The Drosophila *inebriated*-Encoded Neurotransmitter/Osmolyte Transporter: Dual Roles in the Control of Neuronal Excitability and the Osmotic Stress Response

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Manuscript received July 11, 2001
Accepted for publication November 13, 2001

ABSTRACT

Water reabsorption by organs such as the mammalian kidney and insect Malpighian tubule/hindgut requires a region of hypertonicity within the organ. To balance the high extracellular osmolarity, cells within these regions accumulate small organic molecules called osmolytes. These osmolytes can accumulate to a high level without toxic effects on cellular processes. Here we provide evidence consistent with the possibility that the two protein isoforms encoded by the *inebriated* (*ine*) gene, which are members of the Na^+/Cl^--dependent neurotransmitter/osmolyte transporter family, perform osmolyte transport within the Malpighian tubule and hindgut. We show that *ine* mutants lacking both isoforms are hypersensitive to osmotic stress, which we assayed by maintaining flies on media containing NaCl, KCl, or sorbitol, and that this hypersensitivity is completely rescued by high-level ectopic expression of the *ine-RB* isoform. We provide evidence that this hypersensitivity represents a role for *ine* that is distinct from the increased neuronal excitability phenotype of *ine* mutants. Finally, we show that each *ine* genotype exhibits a "threshold" [NaCl]: long-term maintenance on NaCl-containing media above, but not below, the threshold causes lethality. Furthermore, this threshold value increases with the amount of *ine* activity. These data suggest that *ine* mutations confer osmotic stress sensitivity by preventing osmolyte accumulation within the Malpighian tubule and hindgut.

TERRESTRIAL organisms must be able to accommodate variations in the availability of water. Organisms maintained under conditions of water deprivation (which is termed osmotic stress) must increase water retention to maintain the proper osmolarity within tissues. Much of this accommodation is carried out by the organs that perform fluid reabsorption, such as the mammalian kidney or the insect Malpighian tubule/hindgut. When water is plentiful, these organs reabsorb little water and release a dilute urine. However, during water deprivation, these organs increase the reabsorption of water and release a more concentrated urine. Although the specific mechanics of water reabsorption differ between the mammalian kidney and the insect Malpighian tubule/hindgut, in each organ, water reabsorption requires a region of hypertonicity within the organ (which is the inner medulla in the mammalian kidney). Furthermore, the magnitude of this hypertonicity increases as the rate of water reabsorption increases (Wharton 1985). Thus, the cells within these regions must adapt to a hypertonic extracellular environment in which the magnitude of hypertonicity varies according to water availability.

Cells respond to a hypertonic environment in complex ways that are as yet incompletely understood. Initial exposure to a hypertonic environment causes short-term responses, which include a transient cell shrinkage as a consequence of water loss followed rapidly by an influx of Na^+ and K^+. This influx is accompanied passively by water, which enables a recovery of cell volume. Prolonged exposure to elevated intracellular [Na^+] and [K^+] is toxic to cells, possibly due in part to the inhibition of the activities of many enzymes by these ions. For example, the maximum reaction velocity of algal glucose-6-phosphate dehydrogenase is significantly reduced, and the substrate-binding affinity of crab pyruvate kinase is significantly increased by [Na^+] and [K^+] in the 0.5–1.0 M range (reviewed by Yancey *et al.* 1982). Many other enzymes or cellular processes are predicted to be similarly affected. Thus, to survive long-term exposure to hypertonicity, cells must initiate an adaptive response. This adaptive response involves in part the replacement of the elevated intracellular Na^+ and K^+ with certain organic solutes called compatible osmolytes, which, unlike Na^+ and K^+, can accumulate to high concentrations without toxic effects on cellular processes (Yancey *et al.* 1982; Garcia-Perez and Burg 1991). The identity of the

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osmolytes accumulated and the mechanism of accumulation can vary among species. For example, cells of the mammalian kidney epithelium respond to osmotic stress by accumulating osmolytes such as betaine (a derivative of glycine in which the three hydrogens of the NH_3^+ are replaced with methyl groups), which is accomplished by transport from the extracellular medium, and sorbitol, which is accomplished by *de novo* synthesis (Uchida *et al.* 1989, 1993). In contrast, the yeast *Saccharomyces cerevisiae* responds to osmotic stress by accumulating the osmolyte glycerol, which is accomplished by *de novo* synthesis. In each case, however, hypertonic stress increases osmolyte accumulation at least in part by transcriptional induction of the genes enabling osmolyte accumulation (Meikle *et al.* 1988; Smardo *et al.* 1992; Uchida *et al.* 1993; Yamauchi *et al.* 1993; Ferraris *et al.* 1994). Thus, the hypertonic stress-induced accumulation of betaine within kidney cells and glycerol within yeast requires the transcriptional induction of the gene encoding the GABA/betaine transporter (BGT1) and the genes encoding the yeast glycerol synthetic enzymes, respectively. The mechanism(s) by which hypertonic stress triggers these transcriptional responses is unclear. Mechanical forces on the cell membrane caused by the hypertonicity-induced cell volume changes or the increases in intracellular $[Na^+]$ and $[K^+]$ have each been implicated (Uchida *et al.* 1989; Smardo *et al.* 1992). In addition, in both yeast and mammalian kidney cells, initiation of this response requires activation of the *HOG*/p38 family of mitogen-activated protein (MAP) kinases, perhaps other MAP kinases, and a cyclosporin-sensitive molecule (Brewster *et al.* 1993; Sheikh-Hamad *et al.* 1998).

