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Isoform- and Cell-Specific Function of Tyrosine Decarboxylase in the *Drosophila* Malpighian Tubule

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Summary

The biogenic amine tyramine (TA) is a potent diuretic factor when applied to the Malpighian tubule (MT) of *Drosophila melanogaster*, stimulating both urine production and transepithelial chloride conductance. Isolated MTs can respond not only to TA but also to its precursor, tyrosine; this observation led to the proposal that MTs are able to synthesize TA from applied tyrosine through the action of the enzyme tyrosine decarboxylase (TDC). In the current study it is shown that the non-neuronal isoform of TDC, *Tdc1*, is expressed in the principal cells of the MT. A mutant allele of *Tdc1*, $Tdc1^{f03311}$, was identified that reduced expression of the mature *Tdc1* transcript by greater than 100-fold. MTs isolated from $Tdc1^{f03311}$ homozygous flies showed no significant depolarization of their transepithelial potential (TEP) or diuresis in response to tyrosine while retaining normal sensitivity to TA. By contrast, a previously identified null mutant allele of the neuronal TDC isoform *Tdc2* had no effect on either tyrosine or TA sensitivity. To determine in which cell type of the MT *Tdc1* expression is required, flies were generated carrying a *UAS-Tdc1* transgene and cell-type-specific Gal4 drivers on a $Tdc1^{f03311}$ homozygous background. Rescue of *Tdc1* expression in principal cells fully restored sensitivity to tyrosine whereas expression of *Tdc1* in stellate cells had no rescuing effect. It is concluded that synthesis of TA by *Tdc1* in the principal

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cells of the MT is required for physiological responses to tyrosine. TA synthesis in the MT is the first reported physiological role for *Drosophila Tdc1*.

Introduction

The biogenic amine tyramine (TA) has become recognized as an important modulator of many aspects of invertebrate physiology. Synthesized *via* the decarboxylation of tyrosine, TA has long been known to be a precursor for the synthesis of octopamine (OA); however, the past decade has seen growing evidence that TA has physiological roles independent of OA (Lange, 2009; Roeder, 2005). These include effects on olfactory behavior (Kutsukake et al., 2000), locomotion and flight (Brembs et al., 2007; Saraswati et al., 2004; Vierk et al., 2009), neuromuscular transmission (Kutsukake et al., 2000; Nagaya et al., 2002), sleep (Crocker and Sehgal, 2008), appetite (Nisimura et al., 2005), behavioral responses to cocaine and ethanol (Hardie et al., 2007; McClung and Hirsh, 1999; Scholz, 2005) and muscular contractions (da Silva and Lange, 2008; Donini and Lange, 2004; Huddart and Oldfield, 1982). Receptors have been cloned from *Drosophila* and other insect species that are either specific for TA and not OA (Cazzamali et al., 2005) or preferentially activated by TA over OA (Arakawa et al., 1990; Blenau et al., 2000; Molaei et al., 2005; Ohta et al., 2003; Saudou et al., 1990; Vanden Broeck et al., 1995). Neurons have been identified in both *Drosophila* and locust that display immunoreactivity against TA and not OA, suggesting that they are tyraminergic (Donini and Lange, 2004; Kononenko et al., 2009; Nagaya et al., 2002). Recently, a TA-gated ion channel has been cloned in the nematode *Caenorhabditis elegans* that is responsible for modulating behavioral responses to touch (Alkema et al., 2005; Pirri et al., 2009; Ringstad et al., 2009). In addition to the TA-mediated responses listed above, which all involve the nervous system and neuromuscular transmission, TA has a well-defined role in a non-neural tissue, the Malpighian tubules (MTs) of *Drosophila*.

The insect MTs are blind-ended epithelial tubes that form the initial component of the excretory system and produce urine by transporting ions, small molecules and water from the surrounding hemolymph into the tubule lumen (Beyenbach, 1995; Bradley, 1985; Dow and Davies, 2001; Wessing and Eichelberg, 1978). The main segments of the *Drosophila* MTs, which are responsible for urine

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secretion, consist of two cell types, the principal cells (approximately 85% of the cells in the MT) and the stellate cells (the remaining 15%) (Dow et al., 1994; Dow and Davies, 2001; Sözen et al., 1997). Urine secretion is driven by the coupled action of a H^+ pump and a H^+/alkali metal antiporter in the apical membrane of the principal cells, resulting in the net active transport of potassium into the tubule lumen and the generation of a lumen-positive transepithelial potential (TEP) (Beyenbach, 1995; Dow and Davies, 2001; O'Donnell et al., 1996; Pannabecker, 1995). Chloride ions and water move passively into the lumen down their electrochemical and osmotic gradients (O'Donnell et al., 1996; O'Donnell et al., 1998).

