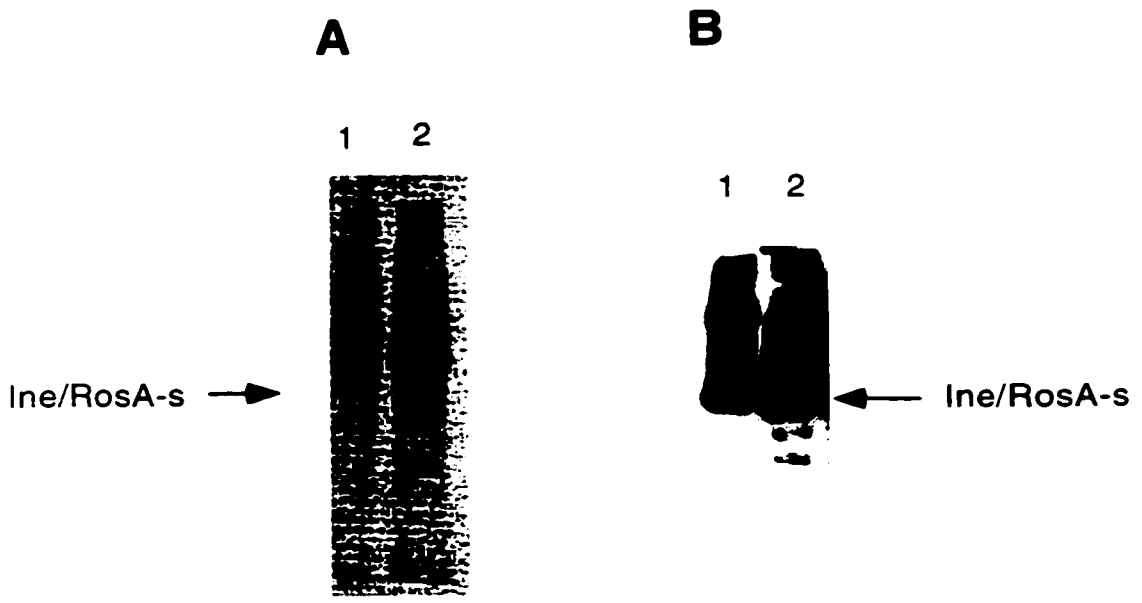


Figure 5.7 Induced Ine/RosA-s expression in the S2 cells is not detectable by Coomassie staining. A. Lane 1 and 2 are lysates from S2 cells expressing Ine/RosA-s after Cu++ induction. The most conspicuous band on SDS-PAGE is larger than the size of Ine/RosA-s B. The induced Ine/RosA-s is readily detectable by Western blotting using anti-V5 antibody (Invitrogen).

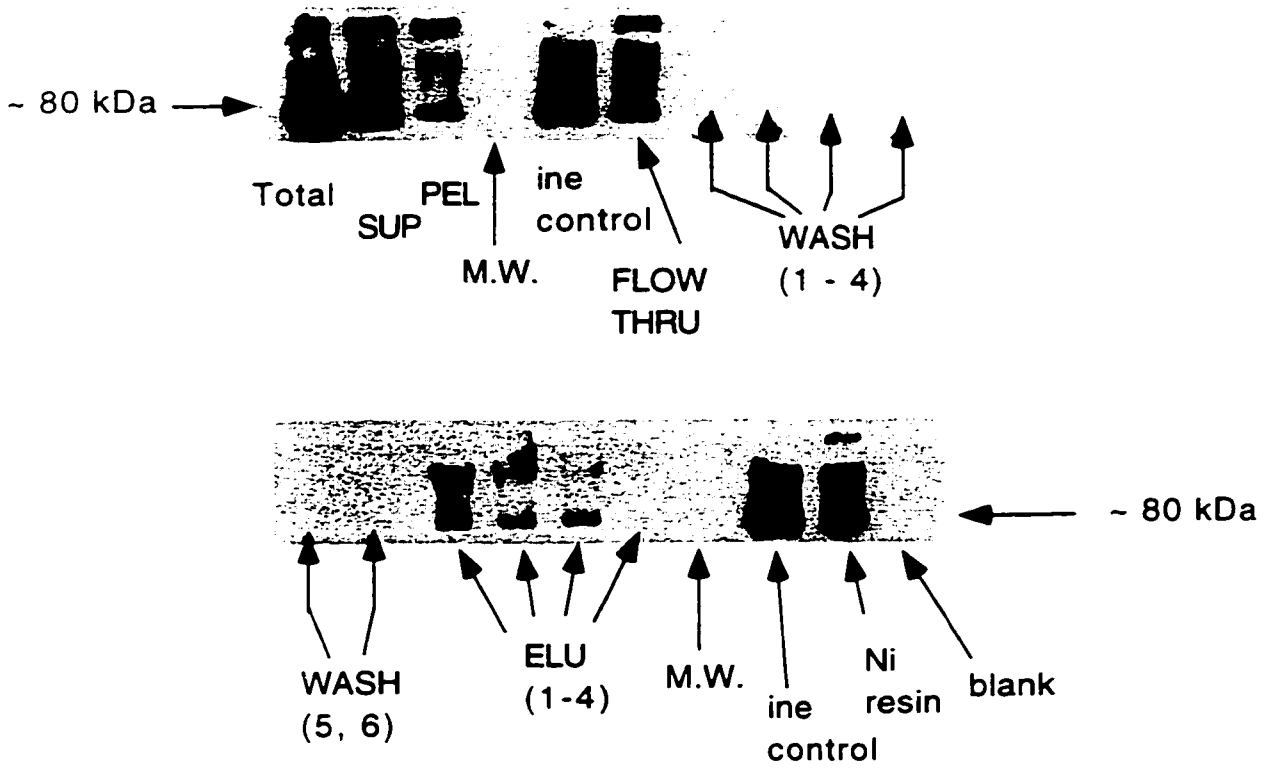
terminus of the recombinant protein. The overexpression of Ine/RosA-s after Cu^{++} induction of stably transfected S2 cells, as detected by the very specific anti-V5 monoclonal antibody, can not be detected by Coomassie blue staining after SDS-PAGE (figure 5.7). Also there is no difference in Coomassie staining between Cu^{++} induced and uninduced S2 cells (data not shown). This shows that the overexpressed protein in S2 cells is not nearly as abundant as it is in *E. coli* strains.

A crucial step in purifying a transmembrane protein is to bring the protein from the membrane to the aqueous solution. This solubilization is achieved by use of detergents. We have tested seven detergents, deoxycholic acid, NP-40, SDS, urea, decylmaltoside, tween 20 and Triton X-100. We found that Triton X-100 is capable of solubilizing Ine/RosA-s after high speed centrifugation, which pellets membrane particles (figure 5.8).

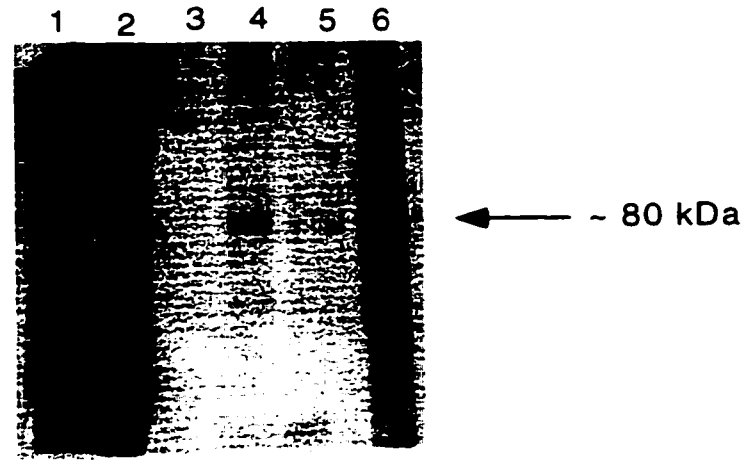
A number of experiments were performed to purify the Ine/RosA-s protein. Figure 5.8 shows one example of such experiments. There were two main problems with our purification procedure, which caused the loss of Ine/RosA-s. First, a considerable portion of solublized Ine/RosA-s didn't bind to Ni resin and ended up in the flow through. Second, most of Ine/RosA-s bound to the Ni resins could not be eluted by 1 M imidazole. As a result, very little Ine/RosA-s was obtained as shown in silver staining and Western blots. Nothing could be detected by Coomassie blue staining on the eluted fractions. The eluted fractions seemed to have a single band in SDS-PAGE after silver staining. However, this was not consistent with Western blots which showed at least two Ine/RosA-s bands: these bands possibly represented monomer and dimers. Therefore the single band seen in the silver staining might not be Ine/RosA-s. After several more tries with increased number of S2 cells to start with, we still could not purify enough Ine/RosA-s detectable by Coomassie blue staining.

Figure 5.8 Purification of Ine/RosA-s expressed in the S2 cells. This figure showed the 14th experiment (E14) from a total of 20 to purify the protein. Total indicates S2 cell lysate before purification steps. After sonicating the S2 cells in buffer A containing 1% TritonX-100 (Materials and Methods), the sonicate was centrifuged and the supernatant (SUP) and the pellet (PEL) were collected. To the supernatant, Ni resin was added, mixed and precipitated, and the flow through (FLOW THRU) was collected. Ni resin was then washed 6 times (WASH), eluted four times (ELU) with 1M imidazole and boiled in 2% SDS buffer (Ni resin) to completely disengage Ine/RosA-s protein from the resin. A. A Western blot using anti-V5 antibody to monitor samples collected from different purification steps. The "ine control" is the lysate from S2 cells expressing Ine/RosA-s used as a control for Cu++ induction and antibody quality for each experiment. B. Silver staining of some samples from A. 1: Flow through. 2: First wash. 3: Sixth wash. 4. First elution. 5. Second elution. 6. Ni resin boiled in 2% SDS buffer after elution. The arrow indicates a band of about 80 kDa.

A


~ 80 kDa
Total
SUP
PEL
M.W.
ine control
FLOW THRU
WASH (1 - 4)
~ 80 kDa
WASH (5, 6)
ELU (1-4)
M.W.
ine control
Ni resin
blank

B


1 2 3 4 5 6
~ 80 kDa

ii. Chemical modifications of antigen

In order to improve the titer of anti-ROSA-N antibodies, I have used performic acid (a mixture of formic acid and hydrogen peroxide) and dinitrobenzene sulfonic acid to modify the ROSA-N antigen (Materials and Methods). The former transforms the thiol ether side chain of methionine into a sulfonic acid group. The latter adds dinitrobenzene groups to a peptide. The ROSA-N protein antigens thus modified were prepared as precipitate suspension and sent to Cocalico, Inc. for rabbit immunization. Two rabbits previously vaccinated with ROSA-N and two naive rabbits were injected with modified antigens. I am in the process of testing different bleeds from these rabbits.

iii. Affinity purification of anti-sera

GST and GST-NT were immobilized onto cyanogen bromide activated agarose resin in order to purify anti-NT antibody in antiserum from RC36. Peptide-NT was also immobilized to the same support to affinity purify anti-Peptide-NT antibodies from RC40 rabbit.

iv. Testing of anti-sera

Antisera obtained from guinea pigs, RCGP1 and RCGP2, immunized with GST-CT were tested on both *E. coli* lysates and fly lysates for their efficacy as antibodies. Antisera from RCGP1 (third bleed) recognizes GST and GST-CT expressed in *E. coli*, but bleed from neither guinea pig can recognize a band in wild type fly lysates that is absent from ine^3 lysates (figure 5.9).

Antisera obtained from rabbits injected with GST-CT (RC35), GST-NT(RC36, 37), Peptide-CT (RC38, 39) and Peptide-NT (RC40, 41) were tested on fly lysates and none of them were able to recognize Ine/RosA proteins. Antisera from RC36 and

Figure 5.9 Testing of antisera from guinea pigs. Western blots on *E. coli* lysates (A) and fly lysates (B) using the third bleed antisera from guinea pigs, RCGP1 and RCGP2, immunized with GST-CT are shown. Goat anti-guinea pig IgG conjugated with peroxidase was used as secondary antibody and colorimetric visualization was performed (Materials and Methods). About 30 μg of total protein from fly and *E. coli* lysates was loaded in each lane and separated in a 12 % SDS polyacrylamide gel.