In Drosophila, it was previously shown that the *inebriated* (*ine*) gene, which affects neuronal excitability, encodes two protein isoforms that share certain similarities to BGT1 (Burg *et al.* 1996; Soehnge *et al.* 1996). In particular, both BGT1 and the Ine proteins are members of the Na^+/Cl^--dependent neurotransmitter/osmolyte transporter family (reviewed by Amara and Kuhar 1993). Members of this family of proteins share sequence similarity, a common structure of 12 transmembrane domains, intracellular domains at the N and C termini, and a 60- to 70-amino-acid-long extracellular loop between the third and fourth transmembrane domains. Substrates of members of this family include a variety of small-molecule neurotransmitters, metabolites such as creatine, and osmolytes such as betaine or taurine. In addition, the *ine* isoforms and BGT1 are each expressed both in the central nervous system and in organs that perform water reabsorption: the Malpighian tubule/hindgut and kidney for *ine* and BGT1, respectively (Borden *et al.* 1992; Rasola *et al.* 1995; Soehnge *et al.* 1996). Although the Drosophila protein encoded by the predicted gene CG 1732 (Flybase curation FBrf0105495) has greater homology to BGT1 than Ine (52% identity for CG 1732 *vs.* 40% identity for Ine), the similar expression patterns for BGT1 and *ine* raised the possibility that the Ine transporter might similarly perform dual roles: neurotransmitter reuptake in the central nervous system and osmolyte transport in the Malpighian tubule/hindgut. If so, then *ine* mutants would be expected to be defective in osmolyte accumulation and thus should be more sensitive than wild type to maintenance on a hypertonic medium.

Here we show that *ine* mutants are hypersensitive to maintenance on media containing elevated [NaCl], [KCl], and [sorbitol] and provide evidence that this hypersensitivity represents a role for *ine* that is distinct from its role in the control of neuronal excitability. Furthermore, we show that both wild-type and *ine* mutants exhibit a "threshold" response to osmotic stress: For each genotype, maintenance on media containing an [NaCl] above this threshold causes inviability. We also assess the relative contributions to the osmotic stress response of each of the two isoforms of *ine*. We find that each protein isoform, when independently overexpressed with the *GAL4* system (Brand and Perrimon 1993), can rescue the osmotic stress response defect of *ine* mutants, suggesting that the two isoforms have similar functions. However, Ine-P2 alone, when expressed from its normal chromosomal position, is sufficient for only a small degree of resistance to osmotic stress. These results demonstrate a role for the *ine*-encoded transporters in the osmotic stress response and introduce this response to genetic analysis in Drosophila.

MATERIALS AND METHODS

Fly stocks: Isolation of ine^1, ine^2, and ine^3 was described previously (Stern and Ganetzky 1992; Soehnge *et al.* 1996). The wild-type parent chromosome of each *ine* mutant carries the eye color mutation *bw* and was isogenized prior to use. Flies bearing the yeast *GAL4* gene driven transcriptionally by the heat-shock promoter (called *hs-GAL4*) were kindly provided by Dan Kalderon. The Sh^{133} and eag^1 mutants were provided by Bob Kreber and Barry Ganetzky. Flies bearing the duplication *Dp (1:4)* r^+f^+, in which cytological region 14A-16A from the X chromosome was appended to the fourth chromosome, were also provided by Barry Ganetzky. This duplication includes the sodium channel gene *para* and is thus called *Dp para*. This duplication is maintained by complementation of the X chromosome deficiency *Df(1)* r^{D27}.

***In situ* hybridization:** To study the localization of each *ine* isoform independently, we amplified by PCR DNA sequences at the 5′ end that are unique to each isoform: the first exon of *ine-RB* and the first three exons of *ine-RA*. These PCR products were subcloned into the pcDNA1 vector (Invitrogen, San Diego). T7 and Sp6 RNA polymerases were used to synthesize both antisense and sense RNA probes, which were labeled with digoxygenin. Hybridizations *in situ* to whole-mount developing embryos were performed as described previously (Soehnge *et al.* 1996).

Identification of the ine^2 mutation: Previous experiments failed to identify the ine^2 mutation within the coding region of the *ine-RB* transcript. Therefore, we sequenced the three exons specific for *ine-RA* from genomic DNA from both ine^2 and *iso bw* (isogenic ine^+ fly line). Four pairs of PCR primers (sequences listed below) were designed to amplify four fragments of genomic DNA that completely cover exons I, II, and

III of *ine*. PCR products amplified from the ine^2 mutant and *iso bw* were sequenced directly and compared with the BESTFIT program of the GCG package.

The primers used were the following: ine2F1, CACTCCGA CATGCTAATG; ine2R1, CACACGGTGATTGGATCAG; ine2F2, CTCCGCCATTTGGAAGG; ine2R2, CTGAAGAGCTCGAGCTG; ine2F3, CACCAACCTGAGACTAC; ine2R3, GCAACACATC AAGTGTC; ine2F4, GGCTGAGTACTGAGTAC; and ine2R4, CAGCATGGAGCTGAAGTC.

Construction of transgenic flies carrying *UAS-ine-RB*: The full-length *ine-RB* cDNA was introduced into the *Eco*RI site of the pUAST vector (Brand and Dormand 1995) and injected into $y\ w^{67c23}$ embryos for *P*-element-mediated germline transformation as described previously (Spradling 1986). One transformant carrying *UAS-ine-RB* on the third chromosome was obtained.