Exposure of isolated *Drosophila* MTs to nanomolar concentrations of TA causes an increase in transepithelial chloride conductance, resulting in a depolarization of the TEP and an increase in urine production (Blumenthal, 2003). This response is similar to that of the diuretic peptide leucokinin, which has been shown to activate receptors on the stellate cells, causing production of inositol trisphosphate, a rise in intracellular calcium levels and a subsequent increase in transepithelial chloride conductance (O'Donnell et al., 1996; O'Donnell et al., 1998; Pollock et al., 2003; Radford et al., 2002; Terhzaz et al., 1999). The responses to TA and leucokinin display cross-desensitization and are both modulated by changes in peritubular osmolality, suggesting that the two factors activate a common signaling pathway (Blumenthal, 2001; Blumenthal, 2005). I have previously shown that MTs can respond not only to TA but also to its synthetic precursor tyrosine, and have hypothesized that the response to tyrosine is due to the synthesis of TA from applied tyrosine within the tubule (Blumenthal, 2003). Three lines of evidence support this hypothesis. First, the MT contains significant levels of tyrosine decarboxylase (TDC) enzymatic activity. Second, the response to tyrosine is blocked by antagonists of the TA receptor, suggesting that it is mediated by this receptor and not a novel tyrosine-activated receptor. Third, the response to tyrosine, but not to TA, is antagonized by D-tyrosine, suggesting that tyrosine does not act as a direct agonist of the TA receptor.

It has previously been shown that the *Drosophila* genome contains two genes that encode TDC: *Tdc1* and *Tdc2* (Cole et al., 2005). The second isoform, *Tdc2*, is expressed specifically in the

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nervous system (Cole et al., 2005). Disruption of its expression results in a variety of behavioral phenotypes. Some of these phenotypes, such as effects on aggression and egg laying, are entirely explainable by the elimination of OA synthesis (Hoyer et al., 2008; Middleton et al., 2006) whereas changes in sleep, locomotion and flight do not phenocopy OA deficiency and suggest an independent role for neuronal TA (Brembs et al., 2007; Crocker and Sehgal, 2008; Hardie et al., 2007). The first TDC isoform, *Tdc1*, is expressed primarily outside of the nervous system, including in the MTs (Cole et al., 2005), but no physiological roles have yet been reported for this gene. The purpose of the current study is to identify the gene encoding TDC in the MT and to test the hypothesis that expression of a TDC gene in the tubule is required for tyrosine-mediated depolarization and diuresis.

Materials and Methods

Fly stocks

Drosophila melanogaster Meigen were maintained on cornmeal/molasses/agar food at 24°C on a 12 h:12 h light:dark cycle. Fly stocks used in these experiments include Canton S, *w;Tdc1*^{f03311} (FBti0051154, FBst1018391, Harvard Exelixis Stock Center, Boston, MA, USA), *w;c42-gal4* (FBti0007122) and *w;c710-gal4* (FBti0009567) (gifts of Dr Julian Dow), *w;P[UAS-Tdc1.C]18.6* (FBst0009314) and *w;P[UAS-Tdc2.C]7.2* (FBst0009315) (Bloomington *Drosophila* Stock Center, Bloomington, IN, USA), and *Tdc2*^{RO54} *cn bw* (FBal0189582), *w;P[Tdc1-GAL4.C]3/TM3, Ser*^{1} (FBst0009312), and *w;P[Tdc2-GAL4.C]2* (FBst0009313) (gifts of Dr Jay Hirsh). *Drosophila* genes studied in these experiments include *rp49* (FBgn0002626), *Tdc1* (FBgn0259977) and *Tdc2* (FBgn0050446).

To eliminate effects of genetic background on TA sensitivity, all lines used in this study were backcrossed for six generations either against Canton S or against a *w* line that had previously been backcrossed against Canton S. With the exception of *Tdc2*^{RO54}, transgenes and insertions were followed during backcrossing by eye color. The *Tdc2*^{RO54} allele was followed by genotyping individual flies with PCR primers specific to the mutant allele: forward primer CCCCACTACGCTGATTCATT, reverse primer TGATCTGGTCCCAGTCTTT.

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It should be noted that after six generations of backcrossing, the *Tdc2*^{RO54} stock still carried the closely-linked *cn*^{1}allele.

All experiments were performed on posterior MTs acutely dissected from adult female flies 6-8 days post-eclosion.

The saline used for dissections and for electrophysiological and urine secretion assays contained (in mmol l^{-1}): 85 NaCl, 20 KCl, 3 $CaCl_2$, 12 $MgSO_4$, 7.5 $NaHCO_3$, 4 NaH_2PO_4, 15 glucose, 10 Hepes, pH 6.75. The osmolality of this saline was 255-265 mosmol as measured with a vapor-pressure osmometer (Wescor, Logan, UT, USA).

