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A putative amino acid transporter of the SLC6 family is up-regulated by lithium and is required for resistance to lithium toxicity in *Drosophila*

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Abstract

Lithium is an efficacious drug for the treatment of mood disorders, and its application is also considered a potential therapy for brain damage. However, the mechanisms underlying lithium's therapeutic action and toxic effects in the nervous system remain largely elusive. Here we report on the use of a versatile genetic model, the fruit fly *Drosophila melanogaster*, to discover novel molecular components involved in the lithium-responsive neurobiological process. We previously identified *CG15088*, which encodes a putative nutrient amino acid transporter of the solute carrier 6 (SLC6) family, as one of the genes most significantly up-regulated in response to lithium treatment. This gene was the only SLC6 gene induced by lithium, and was thus designated as *Lithium-inducible SLC6 transporter* or *List*. Either RNAi-mediated knockdown or complete deletion of *List* resulted in a remarkable increase in the susceptibility of adult flies to lithium's toxic effects, whereas transgenic expression of wild-type *List* significantly suppressed the lithium hypersensitive phenotype of *List*-deficient flies. Other ions such as sodium, potassium and chloride did not induce *List* up-regulation, nor did they affect the viability of flies with suppressed *List* expression. These results indicate that lithium's biochemical or physical properties, rather than general osmotic responses, are responsible for the lithium-induced up-regulation of *List*, as well as for the lithium-susceptible phenotype observed in *List* knockdown flies. Interestingly, flies became significantly more susceptible to lithium toxicity when *List* RNAi was specifically expressed in glia than when it was expressed in neurons or muscles, which is consistent with potential glial expression of *List*. These results show that the *List* transporter confers resistance to lithium toxicity, possibly as a consequence of its amino acid transporter activity in CNS glia. Our results have provided a new avenue of investigation toward a better understanding of the molecular and cellular mechanisms that underlie lithium-responsive neurobiological process.

Keywords

sodium neurotransmitter symporter family; GAL4/UAS system; RNA interference; glia

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The alkaline metal lithium is one of the most effective prophylactic and therapeutic drugs for bipolar affective disorder (also known as manic-depressive illness) (Schou, 2001). Recent studies have indicated that lithium is also effective in preventing apoptosis-dependent neuronal death, raising the promising prospect that lithium can be used to treat or prevent brain damage, either following injury or during the progression of neurodegenerative diseases (Chuang, 2004). The application of lithium at therapeutically relevant concentrations is known to inhibit various enzymes, including inositol monophosphatase (IMPase) (Berridge et al., 1989) and glycogen synthase kinase 3 (GSK-3) (Klein and Melton, 1996). However, it remains unclear which molecular signaling cascade related to these potential lithium targets is most relevant to lithium's beneficial effects on the nervous system.

Although lithium is a valuable drug, the therapeutic window—of doses that are highly effective yet of low toxicity—is narrow. Adverse side effects are also common, even within the therapeutic dose range, and prolonged lithium treatment often cause dysfunction in the digestive, renal, endocrine and nervous systems (Bocchetta et al., 1991; Tam et al., 1996; Lazarus, 1998; Turan et al., 2002; Adityanjee et al., 2005). These adverse effects of lithium at certain concentrations may be caused by the cellular machinery that is involved in lithium's therapeutic actions. Alternatively, lithium's toxicity may be mediated by mechanisms distinct from those that underlie its beneficial actions. Advancing our knowledge of the biological processes triggered by lithium treatment is expected to improve the efficiency of lithium therapy and to minimize its side effects. In addition, such knowledge may lead to a better understanding of the molecular and cellular mechanisms that underlie the neurobiological processes that are affected by lithium treatment, for example mood regulation and apoptosis-dependent neuronal death.

Accumulated evidence has suggested that the physiological effects of lithium and its molecular underpinnings are shared at least partly among evolutionarily diverse organisms such as mammals and the fruit fly *Drosophila melanogaster*, an animal model that has been proven valuable in the study of a variety of complex biological processes. For example, the study of *Drosophila* as well as mammals has demonstrated that long-term administration of lithium lengthens the free-running period of circadian locomotor activity as a consequence of lithium-dependent inhibition of GSK-3 (Padiath et al., 2004; Dokucu et al., 2005; Iitaka et al., 2005). In addition, as in mammalian cell culture models of Huntington's disease (Carmichael et al., 2002), lithium can protect against polyglutamine-mediated neuronal toxicity in *Drosophila* (Berger et al., 2005). Furthermore, lithium has been suggested to restore function in fragile X syndrome patients (Berry-Kravis et al., 2008), consistent with its improvement of behavioral deficits displayed by the *Drosophila dfmr1* mutant, a model for fragile X syndrome (McBride et al., 2005). These findings indicate that studies using *Drosophila* will likely offer valuable insights into evolutionarily conserved molecular components of the lithium-responsive biological process.

To gain insight into the basic neurobiological processes that are regulated by lithium, we recently examined lithium-induced changes in genome-wide gene expression profiles in the *Drosophila* head using Affymetrix Genome Arrays (Kasuya et al., 2009). In this analysis *CG15088* was identified as one of the genes whose expression is most significantly affected by lithium treatment, as assessed by the fold-change and *P*-value (Kasuya et al., 2009). *CG15088* encodes a putative Na⁺/Cl⁻-dependent nutrient amino acid transporter of the solute carrier 6 transporter (SLC6, a.k.a. sodium neurotransmitter symporter) family (Thimman et al., 2006; Romero-Calderon et al., 2007; Miller et al., 2008). The SLC6 family includes transporters of neurotransmitters, neuromodulators, osmolytes, and energy substrates as well as neutral and cationic amino acids. It also includes a number of "orphan transporters" with unknown substrates or physiological functions (Castagna et al., 1997; Chen et al., 2004). The founding members of the SLC6 family, such as SLC6A1 (GABA transporter), SLC6A3

(dopamine transporter) and SLC6A4 (serotonin transporter), terminate synaptic transmission by removing neurotransmitter molecules from the synaptic cleft. Mutations affecting these SLC6 neurotransmitter transporters often lead to disruption of neurotransmission, and may significantly influence behavior and mental functions (Gainetdinov et al., 2002; Hahn and Blakely, 2002). In particular, SLC6 transporters are the likely targets of various drugs used to treat different mood disorders (Rothman and Baumann, 2003). However, the involvement of SLC6 transporters in lithium's beneficial and/or adverse actions in the nervous system is not known. In the present study, we utilized *Drosophila* genetics to investigate functional roles of the lithium-inducible SLC6 transporter gene, *CG15088*.

