**Drosophila** divalent metal ion transporter *Malvolio* is required in dopaminergic neurons for feeding decisions

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Members of the natural resistance-associated macrophage protein (NRAMP) family are evolutionarily conserved metal ion transporters that play an essential role in regulating intracellular divalent cation homeostasis in both prokaryotes and eukaryotes. *Malvolio* (*Mvl*), the sole NRAMP family member in insects, plays a role in food choice behaviors in *Drosophila* and other species. However, the specific physiological and cellular processes that require the action of *Mvl* for appropriate feeding decisions remain elusive. Here, we show that normal food choice requires *Mvl* function specifically in the dopaminergic system, and can be rescued by supplementing food with manganese. Collectively, our data indicate that the action of the *Mvl* transporter affects food choice behavior via the regulation of dopaminergic innervation of the mushroom bodies, a principle brain region associated with decision-making in insects. Our studies suggest that the homeostatic regulation of the intraneuronal levels of divalent cations plays an important role in the development and function of the dopaminergic system and associated behaviors.

Keywords: *D. melanogaster*, dopamine, food choice, fruit fly, ionomics, manganese, *Mvl*, NRAMP, Slc11a, vinegar fly

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Correct food choices are essential for the survival and fitness of all animals. However, the genetic, neuronal and environmental factors that regulate these basal behaviors are still not well understood. Previous studies in *Drosophila melanogaster* indicated that flies carrying a mutation in the gene *Malvolio* (*Mvl*), the insect homolog of the evolutionary-conserved protein family of natural resistance-associated macrophage proteins (NRAMPs) divalent metal ions transporters (*D’Souza* et al. 1999), lead to a reduced appetitive discrimination between high and low sugar-containing foods (*Rodrigues* et al. 1995). The effects of the *Mvl* mutation on food choice behavior could be rescued by supplementing food with manganese or iron (*Orgad* et al. 1998). In addition, studies in the honeybee *Apis mellifera* showed that expression levels of *Mvl* in the brain differ between bees, which perform different tasks, and are associated with changes in the behavioral response threshold to sugar stimuli in bees. Similarly, feeding manganese to young, pre-foraging bees leads to a lower response threshold to sugar stimuli, and a precocious transition to foraging behavior (*Ben-Shahar* et al. 2004; *Søvik* et al. 2015). The effect of the *Mvl* mutation on taste behavior is not because of abnormal gustatory sensory signaling. These data suggest that the effects of *Mvl* mutations on food choice are mediated via central elements of the feeding decision neuronal circuit or non-neuronal tissues (*Rodrigues* et al. 1995). The NRAMP genes are also expressed in the nervous systems of vertebrates, but their physiological roles in these tissues are not well understood (*Evans* et al. 2001; *Ke* et al. 2005; *Skjorringe* et al. 2015).

In addition to the genetic support for the role of *Mvl* in regulating feeding, other studies indicated that trace metals such as manganese and iron contribute to the regulation of organismal energy homeostasis via diverse mechanisms. For example, manganese can act as an insulin-mimetic *in vitro* and *in vivo* in mammalian models via unknown mechanisms (*Nakai* et al. 2005). Similarly, environmental exposure to manganese, iron and other trace metals has been implicated in the development of both type I and type II diabetes (*Meyer* & *Spence* 2009), and genetic variations in the human *SLC11A1* (NRAMP1) gene are associated with the development of type I diabetes in humans (*Yang* et al. 2011). Independently, iron deficiency has been implicated in obesity (*Garcia* et al. 2009; *McClung* & *Karl* 2009). Manganese and iron are also essential for mitochondrial respiration and cellular metabolism (*Chen* et al. 2015b; *Levi* & *Rovida* 2009; *Mena* et al. 2015; *Mühlenhoff* et al. 2015; *Pierrel* et al. 2007). Together, these studies by others and us indicate that metal transporters play an important role in regulating diverse cognitive and metabolic functions in animals, and suggest that disruptions in the function of such transporters could lead to abnormal behavioral outcomes (*Ávila* et al. 2014; *Dusek* et al. 2015).
Materials and methods

Fly stocks and maintenance

Flies were reared on standard Drosophila cornmeal medium at 25°C and 60% humidity under a 12 h:12 h light/dark cycle. GAL4 lines, such as UAS-Dcr2, UAS-Mvl-RNAi and UAS-tetanus toxin (TNT) lines, were from the Bloomington Drosophila Stock Center (Bloomington, IN, USA).