Real-time PCR

RNA was isolated from tubules dissected from eight P6-8 female flies using the RNaqueous micro kit (Ambion/Applied Biosystems, Austin, TX, USA) and cDNA prepared using the iScript (BioRad Laboratories, Hercules, CA, USA) or qScript (Quanta Biosciences, Gaithersburg, MD, USA) reverse transcription kits. Conventional PCR was performed with platinum taq (Invitrogen, Carlsbad, CA, USA), and real-time PCR was performed with SYBR Green Fastmix (Quanta Biosciences), 300 nmol l^{-1} each primer and 0.1 µl cDNA in a MyiQ single-color thermocycler (BioRad Laboratories). Real-time data were quantified by the dilution curve method using MyiQ software. Primers for PCR amplification (Integrated DNA Technologies, Coralville, IA, USA) included *rp49* forward AAGATCGTGAAGAAGCGCACCAA, *rp49* reverse CTGTTGTCGATACCCTTGGGCTT, *Tdc1* forward A TTGAGGTTCGCAACGATGTTC, *Tdc1* reverse A AAGCACTTTATCTGGGTCCAAGC, *Tdc1* forward B CCAAGCACCAACAGATACTTCT, and *Tdc1* reverse B GGGAGCGTCGGCTGGTAG.

Confirmation of the Tdc1^{f03311} *insertion*

DNA was isolated from individual female *Tdc1*^{f03311} homozygotes as described (Gloor et al., 1993). PCR was performed with a forward primer (GTCGAGTGTGCCCCAATTAT) specific to the first intron of *Tdc1* and a reverse primer (CAGTGACACTTACCGCATTGACAAGCACGC)

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specific to the piggyback transposon. Based on the reported position of the insertion site, a 426 bp amplification product was expected.

Electrophysiological recording

Tubules were dissected under saline and placed in a tissue culture dish in which a 100 µl drop of 0.03 mg ml^{-1} poly-L-lysine had been dried. This concentration of poly-L-lysine is 8-fold more dilute than had been used previously (Blumenthal, 2003) and results in more consistently healthy tubules. The TEP was recorded immediately after dissection by impaling the tubule lumen with a sharp electrode (R>25 MΩ) pulled from theta-glass (Sutter Instruments, Novato, CA, USA) and filled with 3 mol l^{-1} KCl. Potentials were amplified (IX1, Dagan Corporation, Minneapolis, MN, USA), filtered at 20 Hz, digitized at 100 Hz and stored online. Recording and analysis was conducted using pClamp 9 software (Axon Instruments, Sunnyvale, CA, USA). The peritubular bath was continuously perfused during recording. The bath input was positioned close to the tubule and drugs were applied by switching the perfusate with a valve controller (Warner Instruments, Hamden, CT, USA). Drugs were applied for 45 s, and multiple applications were separated by 3 min (from the end of one application to the beginning of another). To reduce noise, all electrophysiological traces shown in figures underwent 10-fold data reduction, substituting the mean value.

The response index, a measure of the TEP response to pulses of drugs was calculated as follows. The area under the voltage curve was calculated for a period beginning 5 s after drug application began and ending 5 s after drug application stopped, using −10 mV as the baseline (drug area). For the same period, the area was calculated under a line extrapolated from the voltage trace for the 30 s immediately preceding drug application (control area). The response index was calculated as (control area—drug area)/control area. This technique is modified slightly from a previous report (Blumenthal, 2003), in which the analysis period began and ended 15 s after the solution changes.

Because the response index is a percentage and is therefore constrained in its distribution, data were transformed prior to statistical analysis by taking the arcsin of the square root of each

response index value. Negative values were transformed by taking the negative arcsin of the square root of the absolute value of the response index. When a tubule was treated with both tyrosine and TA, the ratio of the two response indices was calculated and then transformed. For the figures, the mean, mean+s.e.m. and mean—s.e.m. for each transformed data set were reverse-transformed and used for the graphs.

Urine secretion assays

Urine secretion rates were measured as described (Blumenthal, 2003; Dow et al., 1994). Tubules were dissected in saline and placed in a 15 µl droplet of saline under mineral oil. One branch of the tubule was pulled out of the droplet and wrapped around a pin such that the cut end of the ureter and lower section of the other branch were out of the aqueous droplet. At intervals of 12-20 min, the secreted urine droplet was removed from the ureter with a glass rod and its diameter measured with an ocular micrometer. The volume of the urine droplet was calculated assuming spherical geometry. Solution changes were made immediately before the removal of the urine droplet for the second interval by twice removing 12 µl of saline and replacing it with 12 µl of new solution. Secretion rates for each tubule were normalized to the mean rate for that tubule over the first two collection intervals.