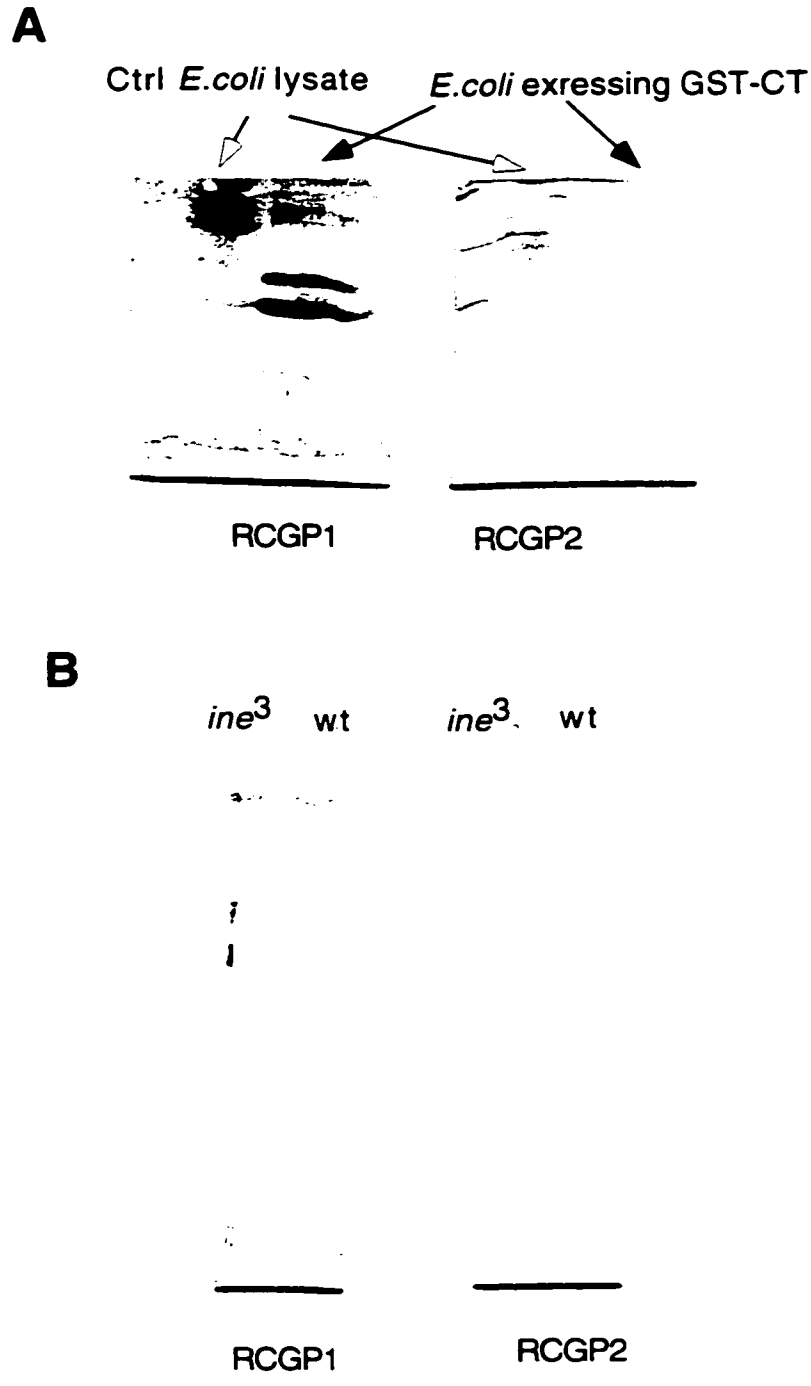
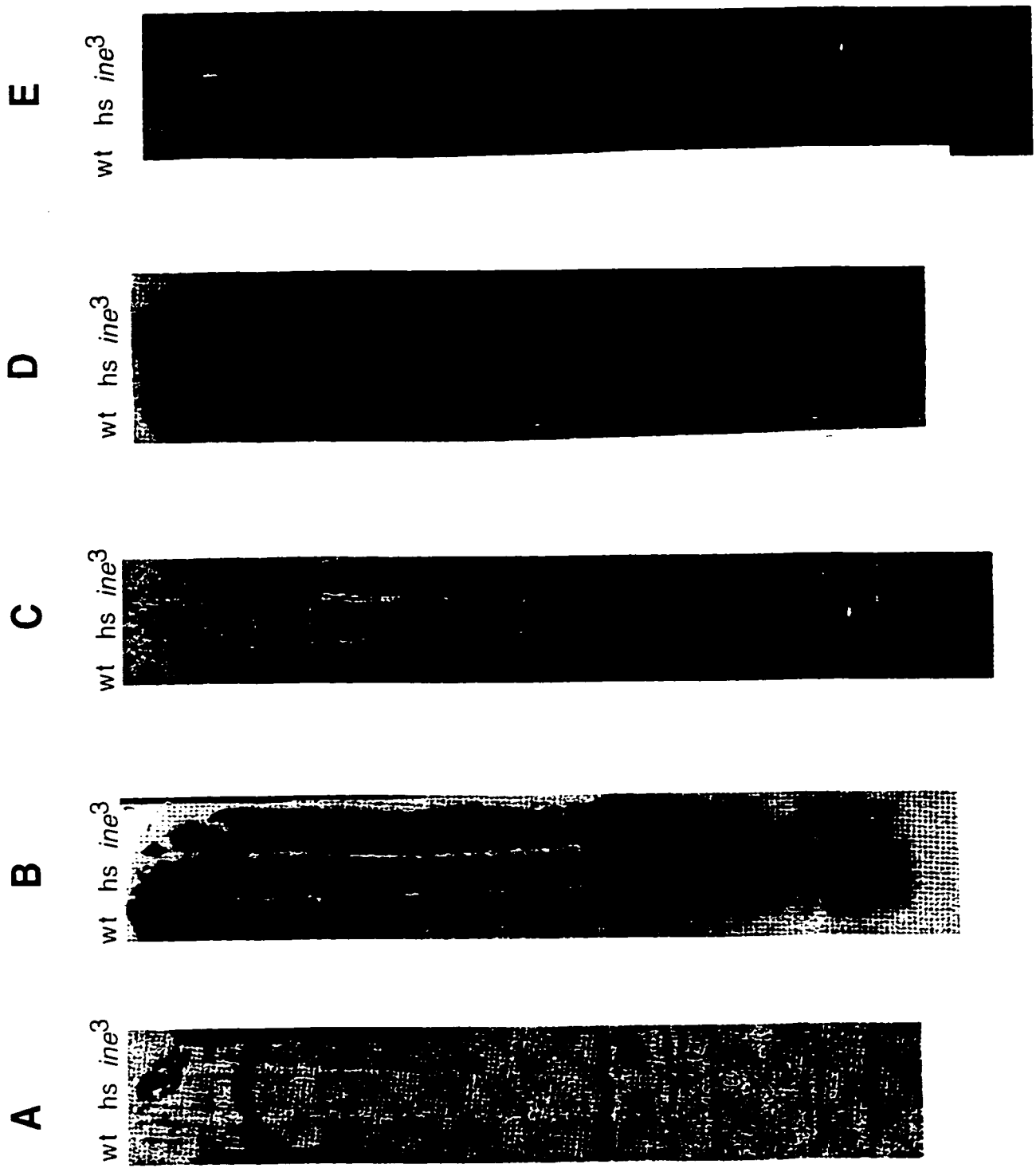

A
Ctrl E.coli lysate
E.coli exressing GST-CT
RCGP1
RCGP2
B
ine3
wt
ine3
wt
RCGP1
RCGP2

Figure 5.10 A series of Western blots on fly lysates using antisera developed against GST fusion proteins in rabbits. About 200 μg of total protein from Drosophila lysates was loaded in each lane in a 10% SDS polyacrylamide gel. After electrophoresis, proteins were transferred from the gel onto nitrocellulose membrane. In each membrane strip, lysates from wildtype flies (wt), wildtype flies under hypertonic stress for 2 days (hs) and *ine*^{3} flies (*ine*^{3}) were loaded from left to right. Antisera used in each strip are as follows. A: Anti Peptide-NT from RC41. B: Anti Peptide-NT from RC40 and affinity purified. C: Anti GST-NT from RC 36 and affinity purified. D: Anti GST-NT from RC36. E: Anti Peptide-CT from RC38. Goat anti-rabbit IgG was used as secondary antibody. Chemiluminescent visualization was used.


A
wt hs ine3
B
wt hs ine3
C
wt hs ine3
D
wt hs ine3
E
wt hs ine3

RC40 were affinity purified and the resulting antibody solution still failed to recognize Ine/RosA in flies. Some results from these tests are shown in figure 5.10. These Western blots showed many bands that can be considered non-specific because I have used high concentration of primary antibodies and longer exposure times to ensure that anti-Ine/RosA antibodies with very low titer could be identified.

I also tested antisera from these animals by performing Western Blotting on lysates of S2 cells expressing Ine/RosA-s. None of the sera tested could pick up any band(s) that appeared only in lysates from *ine/rosA*-s transfected S2 cells but not in the untransfected controls (figure 5.11). These tests demonstrated that these antisera contain almost no anti- Ine/RosA-s antibody.

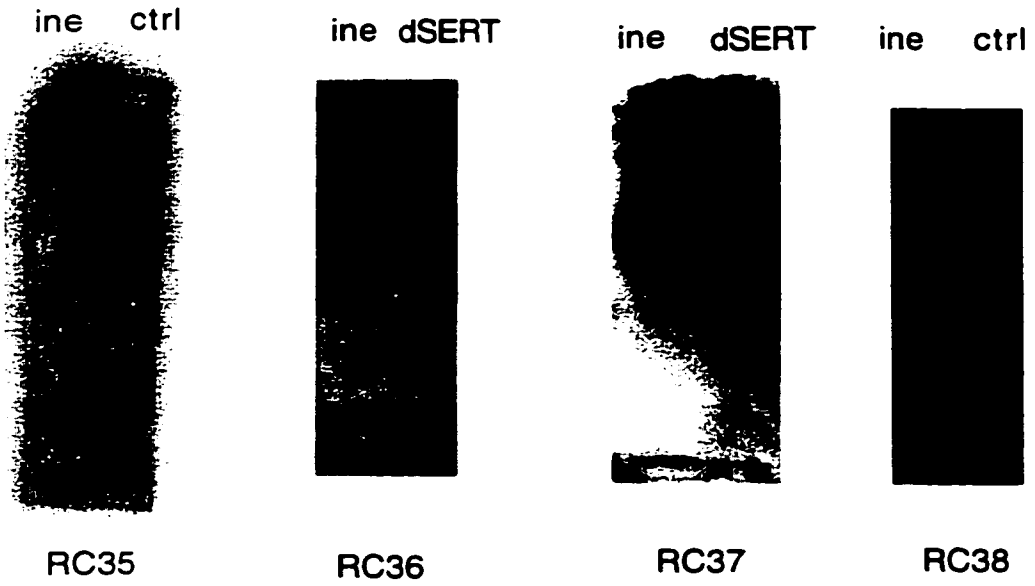
Antiserum obtained from the rabbit injected with ROSA-N in gel slices could recognize Ine/RosA-l expressed in S2 cells well, but failed to do so in *Drosophila* lysates (figure 5.12). Antisera against chemically modified ROSA-N are currently being tested.

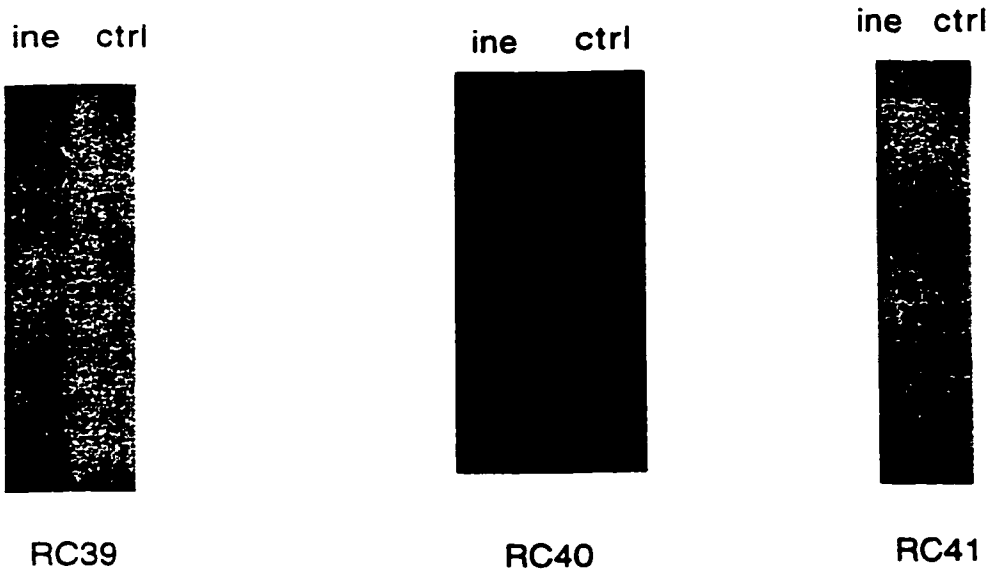
3. Discussion

Na^+/Cl^- dependent neurotransmitter transporters have the largest external loop between the third and the fourth transmembrane domains. This loop may be a very good antigen. Dr. Sarjeet Gill's group at University of California, Riverside, California have developed antibodies against the loop region of the protein encoded by the Manduca *ine/rosA* homologue. My effort to subclone the loop DNA sequence into the pGEX vector was not successful because of mutations introduced during PCR.

The lack of expression of Ine-R1 in *E. coli* suggests that Ine-R1 may be detrimental to *E. coli* and destroyed by proteases or inhibited at the transcriptional level. Due to their small sizes, the lack of CT and NT expression from pET vectors is

Figure 5.11 Testing of rabbit antisera on S2 cell lysates. A series of Western blots showed that the antisera developed in rabbits against GST fusion proteins and synthetic peptide fail to recognize the Ine/RosA-s protein expressed in S2 cells. Each strip represents a Western blot using antiserum obtained from a rabbit indicated at its bottom. For antigens used to immunized each rabbit see table 5.1. "ine" is used to indicate lysates from S2 cells expressing Ine/RosA-s, "dSERT" indicates lysates S2 cells transfected with dSERT cDNA, and "ctrl" indicates untransfected S2 cell lysate. Goat anti-rabbit IgG was used as secondary antibody. Chemiluminescent visualization was used. About 20 μg of total protein from S2 cell lysates was loaded in each lane.


ine ctrl
ine dSERT
ine dSERT
ine ctrl
RC35
RC36
RC37
RC38


ine ctrl
ine ctrl
ine ctrl
RC39
RC40
RC41

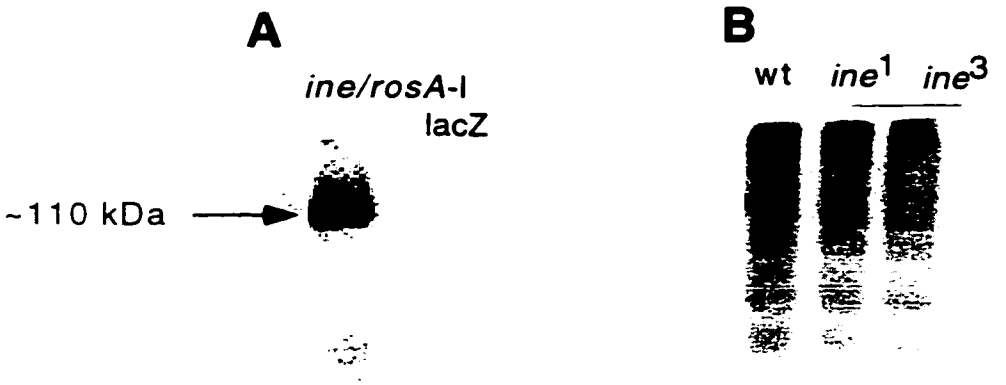


Figure 5.12 Testing of antisera against ROSA-N. The 4th bleed antiserum developed against ROSA-N in rabbit RC 48 was used as primary antibodies. A. A Western blot on lysates from S2 cells expressing Ine/RosA-l (left lane) and LacZ (right lane) protein. Protein from bout 10^5 cells was loaded in each lane. B. A Western blot on lysates from wild type (wt) and *ine* mutant flies. About 30 µg of protein was loaded in each lane. Goat anti-rabbit IgG was used as secondary antibody. Chemiluminescent visualization was used.

not a total surprise. GST fusion proteins expressed well in *E. coli*. However, GST may be more antigenic than CT and NT sequences and animals injected with GST fusion proteins produced good anti-GST antibodies and but not antibodies against Ine/RosA.

Visualization of signals on Western blots using chemiluminescent substrates is much better than using colorimetric substrates in for two reasons. The chemiluminescent method is more sensitive and offers better leverage to adjust signal-noise ratio by using different exposure times.

The fact that Ine/RosA-s expressed in S2 cells and bound to Ni resins could not be eluted with 1 M imidazole may indicate that the solublized Ine/RosA-s was binding to the agarose support of Ni resin instead of Ni^{+}. Transmembrane proteins tend to stick to surfaces, including container walls and matrices, and are lost in many purification steps.

Chapter 6. Study of the role of the *inebriated/rosA* transporter in water and salt metabolism

1. Introduction

It is well established that Na^+/Cl^- dependent neurotransmitter transporters, such as the GABA/betaine, taurine and inositol transporters, function in the mammalian kidney to accumulate osmolytes and regulate the osmotic stress response. The *ine/rosA* gene encodes a protein that has about 40 % amino acid sequence identity to members of this neurotransmitter transporter family and has high transcriptional levels in the Malpighian tubules and the hindgut, which is the insect homologue of the mammalian kidney. From these observations, we hypothesized that Ine/RosA may accumulate osmolyte(s) in the Drosophila "kidney" and facilitate fluid uptake. Furthermore, we predicted that *ine/rosA* mutant flies be more sensitive to a hypertonic environment than wild type flies.

Several approaches were adopted to test the aforementioned hypothesis and prediction. Osmolarity sensitivity assays were used to study the sensitivity of *ine/rosA* mutants to hypertonicity. If the resistance of the fly to a hypertonic environment was impaired by the lack of *ine/rosA* function, the ectopic expression of the *ine/rosA*-s cDNA could be induced to see if the presence of this cDNA in the *ine/rosA* mutant flies could rescue the lowered salt resistance. Other physiological processes might also be involved in the osmotic stress response, such as intake of water from sources other than food and functions of the nervous system. Several experiments were performed to address these issues. Because enhancement of osmolyte uptake through the upregulation of neurotransmitter transporter gene expression is repeatedly observed in the mammalian osmotic stress response, Northern blotting was utilized to address the possibility of *ine/rosA* transcription induction by hypertonicity.