Experimental procedures

Fly stocks and culture conditions

Flies were reared at 25°C in a 12 hr light: 12 hr dark cycle, on a conventional cornmeal/glucose/yeast/agar medium supplemented with the mold inhibitor methyl 4-hydroxybenzoate (0.05 %). The Canton-S (CS) strain was used as the wild-type control. *daughterless* (*da*)-GAL4, *Actin5C* (*Act5C*)-GAL4, and *tubulin* (*tub*)-GAL4 were obtained from Wayne Johnson (University of Iowa). *embryonic lethal abnormal vision* (*elav*)-GAL4 on the X chromosome was obtained from Pam Geyer (University of Iowa). *reversed polarity* (*repo*)-GAL4 and *Myosin heavy chain* (*Mhc*)-GAL4 were obtained from Chun-Fang Wu (University of Iowa). *nervana 2* (*nrv2*)-GAL4 were obtained from Paul Salvaterra (City of Hope, CA). UAS-GFP on II chromosome was obtained from the Bloomington stock center. RNAi lines for *CG15088* (VDRC IDs 44909 and 4993) were obtained from the Vienna *Drosophila* RNAi Center (VDRC). Two deficiency lines, *Df(2R)Exel7157* and *Df(2R)Exel7158*, were obtained from the Exelixis Collection at Harvard University.

RT-PCR analysis

Wild-type virgin females (0–24 hr-old) were kept in a vial containing regular cornmeal-based food with or without 50mM LiCl, NaCl or KCl for 24 hr. Total RNA was extracted from heads and bodies (Fig. 1), head (Fig. 5A) or whole flies (Fig. 6B), using the TRIzol Reagent (Invitrogen, Carlsbad, CA), and this was followed by a DNase I digestion step and an RNeasy (Qiagen, Valencia, CA) cleanup step. cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The primers used in this study are listed in Supplementary Table 1. The amount of template cDNA and amplification cycle number for each target gene were optimized through pilot experiments, such that the PCR amplification was carried out in a linear range (i.e., the PCR amplification remains in the exponential phase). For *List*, *pncr016*, *CG15087*, *Hs3st-A* and *CG15073*, an equivalent amount of cDNA converted from approximately 50 ng of total RNA was subjected to a 30-cycle PCR amplification. As an internal control, *ribosomal protein L32* (a.k.a., *rp49*) was amplified for 30 cycles using an amount of cDNA corresponding to 1 ng of total RNA. The PCR products were analyzed on an agarose gel and signal intensities were quantified by using the ImageJ software (<http://rsbweb.nih.gov/ij/>). Signal intensities were normalized to those of lithium-untreated wild-type samples, and the results were statistically analyzed using the Mann-Whitney Rank Sum Test for single comparisons and the Kruskal-Wallis ANOVA for multiple comparisons.

Salt treatment

Virgin females (0–24 hr-old) were grouped into sets of 15–20 and transferred to vials with cornmeal-based food containing a desired concentration of LiCl (Sigma, St. Louis, MO). For salt comparison experiments, food medium composed of 5% glucose and 1% agarose, supplemented with a desired concentration of a particular salt, was used. For life span

experiments, flies were transferred to a new vial every 3–4 days, and the live flies were counted daily.

Reactive climbing assay

The reactive climbing assay was performed essentially as previously described (Greene et al., 2003), using a countercurrent apparatus that was originally invented by Seymour Benzer (Benzer, 1967). Briefly, groups of 20 flies were placed into one tube (tube #0), tapped to the bottom and allowed 15 sec to climb, at which point the flies that had climbed were transferred to the next tube. This process was repeated a total of five times. After the fifth trial, the flies in each tube (#0 ~ #5) were counted. The climbing index (CI) was calculated using the following formula: $CI = \sum(N_i \times i) / (5 \times \sum N_i)$, where i and N_i represent the tube number (0–5) and the number of flies in the tube, respectively.

Generation of *List-lacZ* and *UAS-List* transformants

To generate *List-LacZ* transformants, the 4 kb 5'-flanking region immediately upstream of the predicted transcription start site of *List* was amplified using the *List* 5'-flanking primers (Supplementary Table 1). The PCR products were verified for sequence accuracy and cloned into the pPelican vector (Barolo et al., 2000) (*Drosophila* Genome Resource Center, Bloomington, IN). The resultant pPelican-*List-LacZ* DNA was then injected into the *w* strain to obtain transformants (Spradling and Rubin, 1982). Five independent *List-LacZ* transformants were obtained and analyzed for *lacZ* expression.

For *UAS-List* transformants, the *List* cDNA was PCR amplified using RNA isolated from fly heads (0–1 day-old CS) with appropriate primers (Supplementary Table 1) and subcloned into pUAST. The sequence of the cloned *List* cDNA was deposited into Genbank (accession number GQ292542). Five independent *UAS-List* transgenic lines were generated. The *UAS-List* transgene on the third chromosome was combined with *Df(2R)Exel7157* to create *w ; Df(2R)Exel7157/CyO ; UAS-List*. The *da-GAL4* driver was combined with *Df(2R)Exel7158* to create *w ; Df(2R)Exel7158/CyO ; da-GAL4*. For rescue experiments, these two stocks were crossed to generate *List*-deficient flies carrying both *UAS-List* and *da-GAL4* transgenes (*w ; Df(2R)Exel7157/Df(2R)Exel7158 ; UAS-List/da-GAL4*).

Histochemical analysis

For LacZ staining, the heads and bodies of *List-lacZ* transformants were fixed with 4% formaldehyde in phosphate-buffered saline (PBS)/0.1% Triton X-100 (PBS/Triton), for 10 min at room temperature. After rinsing in PBS/Triton, specimens were submerged in X-gal staining solution (10 mM Tris-HCl, pH 7.4, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM NaCl, 2 mM MgCl₂, 0.2% X-gal) at 37°C for 3 hours. Specimens were washed in PBS and dehydrated through graded steps of ethanol, cleared with xylene and mounted in Permount (Fisher Scientific Company, Pittsburgh, PA). Stained specimens were viewed by bright-field microscopy.