Behavioral experiments

The single-dye feeding decisions paradigm was as previously described by us and others (Lu et al. 2012; Orgad et al. 1998). In short, the ‘feeding error index’ represents the proportion of flies with red abdomens relative to total number of flies in a single assay. As single-dye feeding assays cannot discriminate between flies that made a correct feeding choice and flies that did not eat at all (both will have no dye in the abdomen), we also measured the effect of genotypes and treatments on the general feeding drive of flies by allowing groups of animals to feed on plates in which all wells had 10 mM trehalose with the same red dye as in feeding choice assays.

Determination of the elemental composition of fly tissues by ICP-MS

Four independent samples of 10 pooled fly heads per genotype were analyzed for B, Na, Mg, Al, P, S, K, Ca, Mn, Fe, Co, Cu, Zn, As, Se, Rb, Mg and Cd. Inductively coupled plasma mass spectrometry (ICP-MS) analyses followed the standard Baxter laboratory protocol as previously described (Ziegler et al. 2013).

Quantification of biogenic amines from fly brains using high-pressure liquid chromatography

Adult fly brains were dissected in chilled PBS. Samples consisting of 10 pooled brains were flash-frozen in liquid nitrogen and stored at −80°C until analysis. Biogenic amine content was extracted in 15μl of 0.2 M perchloric acid containing 10μgμl of the high-pressure liquid chromatography (HPLC) standard dihydroxybenzylamine (Sigma-Aldrich, St. Louis, MO, USA) as previously described (Scheiner et al. 2014; Sevik et al. 2013). The supernatant of each sample separated across an HR-80 column (ESA Biosciences, Inc., Chelmsford, MA, USA) using an Agilent 1200 Series HPLC system (Agilent Technologies, Santa Clara, CA, USA). Levels of biogenic amines were quantified using an ESA Coulochem III electrochemical detector with an ESA 5011A analytical cell. Standard curves for octopamine, dopamine and serotonin (Sigma-Aldrich) were used to calculate the total amount of each amine in the tested samples.

Quantification of mRNA expression levels by real-time qRT-PCR

Total RNA was extracted from pools of approximately 30 adult heads using the TRIzol reagent (Thermo Fisher Scientific, Waltham, MA USA). First strand cDNA was prepared using SuperScript II reverse transcriptase (Thermo Fisher Scientific, Waltham, MA USA). Gene-specific assays were conducted with a SYBR Green kit on a 7500 real-time PCR System (Thermo Fisher Scientific, Waltham, MA USA). Each cDNA sample was run in triplicate PCR reactions. The housekeeping gene rp49 was used as a loading control. Relative expression data were analyzed and presented as expression fold-difference by using the ΔΔCT method, as we have previously described (Lu et al. 2012; Lu et al. 2014; Sevik et al. 2013; Zheng et al. 2014).

Immunohistochemistry

Antibody staining of dopaminergic neurons was as previously described (Lu et al. 2012; Lu et al. 2014; Zelle et al. 2013; Zheng et al. 2014). Brains were co-stained with anti-GFP (Thermo Fisher Scientific, Waltham, MA USA) and nc82 antibodies (Developmental Studies Hybridoma Bank, Iowa City, IA, USA), and mounted using the VECTASHIELD HardSet Antifade Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Imaging was performed using Nikon A1 confocal microscope. Figures were generated from maximal intensity Z-stacks using the Nikon NIS-Elements software package.