2. Results

i. The *inebriated/rosA* mutant flies exhibit increased sensitivity to hypertonic environment than wild type flies

Osmolarity sensitivity assays on wild type and ine^1 mutant flies performed by Laura Huff (figure 6.1) indicate that *ine* mutants die at a lower salt concentration (0.2M NaCl) than wild type flies. It is also observed that *rosA* mutants are also more sensitive to hypertonic stress than wild type flies (Yan-mei Huang and Dr. Stern, personal communication). This decreased viability did not seem to be sodium specific, because *ine* mutant flies also exhibit lower viability when maintained on media containing high concentrations of sorbitol (figure 6.2) or KCl (Dr. Michael Stern, personal communication).

Rescue tests are another important approach to establish the role of *ine/rosA* gene in Drosophila hypertonic stress response. In Drosophila, ectopic expression of a gene can be achieved by using a transposable element called P-element that can be inserted into the Drosophila genome at a random position. The P-element can carry a construct containing a gene of interest. We used the GAL4-UAS system to express the *ine/rosA* cDNAs. In this system, two fly lines with two P-element constructs were used. The first contains a P-element construct with the GAL4 gene encoding a transcription activator either under heat-shock promoter or behind tissue or cell type specific enhancers or promoters (Brand *et al.*, 1994). The former can express the GAL4 protein throughout Drosophila body under heat shock; the latter expresses GAL4 protein in specific organs and cells. The second line has a desired DNA sequence behind the UAS sequence, the cis element recognized by the GAL4 protein, inserted in their genome. A cross between these two lines can generate progeny that have both constructs and can express the desired protein at desired time or place in Drosophila (figure 6.3).

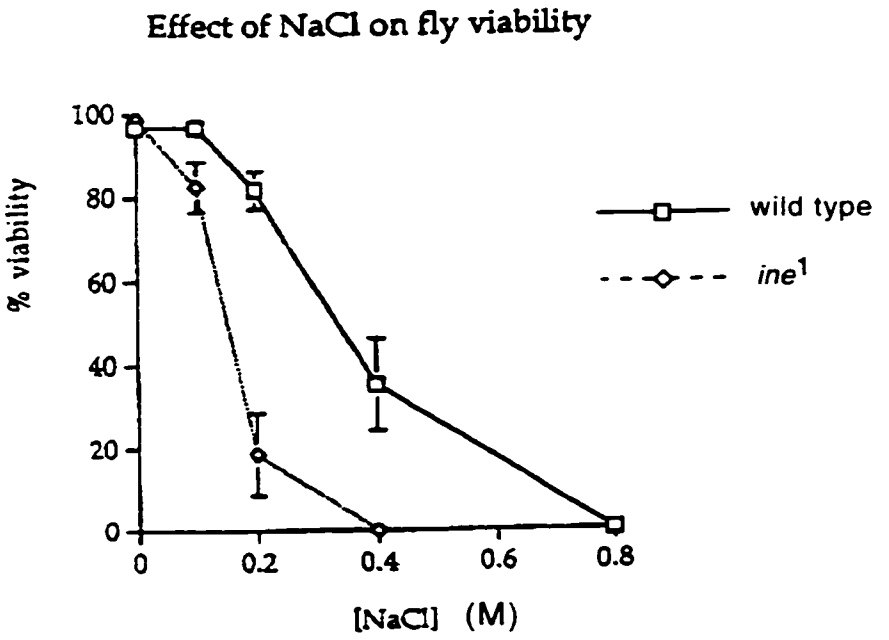


Figure 6.1 *ine*^{1} mutant flies are more sensitive to high concentrations of NaCl than wild type flies. Adult flies were transferred to media containing different concentrations of NaCl and the percentage of flies alive were calculated after 6 days. Two hundred and forty flies were used for each data point. Each error bar indicates standard error of the mean. This experiment was performed by Laura Huff.

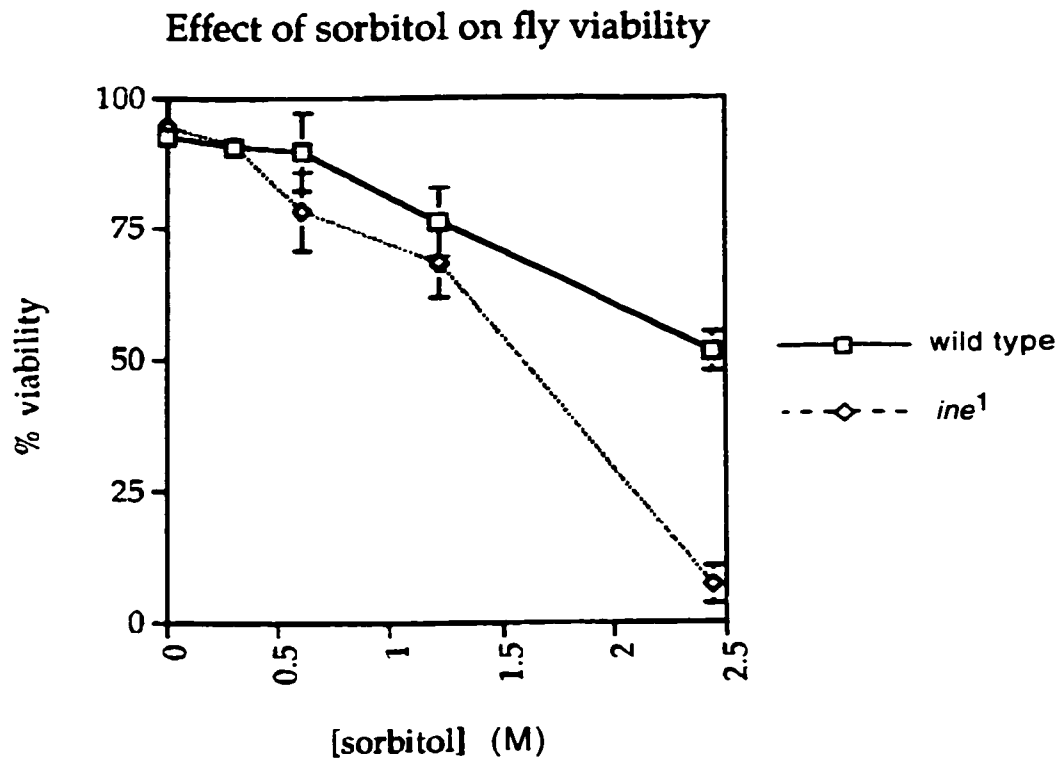
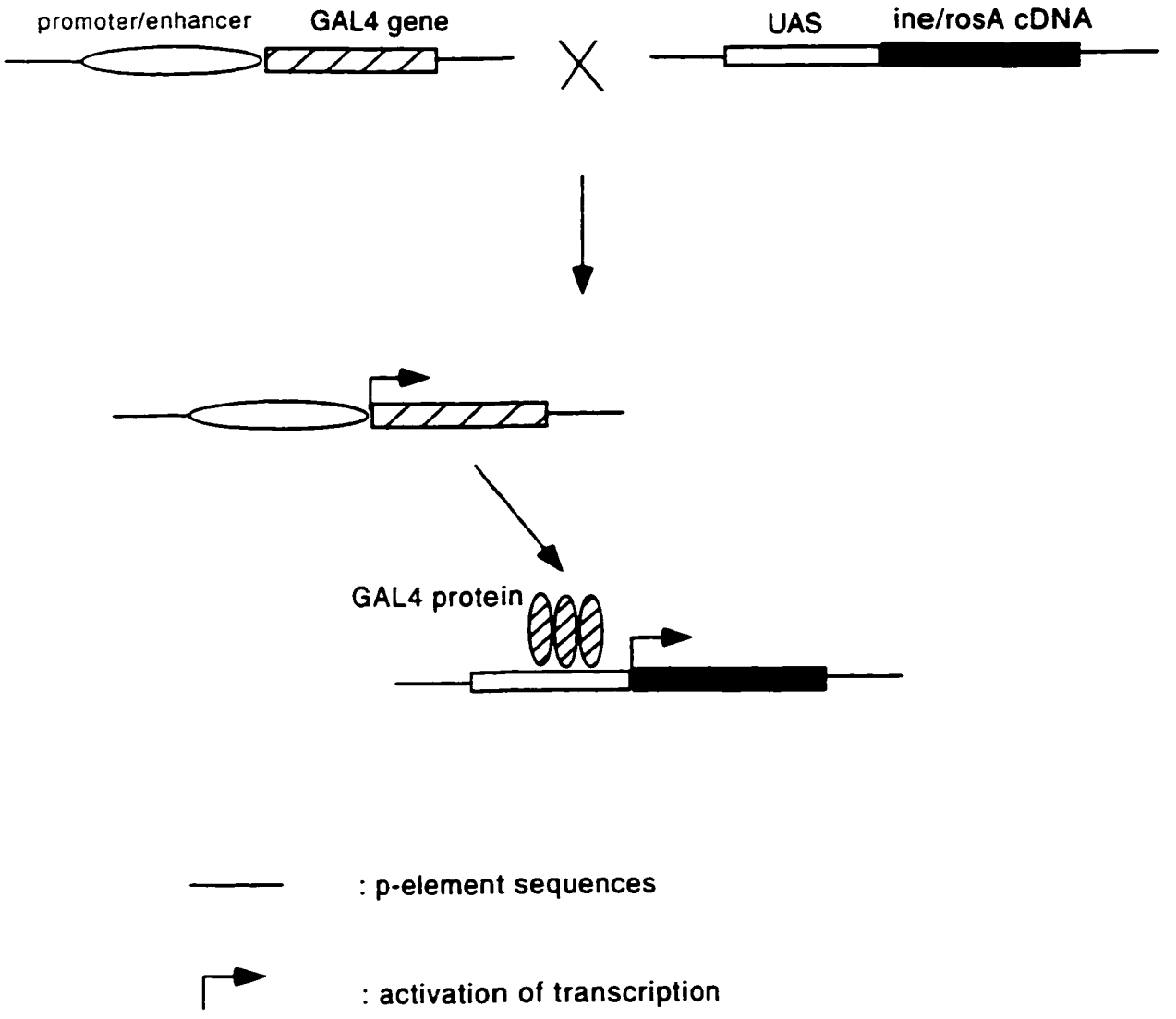


Figure 6.2 *ine*^{1} mutant flies are more sensitive to high concentrations of sorbitol than wild type flies. Adult flies were transferred to media containing different concentrations of sorbitol and the percentage of flies alive were calculated after 6 days. Two hundred and forty flies were used for each data point. Each error bar indicates standard error of the mean. This experiment was performed by Laura Huff.

Figure 6.3 The GAL4-UAS system used to express *ine/rosA* cDNAs in *ine* mutant flies. The enhancer/promoter sequence could be the heat shock promoter or tissue or cell type specific enhancers, driving the expression of GAL4 protein in a temporally or spatially controlled manner.


promoter/enhancer
GAL4 gene
UAS
ine/rosA cDNA
GAL4 protein
: p-element sequences
: activation of transcription

Yan mei Huang and I generated P-element constructs containing *ine/rosA*-s and *ine/rosA*-l cDNA behind the UAS sequence. These constructs were then introduced into the Drosophila genome by embryonic germ line transformation (Yan mei Huang). Flies carrying these constructs were crossed to flies with hs-GAL4 construct. The progeny were collected to be used in rescue assays (Yan mei Huang, Dr. Michael Stern and Raj Chinnappan). Due to leaky expression of the hs-GAL4 construct at room temperature, heat shock was not required to express the GAL4 protein and subsequently the Ine/RosA transporter at a high enough level to rescue salt sensitivity of ine^3 mutant flies (figure 6.4). This leaky expression of the GAL4 protein without heat shock works to our advantage because it eliminates the possibility that the enhancement of ine^3 mutants' salt resistance is a secondary effect of a heat shock response.

Results from these experiments show that mutations in the *ine/rosA* gene impair the ability of Drosophila to respond to hypertonicity, and that *ine/rosA*-s cDNA is sufficient to restore the normal resistance to hypertonicity.

ii. Evidence that the increased sensitivity to hypertonic environments is caused by reduced water reabsorption among *inebriated/rosA* mutants.

There are two major water sources for insects, food and moisture in the air. Some species of beetles can use specialized structures on their body surfaces to trap and condense water. Other insects are also known to have the ability to absorb moisture in the air to survive on dry food with undefined mechanisms (Schmidt-Nielsen, 1975).

Is it possible that *ine/rosA* mutations affect the ability of Drosophila to obtain water from the air and render it more vulnerable to hypertonic stress? If this hypothesis is true, then wildtype flies should live longer than *ine/rosA* mutant flies

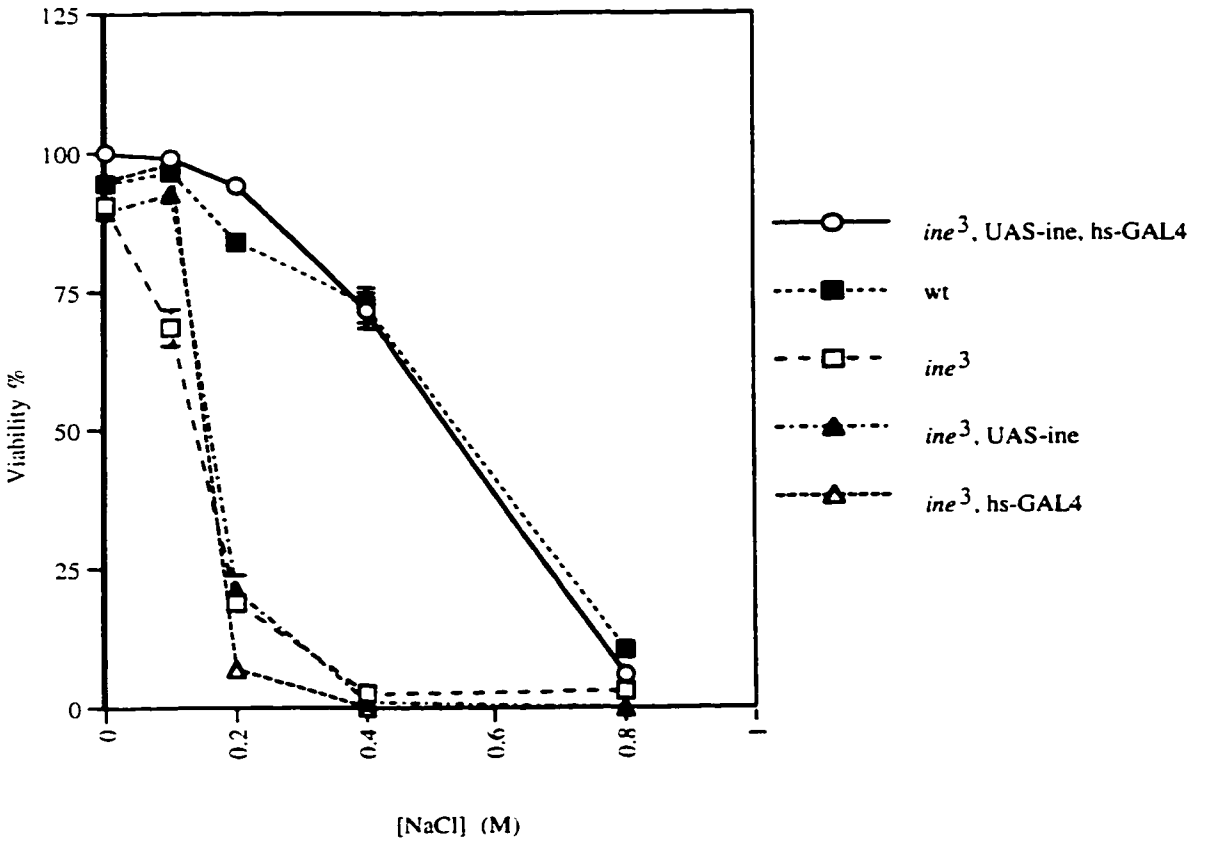


Figure 6.4 The osmolarity sensitivity phenotype can be rescued by the expression of the *ine/rosA*-s cDNA. In the legend, P-element introduced constructs are indicated after the background genotype (*ine*^{3}) and a comma. Two hundred flies were used for each data point. Each error bar indicates standard error of the mean.