To examine glial expression of the *List-lacZ* reporter gene, flies carrying both *nrv2-GAL4* and *UAS-GFP* transgenes were crossed to the *List-lacZ* transformant. The brains of the progeny carrying these three transgenes were fixed with 4% formaldehyde in PBS for 15 min, washed three times with PBS/0.3% Triton X-100 and were incubated overnight at 4°C with rabbit anti-β-galactosidase (1:1000) (Rockland Immunochemicals, Gilbertsville, PA) in PBS/0.3% Triton X-100 with 1.5% normal goat serum. Goat anti-rabbit IgG conjugated with alexa647 (Invitrogen, Carlsbad, CA) was used to visualize immunostaining. Anti-LacZ immunostaining and glia-specific GFP fluorescence driven by *nrv2-GAL4* were observed in the same brain specimens using confocal microscopy (Zeiss 510). Z sections were collected at 2 μm intervals.

Different GAL4 lines (*da*-GAL4, *nrv2*-GAL4, *repo*-GAL4, *elav*-GAL4 and *Mhc*-GAL4) were crossed to the UAS-GFP strain to visualize GAL4 expression levels and patterns. Overall expression levels of GFP were examined under a Leica MZIII fluorescence stereomicroscope. GFP expression levels and patterns in the brain were examined under a confocal microscope (Zeiss 510) as described above. To compare expression levels among different GAL4 lines, all digital pictures were taken and processed under identical conditions.

Measurement of internal lithium

25 virgin female flies (0–24 hr-old) were placed in a vial with food containing 50 mM LiCl for 24 hr, and were then homogenized in 350 μ l of PBS (pH 7.4). The homogenate was centrifuged at 15,000 rpm for 15 min, and filtered through a nanosep spin filter cartridge (Pal Cooperation, East Hills, NY) (0.2 μ m pore size). The supernatant was subjected to lithium analysis by spectrophotometry, using the Infinity™ lithium single liquid stable reagent (Thermo Fisher Scientific, Waltham, MA).

Results

An RNAi transgene effectively suppresses expression of the lithium-inducible SLC6 family transporter gene *List*

In our recent microarray-based gene expression analysis, *CG15088* was identified as one of the genes in the *Drosophila* head that are most significantly up-regulated by lithium treatment (Kasuya et al., 2009). *CG15088* encodes a member of the SLC6 transporter family (Thimman et al., 2006; Romero-Calderon et al., 2007). In the previous report (Miller et al., 2008), it was tentatively referred to as *dmNAT2* for *Drosophila melanogaster* nutrient amino acid transporter 2, based solely on its sequence similarity to the nutrient amino acid transporter 1 gene, *dmNAT1*. Our microarray analysis showed that, of the 21 *Drosophila* SLC6 genes identified by bioinformatics analysis (Thimman et al., 2006), *CG15088* is the only one whose transcript level was considerably altered in response to lithium treatment (Kasuya et al., 2009) (Supplementary Table 2). Based on this characteristic feature, which was verified experimentally, we here designate *CG15088* as *List*, for *lithium-inducible SLC6 transporter*.

To investigate the functions of *List*, in particular, its role in the physiological response to lithium, we suppressed *List* expression by GAL4-mediated RNA interference (RNAi) (Dietz et al., 2007). A transgenic strain carrying the GAL4-inducible UAS-RNAi construct for *List* (*List*-RNAi-1, VDRC ID 44909) was used in combination with a strong, ubiquitous GAL4 driver, *da*-GAL4 (Monferrer and Artero, 2006), to knockdown *List* expression. In 2-day-old *List* knockdown female flies (*List*-RNAi-1/+ ; *da*-GAL4/+, hereafter referred to as *List*-*da*-KD-1), the endogenous levels of *List* transcript were significantly reduced, in both the head and body, to approximately 40% of the levels in wild-type flies judged by semi-quantitative RT-PCR; in contrast, the levels of the *rp49* transcript, which served as an internal control, remained unchanged (Fig.1). Unlike wild-type flies, *List*-*da*-KD-1 flies failed to display the lithium-induced up-regulation of *List* transcripts in the head and body (Fig.1). Consequently, the post lithium-treatment differences in *List* expression levels between wild-type (CS) and *List*-*da*-KD-1 flies were approximately 9-fold, in both the head and body (Fig. 1). These results demonstrate that when the expression of the *List*-RNAi transgene is driven by *da*-GAL4, this effectively suppresses endogenous expression, as well as lithium-induced up-regulation, of *List* in the adult head and body.

RNAi-mediated *List* knockdown results in increased susceptibility of adult flies to lithium toxicity

In spite of the significant reduction in endogenous *List* expression in *List*-*da*-KD-1 flies, these animals appeared normal with respect to development and morphology. Approximately 90%

of *List-da-KD-1* eggs survived to adulthood, and *List-da-KD-1* adult females were able to mate and produce progeny. No apparent morphological defects were observed in *List-da-KD-1* adult flies. In addition, in a reactive climbing test (Greene et al., 2003), which is an indicator of general motor activity of adult flies, 2 day-old *List-da-KD-1* flies exhibited a climbing index (C.I.) comparable to that of wild-type flies, as well as of two other controls that carried either *da-GAL4* or *UAS-List RNAi* (Fig. 2, gray bar). The life span of *List-da-KD-1* flies was also comparable to that of control flies cultured under standard conditions. The median life spans of wild-type and *List-da-KD-1* flies were 55.7 and 61.8 days, respectively (Fig. 3A and B), and those of the other control flies, *da-GAL4/+* and *UAS-List RNAi/+*, were 62 and 66 days, respectively (Fig. 3C and D).

When *List-da-KD-1* flies were treated with lithium, their remarkable phenotype was revealed. One day after being transferred onto fly food containing 50 mM LiCl, they became uncoordinated and their climbing behavior was severely impaired, as indicated by low the C.I. levels of 0.06 ± 0.04 (Fig. 2, black bar). Lithium had little effect on the climbing behavior of the control flies under the same conditions (Fig. 2, black bar).