Statistical analyses

Feeding behavioral data and brain ionomics were analyzed with Student’s t-tests (StatPlus, AnalystSoft, WALNUT, CA USA). All values for the statistical tests are described in Tables S1–S5. Supporting Information. Biogenic amines levels were analyzed by a linear mixed model with genotype as a categorical factor. The HPLC analysis batch was controlled for in the model as well. Dry weight and glucose levels were analyzed by one-way analysis of variance followed by a Tukey post hoc test (P < 0.05).

Results

Mvl is required in neuronal tissues for normal food choice behavior

Analysis of the spatial Mvl mRNA expression data from the FlyAtlas database (Robinson et al. 2013) indicated that it is broadly expressed throughout the adult body (Fig. 1a). Therefore, to identify the specific tissues in which Mvl is required for feeding decisions, we employed a tissue-specific RNAi-based gene knockdown approach (Brand & Perrimon 1993). To test whether the available UAS-Mvl-RNAi line can effectively knockdown endogenous mRNAs, we first showed the effect of the UAS-Mvl-RNAi on the global expression of Mvl with the ubiquitous aTub84B-GAL4 line (Fig. 1b).

Previous studies indicated that the abnormal feeding phenotype in Mvl mutant flies can be rescued on a physiological timescale with manganese or iron food supplements (Orgad et al. 1998). However, because Mvl is broadly expressed throughout development (not shown), it is still impossible to know whether the behavioral phenotype of adult mutants is due, at least in part, to abnormal developmental processes. Therefore, we investigated the impact of global Mvl knockdown on feeding decisions specifically in the adult stage by expressing the UAS-Mvl-RNAi with the conditional heat-induced hsp70-GAL4 driver. We found that under heat-shock conditions, adult flies exhibited abnormal feeding behavior (Fig. 1c and Tables S1 and S2). These data further suggest that the effects of the Mvl gene knockdown on behavior are not solely dependent on post-embryonic development.

Because Mvl is broadly expressed (Fig. 1a), we next used specific organ-enriched GAL4 drivers to investigate whether the effects of Mvl knockdown on food choice behavior are tissue-specific. The tissue-level RNAi screen showed that...
Mvl expression in the nervous system (elav-GAL4) is necessary for normal food choice behavior (Fig. 1d). In contrast, expressing the Mvl-RNAi with GAL4 drivers enriched in the fat body (ppl), hemocytes and fat body (cg), salivary glands and fat body (R4), gut (drm), glia (repo) or everywhere outside the nervous system (DJ761), did not affect feeding decisions (Fig. 1d and Table S1). Together, these data indicate that normal feeding decisions require Mvl activity in the nervous system. We did not observe any effects of the Mvl gene knockdown on general feeding drive in any of the tested genotypes (Fig. 1f and Table S2).

**Effect of neuronal Mvl knockdown on behavior can be rescued by manganese**

Previous work showed that manganese supplements can rescue the effect of the Mvl mutation on feeding decisions (Orgad et al. 1998). Therefore, we next asked whether the effect of neuronal-specific Mvl knockdown on feeding behavior could be rescued by a manganese supplement as well. We found that supplementing fly food with 5 mM Mn^{2+} was sufficient to rescue the effects of the neuronal Mvl-RNAi downregulation on feeding decisions (Fig. 2a). However,
neither genetic background nor Mn^{2+} treatment had an effect on the overall feeding drive of adult flies (Fig. 2b). We also found that the Mn^{2+} supplement rescued a small, but significant, decrease in total dry body weight observed in flies with the pan-neuronal Mvl knockdown (Fig. 2c), but not in total glucose content (Fig. 2d), which possibly reflects the increased consumption of non-nutritive food in Mvl knockdown flies. Together, our data suggest that Mvl-dependent regulation of manganese in neuronal tissues plays a role in food choice behaviors in the fly. These data also indicate that manganese, and possibly other divalent cations, can enter cells via alternative, NRAMP-independent pathways as has been reported in other model systems (Chen et al. 2015a; Garcia-Rodriguez et al. 2015; Jenkins et al. 2016).