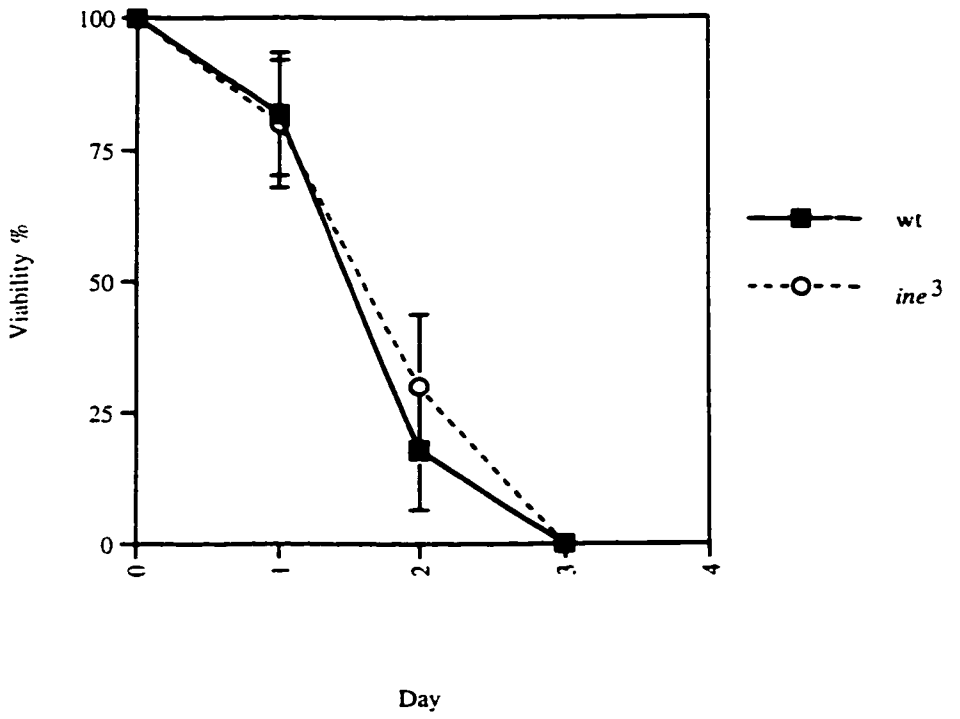


Figure 6.5 Dehydration assay on wildtype and *ine* mutant flies. Eleven wild type flies and ten *ine*^{3} mutant flies were maintained in vials without food. The number of surviving flies was counted everyday. Each error bar indicates standard error of the mean.

when water can only be obtained from the air. To test this possibility, I performed dehydration tests wherein flies were maintained in vials with no food. Results shown in figure 6.5 indicate that *ine* mutants and wildtype flies have very similar death rate during dehydration. We can conclude that *ine* mutations affect osmotic stress response by impairing a fly's ability to effectively reabsorb water from food instead of from the air.

iii. The osmotic sensitivity phenotype may be independent of neuronal defects

We hypothesized that *ine/rosA* transporter accumulates osmolyte(s) and facilitates fluid absorption in the hindgut and Malpighian tubules. An alternative hypothesis is that mutations in the *ine/rosA* gene affect the nervous system function of Drosophila and render it unable to take in food at all under hypertonic stress.

To test this possibility, I studied the eating behavior of wild type and *ine* mutants. Using a mixture of two dyes that stains the midgut of Drosophila (Materials and Methods), I found that *ine* flies and wild type flies have very similar eating behaviors. Both genotypes eat when maintained on normal food and food containing 0.2 M NaCl. Neither eats much when maintained on food with 0.4M NaCl (figure 6.6).

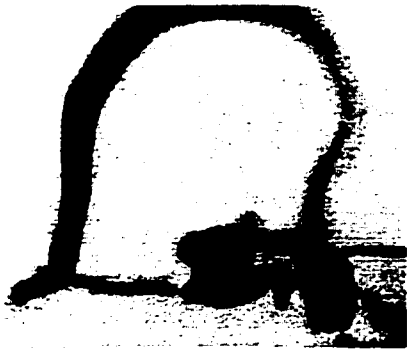
Another way to tell if the osmotic sensitivity phenotype is caused by neuronal defects is to test if other mutations affecting neuronal excitability have an effect on osmotic sensitivity similar to that of *ine/rosA*. Flies carrying mutations affecting neuronal function, such as *Sh* (a potassium channel mutation), *Dp para* (duplication of a sodium channel gene), and *eag* (a mutation in a potassium channel), have survival rates similar to that of wild type flies when maintained on hypertonic food (figure 6.7).

Figure 6.6 Comparison of eating behaviors of wild type flies and *ine* mutants. Each picture is showing the midgut and Malpighian tubules isolated from a fly maintained in food containing concentrations of NaCl as indicated in the center.

wt

ine^3

H_2O



0.2 M NaCl

0.4 M NaCl

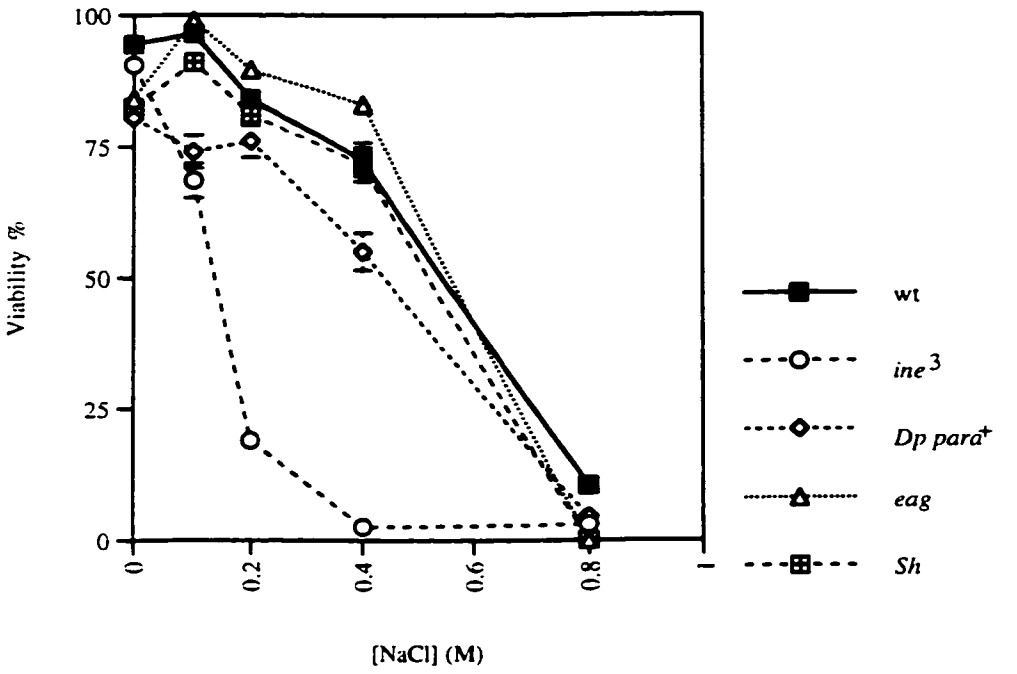


Figure 6.7 Effects of mutations affecting neuronal excitability on Drosophila osmotic stress response. Two hundred flies of each genotype were used to test their survival on hyperosmotic food. Each error bar indicates standard error of the mean. For more explanation see text.

These results suggested that the *ine/rosA* mutation is affecting the ability of Malpighian tubules and the hindgut to absorb water from ingested food, and that this impairment is not necessarily caused by defects in the nervous system.

iv. Overexpression of *inebriated/rosA* cDNA increases salt resistance to hypertonicity in Drosophila

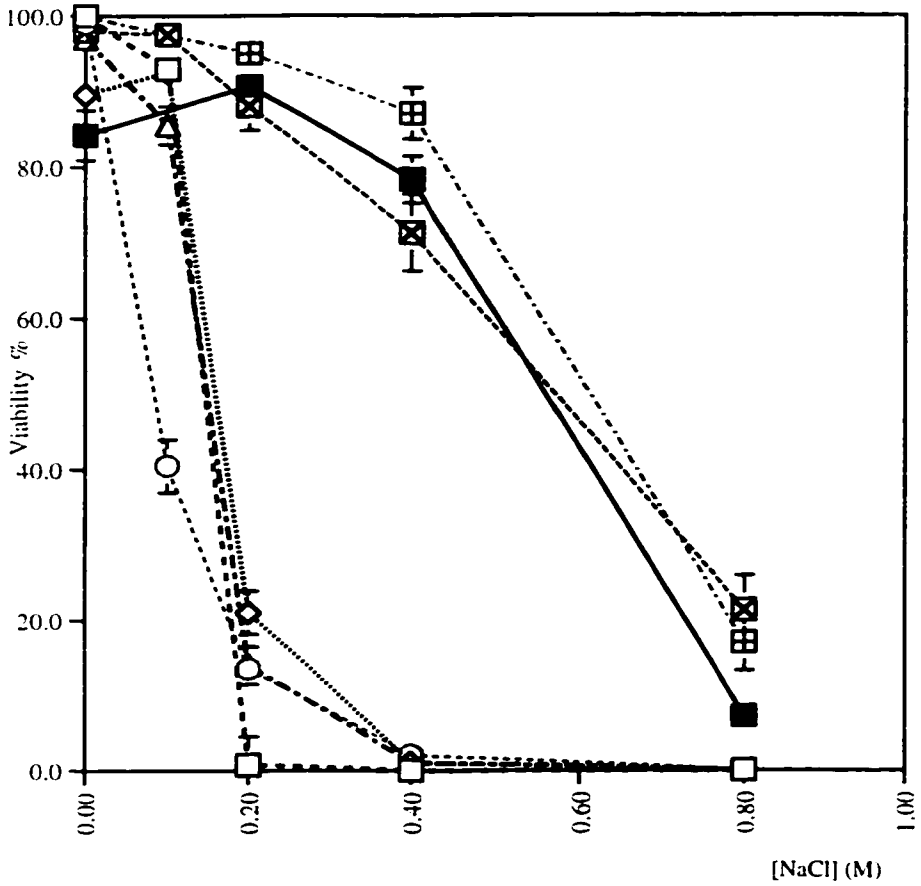
In order to further test the possibility that *ine/rosA* function is required in the fluid absorption organs such as the hindgut and Malpighian tubules to respond to hypertonic stress, Dr. Michael Stern obtained two GAL4 lines, el3c and L729 that expresses GAL4 protein in Malpighian tubules (MT-GAL4) (Harrison *et al.*, 1995). Yan mei Huang used a UAS-lacZ line to confirm that these lines express GAL4 in Malpighian tubules and crossed these lines with line carrying UAS-*ine/rosA*-s (UAS-ine) constructs.

The resulting rescue lines expressing *ine/rosA*-s cDNA in the Malpighian tubules fully rescue the salt sensitivity of ine^3 flies when flies were maintained on food containing 0.2 M or 0.4 M NaCl (figure 6.8). Strikingly, both lines conferred higher salt resistance than wildtype flies when maintained on food containing 0.8 M NaCl. About 7% wild type flies survived after 4 days on 0.8 M NaCl, 21 % ine^3 flies with MT-GAL4(L729) and UAS-ine survived and 17% ine^3 flies with MT-GAL4(el3c) and UAS-ine survived. The differences between the viability of the wild type and rescue lines on 0.8 M NaCl are statistically significant, with p value being smaller than 0.002 and 0.001 for el3c and L729 rescue lines respectively. Further experiments are underway to test the effect of the expression of *ine/rosA*-s cDNA in Malpighian tubules in wild type background.

These results showed that expression of *ine/rosA*-s cDNA in Malpighian tubules is sufficient to restore the salt resistance of *ine* mutant flies and, when maintained under 0.8 M NaCl, even to a level above that of wild type.

Figure 6.8 Expression of *ine/rosA*-s cDNA in Malpighian tubules enhances salt resistance of flies. L729 and el3c are two lines that express GAL4 in the Malpighian tubules due to insertion of p-elements containing the GAL4 gene (MT-GAL4). In the legend, P-element introduced constructs are indicated after the background genotype and a comma. Two hundred flies were used for each data point except that 180 wild type flies were used for "wt". Each error bar indicates standard error of the mean.

Increased salt resistance rendered by overexpression of *ine/rosA*-s cDNA in the Malpighian tubules



e13c, MT-GAL4

ine^3

wt

L729, MT-GAL4

ine^3, UAS-ine

ine^3, MT-GAL4(L729), UAS-ine

ine^3, MT-GAL4(e13c), UAS-ine

v. Study of *inebriated/rosA* transcriptional levels under hypertonic stress.

In the winter of 1997, Lei Tang and I observed increased *ine/rosA* transcription levels when wild type flies were maintained in medium containing 0.4 M and 0.8M NaCl for 48 hours (figure 6.9).

When I tried to delineate the time course of *ine/rosA* induction in a high salt environment, I failed to observe the increase of *ine/rosA* mRNA levels in flies maintained in hypertonic food. One possible reason of not being able to repeat the earlier result is that the humidity level in Houston in the summer is far higher than in the winter when the induction of *ine/rosA* transcription was first observed. The high humidity may allow flies to take more water from moisture in the air instead of solely from food, therefore no induction of *ine/rosA* is needed for extracting water from salty food. I repeated the experiment in a chamber whose humidity was controlled at 25% to 33%, whereas the ambient humidity was 65% to 70%. Still no induction of *ine/rosA* transcript was observed (figure 6.10).

One possible explanation of the lack of induction is that flies raised in high humidity to adulthood may have lost the ability to upregulate the transcription of *ine/rosA* in response to hypertonic stress and low humidity. To test this possibility, flies were raised in controlled low humidity, 20% - 35%, to adulthood and then subjected to hypertonic food in the same humidity. Still, no induction of *ine/rosA* transcription was observed (figure 6.11).

It could also be imagined that wild type flies are already expressing a certain level of Ine/RosA transporter before hypertonic stress. When under hypertonic stress, no further induction of transcription happens. It is possible that if the *ine/rosA* gene products are not functional, the regulatory mechanisms at the transcriptional level could be activated because of a greater need for transporter functions. I therefore used ine^3 flies that carry a deletion of the 3' of the

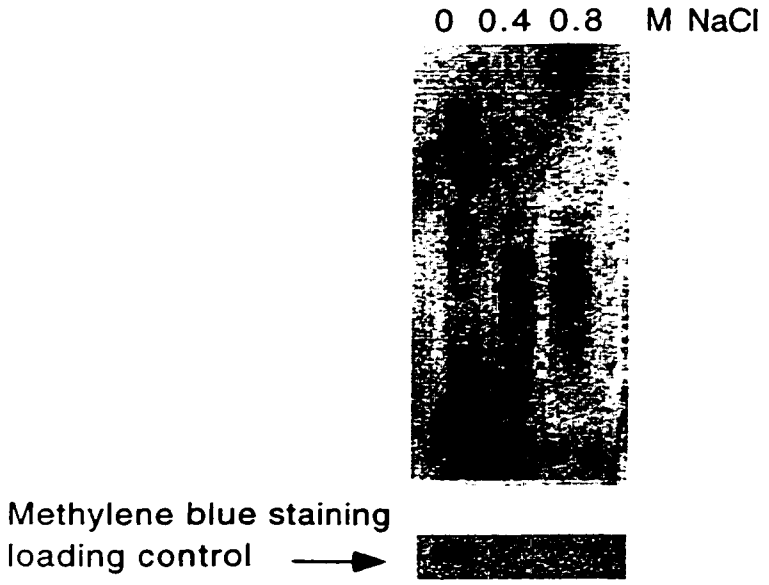


Figure 6.9 Analysis of *ine/rosA* transcriptional levels in wild type flies maintained on hypertonic food for two days. About 5 μg mRNA was from wild type flies was loaded in each lane. The upper panel shows the signals recognized by a probe with the *ine/rosA*-s open reading frame; the lower panel shows the methylene blue staining loading control.