Data analysis and statistics

Data were graphed and analyzed using GraphPad Prism 4.03 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com) software. Statistical significance was defined as $P \leq 0.05$. Each data set was tested for a Gaussian distribution using the Kolmogorov—Smirnov test. Data sets which were non-Gaussian were compared with other groups using non-parametric tests as described in the text.

Results

The two isoforms of TDC in *Drosophila, Tdc1* and *Tdc2*, are reported to be expressed primarily in the periphery and central nervous system (CNS), respectively (Cole et al., 2005). According to

the FlyAtlas microarray database (Chintapalli et al., 2007), *Tdc1* is expressed at approximately 35-fold higher levels than *Tdc2* in the adult MT; indeed, the MT, along with the crop and hindgut, is one of the three tissues with the highest expression of *Tdc1*. The Gal4-UAS binary expression system (van Roessel and Brand, 2000) was used to determine in which cell type within the MT *Tdc1* is expressed. Expression of the Green Fluorescent Protein (GFP) was driven by a *Tdc1-gal4* transgene, in which expression of the Gal4 transcriptional activator is controlled by the *Tdc1* promoter (Cole et al., 2005). As shown in Fig. 1, strong GFP expression was observed in the principal cells of the tubule, and expression was absent in the stellate cells. When GFP expression was driven by a *Tdc2-gal4* transgene, fluorescence was observed in the ventral nerve cord and in neuronal processes innervating the oviduct, as previously reported (Cole et al., 2005), but no fluorescence was observable in the MT (data not shown).

A stock was obtained from the Exelixis transposon insertion collection that is reported to carry a piggyBac transposon inserted into the first intron of *Tdc1* (Fig. 2A) (Thibault et al., 2004). The location of this insertion, $Tdc1^{f03311}$, was confirmed by PCR on genomic DNA using one primer specific to the transposon and a second specific to the intron; a PCR product of the predicted size was obtained (data not shown). Flies homozygous for $Tdc1^{f03311}$ were viable and did not exhibit any gross morphological or behavioral abnormalities; however, homozygous females were sterile. To determine whether the transposon insertion disrupted splicing or expression of the *Tdc1* transcript, conventional reverse transcription PCR (RT-PCR) was performed on MT-specific cDNA. As shown in Fig. 2, a primer set spanning the second intron (primer set A) gave a very weak but detectable product from $Tdc1^{f03311}$ homozygote cDNA. Real-time RT-PCR using this primer set gave a single specific product from wild-type and heterozygote cDNA, but the product from two independent homozygous cDNA preparations was contaminated with primer—dimer, making precise quantification impossible (data not shown). However, because amplification of heterozygous cDNA did not yield primer—dimer even when it was diluted 100-fold, I conclude that the level of *Tdc1* transcript in the $Tdc1^{f03311}$ homozygotes is reduced by a factor of >100 relative to heterozygotes. A second primer set (primer set B) that was designed to yield an amplification product only when exons 1

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and 2 were properly spliced was also used. As shown in Fig. 2, this primer set failed to produce any amplification product from homozygous cDNA in conventional RT-PCR; primer set B could not be used for real-time RT-PCR, however, as it did not produce a single specific product, even from wild-type or heterozygous cDNA. Thus, although the effect of the *Tdc1*^{f03311} insertion on mRNA expression and splicing could not be quantified precisely, the data demonstrate that it is either an extremely hypomorphic or null allele.

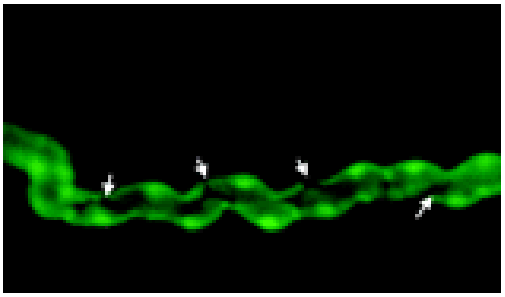

Fig. 1. Fluorescence image of a MT dissected from a P7 *w;UAS-GFP/+;Tdc1-Gal4/+* female. Staining is specific to the principal cells; arrows indicate unstained stellate cells. This pattern of staining was observed in MTs dissected from six flies from three independent crosses.