Viability assays on hypertonic media: Flies were grown in uncrowded half-pint bottles and collected for only the first 4 days following the initial eclosions. Following etherization, flies were aliquoted into groups of 20, placed into vials, and allowed 1 day for recovery from etherization. Then flies were transferred into vials containing instant medium (Carolina) prepared according to the manufacturer's instructions, except that the appropriate salt solution was used in place of water. Fly viability was determined 4 days later by visual inspection. For the threshold viability assays shown in Figure 5, all fly manipulations and assays were conducted at 18° and 70% humidity, and fly viability on salt media was determined every day for 10 days. For all other fly viability experiments, fly manipulations and assays were conducted at room temperature and ambient humidity.

RESULTS

Molecular and functional properties of *inebriated*: Mutations in *ine* confer a number of nervous system phenotypes, including increased motor neuron excitability, oscillations of the light-induced photoreceptor potential, and increased perineurial glial thickness in combination with mutations in *pushover* (*push*) and *NF1* (Wu and Wong 1977; Stern and Ganetzky 1992; Burg *et al.* 1996; Yager *et al.* 2001). Two distinct *ine* cDNAs were previously identified and sequenced (Burg *et al.* 1996; Soehnge *et al.* 1996). As shown in Figure 1, A and B, both cDNAs encode identical sequences in the region of the protein homologous to neurotransmitter transporters; however, the longer cDNA (*ine-RA*) encodes a 943-amino-acid protein called Ine-P1, which contains an N-terminal intracellular domain that is ~300 amino acids longer than the shorter cDNA (*ine-RB*, which encodes Ine-P2). Figure 1B shows the *ine* gene organization. These two isoforms most likely arise from the use of distinct promoters.

The extremely long N-terminal intracellular domain observed in Ine-P1 is not commonly observed in members of this transporter family. This observation raised the possibility that this extended intracellular domain reflected an additional Ine activity distinct from neurotransmitter transport. If so, then Ine-P1 and Ine-P2 might perform distinct functions in Drosophila and thus might exhibit different expression patterns. To test this possibility, we constructed probes for *in situ* hybridization that were specific for either the *ine-RA* or the *ine-RB* cDNAs. As shown in Figure 1C, the embryonic expression patterns of the two cDNAs are virtually indistinguishable, suggesting that the two cDNAs function in the same cells.

An osmotic stress-sensitive phenotype of *ine* mutants: Two observations raised the possibility that *ine* might be required for osmolyte transport and thus for the Drosophila osmotic stress response. First, both forms of *ine* are expressed robustly in fluid reabsorption tissues such as the Malpighian tubule, hindgut, and anal plate (Soehnge *et al.* 1996; Figure 1C), which together comprise the invertebrate analog of the kidney. Second, transport of the osmolytes betaine, taurine, and β-alanine into cells in the mammalian renal medulla is accomplished by transporters such as BGT1 that are members of the same transporter family as *ine* (Burg 1995). These observations raised the possibility that the Ine transporter might function to transport osmolytes as well.

If Ine performs osmolyte transport in the Malpighian tubules and hindgut, then *ine* mutants, which would be defective in such transport, would be expected to be more sensitive to osmotic stress than wild-type flies. To test this possibility, we maintained three independently isolated *ine* mutants and wild-type flies on media containing various [NaCl] (Figure 2A). We found that *ine* mutants exhibited viability similar to wild type when maintained for 4 days on 0 M or 0.1 M [NaCl]. However, ine^1 and ine^3 mutants exhibited significantly greater lethality than wild-type or ine^2 mutants when maintained for 4 days on 0.2 M [NaCl]. Furthermore, whereas ~90% of wild-type flies could survive maintenance on 0.4 M [NaCl], ine^1 and ine^3 mutants exhibited essentially complete inviability on this [NaCl], and ine^2 mutants exhibited only slight viability. The abdomens of both wild-type and *ine* mutants became progressively thinner during their maintenance on lethal, but not sublethal, [NaCl] (data not shown). This observation is consistent with the possibility of desiccation, which could have contributed to the observed lethality.

To confirm that this reduced viability reflected increased sensitivity to a hypertonic medium, rather than increased sensitivity specific to NaCl, we tested the sensitivity of *ine* mutants to elevated [KCl] and [sorbitol]. We found that *ine* mutants displayed increased sensitivity to both, although the sensitivity of both wild-type and *ine* mutants to sorbitol was considerably less than the sensitivity to NaCl and KCl (Figure 2, B and C). This significantly reduced sensitivity to sorbitol compared to NaCl and KCl suggests that the observed lethality in NaCl and KCl might not arise solely from desiccation. One possibility is that some of the NaCl and KCl provided to the flies might accumulate intracellularly and contribute to lethality. Alternatively, the reduced sensitivity to sorbitol might result from some ability of sorbitol to cross the cell membrane, which would give sorbitol a partial osmoprotective effect. As with NaCl, ine^2 mutants