Continuous exposure of *List-da-KD-1* flies to 50 mM LiCl resulted in 50% and 100% mortality within 3.1 and 5.0 days, respectively (Fig. 3B). Although 50 mM LiCl also had a toxic effect on wild-type flies over the longer term, it took 39.3 and 65.0 days for them to reach 50% and 100% mortality, respectively (Fig. 3A). The toxic effect of lithium on the *List-da-KD-1* flies was dose-dependent. When the flies were treated with 5 and 10 mM of LiCl, the median time to lethality in *List-da-KD-1* flies was 11.0 and 6.2 days, respectively (Fig. 3B). On the 5 mM LiCl food, all *List-da-KD-1* flies were dead by day 13 (Fig. 3B). In contrast, 95% of wild-type flies survived through day 13, even when maintained on food containing 50 mM LiCl (Fig. 3A). The lithium sensitivity of other control flies carrying either *da-GAL4* or *UAS-List RNAi* alone was comparable to that of wild type flies (Fig. 3C and D), confirming that the lithium-sensitive phenotype of *List-da-KD-1* flies is due to *List-RNAi* expression driven by *da-GAL4*.

The independently derived *List-RNAi* transgenic strain, *List-RNAi-2*, also showed the lithium-susceptible phenotypes when it was combined with *da-GAL4* (*List-da-KD-2*, Fig. 4). In addition, a similar lithium-susceptible phenotype (significant reduction in viability after 4-day treatment with 50 mM LiCl) was observed when other ubiquitous GAL4 drivers, such as *Act5C-GAL4* or *tub-GAL4*, were used to direct widespread expression of the *List-RNAi* transgene (*List-Act5C-KD* and *List-tub-KD*, Fig. 4). In contrast, control flies bearing only one of the transgenes (*da-GAL4/+* or *List-RNAi/+*) were indistinguishable from wild-type flies with respect to their sensitivity to lithium (Fig. 4), confirming that the lithium-susceptible phenotype was induced by GAL4-dependent *List-RNAi* expression.

List expression in, and viability of, List-KD flies are affected specifically by lithium

We tested whether ions other than lithium affect the expression of *List* and the viability of *List-da-KD-1* flies. In this experiment, 1% agarose/5% sucrose medium was used instead of the standard fly food, in order to avoid any effects the cornmeal-yeast-based food might have on the concentrations of the ions of interest. In wild-type flies, *List* up-regulation was specific to lithium treatment; treatment with 50 mM NaCl or 50 mM KCl for 24 hr did not lead to an increase in the level of the *List* transcript (Fig. 5A). Moreover, the expression of *List* did not increase even when the flies were exposed to a significantly higher concentration (500 mM) of NaCl or KCl for 24 hr (data not shown).

We found that treatment with 50 mM NaCl or 50 mM KCl for 4 days had little effect on the viability of *List-da-KD-1* flies (Fig. 5B). Continuous treatment with these salts for up to 12 days also did not cause significant lethality in either *List-da-KD-1* flies or wild-type flies (data not shown). Furthermore, even when a higher salt concentration (500 mM) was used, *List*-

da-KD-1 flies were not more susceptible to NaCl or KCl treatment than wild-type flies (Fig. 5C). On the contrary, *List-da*-KD-1 flies were slightly more resistant to treatment with 500mM NaCl than were wild-type flies; in the presence of 500 mM NaCl, the median time to lethality for *List-da*-KD-1 flies (7.0 days) was slightly longer than that for wild-type flies (6.0 days) (Mann-Whitney Rank Sum test, $P < 0.001$) (Fig. 5C). The median time to lethality in the presence of 500 mM KCl did not differ between *List-da*-KD-1 flies and wild-type flies (Fig. 5C).

A *List* gene deficiency results in increased susceptibility to lithium toxicity

The experiments using *List*-specific RNAi strongly indicated that reduced *List* transcript levels cause the lithium-susceptible phenotype. However, it remained possible that the *List* RNAi unexpectedly down-regulates not only the expression of *List*, but also that of other gene(s), and that such “off-target” gene suppression was the actual cause of the lithium-susceptible phenotype. To rule out this possibility, we generated flies null for the *List* gene, using two molecularly defined genetic deficiencies: *Df(2R)Exel7157* and *Df(2R)Exel7158*. These flies were then examined for their sensitivity to lithium. As shown in Fig. 6A, the 25.2 kb genomic region that contains the *List* gene is absent in flies that are trans-heterozygous for the two molecularly defined deficiencies (thus we refer to these flies as *List*-null herein) (Fig. 6A). In *List*-null flies, three additional genes (*pncr016*, *CG15073* and *CG15087*) and a portion of *Hs3st-A* are missing. In contrast to *List*, whose expression was up-regulated by lithium and down-regulated by *List* RNAi, the transcript levels of these four genes were influenced neither by lithium treatment nor expression of *List* RNAi, as judged by RT-PCR analysis (Fig. 6B).

List-null adult flies produced from a cross between *Df(2R)Exel7157/CyO* and *Df(2R)Exel7158/CyO* were viable but were not observed at the expected Mendelian ratios; the ratio of adult *List*-null flies to the total F1 progeny was approximately 1:10 rather than the expected 1:3. Thus, a subpopulation of *List*-null animals failed to become adults because *List* or other gene(s) deleted in *List*-null flies are required for full viability during development. Nevertheless, *List*-null flies that survived to adulthood showed no obvious morphological abnormalities. They were apparently healthy, and their viability was not at all compromised for at least for 2 weeks in the absence of lithium treatment (Fig. 6C, open circle). However, when *List*-null flies received food containing 50 mM LiCl, their general activity was severely impaired within 1 day, followed by death within the next few days (Fig. 6C, closed circle). The median time to lethality of *List*-null flies with the 50 mM LiCl food was 2.3 days. In contrast, the lithium sensitivity of flies heterozygous for either *Df(2R)Exel7157* or *Df(2R)Exel7158* (Fig. 6C, closed square or diamond) was indistinguishable from that of wild-type flies (Fig. 6C, closed triangles), for at least two weeks, suggesting that a 50% reduction in *List* expression does not significantly affect resistance to lithium toxicity. However, like the *List-da*-KD-1 flies, *List*-null flies displayed lithium-specific hypersensitivity. Few, if any, *List*-null adult flies survived for 4 days or more after being transferred onto food containing 50 mM LiCl (Fig. 6D). In contrast, treatment with either 50 mM NaCl or KCl had little effect on their viability under conditions that were otherwise the same (Fig. 6D).