If the response of MTs to tyrosine is dependent upon the synthesis of TA by TDC, then one would predict that TDC-deficient tubules would be selectively insensitive to tyrosine but not TA. Fig. 3 shows the TEP depolarization responses of heterozygous and homozygous mutant tubules to applications of 1 mmol l^{-1} tyrosine and 100 nmol l^{-1} TA. While the tubule isolated from a *Tdc1*^{f03311}*/CyO* heterozygote responded to both agonists (Fig. 3A left), the homozygous mutant tubule responded only to the TA application (Fig. 3A right). On average, the ratio of responses to 1 mmol l^{-1} tyrosine and 100 nmol l^{-1} TA was significantly lower in *Tdc1*^{f03311} homozygotes than in heterozygotes, and that ratio was not significantly different from zero in the homozygotes (Fig. 3C, see figure legend for statistics). Depolarizing responses to 1 mmol l^{-1} tyrosine were occasionally observed in homozygous mutant tubules, but these responses were always tonic depolarizations that did not resemble the oscillatory responses always seen in heterozygotes. In contrast to the dramatic effect of the *Tdc1*^{f03311} mutation, tubules homozygous for a null mutation in *Tdc2, Tdc2*^{RO54} (Cole et al., 2005), responded to both

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tyrosine and TA to an extent identical to *Tdc2*^{RO54}*/CyO* heterozygotes (Fig. 3B,C). To more accurately quantify the effects of the *Tdc* mutants, dose—response curves were generated to tyrosine and TA for tubules heterozygous and homozygous for the mutations in each isoform. As shown in Fig. 4, the dose—response curves to TA and tyrosine were identical among *Tdc1*^{f03311} heterozygotes and *Tdc2*^{RO54} homozygotes and heterozygotes. By contrast, *Tdc1*^{f03311} homozygotes showed a dose—response curve to TA that was indistinguishable from the other three genotypes but failed to produce a significant depolarizing response to either 0.1 mmol l^{-1} or 1 mmol l^{-1} tyrosine (see Fig. 4 legend for statistics).

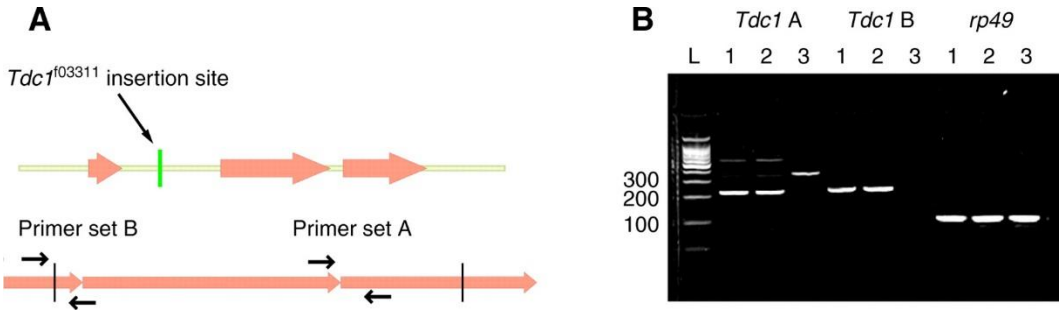

Fig. 2. (A) Schematic of the *Tdc1* gene, showing approximately 3.5 kb of genomic sequence (top) and 2.2 kb of transcript (bottom). The exons (orange arrows), translational start and stop sites (vertical lines on lower schematic) and site of the insertion of a PiggyBac transposon in the *Tdc1*^{f03311} allele are shown. (B) Loss of properly spliced *Tdc1* transcript in the *Tdc1*^{f03311} mutant. Reverse transcription PCR (RT-PCR) was performed from Malpighian tubule (MT)-specific cDNA isolated from Canton S (1), w;*Tdc1*^{f03311}*/CyO* (2), and w;*Tdc1*^{f03311}*/Tdc1*^{f03311} (3) females with two sets of primers to *Tdc1*: primer set A, which amplifies a 217 bp product from spliced cDNA and a 339 bp product from genomic DNA, and primer set B, which amplifies a 220 bp product only when exons 1 and 2 are correctly spliced. Primers to the ribosomal gene *rp49* were used as a control.