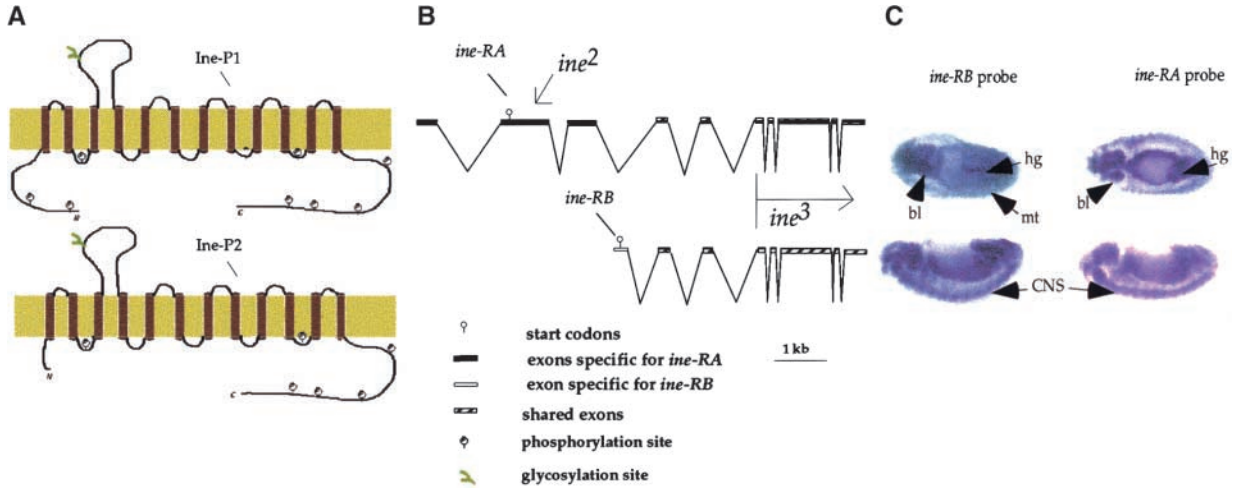

FIGURE 1.—Organization and expression of *ine*. (A) The putative membrane topology of the two Ine protein isoforms. The two isoforms are identical except that Ine-P1 (encoded by *ine-RA*) contains an N-terminal intracellular region that is ~300 amino acids longer than the corresponding region in Ine-P2, encoded by *ine-RB*. This 300-amino-acid extension, unlike the region common to both proteins, has no similarity to other transporters. Potential sites of N-linked glycosylation and phosphorylation are indicated. (B) Map of the *ine* region showing the exons of the two *ine* isoforms and the location of two identified mutations. The ine^2 mutation is a nonsense mutation at codon 126 of *ine-RA*, whereas ine^3 is a deletion that removes most of the open reading frame common to both transcripts and begins at codon 293 of *ine-RA*. Although ine^1 has not been localized, it was previously shown by Northern blot analysis that transcripts of both *ine* isoforms are undetectable in the ine^1 mutant (SOEHNGE *et al.* 1996). (C) Whole-mount *in situ* hybridization using *ine-RB*- and *ine-RA*-specific probes. Both isoforms exhibit very similar expression patterns in these developing embryos. Expression is found in the hindgut (hg), Malpighian tubules (mt), and the central nervous system (CNS) along the ventral midline and brain lobes (bl). (Bottom) Lateral view. (Top) Dorsal view.

exhibited slightly better survival than ine^1 and ine^3 mutants when maintained on media containing 0.2 M [KCl], although the difference was less extreme than the difference observed on NaCl-containing media.

To test the possibility that *ine* mutants might be hypersensitive to any environmental stress, rather than specifically sensitive to hypertonic stress, we compared the sensitivity of ine^3 flies and wild type to two types of heat-shock stresses: long-term maintenance at a temperature of 34° and 3-hr heat shocks at 37° during long-term maintenance at room temperature. We found that ine^3 flies displayed the same viability as wild type to these stresses (not shown).

Specific elimination of Ine-P1 in ine^2 mutants: The phenotype of ine^1 and ine^3 mutants most likely represents the null phenotype: ine^3 is a deletion mutation that removes most of the *ine* open reading frame, and ine^1 mutants produce undetectable levels of mRNA from either of the *ine* isoforms (SOEHNGE *et al.* 1996), although the ine^1 sequence change was not identified. The observation that ine^2 mutants survive significantly better than ine^1 and ine^3 mutants on media containing 0.2 M NaCl or 0.2 M KCl suggested that the ine^2 mutation does not completely eliminate Ine activity. To identify the ine^2 mutation, we compared the sequence of *ine* in the ine^2 mutant and in the isogenic wild-type strain. We found that ine^2 is a nonsense mutation in codon 125 of the Ine-P1 isoform (see Figure 1). This mutation is expected to eliminate Ine-P1, but as this mutation lies in an exon that is not present in the Ine-P2 isoform, it is expected to leave Ine-P2 unaffected. The observation that the ine^2 mutant retains partial activity for the osmotic stress response demonstrates that Ine-P1 is required for most of, but not all of, the osmotic stress response. Ine-P2 alone is sufficient for a small amount of osmotic stress response.