Effects of the neuronal Mvl knockdown on the head ionome

Because Mvl is a diveral metal ion transporter, we next examined the elemental composition of fly heads in elav>Mvl-RNAi flies relative to wild-type controls. Surprisingly, we found that levels of Mn^{2+}, Fe^{2+} and Cu^{2+}, which are ions known to be transported by Mvl (Ben-Shahar et al. 2004; Bettedi et al. 2011; Folwell et al. 2006; Orgad et al. 1998; Southon et al. 2008), were not affected by the neuronal Mvl knockdown (Fig. 2 and Table S3). In contrast, levels of Na^{+}, K^{+} and Rb^{+}, three elements known to have similar chemical properties, showed a small but significant reduction in the Mvl knockdown animals (Fig. 3 and Table S3). These data indicate that the neuronal-specific knockdown of Mvl is not sufficient to impact the overall metal homeostasis of trace metals, possibly because levels of these metals in the hemolymph and non-neuronal tissues in the head mask neuronal-specific changes. Whether the small effects of the neuronal Mvl knockdown on the levels of other ions are directly related to the transporter activity of Mvl, or whether they represent indirect, downstream effects of the knockdown on other ionic transport pathways was not investigated further in this study.

Mvl expression in the dopaminergic system is required for normal food choice behavior

Feeding decisions in all animals, including flies, are driven by complex neuronal circuits, and depend on the integration of diverse internal and external signals (Albin et al. 2015; Flood et al. 2013; Huetteroth et al. 2015; Itskov & Ribeiro 2013; LeDue et al. 2015; Perisse et al. 2016; Yapici et al. 2016). To further narrow the possible neuronal populations that require Mvl function for normal feeding decisions, we next drove the Mvl-RNAi construct with GAL4 drivers that are active in specific neuronal subpopulation that have been previously reported to play a role in feeding decisions. Our screen included drivers specific to gustatory receptor (Poxn) neurons (Dahanukar et al. 2001), dopaminergic (TH) neurons (Marella et al. 2012; Melcher & Pankratz 2005), octopaminergic (Tdc2) neurons (Zhang et al. 2013), cholinergic (Cha) neurons (Yapici et al. 2016), neuropeptidergic (dimm) neurons (Kahsai et al. 2010; Taghert & Nitabach 2012; Zhang et al. 2013), Neuropeptide F-expressing (Npf) neurons (Shen & Cai 2001), Adipokinetic hormone-expressing (Akh) neurons (Kim & Neufeld 2015) and the circadian system (Tim) (DiAngelo et al. 2011). This limited screen showed that Mvl knockdown specifically in dopaminergic neurons (TH-GAL4; Fig. 4a and Table S4) phenocopied the abnormal food choice phenotype we observed in animals with pan-neuronal Mvl knockdown (Fig. 1e). In contrast, expression of the Mvl-RNAi in all gustatory receptor neurons (Poxn) or neurons that express the neuropeptide adipokinetic hormone (Akh), which have been previously implicated in the regulation of feeding and metabolism (Itskov & Ribeiro 2013; Pool & Scott 2014), showed a small but significant decrease in feeding error rates relative to wild-type controls (Fig. 4a and Table S4). Unexpectedly, these data indicate that Mvl knockdown has different effects on feeding decisions in different neuronal elements of the feeding decision circuit. Regardless, Mvl gene knockdown in any of the neuronal populations we have examined did not affect the general feeding drive of adult flies, further confirming that the effects observed represents an abnormal decision-making phenotype rather than simply observing the behavior of unhealthy flies (Fig. 4b and Table S5).
Effects of neuronal Mvl knockdown on behavior are not mediated via overall changes in biogenic amine synthesis

Because several of the metal ions that were previously shown to be transported by Mvl function as cofactors in neurotransmitter synthesis, and because of the effect of Mvl knockdown in dopaminergic neurons on food choice behavior, we next investigated the effects of Mvl-RNAi expression on the syntheses of biogenic amines in the fly brain. Using HPLC, we measured levels of dopamine, serotonin and octopamine from the brains of pan-neuronal Mvl knockdown and wild-type flies. Our data show that the overall brain levels of the three primary biogenic amines in the insect brain, dopamine, octopamine and serotonin (Evans 1980; Mesce 2002), were not affected by neuronal Mvl knockdown (Fig. 5a). These data suggest that the effects of Mvl knockdown on behavior are not a direct consequence of dramatic physiological changes in the brain biogenic amines systems.