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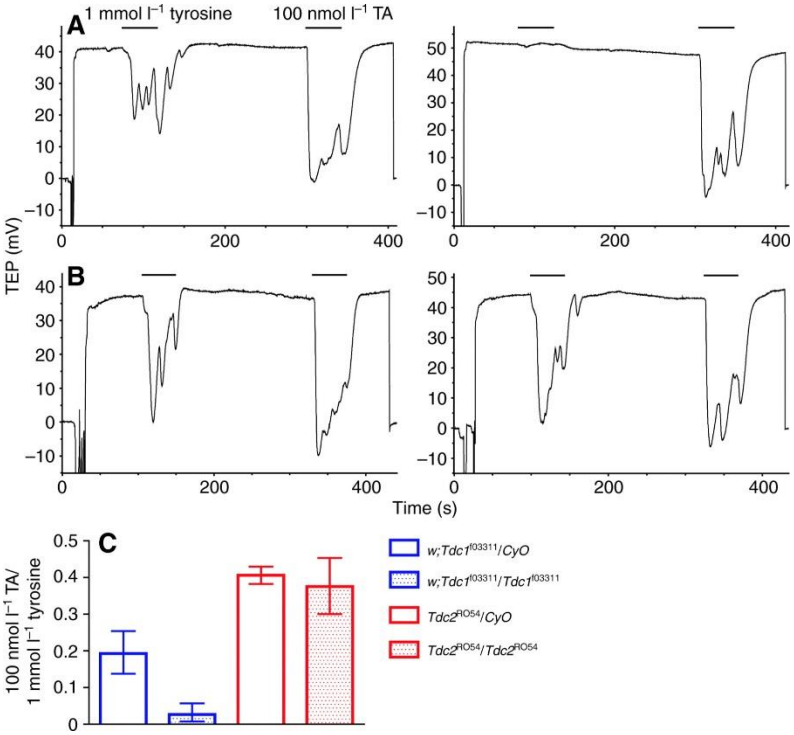

Fig. 3. Transepithelial potential (TEP) recordings from tubules isolated from *Tdc1*^{f03311} (A) and *Tdc2*^{RO54} (B) heterozygotes (left) and homozygotes (right). Each tubule was challenged with 45 s applications of first 1 mmol l^{-1} tyrosine and then 100 nmol l^{-1} tyramine (TA) as indicated by the bars. (C) Ratio of tyrosine and TA responses in individual tubules. The graph shows the ratio of the response indices in individual tubules. *N*=8-12 tubules/genotype, error bars on this and subsequent figures indicate s.e.m. (see Materials and methods for transformation and analysis of electrophysiological data). A one-way analysis of variance (ANOVA) showed a significant difference among the four genotypes ($P<0.0001$), and a Tukey's post-hoc test showed that the *Tdc1*^{f03311} homozygotes were significantly different from the other three groups ($P<0.05$), and there were no differences among the other three groups. A one-sample t-test showed that the ratio in *Tdc1*^{f03311} homozygotes was not significantly different from zero ($P>0.05$).

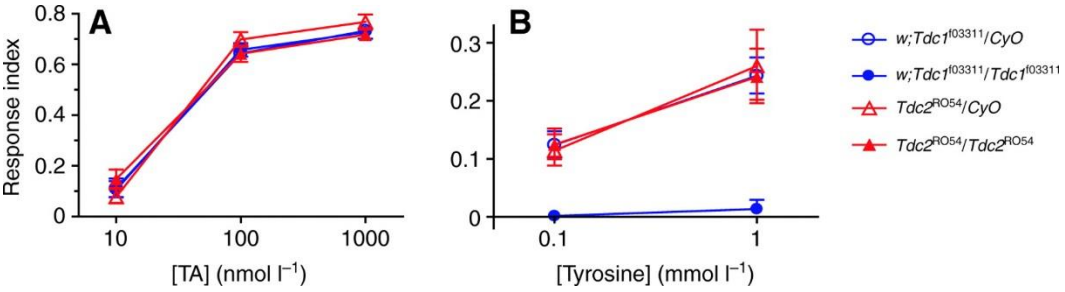

Fig. 4. Dose—response curves for the transepithelial potential (TEP) depolarization of *Tdc1*^{f03311} and *Tdc2*^{RO54} heterozygotes and homozygotes in response to tyramine (TA) (A) and tyrosine (B). Each tubule was challenged with all three TA concentrations or both tyrosine concentrations, in order of increasing concentration. *N*=7-8

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tubules/agonist. (A) Two-way analysis of variance (ANOVA) showed no significant effect of genotype. Because the 1000 nmol l^{-1} data set from the *Tdc1*^{f03311} heterozygotes was non-Gaussian, the 1000 nmol l^{-1} data sets were also compared with a non-parametric Kruskal—Wallis test, which showed no differences among the four genotypes. (B) Two-way ANOVA showed a significant effect of genotype ($P<0.0001$), and a Bonferroni post-hoc test showed significant differences ($P<0.001$) between *Tdc1*^{f03311} homozygotes and all others at both concentrations of tyrosine. Because the 0.1 mmol l^{-1} data set from the *Tdc2*^{RO54} heterozygotes and the 1 mmol l^{-1} data set from the *Tdc2*^{RO54} homozygotes were non-Gaussian, the responses at each concentration were also compared with a Kruskal—Wallis test and Dunn's post-hoc test, which confirmed the significant ($P<0.01$) differences only between *Tdc1*^{f03311} homozygotes and the other three genotypes at both tyrosine concentrations. A one-sample t-test showed that the responses of *Tdc1*^{f03311} homozygotes were not significantly different from zero at either concentration of tyrosine.