Overexpression of Ine-P2 is sufficient for a normal osmotic stress response: An additional way to assess the role of each *ine* isoform is to assay the osmotic stress response in transgenic flies carrying each isoform independently. We showed that *ine* mutants expressing *ine-RA* under transcriptional control of the heat-shock promoter (constructed and kindly supplied by M. Burg and W. Pak; BURG *et al.* 1996) completely rescued the increased sensitivity of *ine* mutants to NaCl, even in the absence of heat shock (not shown). In addition, we constructed flies carrying *ine-RB* under the transcriptional control of the upstream activator sequence of the yeast Gal4 protein (*UAS-ine-RB*; BRAND and PERRIMON 1993). We found that *ine* mutants were completely rescued for the phenotype of NaCl sensitivity in the simultaneous presence of *UAS-ine-RB* and a transgene ubiquitously expressing *GAL4* (called *hs-GAL4,* Figure 3). In contrast, *ine* mutants expressing either the *hs-GAL4* line or the *UAS-ine-RB* line alone exhibited an identical sensitivity to NaCl as *ine* mutants (Figure 3). Thus, expression of Ine-P2, via the *GAL4* system, but not expression of Ine-P2 from its normal chromosomal position, is sufficient for a normal osmotic stress response even in the absence of Ine-P1. We suggest that this ability of Ine-P2

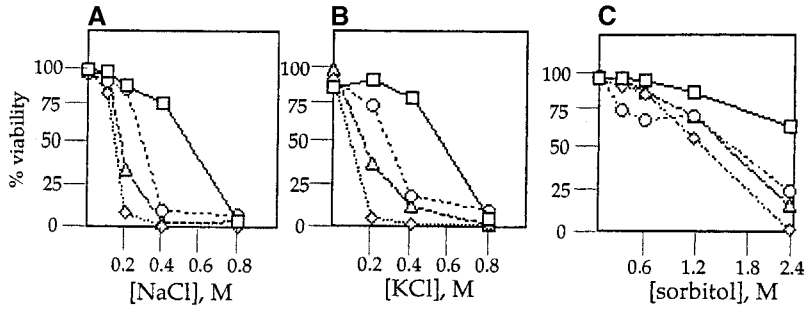

FIGURE 2.—Increased sensitivity of *ine* mutants to NaCl, KCl, and sorbitol. Flies of the indicated genotype (20 per vial) were placed on media containing the NaCl (A), KCl (B), or sorbitol (C) at the indicated concentrations. Viability was determined 4 days later. Error bars represent standard errors, given by the square root of $p(1 - p)/n$, where p is the fraction of surviving flies (BAILEY 1995), and are shown only when larger than the radius of the data point. (A) Viability in the presence of NaCl. For wild type, $n = 280$; for ine^1, $n = 160$; for ine^2 and ine^3, $n = 200$. (B) Viability in the presence of KCl. For wild type, $n = 180$; for ine^1, $n = 120$; for ine^2 and ine^3, $n = 200$. (C) Viability in the presence of sorbitol. For wild type and ine^3, $n = 240$; for ine^1 and ine^2, $n = 200$. (□) Wild type, (◇) ine^1, (○) ine^2, (△) ine^3.

to rescue is a result of its overexpression by the *GAL4* system, although this overexpression has not been demonstrated.

Independent roles for *ine* in the control of neuronal excitability and the osmotic stress response: The osmotic stress-sensitive and neuronal excitability phenotypes of *ine* could reflect independent roles for *ine* in these two processes. In this view, *ine* expression in the central nervous system (CNS) controls neuronal excitability, whereas *ine* expression in the Malpighian tubules and hindgut is required for a proper osmotic stress response. However, it is also possible that these two types of defects reflect a common underlying cause. For example, it is possible that the behavioral changes caused by hyperexcitability in *ine* mutants in some way reduce the ability of the fly to survive a hypertonic environment (for example, by altering feeding behavior).

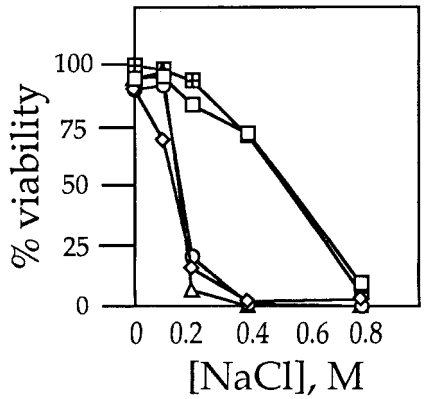

FIGURE 3.—Transformation rescue of the *ine* mutant salt sensitivity. The *GAL4*-expressing line used for the transformation rescue experiment shown here, kindly provided by Dan Kalderon, was driven by the heat-shock promoter and is described as *hs-GAL4*. This construct generates ubiquitous Gal4 protein even when the flies are reared at room temperature (D. KALDERON, personal communication). A total of 200 flies were assayed for each genotype. Error bars represent standard errors, given by the square root of $p(1 - p)/n$, where p is the fraction of surviving flies, and are shown only when larger than the radius of the data point. Viability was determined after 4 days of maintenance on media of the indicated salt concentration. (□) Wild type, (◇) ine^3, (○) ine^3; *UAS-ine-RB/+*, (△) ine^3; *hs-GAL4/+*, (⊞) ine^3; *UAS-ine-RB hs-GAL4/+*.

To distinguish between these possibilities, we tested other mutants with neuronal excitability defects similar to *ine* for osmotic stress sensitivity. Flies tested were mutant for either *Shaker* (*Sh*) or *ether a go-go* (*eag*), which each encode distinct potassium channel α-subunits, or flies carrying a duplication of the *paralytic* (*para*) gene (which we term *Dp para*$^+$), which encodes a sodium channel. Each genotype confers a motor neuron hyperexcitability phenotype that is similar to the phenotype of *ine* mutants (JAN *et al.* 1977; GANETZKY and WU 1983; STERN *et al.* 1990; STERN and GANETZKY 1992). The osmotic stress-sensitive phenotypes of *eag* mutants and flies bearing *Dp para*$^+$ were of particular interest, because *ine* mutations, *eag* mutations, and *Dp para*$^+$ each interact with *Sh* mutations in an identical manner: *eag Sh* and *Sh; ine* double mutants, and *Sh* mutants carrying *Dp para*$^+$, each show a characteristic "downturned wings and indented thorax" phenotype (STERN *et al.* 1990; STERN and GANETZKY 1992), which is not exhibited by any of the single mutants described above. The notion that the osmotic stress-sensitive defect of *ine* mutants is causally related to the neuronal hyperexcitability defect would be supported by observing a similar osmotic stress-sensitive defect in *eag*, *Sh*, or *Dp para*$^+$ flies.