GAL4-mediated expression of the *List* gene decreases the sensitivity of *List*-null flies to lithium toxicity

In addition to *List*, four genes are partially or completely deleted in *List*-null flies. To examine if the lithium-sensitive phenotype of *List*-null flies is attributable to the absence of *List*, a rescue experiment was performed using the *List* cDNA transgene in combination with the GAL4/UAS system. Through genetic crossing we introduced two transgenes, UAS-*List* and the ubiquitous GAL4 driver, *da*-GAL4, into *List*-null flies. The resultant flies (*Df(2R)Exel7157/Df(2R)Exel7158*; UAS-*List/da*-GAL4) expressed high levels of *List* transcripts regardless of lithium treatment, whereas no *List* transcript was observed in either *List*-null flies or *List*-null flies with

only one of the two transgenes (Fig. 7A). We found that transgenic expression of the *List* gene significantly alleviates the lithium-sensitive phenotype of *List*-null flies. Approximately half of the *List*-null flies carrying both UAS-*List* and *da*-GAL4 survived for four days. The original *List*-null flies and *List*-null flies carrying either *da*-GAL4 or UAS-*List* alone remained susceptible to lithium toxicity, and these flies were barely viable after 4 days of treatment with 50 mM LiCl. These results unequivocally show that the absence of *List* function is primarily responsible for the elevated sensitivity of *List*-null flies to lithium toxicity.

The involvement of glia in *List*-mediated resistance to lithium toxicity

Among the 21 *Drosophila* SLC6 transporter genes defined by Thimman et al., *List* is one of only seven that are detected in the CNS by *in situ* hybridization analysis (Thimman et al., 2006). In that study, *List* mRNA was detected in the brain glial cells (Thimman et al., 2006). Consistent with this finding, FlyAtlas, an online microarray-based resource on gene expression in adult *Drosophila* tissues (<http://www.flyatlas.org/>) (Chintapalli et al., 2007), shows that *List* transcripts are highly enriched in the brain and thoracoabdominal ganglion.

To gain further insight into *List* expression patterns, we generated germline transformants carrying the *lacZ* reporter gene fused to the 4 kb genomic DNA immediately 5'-upstream of the predicted *List* transcript start site (*List-lacZ*) (Fig. 8A). The *List-lacZ* adult transformants showed highly restricted *lacZ* expression in the brain (Fig. 8B), thoracoabdominal ganglion and gut (Fig. 8C). Immunohistochemical analysis revealed that the expression pattern of the *List-lacZ* reporter in the brain (Fig. 8D and G) significantly overlaps with that of GFP induced by the glia-specific driver, *nrv2*-GAL4 (Fig. 8E and H) (Sun et al., 1999; Peraanu et al., 2007), indicating that the 5'-upstream region of the *List* gene contains the regulatory information necessary for the expression of *List* in brain glia.

We next examined which cell types are involved in the hypersensitivity to lithium toxicity that had been observed in *List-da*-KD and *List*-null flies. In light of the probable glial expression of *List*, we hypothesized that a reduction of *List* expression in glial cells might contribute to the lithium-susceptible phenotype. To test this hypothesis, we suppressed *List* expression specifically in glia, using *List* RNAi in combination with the glia-specific GAL4 driver *nrv2*-GAL4 (Sun et al., 1999; Peraanu et al., 2007). Flies carrying *List*-RNAi and *nrv2*-GAL4 (*List-nrv2*-KD) appeared normal with respect to development and morphology. Moreover, the climbing ability and viability of *List-nrv2*-KD flies was comparable to that of wild-type control flies, for at least the first 15 days as an adult, when raised without lithium treatment (Fig. 9A and C). However, in the presence of 100 mM LiCl, *List-nrv2*-KD flies were significantly more susceptible to lithium toxicity than were control wild-type flies (Fig. 9B); the median life span of *List-nrv2*-KD flies subjected to treatment with 100 mM LiCl was only 7 days (Fig. 9B), and the climbing ability of the surviving *List-nrv2*-KD flies after a 4-day treatment was severely impaired (Fig. 9C). In contrast, when the *List*-RNAi transgene was expressed in neurons or muscle cells, using *elav*-GAL4 or *Mhc*-GAL4, respectively, their sensitivity to lithium was not considerably different from that of control flies, with respect to either survival (Fig. 9B) or climbing ability (Fig. 9C). The lithium hypersensitivity of *List-nrv2*-KD flies was not simply due to stronger GAL4 activity of the *nrv2*-GAL4 driver. Overall, the induction of GFP expression by *nrv2*-GAL4, *elav*-GAL4 and *Mhc*-GAL4 in glia, neurons and muscle cells, respectively, was comparable (Fig. 9E, G and H). Also, *da*-GAL4 displayed the most intense GFP expression (Fig. 9D and H) and had the strongest effect on viability when used to drive *List* RNAi expression ubiquitously (Fig. 9B). Finally, although a second glia-specific GAL4 line, *repo*-GAL4 (Campbell et al., 1994), was found to drive much weaker UAS-transgene expression than other GAL4 lines used in this experiment (Fig. 9F and H), its use in driving *List*-RNAi expression led to a slightly higher sensitivity to lithium than when the stronger, but non-glia expressing *elav*-GAL4 and *Mhc*-GAL4 drivers were used (Fig. 9B). These results

further support the conclusion that *List* expression in glia is important in conferring resistance to lithium toxicity.