Since Mvl knockdown in dopaminergic neurons does not seem to affect overall dopamine synthesis, we next asked whether the effects of dopaminergic Mvl knockdown are because of a reduced dopamine release. Previous studies indicated that disruptions of the dopaminergic signaling pathway can lead to a general low appetite (hypophagia) (Krashes et al. 2009; Riemensperger et al. 2011). In agreement with the earlier findings, we found that blocking dopamine release by the ectopic expression of the TNT with the dopaminergic TH-GAL4 driver resulted in general hypophagia (Fig. 5b,c), which is unlike the phenotype we observed in Mvl knockdown animals. We also found that the mRNA levels of ple (the gene encoding TH) were not affected by the expression of the Mvl-RNAi in the dopaminergic system. Together, these data further indicate that the effects of Mvl knockdown in the dopaminergic system on food choice behavior are not likely to be directly mediated via modulation of dopamine synthesis or release (Fig. 5d).

Since Mvl activity does not seem to directly affect dopamine synthesis or release, we also examined the effects of the Mvl knockdown on dopaminergic neuron morphology. As in other animals, the dopaminergic system in the fly is complex and heterogeneous. Previous studies have identified the protocerebral anterior medial neuronal cluster, which innervates the mushroom bodies, as the primary modulatory pathways for the integration of food-rewards in long-term memory (Huetteroth et al. 2015; Liu et al. 2012). Independently, the activity of TH-VUM, a single dopaminergic neuron that innervates the subesophageal ganglion (SOG) taste center, was shown to regulate the appetitive acceptance of sugar rewards (Marella et al. 2012). To test whether Mvl knockdown affected dopaminergic morphology, we expressed a membrane tethered GFP (UAS-CD8::GFP) with the TH-GAL4 line, with or without the UAS-Mvl-RNAi. These studies showed that Mvl knockdown animals exhibit a dramatic reduction in the dopaminergic innervation of both the mushroom bodies and the SOG (Figs. 5f and S2b). By contrast, control animals show extensive innervation of the mushroom bodies in the protocerebrum and TH-VUM signal in the SOG (Figs. 5e and S2a). To further establish a causal link between the action of Mvl in the dopaminergic system and food-choice behaviors, we also attempted to study dopaminergic neuronal morphology and food-choice behavior in flies carrying the previously published Mvl<sup>RTH</sup> allele (D’Souza et al. 1999; Orgad et al. 1998; Rodrigues et al. 1995). However, in our hands, the observed phenotype of flies carrying this allele in two different genetic backgrounds.
was weak and inconsistent (not shown). Nevertheless, our findings suggest that Mvl activity in dopaminergic neurons play a critical role in modulating synaptic connectivity in the dopaminergic system, which possibly affects specific neuronal pathways associated with food-choice decisions.

Discussion

In recent years, studies of the dopaminergic system across animal phyla have indicated that it plays a conserved, important role in regulating cognitive processes associated with decision-making in general, and feeding decisions in particular (Barron et al. 2015; de Bivort & van Swinderen 2016; Khani & Rainer 2016; Stopper & Floresco 2015). Consequently, the highly conserved cellular and biochemical properties of the dopaminergic system across mammals and invertebrates indicate that mechanistic insights gained here are likely relevant to neuronal and behavioral functions of metal transporters in many other animal species, including humans.