As shown in Figure 4, none of the other excitability mutants tested exhibited a sensitivity to [NaCl] comparable to *ine* mutants. These results indicate that neuronal hyperexcitability by itself fails to confer sensitivity to NaCl and thus that neuronal excitability and osmotic stress response are separable processes. These results are consistent with the hypothesis that the *ine* phenotype of osmotic stress sensitivity reflects loss of osmolyte transport in the hindgut and Malpighian tubule, rather than a secondary consequence of a neuronal excitability defect. This possibility is supported by the observation that flies in which *ine* expression is driven only in neurons by the *elav-GAL4* construct exhibit rescue of the "downturned wings" phenotype of *Sh; ine* double mutants, but fail to exhibit any rescue of the osmotic stress-sensitive phenotype (not shown).

Wild-type flies and *ine* mutants exhibit different thresholds for maintenance on NaCl: The data shown in Figures 2–4 above demonstrate that *ine* mutants, but not

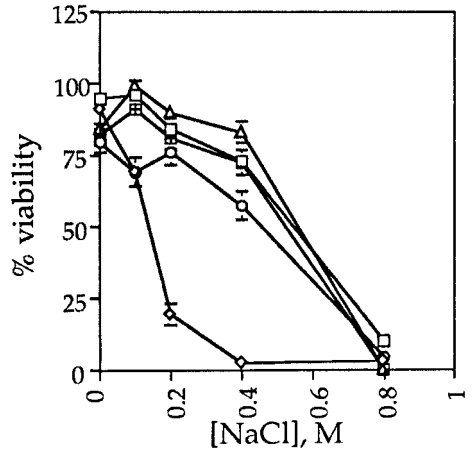

FIGURE 4.—Normal sensitivity of excitability mutants to NaCl. For wild type (□), ine^3 (◇), *Dp para*$^+$ (○), and eag^1 (△), $n = 200$; for Sh^{133} (⊞), $n = 180$. Error bars represent standard errors, given by the square root of $p(1 - p)/n$, where p is the fraction of surviving flies, and are shown only when larger than the radius of the data point. Viability was determined after 4 days of maintenance on media of the indicated salt concentration.

wild-type flies, die following maintenance on media containing 0.2 or 0.4 M NaCl. However, because these data represent viability at only a single time point, no information on mortality kinetics is contained. We compared the rate of death of ine^1, ine^2, and wild-type flies on media containing varying [NaCl]. We found that each genotype exhibited a "threshold" [NaCl]: Flies maintained on media containing [NaCl] below the threshold exhibited very little lethality, even after 9 days of maintenance on the hypertonic medium. However, flies maintained on media containing [NaCl] above the threshold died quickly (death typically began within 3–5 days following addition to the hypertonic media) and continuously until, after 9 days upon NaCl-containing media, <10% of the flies remained alive. The [NaCl] at which this threshold response occurred depended on the allele present at *ine*. As shown in Figure 5, wild-type flies exhibited an [NaCl] viability threshold between 0.5 and 0.6 M [NaCl]. In contrast, ine^1 mutant flies exhibited a [NaCl] viability threshold between 0.15 and 0.2 M [NaCl]. Finally, ine^2 mutants exhibited a threshold concentration between 0.2 and 0.25 M [NaCl], which is intermediate between wild type and ine^1 and mutants. Thus, there is a close correlation between the strength of the mutant allele at *ine* and the sensitivity of the fly to osmotic stress. This observation suggests that threshold [NaCl] is determined, at least in part, by the amount of osmolyte accumulation that can be performed in the Malpighian tubule and hindgut.

DISCUSSION

A role in osmolyte accumulation for the *ine*-encoded transporter: Osmolyte accumulation and neurotransmitter reuptake can each be performed by members of the Na^+/Cl^--dependent family of neurotransmitter/osmolyte transporters (BORDEN *et al.* 1995; RASOLA *et al.* 1995). In fact, one member of this transporter family, the mammalian betaine/GABA transporter BGT1, appears to have a dual function. BGT1 is expressed in both the kidney and the brain: In the brain, this transporter presumably performs reuptake of GABA following release into synapses, whereas in the kidney this transporter presumably enables the accumulation of the osmolyte betaine. The previous observation that the Drosophila *ine*-encoded transporter is expressed in the Malpighian tubule and hindgut as well as in certain cells within the central nervous system raised the possibility that this transporter might also perform such a dual function. In this study, we provide functional evidence that supports this possibility. We show that *ine* mutants are more sensitive than wild type to maintenance on a hypertonic medium, that this hypersensitivity apparently occurs independently of the role of *ine* in the control of neuronal excitability, and that levels of *ine* transporter define a threshold [NaCl] above which the flies cannot survive for prolonged periods. We also show that the Ine short form, Ine-P2, can confer some osmotic stress resistance in the absence of Ine-P1, but overexpression is required to confer full resistance. We propose that the osmotic stress-sensitive phenotype of *ine* mutants results from an inability to accumulate osmolytes within the Malpighian tubule and hindgut.