Internal lithium concentration does not differ in wild-type and *List-da-KD* flies

To determine the internal lithium levels in wild-type and *List-da-KD-1* flies, we homogenized whole flies and subjected them to a lithium colorimetric assay (see Experimental Procedures). In the absence of lithium treatment, the lithium concentrations were below the detection limit ($< \sim 0.1$ mM) in flies of both genotypes. After 24 hr of treatment with 50mM LiCl, the lithium concentrations in *List-da-KD-1* flies and wild-type flies were measurable but did not differ significantly (0.67 ± 0.07 mM and 0.68 ± 0.21 mM for *List-da-KD-1* flies and wild-type flies, respectively) (Fig. 10), suggesting that the levels of lithium uptake and clearance were not significantly altered in *List-da-KD-1* flies. The estimated lithium levels in wild-type and *List-da-KD-1* flies were comparable to those reported in two previous studies, in which flies were fed lithium at concentrations similar to those used in this study (Padiath et al., 2004; Dokucu et al., 2005).

Discussion

Here we have shown that the suppression or complete deletion of *List*, the *Drosophila* lithium-inducible SLC6 gene that encodes a putative amino acid transporter, leads to a remarkable increase in the fly's susceptibility to lithium, and that glial expression of *List* is the primary requirement for resistance to lithium toxicity. Our results have identified the *List* transporter as a novel molecular player that is critically involved in mediating the physiological effects of lithium. In addition, they have identified a new avenue of investigation toward a better understanding of the molecular and cellular mechanisms that underlie lithium-responsive neurobiological process.

It is notable that the up-regulation of *List* is specific to lithium treatment, with sodium, potassium or chloride treatment having no effect on the levels of *List* transcripts (Fig. 5). Also, previously published microarray studies did not identify *List* as a gene whose expression is significantly affected in response to sublethal doses of heavy metals (Yepiskoposyan et al., 2006) or various harmful chemicals (Girardot et al., 2004; Willoughby et al., 2006; Willoughby et al., 2007). Collectively, these findings suggest that the lithium-induced up-regulation of *List* is not due to a general response to salt stress or toxic compounds, but rather due to specific chemical or physical properties of lithium.

The lithium-induced increase in steady-state *List* mRNA levels could be caused by lithium-dependent activation of *List* transcription, or alternatively, by post-transcriptional mechanisms. In support of the former possibility, the intracellular signaling pathways influenced by lithium regulate various transcription factors (Ozaki and Chuang, 1997; Yuan et al., 1998; Rowe and Chuang, 2004). In particular, lithium is known to activate Wnt signaling pathways by directly inhibiting GSK-3 β , which leads to an increase in the levels of β -catenin and thereby enhances the activity of TCF/LEF transcription factors (Moon et al., 2002). In *Drosophila*, GSK-3 β , β -catenin and TCF/LEF are encoded by *shaggy* (*sgg*), *armadillo* (*arm*) and *pangolin* (*pan*), respectively. These are prime candidate genes for involvement in the lithium-induced increase in *List* transcription. Support for the notion that post-transcriptional mechanisms are responsible for the up-regulation of *List* mRNA levels by lithium comes from the fact that lithium has been shown to stabilize transcripts of the melanin-concentrating hormone in PC12 cells, and to increase their steady state levels of this hormone (Presse et al., 1997). Lithium also increases the levels of adenylate/uridylylate (AU)-specific RNA binding protein in the vertebrate brain, and this change may play a role in the regulation of mRNA stability (Chen et al., 2001). Although our study revealed that the 4 kb *List* 5'-upstream DNA is able to drive *lacZ* reporter gene expression in glia of the adult CNS (Fig. 8), we did not observe a lithium-

induced increase in *lacZ* mRNA levels (data not shown). Thus, the 4 kb *List* 5'-flanking DNA does not appear to contain sufficient regulatory information to activate *List* transcription in response to lithium, and a clearer understanding of the transcriptional and/or post-transcriptional mechanisms responsible for the lithium-induced up-regulation of *List* will require more detailed reporter analyses. In the case of transcriptional mechanisms, for example, it may be necessary to test the intronic sequences of *List* for their potential regulatory function. Indeed, a recent report has demonstrated that expression of the human serotonin transporter gene (SLC6A4) is regulated by lithium through transcription factors that bind to the variable number tandem repeat (VNTR) polymorphic region in the second intron of the gene (Roberts et al., 2007).

Experiments employing RNAi, chromosome deficiencies and the wild-type *List* transgene have demonstrated that the *List* transporter plays an important role in conferring resistance of adult flies to lithium toxicity. A key question that remains to be answered, however, is how this transporter affects lithium sensitivity. We know that expression in glia is pivotal in establishing resistance to lithium toxicity, as *List* is expressed in the CNS glia, and glia-specific suppression of *List* leads to the lithium-sensitive phenotype. However, other cell types or tissues may also be involved, because flies were more sensitive to lithium when *List* RNAi was ubiquitously expressed using *da*-GAL4 than when it was expressed only in glia, under the control of *nrv2*-GAL4. Based on tissue-specific microarray (FlyAtlas) and reporter gene analyses (Fig. 8C), the secondary site most likely to be involved in *List*-mediated resistance to lithium is the digestive system. Although we were not able to show lithium hypersensitivity when *List*-RNAi expression was directed to the adult digestive system using *drm*-GAL4 (Green et al., 2002) (data not shown), this failure could have been due to the low GAL4 expression level in *drm*-GAL4 flies, as revealed by expressing the GFP reporter gene with *drm*-GAL4 driver.

At least two distinct mechanisms may contribute to *List*-mediated resistance to lithium toxicity. Firstly, the *List* transporter might regulate the extracellular concentration of lithium, preventing lithium-sensitive cells from being exposed to toxic levels of this metal. Secondly, unidentified substrates of the *List* transporter may directly or indirectly counteract the toxic effects of lithium. Such substrates could compensate for the enzymatic activities inhibited by lithium, or antagonize lithium's adverse effects on particular cellular processes.

How could the *List* transporter regulate extracellular concentrations of lithium? The SLC6 transporters move compounds across the plasma membrane against their concentration gradient, and this uptake process is coupled with the co-transport of sodium (Amara and Arriza, 1993; Nelson, 1998) and/or potassium (Castagna et al., 1998; Feldman et al., 2000; Boudko et al., 2005). Interestingly, other ions can permeate certain SLC6 transporters without causing the uptake of any substrate, and thereby produce an inward un-coupled leak current (Sonders and Amara, 1996). For example, Bossi et al. reported that a *Maduca sexta* SLC6 transporter, the potassium-coupled amino acid transporter-1 (KAAT1), is highly permeable to lithium even in the absence of amino acid substrates (Bossi et al., 1999). The *List* transporter may share this property, and contribute to the regulation of the extracellular lithium concentration through this "lithium channel-like" activity. As a result, neurons or other cell types that may be susceptible to lithium toxicity could be protected from exposure to high lithium concentrations.