We have previously showed that chronic feeding of wild-type flies and honey bees with manganese increased the overall brain levels of dopamine, and adversely affected bee foraging decisions (Sevik et al. 2015). We have interpreted these published data to suggest that the effects of environmental exposure to manganese were mediated via a direct effect on the dopaminergic synthesis pathway. Therefore, we originally expected that the effects of knocking down Mvl, an established manganese transporter, on feeding behavior will be associated with changes in dopaminergic levels as well. Our finding that the adverse behavioral outcomes of dopaminergic Mvl knockdown are not associated with overall changes in dopamine levels could be possibly explained by the insensitivity of currently available assays to very small changes in dopamine levels. Alternatively, these data could indicate that the interaction between the dopaminergic system and metal ion homeostasis is more complex than we originally expected, and may independently affect brain functions at both the developmental and physiological timescales. Nonetheless, our finding that Mvl knockdown in dopaminergic neurons changes their innervation patterns further supports this alternative interpretation.

Previous studies showed that the dopaminergic system in the fly brain is remodeled at the pupal stage during metamorphosis (Budnik & White 1988). However, we still do not know whether the effect of Mvl knockdown on the morphology and innervation patterns of dopaminergic neurons is because of its requirement at the pupal neuronal remodeling stage, or whether it reflects an interaction between Mvl activity and post-pupal neuronal plasticity. In addition, although the focus of this study

Figure 4: Effect of Mvl knockdown in specific neuronal populations. (a) Feeding choice assay. GAL4 drivers: Poxn, all gustatory receptor neurons; TH, dopaminergic neurons; Tdc2, octopaminergic neurons; Cha, cholinergic neurons; dimm, neuropeptidergic neurons; Npf, Neuropeptide F neurons; Akh, Adipokinetic hormone neurons; Tim, circadian neurons. **, p < 0.01. (b) General feeding-drive control experiment. Genotypes as in (a). See Tables S4 and S5 for statistics.
is on the interaction between Mvl and the dopaminergic system, we cannot exclude a possible effect of Mvl knockdown on the morphology of other neuronal classes, and therefore, on other, dopamine-independent behavioral phenotypes.

We found that knocking down Mvl expression specifically in the dopaminergic system is sufficient to phenocopy the pan-neuronal knockdown phenotype. We interpret these data to suggest that, in agreement with previous studies, the dopaminergic system acts as a ‘master switch’, which determines the probability that an animal will respond behaviorally to an appetitive stimulus. The concept of a dopaminergic ‘master switch’ is further supported by the contrary effects of Mvl knockdown specifically in the gustatory sensory system and Npf-expressing neurons, which resulted in an increase in the appetitive preference for sugar relative to control animals. However, to completely understand how Mvl-dependent changes in dopaminergic innervation affect feeding-related decision-making behaviors will require the identification and characterization of the relevant neuronal elements that receive inputs from Mvl-expressing dopaminergic neurons. Nevertheless, these data suggest that Mvl, and its possible effects on metal ion homeostasis, play independent roles in different elements of the neuronal circuit associated with feeding decisions.

Data presented here strongly support the idea that metal ion homeostasis in specific neuronal circuits could have a dramatic impact on neuronal functions and behavior. The conserved biochemical properties of the molecules involved in metal ion homeostasis across distant phylogenetic clades indicate that insights gained in the current studies are likely conserved in other animal species, including humans.


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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher’s web-site:

**Table S1**: Statistical analyses of % food choice error in *Mvl* knockdown vs. wild type flies (Fig. 1c–f).

**Table S2**: Statistical analyses of % feeding controls in *Mvl* knockdown vs. wt flies (Fig. 1c–f).

**Table S3**: Effect of neuronal *Mvl* knockdown on elemental composition of fly heads (Fig. 3).

**Table S4**: Statistical analyses of % food choice error in *Mvl* knockdown vs. wt flies (Fig. 4a).

**Table S5**: Statistical analyses of % feeding controls in *Mvl* knockdown vs. wt flies (Fig. 4b).