Relative roles of the two *ine* isoforms in the osmotic stress response: Two *ine* isoforms have been identified, a short form (Ine-P2) and a long form (Ine-P1). The N-terminal intracellular domain of Ine-P1 is ~300 amino acids longer than the N-terminal intracellular domain of Ine-P2; the two isoforms are otherwise identical. The extremely long N-terminal domain of Ine-P1 is unusual among members of this transporter family, and it is unclear what function, if any, this extended domain might confer upon Ine-P1 activity. Previous work on the rat GABA transporter suggested that both the N- and C-terminal intracellular domains were dispensable for transport activity (MABJEESH and KANNER 1992), although a more recent report on studies on the norepinephrine transporter suggested that the C terminus might be required for activity (LIU *et al.* 1998). It is possible that this domain produces a signal independent of transport activity, such as signal transduction in response to substrate binding (for example, see ALONSO *et al.* 1999). Alternatively, the extended domain might affect transporter localization or activity. Finally, the extended domain might have no discernible effect on protein function.

We have addressed this question by assaying the osmotic stress-sensitive phenotype of mutants expressing or overexpressing Ine-P2 alone. We found that ine^2 mutants, which express only Ine-P2, survive osmotic stress slightly, but significantly, better than *ine* null mutants.

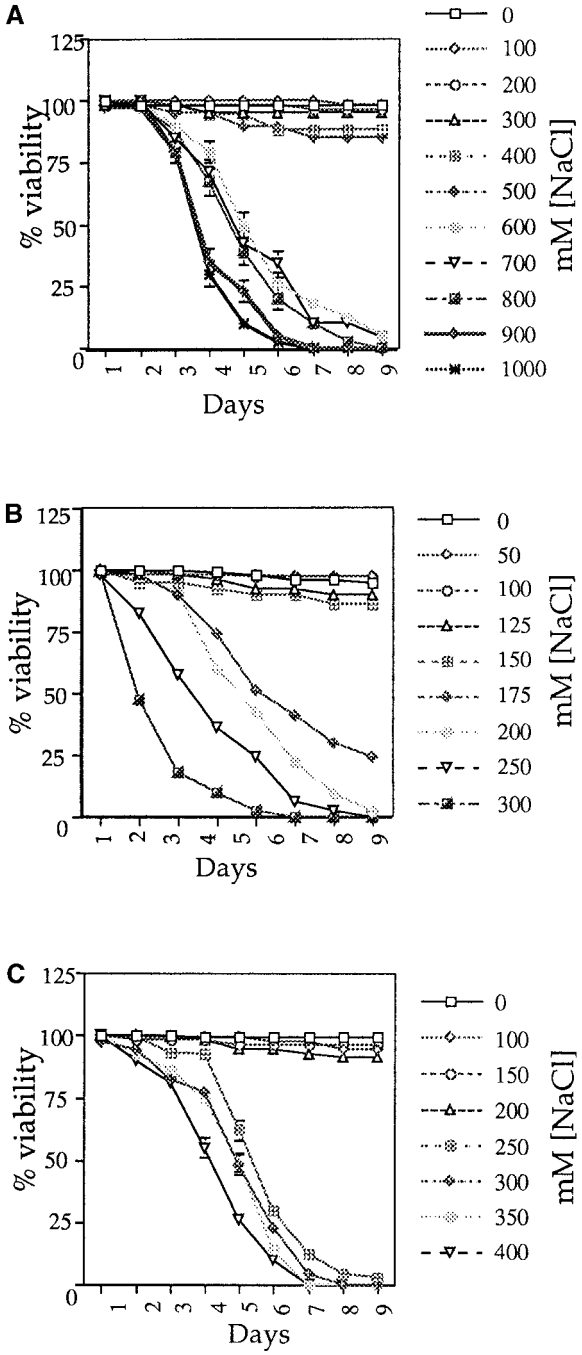

FIGURE 5.—Effects of Ine transporter expression on [NaCl] viability threshold. Flies of the indicated genotype were maintained at 18° and 70% relative humidity on media containing the indicated [NaCl] for 9 days. Live and dead flies were counted daily. For wild type (A) and ine^1 (B), $n = 120$; for ine^2 (C), $n = 160$. Error bars represent standard errors, given by the square root of $p(1 - p)/n$, where p is the fraction of surviving flies, and are shown only when larger than the radius of the data point.

This result demonstrates that Ine-P2 can perform osmotic stress response in the absence of Ine-P1. Furthermore, overexpression of Ine-P2, even in the absence of Ine-P1, confers normal survival under osmotic stress. These results argue against the possibility that Ine-P1 has a novel function, not shared with Ine-P2, that is required for the osmotic stress response. Rather, these results suggest that Ine-P2 can perform all functions required for osmotic stress response that Ine-P1 can perform. The requirement for Ine-P2 overexpression for complete phenotypic rescue could be the result of insufficient translation, insufficient transport activity, or incorrect protein transport or localization. Because a mutation that selectively removes Ine-P2 is not available, we have no way of monitoring the relative ability of each isoform to rescue the osmotic stress phenotype. However, recent experiments on the relative ability of Ine isoforms to rescue the *ine* mutant neuronal phenotypes have suggested that although each isoform is active in the absence of the other, Ine-P1 is more active than Ine-P2 (Y. HUANG and M. STERN, unpublished results; M. BURG and W. PAK, personal communication).