In the second proposed scenario for *List*-mediated resistance to lithium toxicity, unidentified *List* transporter substrates could play a critical role in *List*-mediated resistance to lithium toxicity. *List* is classified as one of the 6 members of the putative amino acid transporter subfamily (Thimman et al., 2006; Romero-Calderon et al., 2007; Miller et al., 2008), which has also been referred to as the insect amino acid transporter (IAAT) subfamily (Thimman et al., 2006) or the nutrient amino acid transporter (NAT) subfamily (Miller et al., 2008). *Drosophila* NAT1 (dmNAT1), which is encoded by *CG3252*, is the only transporter in the

Drosophila IAAT/NAT subfamily that has been experimentally shown to transport a broad set of neutral amino acids (Miller et al., 2008). The insect IAAT/NATs are phylogenetically close to the mammalian B⁰ (broad substrate spectra neutral) amino acid transporters, and they have been proposed to be their functional counterparts (Boudko et al., 2005; Meleshkevitch et al., 2006). Thus, although experimental data regarding the physiological properties of the *List* transporter are lacking, this protein is most likely to function as a transporter for amino acids or their derivatives. Indeed, in mammals lithium has been reported to alter the levels of particular free amino acids in the brain (Eroglu et al., 1980; Dixon et al., 1994) and, although the effect of lithium on amino acid levels in the brain has not been investigated in *Drosophila*, our recent microarray study revealed that the genes involved in amino acid metabolism are those most significantly altered in the *Drosophila* head following lithium treatment (Kasuya et al., 2009). These findings are consistent with the idea that the *List* transporter may support the absorption of amino acids and play a role in maintaining appropriate amino acid levels in different compartments of the nervous system after lithium treatment.

To fully elucidate the mechanisms underlying the *List*-mediated resistance to lithium toxicity, it is necessary to characterize the physiological properties of the *List* transporter and identify its endogenous substrates. Crucial information about functions and mechanisms of action of the *List* transporter would be obtained using biochemical and electrophysiological approaches, such as those recently employed by Miller et al. (2008) for dmNAT1. It will be also important to determine if any of the mammalian SLC6 family members, particularly B⁰ amino acid transporters, can rescue the lithium-sensitive phenotype of *List*-KD and *List*-null flies. Such a finding would lead to the identification of functional mammalian homologs of the *Drosophila* *List* transporter, providing important insights into the molecular mechanisms that underlie the therapeutic, as well as toxic, effects of lithium in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

GSK-3, glycogen synthase kinase 3
 IMPase, inositol monophosphatase
 STD, standard deviation
 SEM, standard error of the mean
 SLC6, solute carrier 6
List, Lithium-inducible SLC6 transporter
dfmr1, *Drosophila fragile X mental retardation 1*
da, daughterless
Act5C, *Actin5C*
tub, tubulin
repo, reversed polarity
elav, embryonic lethal abnormal vision
Mhc, Myosin heavy chain
nrv2, *nervana 2*
 UAS-GFP, Upstream activation sequence-green fluorescent protein
 DEPC, diethyl pyrocarbonate
rp49, RNA binding protein 49
 PBS, phosphate-buffered saline
 X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
 NAT, nutrient amino acid transporter

IAAT, insect amino acid transporter
RNAi, RNA interference
KD, knockdown

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Figure 1. Effects of lithium on *List* (*CG15088*) mRNA levels in wild-type and *List* knockdown flies RT-PCR analyses were performed to estimate *List* transcript levels in the head (A) and body (B) of wild-type (CS) and *List*-knockdown (*List-da-KD-1*) flies, with (black bars) or without (gray bars) 50 mM lithium treatment (Fig. 1A and B, top panels). *rp49* transcript in the same samples served as a control (Fig. 1A and B, bottom panels). The ratios of transcript levels in experimental samples and the corresponding lithium-untreated wild-type samples are presented. Average $\pm$ STD, N=4 for *List*, N=3 for *rp49*. Transcript levels were statistically compared between CS and *List-da-KD-1* flies using the Mann-Whitney Rank Sum Test. * $P < 0.05$. NS: not significant.

Figure 2. Effects of lithium treatment on reactive climbing in wild-type and *List* knockdown flies Wild-type flies (CS), *List-da-KD-1* flies and flies from two control lines, that carry either *da-GAL4* or *UAS-List-RNAi*, were subjected to a reactive climbing assay (see Experimental Procedures). Climbing Indexes were determined from 5 sets of 20 flies that had remained untreated (gray bars) or had been treated with 50mM LiCl for one day (black bars). Average $\pm$ STD. Student's t-test was applied. *** $P < 0.001$. NS: not significant.

Figure 3. Effects of lithium treatment on survival rates of wild-type and *List* knockdown flies
 Wild-type flies (CS), *List-da-KD-1* flies and flies from two control lines carrying either *da-GAL4* or *UAS-List-RNAi* were grouped into 5 sets of 20 flies and remained untreated (control) or were treated with 5 (●), 10 (■) or 50 (▲) mM LiCl. Average survival rates of CS (A), *List-da-KD-1* (B), *da-GAL4/+* (C), and *UAS-List RNAi/+* (D) are shown with SEM.