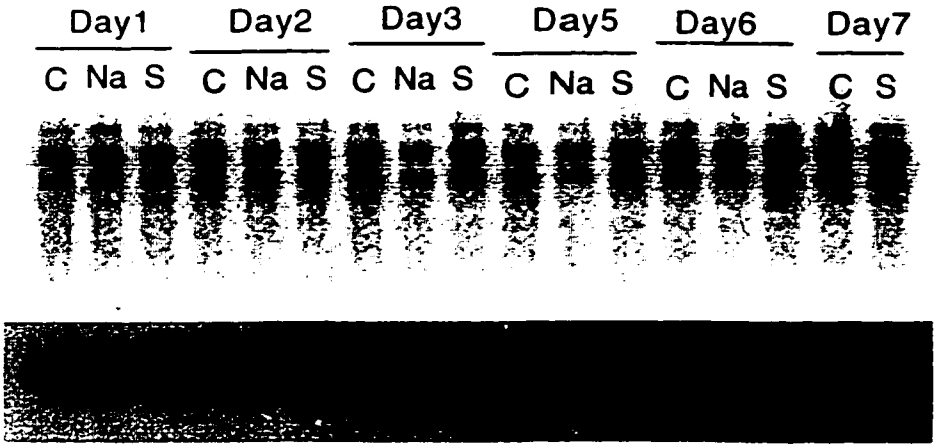


Figure 6.10 Analysis of *ine/rosA* transcriptional levels in hypertonic environment with controlled humidity (25-33%). Wild type mRNAs were isolated and blotted onto the membrane. The upper panel shows the signals recognized by a probe with the *ine/rosA*-s open reading frame; the lower panel shows the loading control using *RpL27a*, a house keeping ribosomal protein gene, as probe.
C: flies maintained on normal food. S: flies maintained on food with 2.4 M sorbitol. Na: flies maintained on food containing 0.8 M NaCl.

ine/rosA gene encoding seven transmembrane domains to see if the transcription of the truncated gene could be induced by hypertonic stress. Results in figure 6.11 show that the transcriptional level of the truncated *ine/rosA* gene was not increased by hypertonicity.

This series of experiments can largely rule out the possibility of the existence of direct transcriptional regulation of *ine/rosA* in response to salt.

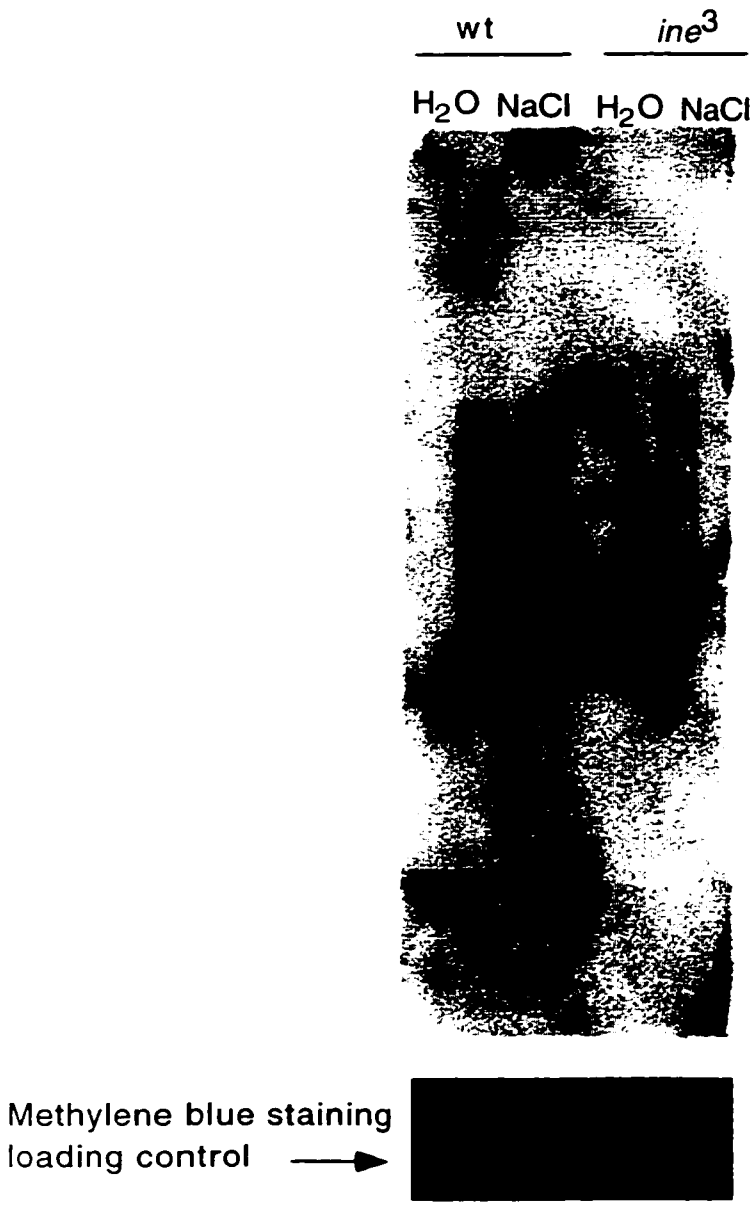
3. Discussion and conclusions

Results from osmolarity sensitivity assays and rescue assays show that the *ine/rosA* gene undoubtedly plays an important role in the osmotic stress response of Drosophila. It is likely that the *ine/rosA* transporter is vital for the fly's ability to reabsorb water from salty ingested food. Several lines of evidence also suggest that this role may be carried out in the hindgut and Malpighian tubules.

The experimental results from the MT-GAL4-UAS-ine system could not entirely exclude possible nervous system effects on the osmolarity sensitivity because the GAL4 protein was found to be expressed in parts of the brain also (Yan mei Huang).

Several reasons could account for the fact that the initial result on the induction of *ine/rosA* transcription could not be repeated. First, the methylene blue staining, used for the loading control, may not have been done properly. For example, the blot might have had methylene blue unevenly distributed on its surface. This may have led to errors in quantification. Second, there might be factors other than humidity in the winter that are different from other seasons that affect flies response to hypertonicity. Third, I can not exclude the possibility that upregulation of *ine/rosA* transcription in certain cells or tissues in Drosophila does occur and is not reflected in Northern blots using whole fly mRNA preparations.

Figure 6.11 A Northern blot examining transcriptional level of *ine/rosA* in wild type and ine^3 mutant flies. Flies were maintained on food containing 0.4 M NaCl for two days. The wildtype flies used here were developed under controlled low humidity (20-35%). The mRNAs (about 5 μg) from wild type and ine^3 mutant flies were isolated and blotted onto the membrane. The upper panel shows the signals recognized by a probe with the *ine/rosA*-s open reading frame; the lower panel shows the methylene blue staining loading control.


wt
ine3
H2O NaCl H2O NaCl
Methylene blue staining
loading control

From results presented in this chapter, it is quite possible that *ine/rosA* plays a vital role in the response of Drosophila to a high salt environment without induction at the transcriptional level. Whereas it is possible that the Ine/RosA transporter activity is constant and not affected by hypertonic stress, we can't rule out the possibility of regulation of Ine/RosA activity in response to hypertonicity. Post-translational mechanisms may be involved in the regulation of the Ine/RosA transporter activity. Phosphorylation and subcellular membrane trafficking have been observed to be involved in the regulation of neurotransmitter transporters (Chapter 1, introduction).

The only knowledge on insect osmotic stress response at a molecular level is that a MAPK, P38, is activated by tyrosine-phosphorylation under hypertonic stress response (Han *et al.*, 1998). Given the advanced genetics in Drosophila, the *ine/rosA* system provides a great opportunity to explore other factors involved in the osmotic stress response pathway.

Chapter 7 Discussion

1. Possible models to explain the neuronal excitability phenotypes of *inebriated/rosA* mutants.

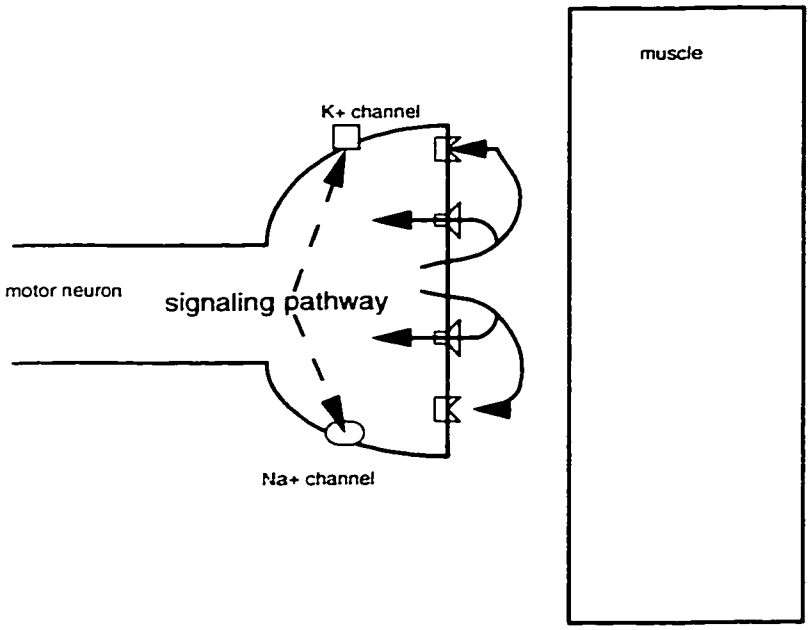
The *ine/rosA* gene encodes a putative neurotransmitter transporter. Mutations in this gene cause increased motor neuron excitability. Several models could be envisioned to explain *ine/rosA* functions and mutant phenotypes in the nervous system.

Two models had been proposed to account for the increased motor neuron excitability seen in *ine/rosA* mutant flies. In the first model, Ine/RosA protein transports neurotransmitters or co-transmitters in the neuromuscular junction (Figure 7.1 A). The *ine/rosA* substrate transmitter acts in an autocrine pathway and has receptors in the presynaptic (motor neuron) terminal where it is released. The binding of transmitter to its receptor triggers the signaling system in the cell that modifies channel activities. Defects in *ine/rosA* transporter means longer transmitter effects on the signaling system leading to the increased excitability (Figure 7.1 A).

In the second model, Ine/RosA transporters are expressed in interneurons that directly or indirectly innervate motor neurons in the central nervous system (Figure 7.1 B). Through its receptors on the motor neuron, the neurotransmitter substrate of *ine/rosA* transporter modifies ion channel activities and neuronal excitability, possibly through signal transduction pathway(s). In *ine/rosA* mutant flies, the effect of the neurotransmitter substrate of Ine/RosA will be enhanced because it can't be eliminated effectively from the synapse. As a result, during the development and possibly through signal transduction pathway(s), the expression of ion channels or the expression of proteins that modify ion channel activities will be altered and increased motor neuron excitability occurs (Figure 7.1 B).

Figure 7.1 Two possible mechanisms to explain *ine/rosA* function in controlling motor neuron excitability. A. The *ine/rosA* transporter is expressed in the motor neuron terminal in the Drosophila neuromuscular junction. Arrows indicate the movement of the *ine/rosA* transporter substrate, which acts as an autocrine. Dashed arrows indicate the signaling pathway. B. The *ine/rosA* transporter is expressed in the Drosophila central nervous system (CNS). The *ine/rosA* transporter may be in glial cells or interneurons that innervate motor neurons. Arrows indicate the signaling pathway. In both A and B, the *ine/rosA* transporter regulates the concentration of its substrate in the synapse. The substrate receptors are present on the motor neuron.

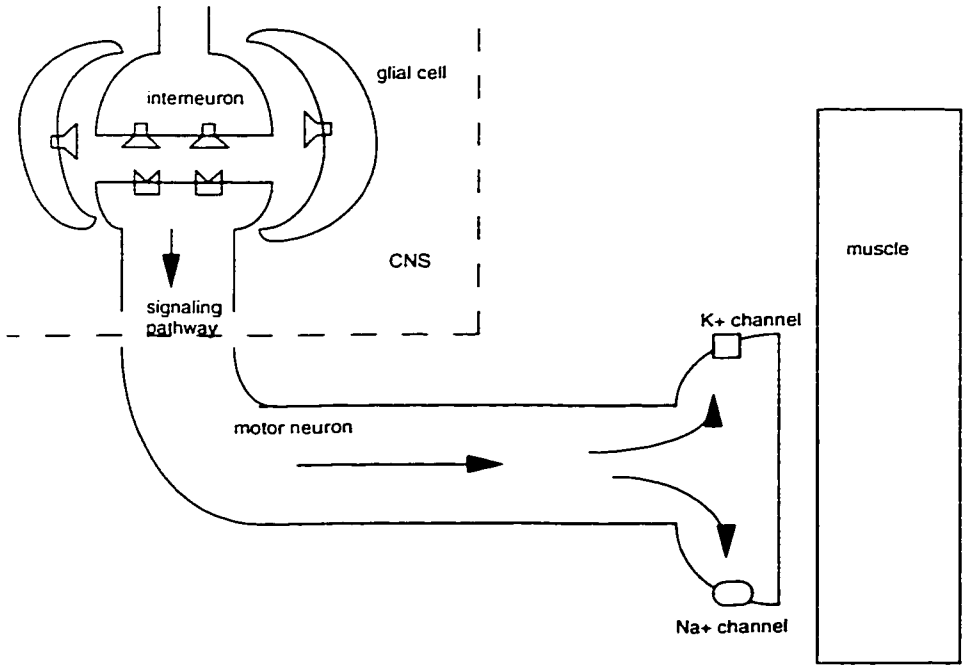
A



: ine/rosA transporter

: receptor for ine/rosA substrate transmitter

B



Currently, we can not determine which, if any, of these two models represents the reality. The *ine/rosA* mRNAs were localized to the central nervous system, but the location of Ine/RosA proteins remain unclear. Proteins functioning in the nerve endings in the peripheral nervous system may be synthesized in cell bodes in the central nervous system. Antibodies against Ine/RosA and immunolocalization experiments would help resolve this issue.

In mammals, the brain tissue is usually isotonic to the plasma. During acute hypernatraemia, the brain volume is maintained by active accumulation of ions and organic osmolytes (Pollock and Arieff, 1980). Under hyperosmotic stress, the brain can actively accumulate organic osmolytes such as alanine, glutamate, glycine, GABA, taurine, and inositol (Law, 1991b). Na^+ dependent amino acid uptake has been observed in astrocyte cell culture (Lehmann and Hansson, 1987). High affinity Na^+ dependent taurine uptake was identified in both neurons and astrocytes (Larsson *et al.*, 1986).