A role for Ine in response to hyperosmolarity has been suggested independently by CHIU *et al.* (2000). These investigators cloned the *inebriated* homolog from *Manduca sexta*, called Masine, and reported two isoforms: a short form and a long form containing an additional 108 amino acids at the N terminus. Injection of the long form, but not the short form, into Xenopus oocytes elicited a hyperosmolarity-induced Cl^- current, which was attributed to a phospholipase C-mediated activation of a Ca^{2+} flux. Furthermore, the additional 108 amino acids in MasIne-long was sufficient to confer a similar Cl^- current when appended to the GABA transporter GAT1. Thus, CHIU *et al.* (2000) also suggest a role for Ine in response to hyperosmolarity, although the mechanism that they suggest (induction of a Ca^{2+}-activated K^+ channel) is quite different from the mechanism of osmolyte accumulation that we propose. One possibility is that Ine and Mas-Ine utilize different mechanisms to respond to hyperosmolarity. The observation that the 108-amino-acid domain of MasIne-long shares only 9 amino acids with Ine-P1 is consistent with this possibility. Alternatively, Ine and Mas-Ine might each use both mechanisms to respond to hyperosmolarity. Further research will be required to distinguish between these possibilities.

A possible mechanism for organismal lethality in response to hypertonic stress: When cells are placed in a hypertonic media, an extremely rapid loss of water is followed by influx of Na^+ and K^+. This influx of ions causes the passive return of water to the cell, thus enabling cell volume to be recovered. However, this influx also increases the intracellular [Na^+] and [K^+] with detrimental consequences to the activity of essential cellular functions. To accommodate to osmotic stress, cells then replace the intracellular Na^+ and K^+ with nonperturbing osmolytes such as betaine, sorbitol, inositol, or glycerol. This replacement thus enables a restoration of normal cell volume and normal intracellular [Na^+] and [K^+]. We propose that the Ine transporter

plays an important role in this replacement by enabling the transport of an osmolyte, the substrate for the Ine transporter, which has not yet been identified. Thus, in *ine* mutants this replacement fails to occur properly, and, following osmotic stress, the elevated Na^+ and K^+ levels within hindgut epithelial cells persist for the duration of the exposure to osmotic stress. This long-term exposure could kill these epithelial cells by either necrotic or apoptotic mechanisms, as suggested by Sheikh-Hamad *et al.* (1998).

Although *ine* mutants are considerably more sensitive to osmotic stress than wild type, even *ine* null mutants can survive exposure to [NaCl] of ~0.15 M. This low-level viability could reflect *ine*-independent osmolyte accumulation mechanisms, which would enable some capacity for Na^+ replacement even in the absence of *ine*. Alternatively, it is possible that *ine* null mutants are completely defective in osmolyte accumulation and the viability of *ine* mutants in low [NaCl] reflects the ability of hindgut epithelial cells to survive elevated intracellular [Na^+] for prolonged periods.

Ability to accumulate osmolytes generates a [NaCl] viability threshold: The mechanism for osmotic stress-induced lethality proposed above is supported by the observation that flies exhibit a sharp [NaCl] survival threshold. In particular, flies maintained on media containing any [NaCl] below the threshold concentration were able to survive prolonged maintenance on medium containing NaCl. In contrast, flies maintained on media containing any [NaCl] above the threshold were unable to survive: these flies showed essentially complete lethality after 6–9 days, depending on the [NaCl] of the media. The [NaCl] at which this viability threshold occurred was controlled by the genotype at *ine*: Mutants null for *ine* exhibited a viability threshold between 0.15 and 0.2 M [NaCl], whereas ine^2 mutants, in which only Ine-P2 is expected to be produced, exhibited a viability threshold between 0.2 and 0.25 M [NaCl], and wild-type flies, in which both Ine isoforms are expected to be present, exhibited a viability threshold between 0.5 and 0.6 M [NaCl]. We speculate that this viability threshold reflects the maximum amount of osmolyte accumulation that is possible for flies of each genotype. When flies are exposed to [NaCl] above this maximum level, residual Na^+ accumulates intracellularly, and death results by mechanisms described above.

Signaling pathways regulating the osmotic stress response: From studies in yeast, and in canine kidney cells grown in culture, some of the signaling pathways required for induction of the osmotic stress response have been identified. In each system, osmotic stress activates the *HOG*/p38 MAP kinase system, and this activation is required for the transcriptional induction of the genes encoding osmolyte-accumulating enzymes (glycerol synthetic enzymes and the betaine transporter for the yeast and canine kidney cells, respectively; Brewster *et al.* 1993; Sheikh-Hamad *et al.*1998). In addition, a role for calcineurin in tolerance to NaCl was identified in yeast (Garrett-Engele *et al.* 1995), whereas osmotic stress response in kidney cells requires a cyclosporine-sensitive molecule (D. Sheikh-Hamad, personal communication). The signaling pathways and mechanisms by which Drosophila respond to osmotic stress remain to be identified. However, the identification of a role for Ine in this process opens the way for this phenomenon to be studied with genetic methods.

We are grateful to Dan Kalderon, Martin Burg, and William Pak for providing fly stocks; Laura Huff for technical assistance; and David Sheikh-Hamad for comments on the manuscript. This work was supported by National Institutes of Health grant GM-46566 to M.S. and National Science Foundation grant MCB-0091236 to M.C.G.

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Communicating editor: M. J. Simmons