Urine secretion rates were measured from tubules of the four genotypes described above to determine whether *Tdc1*^{f03311} homozygotes also lacked the diuretic response to tyrosine. The results are shown in Fig. 5. Mutation of *Tdc2* had no effect on urine secretion; there were no differences between *Tdc2*^{RO54} homozygotes and heterozygotes in their diuretic responses to either 1 mmol l^{-1} tyrosine or 1 μmol l^{-1} TA (see Fig. 5 legend for statistics). However, disruption of *Tdc1* expression resulted in selective insensitivity to tyrosine just as in the TEP measurements above. While MTs from *Tdc1*^{f03311} heterozygotes showed a significant, if transient, diuresis in response to 1 mmol l^{-1} tyrosine, homozygous tubules showed no response. Indeed, the secretion profiles of *Tdc1*^{f03311} homozygotes treated with 1 mmol l^{-1} tyrosine or kept in saline were identical. As expected, there was no difference between *Tdc1*^{f03311} homozygotes and heterozygotes in the diuretic response to 1 μmol l^{-1} TA.

To test whether *Tdc1* expression is required in the principal cells or stellate cells of the MT for normal sensitivity to tyrosine, the Gal4-UAS system was once again used to perform cell-type-specific rescue of *Tdc1* expression. Two different Gal4 drivers were used: *c42-gal4*, which drives expression in the principal cells, and *c710-gal4*, which drives expression in the stellate cells (Sözen et al., 1997). Each driver was combined with a *UAS-Tdc1* transgene on a *Tdc1*^{f03311} homozygous mutant background. The data in Fig. 6 show that when both the *c42-gal4* driver and the *UAS-Tdc1* transgene were present, depolarizing responses to 1 mmol l^{-1} tyrosine were restored to the same amplitude seen in *Tdc1*^{f03311} heterozygotes; by contrast, driving *Tdc1* expression in the stellate cells did not result in any rescue (see Fig. 6 legend for statistics). Similarly, tyrosine-mediated diuresis was also fully rescued

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when *Tdc1* expression was driven in the principal cells whereas no rescue resulted from expression in the stellate cells (Fig. 7). Induction of *Tdc1* expression in the principal cells actually resulted in a more long-lasting diuresis to 1 mmol l^{-1} tyrosine than was seen in heterozygotes (see Discussion). Finally, I wished to test whether the sensitivity of MTs to tyrosine could also be rescued by expression of *Tdc2*, as others have shown the inverse, i.e. that *Tdc1* is able to rescue the effects of the $Tdc2^{RO54}$ mutation in the CNS (Cole et al., 2005; Crocker and Sehgal, 2008; Hardie et al., 2007; Hoyer et al., 2008). Unfortunately, induction of a *UAS-Tdc2* transgene with either of the Gal4 drivers proved to be lethal.