From the roles of *ine/rosA* in the osmotic stress response, an additional model could be proposed to explain the neuronal excitability phenotype of *ine/rosA* mutants. In this model, the *ine/rosA* transporter accumulates osmolyte(s) in neurons or glia. During the development of *ine/rosA* mutant flies, the extracellular ion concentrations have to be changed in order to accommodate the lowered osmotic tolerance of these cells. The altered extracellular ion concentrations will cause changes in the resting membrane potential, in the amount of neurotransmitters released by neurons, and then in neuronal excitability. Electrophysiological studies on invertebrates have shown that extracellular osmolarity and cell volume have a huge effect on neuronal excitability (Pichon and Treherne, 1976).

Finally, another possibility is that *ine/rosA* transporter may affect excitability directly through its substrate dependent or independent ion channel modes. As

stated in the Introduction of this thesis, neurotransmitter transporters can generate currents and change membrane potentials through its ion channel-like activities. In salamander, the regulation of excitability in the photoreceptor is speculated to be achieved by glutamate transporters behaving like glutamate-gated chloride channel (Picaud *et al.*, 1995).

2. Possible models to explain the osmolarity sensitivity phenotypes of *inebriated/rosA* mutants

Two models to explain the *ine/rosA* phenotype in Drosophila osmotic stress response can be envisioned. Both of the models make use of the three-compartment theory often employed to explain how animals absorb water from food with high osmolarity, sometimes even higher than that of their body fluid (see Chapter 1, introduction). In the Drosophila hindgut, the three compartments are (A) the hindgut lumen where urine passes, (B) the hindgut wall with one or more layers of cells and high osmotic extracellular space on the apical side, and (C) the hemolymph that is draining diluted absorbate from the hindgut wall (figure 7.2).

The cells in the hindgut have to endure a very hypertonic environment that is built up by active ion transportation. This hypertonicity is required to absorb water from the hindgut lumen through osmosis. We hypothesize that the *ine/rosA* transporter is called upon to accumulate osmolyte(s) and maintain normal structure and functions of cells in the hindgut wall.

In the first model, during Drosophila development, the osmolarity of the hindgut in *ine/rosA* mutants becomes lower than that of wild type flies. This reduced osmolarity will accommodate the lack of *ine/rosA* transporter function and the consequent higher sensitivity to osmolarity of the hindgut cells. The osmolarity of the hindgut wall is the driving force for water absorption and regulates the osmotic tolerance of Drosophila. The low osmolarity in the *ine/rosA* mutant

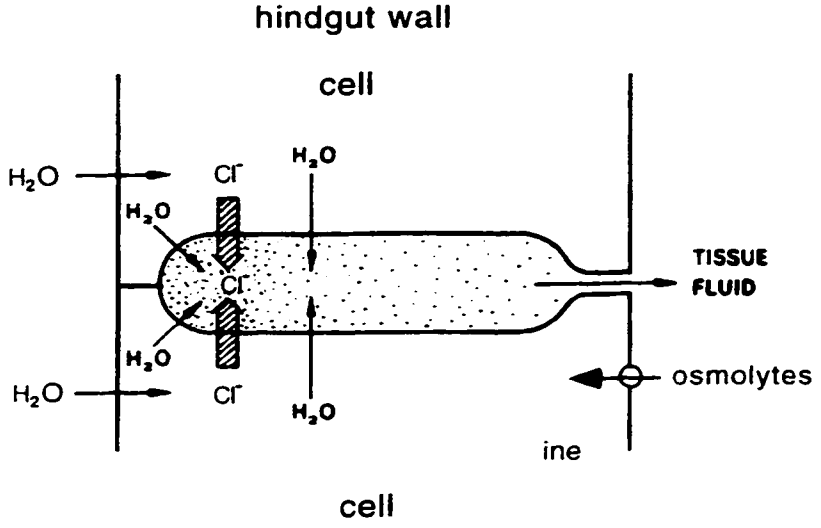


Figure 7.2 Possible role of Ine/RosA in the fluid absorption of the Drosophila hindgut. Two cells are shown in the hindgut wall. Na^+ is actively pumped from the hindgut lumen into the cells and then to the intercellular space in the hindgut wall. The density of dots indicate the level of osmolarity in the intercellular space. In reality, there may be many layers of cells in the hindgut wall and the intercellular space is long and convoluted.

hindgut will still allow fluid absorption from normal food, but not from food with higher osmolarity. The *ine/rosA* mutant flies die faster on high osmotic food because they are unable to concentrate their urine.

In the second model, the hindguts of wild type flies and *ine/rosA* mutants have similar osmolarities when maintained on normal food. When there is an increase in food osmolarity, flies have to increase the osmolarity in the hindgut. Wild type flies can handle the increase in hindgut osmolarity via activation of *ine/rosA* transporter mediated osmolyte accumulation, perhaps through post-translational regulation, whereas *ine/rosA* mutant flies start dying from salt induced toxicity on the hindgut cells.

In the second model, it is implied that the accumulation of Ine/RosA substrate is activated by hypertonic stress. However, it is also possible that many osmolytes are accumulated in Drosophila hindgut and Malpighian tubules and *ine/rosA* transporter is transporting a "house-keeping" osmolyte with a constant intracellular concentration or a concentration dependent on its supply in the hemolymph. Under hypertonic stress, activities of transporters or synthesizing enzymes for other osmolytes was up-regulated by various possible mechanisms, but not that of the *ine/rosA* transporter. Mutations in the *ine/rosA* gene cause lower basal level of house-keeping osmolytes and subsequently a smaller pool of osmolytes that could balance extracellular osmolarity.

In mammals, the inhibition of neurotransmitter transporters or depletion of available osmolytes in the kidney cause kidney failure and inability to concentrate urine. The inhibition of mammalian myo-inositol transport in the kidney causes acute kidney failure characterized by tubular cell injury in outer medulla and increased blood urea content (Kitamura *et al.*, 1998). The kidney failure is more severe when NaCl is administered together with the inositol transport inhibitor. When animals are subjected to sudden hypertonic stress after chronicle hypotonic

conditioning that inactivates transporters and synthetic enzymes for osmolytes in the kidney, the inability to accumulate osmolytes in the medulla makes the kidney unable to concentrate urine normally (Sone *et al.*, 1995).

The ability of overexpression of *ine/rosA*-s cDNA in the Malpighian tubules to enhance salt tolerance in Drosophila is intriguing. Previously, in only one other case did the over expression of one single gene render higher salt tolerance than that of wild type: the overexpression of a Na^+/H^+ antiport in Arabidopsis (Apse *et al.*, 1999). Like the *ine/rosA* gene, the transcription level of this antiport gene is not increased by various levels of salt stress. This may be because there are other requirements in the induction of these genes not satisfied in these experiments. It is also possible that Drosophila does not respond to hypertonic stress by increasing the transcriptional level of Ine/RosA, but by transporting more Ine transporters from intracellular compartments to the plasma membrane. The overexpression of *ine/rosA* may cause mistrafficking and put more Ine/RosA proteins to the plasma membrane. Mistrafficking due to mutations, including those on a gene encoding a putative transmembrane protein, has been observed previously in yeast (Li *et al.*, 1999).

3. Differential rescue abilities of distinct *inebriated/rosA* cDNAs

The *ine/rosA* mutants exhibit an interesting array of phenotypes that are both diverse and closely related. The morphological phenotypes in the *Sh* background (the indented thorax and downturned wings) could be the results of increased motor neuron excitability. Presumably, during pupal eclosion, muscle contractions controlled by motor neurons are required for the normal development of thoracic muscles. Many mutations causing increased excitability in the motor neuron, such as *eag*, *Dp para* and *ine/rosA*, may lead to excessive muscle contractions and result in the morphological phenotypes in a *Sh* mutant background.

It is also possible that defects in photoreceptor properties and in the ability to survive hypertonic environment among *ine/rosA* mutants have the same cause: the inability to accumulate osmolyte(s) and regulate cellular response to changes in osmolarity. Mammalian osmotically responsive osmolyte transporters, such as the inositol transporter, are found in the lens epithelial cells (Zhou and Cammarata, 1997). The fact that the *ine/rosA*-s cDNA can rescue the defects in the photoreceptor and the osmotic sensitivity phenotype, but not the morphological and electrophysiological phenotypes, might be an indication of the relatedness of the two different phenotypes (table 7.1).

One possible explanation of the differences in the rescue capability between *ine/rosA*-s and *ine/rosA*-l cDNAs (table 7.1) is that the common part of Ine/rosA, including all the transmembrane domains of *ine/rosA* transporter, is sufficient to transport substrate(s) and maintain cellular osmolarity in the hindgut and Malpighian tubules, as well as in the eye. The N-terminus of Ine/RosA-l may contain regulatory site(s) that confer substrate selectivity or subcellular localization. In *ine/rosA* mutant flies expressing only Ine/RosA-s, the lack of the N-terminal domain of Ine/RosA-l makes the *ine/rosA* transporter unable to either catalyze the reuptake of a specific substrate or be translocalized to the proper subcellular compartment(s) within neurons.

Different functions rendered by extra amino acid sequence is not unprecedented among neurotransmitter transporters. The glycine transporter GLYT2 is longer by 200 amino acids than GLYT1-a and GLYT1-b. The extra sequence before the first transmembrane domain may have conserved sites for phosphorylation by PKC, PKA and CAM(Liu *et al.*, 1993). GLYT2 is believed to play a major role in inhibitory effect in the brain stem and spinal cord and is found in almost all the glycinergic neurons, whereas GLYT1-a and GLYT1-b are found in

phenotype	increased rate of onset of facilitation in electrophysiology	morphological defects	photoreceptor defects	osmotic sensitivity
rescued by *ine/rosA*-s	no	no	yes	yes
rescued by *ine/rosA*-l	yes	yes	yes	yes

Table 7.1 Different sets of mutant phenotypes rescued by the expression of *ine/rosA*-s or *ine/rosA*-l cDNA. Photoreceptor data are provided by Dr. Burg. Electrophysiology and morphology data are provided by Yan mei Huang and Dr. Stern. Osmotic sensitivity data are collected by Laura Huff. In Drosophila electrophysiology, long term facilitation is also called augmentation.

only a small portion of glycinergic neurons (Jursky and Nelson, 1995; Luque *et al.*, 1995).

4. Speculations on future directions

Measuring the osmolarity by determining the ion content of organs from *ine/rosA* mutant flies and wildtype flies by atomic spectrometry (Apse *et al.*, 1999) will help to examine the two models proposed to explain the *ine/rosA* mutant phenotype. On normal diet, if the ion content in the hindgut wall is the same between wild type flies and *ine/rosA* mutant flies, the second model explaining *ine/rosA* function in fluid absorption holds. Otherwise, the first one should be a more plausible one. A study of the osmolarity of the Drosophila brain can also help to determine if the osmolarity of the central nervous system is affected by the *ine/rosA* mutations and increases neuronal excitability.

Very little is known about the chemical composition of the hindgut, except that it has high levels of glycine and glutamate and low levels of taurine and GABA (Wagner *et al.*, 1991). A survey of the content of potential substrate chemicals in the Drosophila hindgut using infrared spectroscopy or NMR and the identification of those whose concentrations are increased by hypertonicity may provide clues to the identity of the *ine/rosA* substrate(s). Also, viability assays on S2 cells expressing Ine/RosA transporter in a well-defined hypertonic media, with the addition or subtraction of certain organic compounds may also be helpful in the effort to identify Ine/RosA substrates. Compound(s) that enhance S2 cell survival in hypertonic media may potentially be the substrate(s) of Ine/RosA and warrant further tests in uptake assays.

The ion dependence of Ine/RosA is completely unknown. In future uptake assays, it may be necessary to try using a K^+ gradient, or both Na^+ and K^+ gradients.

Also, the uptake assays could be expanded to more potential osmolyte substrates, such as sugar alcohols.

Given the importance of application of antibodies in functional studies, the effort to develop antibodies to Ine/RosA should be continued. The external loop sequence between the third and the fourth transmembrane domain could be a very good antigen. Further, multiple peptides from the same or different soluble regions of Ine/RosA could be fused to form a larger peptide to achieve better expression using the pET vectors in *E. coli*. These larger peptides might also be more antigenic than smaller ones.

The *ine/rosA* transcription is not inducible by osmotic stress and may be regulated by post-translational modification. Activation or inhibition of posttranslational pathways such as PKC and arachidonic acid pathways in a tissue specific manner may provide insights into regulatory mechanisms of osmolyte uptake.

Dr. Stern and others in the lab have isolated a mutation that suppresses the salt sensitive phenotype of *ine/rosA* flies. Identifying this suppressor gene may lead to the discovery of more components in the regulation of osmotic stress response, such as proteins in or modulated by the MAPK cascade. Some companies, such as Affymetrix, are in the process of making Drosophila cDNA microarrays. With these microarrays, genes that are up-regulated or down-regulated by mutations on the *ine/rosA* gene could be identified and further studied.

We have speculated that the N-terminus of Ine/RosA-l (ROSA-N) could be a regulatory domain. The yeast two-hybrid system could be employed to identify proteins that interact with ROSA-N. In the meantime, frequent literature search and bioinformatics research may help identifying newly defined domain sequences in ROSA-N.

References

Amara, S. G., and Kuhar, M. J. (1993). Neurotransmitter transporters: recent progress. *Annu Rev Neurosci* **16,** 73-93.

Apse, M. P., Aharon, G. S., Snedden, W. A., and Blumwald, E. (1999). Salt tolerance conferred by overexpression of a vacuolar Na+/H+ antiport in Arabidopsis. *Science* **285,** 1256-8.

Attwell, D., Barbour, B., and Szatkowski, M. (1993). Nonvesicular release of neurotransmitter. *Neuron* **11,** 401-7.

Bakker, E. P. (1992). Cell K+ and K+ transport systems in prokaryotes. *In* "Alkali Cation Transport Systems in Prokaryotes", pp. 205-224. CRC press.

Barbour, B., Szatkowski, M., Ingledew, N., and Attwell, D. (1989). Arachidonic acid induces a prolonged inhibition of glutamate uptake into glial cells. *Nature* **342,** 918-20.

Beck, F. X., Burger-Kentischer, A., and Muller, E. (1998). Cellular response to osmotic stress in the renal medulla. *Pflugers Arch* **436,** 814-27.

Beckman, M. L., and Quick, M. W. (1998). Neurotransmitter transporters: regulators of function and functional regulation. *J Membr Biol* **164,** 1-10.

Bengel, D., Murphy, D. L., Andrews, A. M., Wichems, C. H., Feltner, D., Heils, A., Mossner, R., Westphal, H., and Lesch, K. P. (1998). Altered brain serotonin homeostasis and locomotor insensitivity to 3, 4- methylenedioxymethamphetamine ("Ecstasy") in serotonin transporter- deficient mice. *Mol Pharmacol* **53,** 649-55.

Bennett, E. R., and Kanner, B. I. (1997). The membrane topology of GAT-1, a (Na+ + Cl-)-coupled gamma- aminobutyric acid transporter from rat brain. *J Biol Chem* **272,** 1203-10.

Berl, T., Siriwardana G, Ao L, Butterfield LM, Heasley LE. (1997). Multiple mitogen-activated protein kinases are regulated by hyperosmolality in mouse IMCD cells. *Am J Physiol* **272(3 Pt 2),** F305-11.

Billups, B., and Attwell, D. (1996). Modulation of non-vesicular glutamate release by pH. *Nature* **379,** 171-4.

Bismuth, Y., Kavanaugh, M. P., and Kanner, B. I. (1997). Tyrosine 140 of the gamma-aminobutyric acid transporter GAT-1 plays a critical role in neurotransmitter recognition. *J Biol Chem* **272,** 16096-102.