Figure 4. Specificity of the lithium-susceptible phenotype induced by RNAi-mediated suppression of *List*

Female flies carrying different combinations of GAL4 and UAS-*List* RNAi transgenes were tested for their sensitivity to lithium. Average survival rates for untreated flies (gray bars) and those for flies that had been treated with 50 mM LiCl (black bars) for 4 days are shown with STD (20 flies for each test, $N \geq 3$). Genotypes of tested flies are *da-GAL4/+* ($w ; +/+ ; da-GAL4/+$), *List-RNAi-1/+* (UAS-*List*-RNAi-1/ $w ; +/+ ; +/+$), *List-RNAi-2/+* (UAS-*List*-RNAi-2/ $w ; +/+ ; +/+$), *List-da-KD-1* (UAS-*List*-RNAi-1/ $w ; +/+ ; da-GAL4/+$), *List-da-KD-2* (UAS-*List*-RNAi-2/ $w ; +/+ ; da-GAL4/+$), *List-Act5C-KD* (UAS-*List*-RNAi-1/ $w ; +/+ ; Act5C-GAL4/+$) and *List-tub-KD* (UAS-*List*-RNAi-1/ $w ; +/+ ; tub-GAL4/+$).

Figure 5. Effects of different ions on *List* mRNA levels and on viability of *List* knockdown flies (A) An RT-PCR analysis for *List* and *rp49* transcript levels in untreated wild-type (CS) flies (-) and wild-type flies treated with 50 mM LiCl (Li), 50 mM NaCl (Na) or 50 mM KCl (K) for 24 hr. (B) The effects of 50 mM LiCl, NaCl or KCl on survival rates of *List-da-KD1* flies were examined after 4 days of treatment. Average survival rates are shown with STD (12–20 flies for each test, N=5). (C) Effects of higher concentrations of NaCl and KCl on survival rates of wild-type (open symbols) and *List-da-KD1* (closed symbols) flies. Average survival rates are shown with SEM (16–20 flies for each test, N=3). Circles, triangles and squares represent control, treatment with 500 mM KCl (K) and treatment with 500 mM NaCl (Na), respectively.

Figure 6. Effects of lithium treatment on survival rates of wild-type and *List*-null flies

(A) Flies trans-heterozygous for the two molecularly defined deficiencies, *Df(2R)Exel7157* and *Df(2R)Exel7158*, lack the 25.2 kb genomic region in which the *List* gene resides (*List*-null). Arrows indicate annotated genes in the chromosome region 55E-55F. In addition to *List*, four genes are completely or partially deleted in *List*-null flies (see text). (B) Outcome of an RT-PCR analysis of the four genes that are completely or partially deleted in *List*-null flies. Transcript levels of each gene in lithium-treated wild-type flies (gray bars) and *List-da-KD-1* flies (black bars) were normalized to those in untreated wild-type flies (open bars). Averages are shown with STD (N=3). Kruskal-Wallis ANOVA was applied for statistical analysis, and no significant differences in the transcript level of each gene was detected among *List-da-KD-1* flies, lithium-treated wild-type flies and untreated wild-type flies. (C) The survival rates of *List*-null flies (●), *Df(2R)Exel7157/CyO* (■), *Df(2R)Exel7158/CyO* (◆), and wild-type flies (▲) in the presence of 50 mM LiCl are shown. The survival rate of *List*-null flies without lithium treatment is also presented (○). Average survival rates are shown with SEM (16–20 flies for each test, N=3). (D) Effects of different ions on viability of *List*-null flies. Shown are average survival rates with STD (16–20 flies for each test, N=3) at 4 days, for *List*-null flies provided with control food (open bar) and counterparts treated with 50mM LiCl (Li), NaCl (Na) and KCl (K) for this period.

Figure 7. Transgenic expression of *List* decreases the sensitivity of *List*-null flies to lithium toxicity
 The *UAS-List* cDNA and *da-GAL4* transgenes were introduced into *List*-null flies through genetic crosses. (A) An RT-PCR analysis to estimate transcript levels of *List* and *rp49* in *List*-null flies with *UAS-List* and the *da-GAL4* transgene in the presence or absence of lithium treatment (50 mM, 24 hr). Shown are representative agarose gel images and average relative signal intensities with STD (N=3). (B) *List*-null flies with *UAS-List* and *da-GAL4* transgene were tested for their sensitivity to lithium toxicity. Presented with SEM are average survival rates of adult flies after 4 days of treatment with 50 mM LiCl (13–20 flies for each test, N=7). The Kruskal-Wallis ANOVA, followed by Dunn's pairwise test was applied. ***P<0.001.

Figure 8. Expression of the *List-lacZ* reporter gene in the adult CNS

(A) Schematic illustration of the *List-lacZ* transgene. The 4 kb *List* 5'-flanking DNA was inserted in front of the *lacZ* cDNA in the pPelican vector. β-galactosidase expression was observed in the brain (B), as well as in the thoracic ganglion and part of the gut (C), in *List-lacZ* transformants. Low (D–F) and high (G–I) magnification images of whole-mount anti-β-galactosidase staining (D, G) and GFP fluorescence (E, F) of the *List-lacZ*/+ ; *nrv2-GAL4* UAS-*GFP*/+ fly brains. The merged images of the two stainings are shown in F and I. Scale bars: 100 μm.

Figure 9. Effects of tissue-specific suppression of *List* on the viability of adult flies treated with lithium

The *List* RNAi transgenic line was crossed to tissue-specific GAL4 drivers, *elav*-GAL4 (neuron, blue triangle), *nrv2*-GAL4 (glia, blue square), *repo*-GAL4 (glia, green square), *Mhc*-GAL4 (muscle, purple diamond) and *da*-GAL4 (ubiquitous, red circle), as well as to wild-type flies (control, pink circle). Average survival rates are shown with SEM in the absence (A) or presence of 100mM LiCl (B). (C) Reactive climbing was examined for different *List*-KD flies after 4-day treatment with control food (gray bars) or food containing 100mM LiCl (black bars). Averages with STD are shown (N=3). Student's t-test was applied. ** $P < 0.01$, NS: not significant. (D–H) Levels and patterns of GFP expression were compared among different GAL4 lines carrying the GFP reporter gene. Images of the whole-mount brain (D–G) and the whole body (H) were taken under the same conditions for different GAL4 lines, using either a confocal microscope (D–G) or a fluorescence stereomicroscope (H).

Figure 10. Internal lithium concentrations in wild-type and *List-da-KD* flies

Lithium content in whole flies after 24 hr of 50 mM LiCl treatment was measured by spectrophotometry (see Experimental Procedures). Average lithium concentrations in fly homogenates are presented with STD (N=3). Student's t-test was applied and no statistically significant difference was detected between the two genotypes.