Bookstein, C., Musch MW, DePaoli A, Xie Y, Villereal M, Rao MC, Chang EB. (1994). A unique sodium-hydrogen exchange isoform (NHE-4) of the inner medulla of the rat kidney is induced by hyperosmolarity. *J Biol Chem* **469,** 29704-29709.

Borden, L. A., Smith, K. E., Gustafson, E. L., Branchek, T. A., and Weinshank, R. L. (1995). Cloning and expression of a betaine/GABA transporter from human brain. *J Neurochem* **64,** 977-84.

Borden, L. A., Smith, K. E., Hartig, P. R., Branchek, T. A., and Weinshank, R. L. (1992). Molecular heterogeneity of the gamma-aminobutyric acid (GABA) transport system. Cloning of two novel high affinity GABA transporters from rat brain. *J Biol Chem* **267,** 21098-104.

Borowsky, B., Mezey, E., and Hoffman, B. J. (1993). Two glycine transporter variants with distinct localization in the CNS and peripheral tissues are encoded by a common gene. *Neuron* **10,** 851-63.

Bouvier, M., Szatkowski, M., Amato, A., and Attwell, D. (1992). The glial cell glutamate uptake carrier countertransports pH-changing anions. *Nature* **360,** 471-4.

Bradley, T. J. (1989). Membrane dynamics in insect malpighian tubules. *Am J Physiol* **257,** R967-72.

Brand, A. H., Manoukian, A. S., and Perrimon, N. (1994). Ectopic expression in Drosophila. *Methods Cell Biol* **44,** 635-54.

Brewster, J. L., de Valoir, T., Dwyer, N. D., Winter, E., and Gustin, M. C. (1993). An osmosensing signal transduction pathway in yeast. *Science* **259,** 1760-3.

Bruns, D., Engert, F., and Lux, H. D. (1993). A fast activating presynaptic reuptake current during serotonergic transmission in identified neurons of Hirudo. *Neuron* **10,** 559-72.

Bruss, M., Hammermann, R., Brimijoin, S., and Bonisch, H. (1995). Antipeptide antibodies confirm the topology of the human norepinephrine transporter. *J Biol Chem* **270,** 9197-201.

Buchner, E. (1991). Genes expressed in the adult brain of Drosophila and effects of their mutations on behavior: a survey of transmitter- and second messenger- related genes. *J Neurogenet* **7,** 153-92.

Burg, M. B., Kwon, E. D., and Kultz, D. (1996). Osmotic regulation of gene expression. *Faseb J* **10,** 1598-606.

Burg, M. G., Geng C., Guan, Y., Koliantz, G., & Pak, W. L. (1996). Drosophila rosA gene, which when mutant causes aberrant photoreceptor oscillation, encodes a novel neurotransmitter transporter homologue. *J. Neurogenetics.* **Vol. 11,** 59-79.

Cammack, J. N., Rakhilin, S. V., and Schwartz, E. A. (1994). A GABA transporter operates asymmetrically and with variable stoichiometry. *Neuron* **13,** 949-60.

Cao, Y., Mager, S., and Lester, H. A. (1997). H+ permeation and pH regulation at a mammalian serotonin transporter. *J Neurosci* **17,** 2257-66.

Casado, M., Bendahan, A., Zafra, F., Danbolt, N. C., Aragon, C., Gimenez, C., and Kanner, B. I. (1993). Phosphorylation and modulation of brain glutamate transporters by protein kinase C. *J Biol Chem* **268,** 27313-7.

Chen, J. G., Liu-Chen, S., and Rudnick, G. (1997). External cysteine residues in the serotonin transporter. *Biochemistry* **36,** 1479-86.

Cherbas, L., Moss, R., and Cherbas, P. (1994). Transformation techniques for Drosophila cell lines. *Methods Cell Biol* **44,** 161-79.

Ciliax, B. J., Drash, G. W., Staley, J. K., Haber, S., Mobley, C. J., Miller, G. W., Mufson, E. J., Mash, D. C., and Levey, A. I. (1999). Immunocytochemical localization of the dopamine transporter in human brain. *J Comp Neurol* **409,** 38-56.

Conradt, M., and Stoffel, W. (1997). Inhibition of the high-affinity brain glutamate transporter GLAST-1 via direct phosphorylation. *J Neurochem* **68,** 1244-51.

Corey, J. L., Quick, M. W., Davidson, N., Lester, H. A., and Guastella, J. (1994). A cocaine-sensitive Drosophila serotonin transporter: cloning, expression, and electrophysiological characterization. *Proc Natl Acad Sci U S A* **91,** 1188-92.

Csoknya, M., Barna, J., Banvolgyi, T., Hiripi, L., Eckert, M., Hamori, J., and Elekes, K. (1997). Octopamine-containing neurons in the alimentary tract of the earthworm (Eisenia fetida). *Brain Res* **778,** 414-7.

Demchyshyn, L. L., Pristupa, Z. B., Sugamori, K. S., Barker, E. L., Blakely, R. D., Wolfgang, W. J., Forte, M. A., and Niznik, H. B. (1994). Cloning, expression, and localization of a chloride-facilitated, cocaine-sensitive serotonin transporter from Drosophila melanogaster. *Proc Natl Acad Sci U S A* **91,** 5158-62.

Diamond, J. S., and Jahr, C. E. (1997). Transporters buffer synaptically released glutamate on a submillisecond time scale. *J Neurosci* **17,** 4672-87.

During, M. J., Ryder, K. M., and Spencer, D. D. (1995). Hippocampal GABA transporter function in temporal-lobe epilepsy. *Nature* **376,** 174-7.

Epstein, W. (1992). Kdp, a bacterial P-type ATPase whose expression and activity are regulated by turgor pressure. *Acta Physiol Scand* **607,** 193-199.

Ferraris, J. D., Williams, C. K., Jung, K. Y., Bedford, J. J., Burg, M. B., and Garcia-Perez, A. (1996). ORE, a eukaryotic minimal essential osmotic response element. The aldose reductase gene in hyperosmotic stress. *J Biol Chem* **271,** 18318-21.

Gainetdinov, R. R., Wetsel, W. C., Jones, S. R., Levin, E. D., Jaber, M., and Caron, M. G. (1999). Role of serotonin in the paradoxical calming effect of psychostimulants on hyperactivity. *Science* **283,** 397-401.

Galcheva-Gargova, Z., Derijard, B., Wu, I. H., and Davis, R. J. (1994). An osmosensing signal transduction pathway in mammalian cells. *Science* **265,** 806-8.

Galli, A., Blakely, R. D., and DeFelice, L. J. (1996). Norepinephrine transporters have channel modes of conduction. *Proc Natl Acad Sci U S A* **93,** 8671-6.

Galli, A., DeFelice, L. J., Duke, B. J., Moore, K. R., and Blakely, R. D. (1995). Sodium-dependent norepinephrine-induced currents in norepinephrine- transporter-transfected HEK-293 cells blocked by cocaine and antidepressants. *J Exp Biol* **198,** 2197-212.

Galli, A., Petersen, C. I., deBlaquiere, M., Blakely, R. D., and DeFelice, L. J. (1997). Drosophila serotonin transporters have voltage-dependent uptake coupled to a serotonin-gated ion channel. *J Neurosci* **17,** 3401-11.

Garcia-Perez, A., and Burg, M. B. (1991a). Renal medullary organic osmolytes. *Physiol Rev* **71,** 1081-115.

Garcia-Perez, A., and Burg, M. B. (1991b). Role of organic osmolytes in adaptation of renal cells to high osmolality. *J Membr Biol* **119,** 1-13.

Gegelashvili, G., Civenni, G., Racagni, G., Danbolt, N. C., Schousboe, I., and Schousboe, A. (1996). Glutamate receptor agonists up-regulate glutamate transporter GLAST in astrocytes. *Neuroreport* **8,** 261-5.

Giros, B., Jaber, M., Jones, S. R., Wightman, R. M., and Caron, M. G. (1996). Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. *Nature* **379,** 606-12.

Grunewald, R. W., Weber, II, and Kinne, R. K. (1995). Renal inner medullary sorbitol metabolism. *Am J Physiol* **269,** F696-701.

Guastella, J., Nelson, N., Nelson, H., Czyzyk, L., Keynan, S., Miedel, M. C., Davidson, N., Lester, H. A., and Kanner, B. I. (1990). Cloning and expression of a rat brain GABA transporter. *Science* **249,** 1303-6.

Hakes, D. J., and Dixon, J. E. (1992). New vectors for high level expression of recombinant proteins in bacteria. *Anal Biochem* **202,** 293-8.

Han, S. J., Choi, K. Y., Brey, P. T., and Lee, W. J. (1998). Molecular cloning and characterization of a Drosophila p38 mitogen- activated protein kinase. *J Biol Chem* **273,** 369-74.

Harlow, E. L., D. (1988). "Antibodies, A Laboratory Manual." Cold Spring Harbor Laboratory,

Harrison, D. A., Binari, R., Nahreini, T. S., Gilman, M., and Perrimon, N. (1995). Activation of a Drosophila Janus kinase (JAK) causes hematopoietic neoplasia and developmental defects. *Embo J* **14,** 2857-65.

Hediger, M. A. (1994). Structure, function and evolution of solute transporters in prokaryotes and eukaryotes. *J Exp Biol* **196,** 15-49.

Hestrin, S., Sah, P., and Nicoll, R. A. (1990). Mechanisms generating the time course of dual component excitatory synaptic currents recorded in hippocampal slices. *Neuron* **5,** 247-53.

Holden, C. (1997). A gene is linked to autism. *Science* **276,** 905.

Huff, R. A., Vaughan, R. A., Kuhar, M. J., and Uhl, G. R. (1997). Phorbol esters increase dopamine transporter phosphorylation and decrease transport Vmax. *J Neurochem* **68,** 225-32.

Isaacson, J. S., Solis, J. M., and Nicoll, R. A. (1993). Local and diffuse synaptic actions of GABA in the hippocampus. *Neuron* **10,** 165-75.

Jayanthi, L. D., Ramamoorthy, S., Mahesh, V. B., Leibach, F. H., and Ganapathy, V. (1994). Calmodulin-dependent regulation of the catalytic function of the human serotonin transporter in placental choriocarcinoma cells. *J Biol Chem* **269,** 14424-9.

Jursky, F., and Nelson, N. (1995). Localization of glycine neurotransmitter transporter (GLYT2) reveals correlation with the distribution of glycine receptor. *J Neurochem* **64,** 1026-33.

Kaback, H. R., Voss, J., and Wu, J. (1997). Helix packing in polytopic membrane proteins: the lactose permease of Escherichia coli. *Curr Opin Struct Biol* **7,** 537-42.

Kamb, A., Iverson, L. E., and Tanouye, M. A. (1987). Molecular characterization of Shaker, a Drosophila gene that encodes a potassium channel. *Cell* **50,** 405-13.

Kanner, B. I. (1983). Bioenergetics of neurotransmitter transport. *Biochim Biophys Acta* **726,** 293-316.

Karihaloo, A., Kato, K., Greene, D. A., and Thomas, T. P. (1997). Protein kinase and Ca2+ modulation of myo-inositol transport in cultured retinal pigment epithelial cells. *Am J Physiol* **273,** C671-8.

Kay, M. A. a. J.-L. (1987). Developmental genetics of ribosome synthesis in Drosophila. *Trends in Genetics* **3,** 351.

Keshet, G. I., Bendahan, A., Su, H., Mager, S., Lester, H. A., and Kanner, B. I. (1995). Glutamate-101 is critical for the function of the sodium and chloride- coupled GABA transporter GAT-1. *FEBS Lett* **371,** 39-42.

Kitamura, H., Yamauchi, A., Sugiura, T., Matsuoka, Y., Horio, M., Tohyama, M., Shimada, S., Imai, E., and Hori, M. (1998). Inhibition of myo-inositol transport causes acute renal failure with selective medullary injury in the rat. *Kidney Int* **53,** 146-53.

Kitayama, S., Shimada, S., Xu, H., Markham, L., Donovan, D. M., and Uhl, G. R. (1992). Dopamine transporter site-directed mutations differentially alter substrate transport and cocaine binding. *Proc Natl Acad Sci U S A* **89,** 7782-5.

Kitayama, S., Wang, J. B., and Uhl, G. R. (1993). Dopamine transporter mutants selectively enhance MPP+ transport. *Synapse* **15,** 58-62.

Kleinberger-Doron, N., and Kanner, B. I. (1994). Identification of tryptophan residues critical for the function and targeting of the gamma-aminobutyric acid transporter (subtype A). *J Biol Chem* **269,** 3063-7.

Ko, B. C. B., Ruepp, B., Bohren, K. M., Gabbay, K. H., and Chung, S. S. (1997). Identification and characterization of multiple osmotic response sequences in the human aldose reductase gene. *J Biol Chem* **272,** 16431-7.

Kojima, R., Randall, J., Brenner, B. M., and Gullans, S. R. (1996). Osmotic stress protein 94 (Osp94). A new member of the Hsp110/SSE gene subfamily. *J Biol Chem* **271,** 12327-32.

Krieg, P., Strachan, R., Wallis, E., Tabe, L., and Colman, A. (1984). Efficient expression of cloned complementary DNAs for secretory proteins after injection into Xenopus oocytes. *J Mol Biol* **180,** 615-43.

Kultz, D., Madhany, S., and Burg, M. B. (1998). Hyperosmolality causes growth arrest of murine kidney cells. Induction of GADD45 and GADD153 by osmosensing via stress-activated protein kinase 2. *J Biol Chem* **273,** 13645-51.

Kutz, D., and Burg, M. (1998). Evolution of osmotic stress signaling via MAP kinase cascades. *J Exp Biol* **201,** 3015-21.

Larsson, O. M., Griffiths, R., Allen, I. C., and Schousboe, A. (1986). Mutual inhibition kinetic analysis of gamma-aminobutyric acid, taurine, and beta-alanine high-affinity transport into neurons and astrocytes: evidence for similarity between the taurine and beta-alanine carriers in both cell types. *J Neurochem* **47,** 426-32.

Launay, J. M., Bondoux, D., Oset-Gasque, M. J., Emami, S., Mutel, V., Haimart, M., and Gespach, C. (1994). Increase of human platelet serotonin uptake by atypical histamine receptors. *Am J Physiol* **266,** R526-36.

Law, R. O. (1991a). Alterations in renal inner medullary levels of amino nitrogen during acute water diuresis and hypovolaemic oliguria in rats. *Pflugers Arch* **418,** 442-6.

Law, R. O. a. B., M. B. (1991b). The Role of Organic Osmolytes in the Regulation of Mammalian Cell Volume. *In* "Advances in Comparative & Evironmental Physiology", Vol. 9, pp. 189-225.

Lehmann, A., and Hansson, E. (1987). Amino acid content in astroglial primary cultures from different brain regions during cultivation. *Neurochem Res* **12,** 797-800.

Li, Y., Kane, T., Tipper, C., Spatrick, P., and Jenness, D. D. (1999). Yeast mutants affecting possible quality control of plasma membrane proteins. *Mol Cell Biol* **19,** 3588-99.

Lin, F., Lester, H. A., and Mager, S. (1996). Single-channel currents produced by the serotonin transporter and analysis of a mutation affecting ion permeation. *Biophys J* **71,** 3126-35.

Liu, Q. R., Lopez-Corcuera, B., Mandiyan, S., Nelson, H., and Nelson, N. (1993). Cloning and expression of a spinal cord- and brain-specific glycine transporter with novel structural features. *J Biol Chem* **268,** 22802-8.

Liu, Q. R., Lopez-Corcuera, B., Nelson, H., Mandiyan, S., and Nelson, N. (1992a). Cloning and expression of a cDNA encoding the transporter of taurine and beta-alanine in mouse brain. *Proc Natl Acad Sci U S A* **89,** 12145-9.

Liu, Q. R., Mandiyan, S., Nelson, H., and Nelson, N. (1992b). A family of genes encoding neurotransmitter transporters. *Proc Natl Acad Sci U S A* **89,** 6639-43.

Liu, Y., Eckstein-Ludwig, U., Fei, J., and Schwarz, W. (1998). Effect of mutation of glycosylation sites on the Na+ dependence of steady-state and transient currents generated by the neuronal GABA transporter. *Biochim Biophys Acta* **1415,** 246-54.

Lucht, J. M., and Bremer, E. (1994). Adaptation of Escherichia coli to high osmolarity environments: osmoregulation of the high-affinity glycine betaine transport system proU. *FEMS Microbiol Rev* **14,** 3-20.

Luque, J. M., Nelson, N., and Richards, J. G. (1995). Cellular expression of glycine transporter 2 messenger RNA exclusively in rat hindbrain and spinal cord. *Neuroscience* **64,** 525-35.

Maeda, T., Wurgler-Murphy, S. M., and Saito, H. (1994). A two-component system that regulates an osmosensing MAP kinase cascade in yeast. *Nature* **369,** 242-5.

Mager, S., Kleinberger-Doron, N., Keshet, G. I., Davidson, N., Kanner, B. I., and Lester, H. A. (1996). Ion binding and permeation at the GABA transporter GAT1. *J Neurosci* **16,** 5405-14.

Mager, S., Min, C., Henry, D. J., Chavkin, C., Hoffman, B. J., Davidson, N., and Lester, H. A. (1994). Conducting states of a mammalian serotonin transporter. *Neuron* **12,** 845-59.

Mager, S., Naeve, J., Quick, M., Labarca, C., Davidson, N., and Lester, H. A. (1993). Steady states, charge movements, and rates for a cloned GABA transporter expressed in Xenopus oocytes. *Neuron* **10,** 177-88.

Melikian, H. E., McDonald, J. K., Gu, H., Rudnick, G., Moore, K. R., and Blakely, R. D. (1994). Human norepinephrine transporter. Biosynthetic studies using a site-directed polyclonal antibody. *J Biol Chem* **269,** 12290-7.

Mizoguchi, T., Irie, K., Hirayama, T., Hayashida, N., Yamaguchi-Shinozaki, K., Matsumoto, K., and Shinozaki, K. (1996). A gene encoding a mitogen-activated protein kinase kinase kinase is induced simultaneously with genes for a mitogen-activated protein kinase and an S6 ribosomal protein kinase by touch, cold, and water stress in Arabidopsis thaliana. *Proc Natl Acad Sci U S A* **93,** 765-9.

Muller, E., Neuhofer, W., Ohno, A., Rucker, S., Thurau, K., and Beck, F. X. (1996). Heat shock proteins HSP25, HSP60, HSP72, HSP73 in isoosmotic cortex and hyperosmotic medulla of rat kidney. *Pflugers Arch* **431,** 608-17.

Nanjo, T., Kobayashi, M., Yoshiba, Y., Sanada, Y., Wada, K., Tsukaya, H., Kakubari, Y., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1999). Biological functions of proline in morphogenesis and osmotolerance revealed in antisense transgenic Arabidopsis thaliana. *Plant J* **18,** 185-93.

Nass, R., and Rao, R. (1998). Novel localization of a Na+/H+ exchanger in a late endosomal compartment of yeast. Implications for vacuole biogenesis. *J Biol Chem* **273,** 21054-60.

Nelson, N. (1994). Energizing porters by proton-motive force. *J Exp Biol* **196,** 7-13.

Nelson, N. (1998). The family of Na+/Cl- neurotransmitter transporters. *J Neurochem* **71,** 1785-803.

Nirenberg, M. J., Vaughan, R. A., Uhl, G. R., Kuhar, M. J., and Pickel, V. M. (1996). The dopamine transporter is localized to dendritic and axonal plasma membranes of nigrostriatal dopaminergic neurons. *J Neurosci* **16,** 436-47.

Nishikawa, T., Aiba, H., and Mizuno, T. (1999). The cta3+ gene that encodes a cation-transporting P-type ATPase is induced by salt stress under control of the Wis1-Sty1 MAPKK-MAPK cascade in fission yeast. *FEBS Lett* **455,** 183-7.

Novagen. (1996). "pET System Manual."

O'Donnell, M. J., and Maddrell, S. H. (1995). Fluid reabsorption and ion transport by the lower Malpighian tubules of adult female Drosophila. *J Exp Biol* **198,** 1647-53.

Pacholczyk, T., Blakely, R. D., and Amara, S. G. (1991). Expression cloning of a cocaine- and antidepressant-sensitive human noradrenaline transporter. *Nature* **350,** 350-4.

Pan, Z. Z., and Williams, J. T. (1989). Differential actions of cocaine and amphetamine on dorsal raphe neurons in vitro. *J Pharmacol Exp Ther* **251,** 56-62.

Peterson, D. P., Murphy, K. M., Ursino, R., Streeter, K., and Yancey, P. H. (1992). Effects of dietary protein and salt on rat renal osmolytes: covariation in urea and GPC contents. *Am J Physiol* **263,** F594-600.

PharmaciaBiotech. (1994). "GST Gene Fusion System."

Phillips, J. E., Audsley, N., Lechleitner, R., Thomson, B., Meredith, J., Chamberlin, M. (1998). Some major transport mechanisms of insect absorptive epithelia. *Comp Biochem Physiol A* **90.**

Picaud, S. A., Larsson, H. P., Grant, G. B., Lecar, H., and Werblin, F. S. (1995). Glutamate-gated chloride channel with glutamate-transporter-like properties in cone photoreceptors of the tiger salamander. *J Neurophysiol* **74,** 1760-71.

Pichon, Y., and Treherne, J. E. (1976). The effects of osmotic stress on the electrical properties of the axons of a marine osmoconformer (Mala squinado. brachyura: crustacea). *J Exp Biol* **65,** 553-63.

Pollock, A. S., and Arieff, A. I. (1980). Abnormalities of cell volume regulation and their functional consequences. *Am J Physiol* **239,** F195-205.

Poolman, B., and Glaasker, E. (1998). Regulation of compatible solute accumulation in bacteria. *Mol Microbiol* **29,** 397-407.

Popping, B., Gibbons, T., and Watson, M. D. (1996). The Pisum sativum MAP kinase homologue (PsMAPK) rescues the Saccharomyces cerevisiae hog1 deletion mutant under conditions of high osmotic stress. *Plant Mol Biol* **31,** 355-63.

Posas, F., and Saito, H. (1997). Osmotic activation of the HOG MAPK pathway via Ste11p MAPKKK: scaffold role of Pbs2p MAPKK. *Science* **276,** 1702-5.

Preston, A. S., Yamauchi, A., Kwon, H. M., and Handler, J. S. (1995). Activators of protein kinase A and of protein kinase C inhibit MDCK cell myo-inositol and betaine uptake. *J Am Soc Nephrol* **6,** 1559-64.

Qian, S., Zhang, J. Y., Kay, M. A., and Jacobs-Lorena, M. (1987). Structural analysis of the Drosophila rpA1 gene, a member of the eucaryotic 'A' type ribosomal protein family. *Nucleic Acids Res* **15,** 987-1003.

Quick, M. W., Corey, J. L., Davidson, N., and Lester, H. A. (1997). Second messengers, trafficking-related proteins, and amino acid residues that contribute to the functional regulation of the rat brain GABA transporter GAT1. *J Neurosci* **17,** 2967-79.

Ramamoorthy, S., and Blakely, R. D. (1999). Phosphorylation and sequestration of serotonin transporters differentially modulated by psychostimulants. *Science* **285,** 763-6.

Ramamoorthy, S., Cool, D. R., Mahesh, V. B., Leibach, F. H., Melikian, H. E., Blakely, R. D., and Ganapathy, V. (1993). Regulation of the human serotonin transporter. Cholera toxin-induced stimulation of serotonin uptake in human placental choriocarcinoma cells is accompanied by increased serotonin transporter mRNA levels and serotonin transporter-specific ligand binding. *J Biol Chem* **268,** 21626-31.

Randall, D., Burggren, W., French, K. (1997). "Animal Physiology, Mechanisms and Adaptations." W. H. Freeman and Company,

Rasola, A., Galietta, L. J., Barone, V., Romeo, G., and Bagnasco, S. (1995). Molecular cloning and functional characterization of a GABA/betaine transporter from human kidney. *FEBS Lett* **373,** 229-33.

Rivosecchi, R., Pongs, O., Theil, T., and Mallart, A. (1994). Implication of frequenin in the facilitation of transmitter release in Drosophila. *J Physiol (Lond)* **474,** 223-32.

Rudnick, G., and Kilic, F. (1999). Functional multimerization of the serotonin transporter. *In* "29th annual meeting of the society for neuroscience", pp. 1037, Miami beach.

Rudnick, G., and Clark, J. (1993). From synapse to vesicle: the reuptake and storage of biogenic amine neurotransmitters. *Biochim Biophys Acta* **1144,** 249-63.

Saier, M. H., Jr. (1994). Convergence and divergence in the evolution of transport proteins. *Bioessays* **16,** 23-9.

Sambrook, J., Fritsch, E. T. & Maniatis, T. (1989). "Molecular Cloning." Cold Spring Harbor Laboratory Press,

Sands, J. M., and Schrader, D. C. (1990). Coordinated response of renal medullary enzymes regulating net sorbitol production in diuresis and antidiuresis. *J Am Soc Nephrol* **1,** 58-65.

Schlosser, A., Meldorf, M., Stumpe, S., Bakker, E. P., and Epstein, W. (1995). TrkH and its homolog, TrkG, determine the specificity and kinetics of cation transport by the Trk system of Escherichia coli. *J Bacteriol* **177,** 1908-10.

Schmidt-Nielsen, K. (1975). "Animal Physiology, Adaptation and Environment." Cambridge University Press,

Schwartz, E. A. (1987). Depolarization without calcium can release gamma-aminobutyric acid from a retinal neuron. *Science* **238,** 350-5.

Sheikh-Hamad, D., Di Mari, J., Suki, W. N., Safirstein, R., Watts, B. A., 3rd, and Rouse, D. (1998). p38 kinase activity is essential for osmotic induction of mRNAs for HSP70 and transporter for organic solute betaine in Madin-Darby canine kidney cells. *J Biol Chem* **273,** 1832-7.

Smardo, F. L., Jr., Burg, M. B., and Garcia-Perez, A. (1992). Kidney aldose reductase gene transcription is osmotically regulated. *Am J Physiol* **262,** C776-82.

Soehnge, H., Huang, X., Becker, M., Conover, D., and Stern, M. (1997). Cloning and sequencing of ribosomal protein L27a and a gene similar to human GS1 in Drosophila. *Gene* **185,** 257-63.

Soehnge, H., Huang, X., Becker, M., Whitley, P., Conover, D., and Stern, M. (1996). A neurotransmitter transporter encoded by the Drosophila inebriated gene. *Proc Natl Acad Sci U S A* **93,** 13262-7.

Sonders, M. S., Zhu, S. J., Zahniser, N. R., Kavanaugh, M. P., and Amara, S. G. (1997). Multiple ionic conductances of the human dopamine transporter: the actions of dopamine and psychostimulants. *J Neurosci* **17,** 960-74.

Sone, M., Ohno, A., Albrecht, G. J., Thurau, K., and Beck, F. X. (1995). Restoration of urine concentrating ability and accumulation of medullary osmolytes after chronic diuresis. *Am J Physiol* **269,** F480-90.

Stephan, M. M., Chen, M. A., Penado, K. M., and Rudnick, G. (1997). An extracellular loop region of the serotonin transporter may be involved in the translocation mechanism. *Biochemistry* **36,** 1322-8.

Stern, M., and Ganetzky, B. (1989). Altered synaptic transmission in Drosophila hyperkinetic mutants. *J Neurogenet* **5,** 215-28.

Stern, M., and Ganetzky, B. (1992). Identification and characterization of inebriated, a gene affecting neuronal excitability in Drosophila. *J Neurogenet* **8,** 157-72.

Stern, M., Kreber, R., and Ganetzky, B. (1990). Dosage effects of a Drosophila sodium channel gene on behavior and axonal excitability. *Genetics* **124,** 133-43.

Takenaka, M., Preston, A. S., Kwon, H. M., and Handler, J. S. (1994). The tonicity-sensitive element that mediates increased transcription of the betaine transporter gene in response to hypertonic stress. *J Biol Chem* **269,** 29379-81.

Tamura, S., Nelson, H., Tamura, A., and Nelson, N. (1995). Short external loops as potential substrate binding site of gamma- aminobutyric acid transporters. *J Biol Chem* **270,** 28712-5.

Trotti, D., Volterra, A., Lehre, K. P., Rossi, D., Gjesdal, O., Racagni, G., and Danbolt, N. C. (1995). Arachidonic acid inhibits a purified and reconstituted glutamate transporter directly from the water phase and not via the phospholipid membrane. *J Biol Chem* **270,** 9890-5.

Uchida, S., Kwon, H. M., Yamauchi, A., Preston, A. S., Marumo, F., and Handler, J. S. (1992). Molecular cloning of the cDNA for an MDCK cell Na(+)- and Cl(-)-dependent taurine transporter that is regulated by hypertonicity [published erratum appears in Proc Natl Acad Sci U S A 1993 Aug 1;90(15):7424]. *Proc Natl Acad Sci U S A* **89,** 8230-4.

Vandenbergh, D. J., Persico, A. M., and Uhl, G. R. (1992). A human dopamine transporter cDNA predicts reduced glycosylation, displays a novel repetitive

element and provides racially-dimorphic TaqI RFLPs. *Brain Res Mol Brain Res* **15,** 161-6.

Wagner, R. M., Cook, B. J., and Cooper, J. F. (1991). Action of taurine, 3-aminopropanesulfonic acid, and GABA on the hindgut and heart of the cockroach, Leucophaea maderae. *Arch Insect Biochem Physiol* **16,** 81-93.

Wall, B. J. a. O., J. L. (1970). Water and solute uptake by rectal pads of Periplaneta americana. *American Journal of Physiology* **218,** 1208-1214.

Warmke, J., Drysdale, R., and Ganetzky, B. (1991). A distinct potassium channel polypeptide encoded by the Drosophila eag locus. *Science* **252,** 1560-2.

Welsh, D. T., and Herbert, R. A. (1999). Osmotically induced intracellular trehalose, but not glycine betaine accumulation promotes desiccation tolerance in Escherichia coli. *FEMS Microbiol Lett* **174,** 57-63.

Wurgler-Murphy, S. M., and Saito, H. (1997). Two-component signal transducers and MAPK cascades. *Trends Biochem Sci* **22,** 172-6.

Yancey, P. H., Clark, M. E., Hand, S. C., Bowlus, R. D., and Somero, G. N. (1982). Living with water stress: evolution of osmolyte systems. *Science* **217,** 1214-22.

Zhang, L., and Reith, M. E. (1996). Regulation of the functional activity of the human dopamine transporter by the arachidonic acid pathway. *Eur J Pharmacol* **315,** 345-54.

Zhou, C., and Cammarata, P. R. (1997). Cloning the bovine Na+/myo-inositol cotransporter gene and characterization of an osmotic responsive promoter. *Exp Eye Res* **65,** 349-63.

Zorumski, C. F., Mennerick, S., and Que, J. (1996). Modulation of excitatory synaptic transmission by low concentrations of glutamate in cultured rat hippocampal neurons. *J Physiol (Lond)* **494,** 465-77.