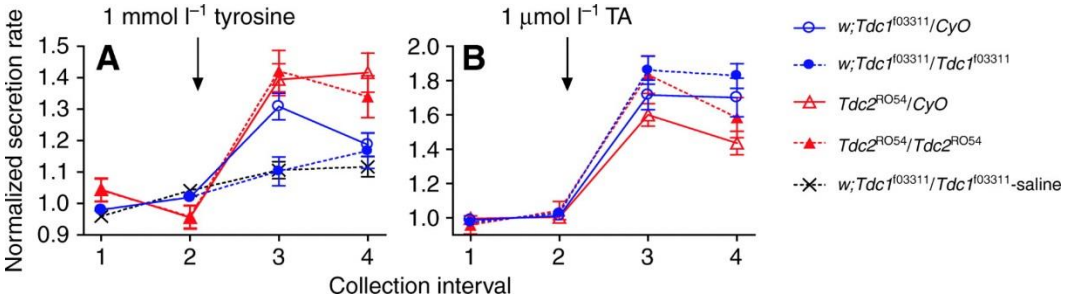

Fig. 5. Urine secretion rates of tubules from $Tdc1^{f03311}$ and $Tdc2^{RO54}$ mutant homozygotes and heterozygotes. At the end of the second collection interval, tubules were treated with either 1 mmol l^{-1} tyrosine (A) or 1 µmol l^{-1} tyramine (TA) (B). One group of $Tdc1^{f03311}$ homozygotes was left in saline as a control (A). N=9-11 tubules/condition. In A, a two-way analysis of variance (ANOVA) showed a significant effect of genotype in the response to tyrosine ($P<0.0001$), and a Bonferroni post-hoc test showed no differences between $Tdc2^{RO54}$ homozygotes and heterozygotes or between $Tdc1^{f03311}$ homozygotes treated with tyrosine and with saline. Because the data sets from intervals 3 and 4 of the $Tdc1^{f03311}$ heterozygotes were non-Gaussian, these data were compared with those of the $Tdc1^{f03311}$ homozygotes by non-parametric Mann-Whitney tests; the homozygotes and heterozygotes differed significantly at interval 3 ($P<0.05$) but not at interval 4. In B, a two-way ANOVA showed no effect of genotype on secretion. Because the data set from interval 3 of the $Tdc1^{f03311}$ heterozygotes was non-Gaussian, the data from this interval were also compared by a non-parametric Kruskal—Wallis test and no significant effect of genotype was found. Initial secretion rates (mean ± s.e.m. in nl min^{-1}) for the groups in A were: $Tdc2^{RO54}/CyO$, 0.38±0.06; $Tdc2^{RO54}/Tdc2^{RO54}$, 0.62±0.09; $w;Tdc1^{f03311}/CyO$, 0.81±0.06; $w;Tdc1^{f03311}/Tdc1^{f03311}$, 0.74±0.06; $w;Tdc1^{f03311}/Tdc1^{f03311}$ (saline), 0.82±0.04. A one-way ANOVA of these rates showed significant differences ($P<0.0001$); a Tukey post-hoc test indicated that the only significant differences were between $Tdc2^{RO54}$ heterozygotes and the three $Tdc1^{f03311}$ groups. Initial secretion rates for the groups in B were: $Tdc2^{RO54}/CyO$, 0.62±0.07; $Tdc2^{RO54}/Tdc2^{RO54}$, 0.68±0.06; $w;Tdc1^{f03311}/CyO$, 0.76±0.07; $w;Tdc1^{f03311}/Tdc1^{f03311}$, 0.84±0.10. A one-way ANOVA showed no significant differences among these rates.

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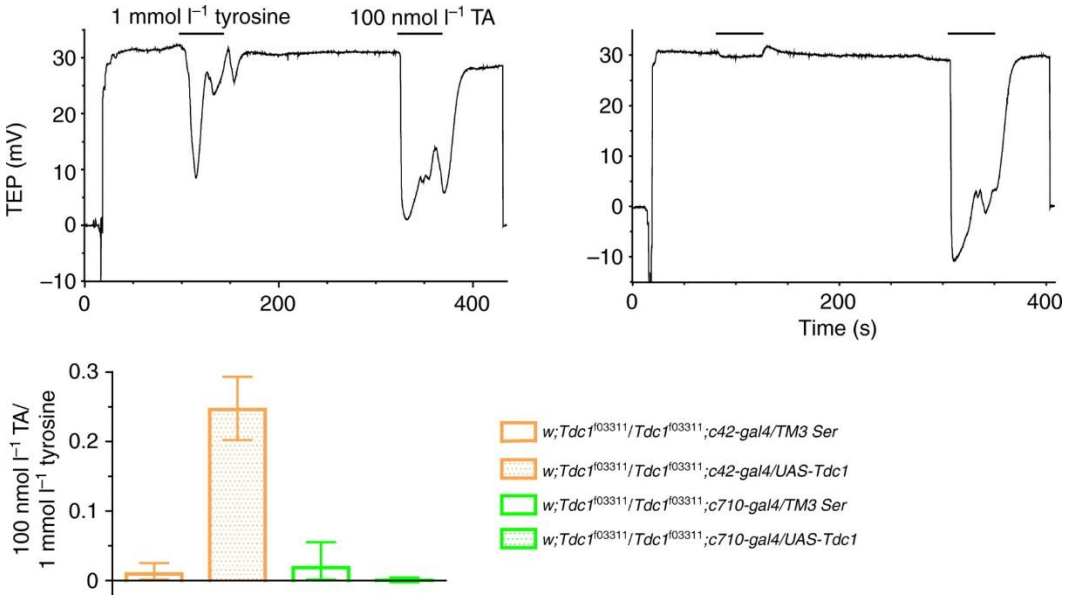

Fig. 6. Cell-specific rescue of the *Tdc1*^{f03311} electrophysiological phenotype. Representative transepithelial potential (TEP) recordings are shown from a *w;Tdc1*^{f03311}*/Tdc1*^{f03311}*;c42-gal4/UAS-Tdc1* (A) and *w;Tdc1*^{f03311}*/Tdc1*^{f03311}*;c710-gal4/UAS-Tdc1* (B) tubule challenged with 1 mmol l^{-1} tyrosine and 100 nmol l^{-1} tyramine (TA) as in Fig. 3. (C) Ratios of tyrosine to TA responses as in Fig. 3. *N*=8 tubules/genotype. A one-way analysis of variance (ANOVA) showed a significant effect of genotype (*P*=0.0001), and a Tukey's post-hoc test showed a significant difference only between *w*;*Tdc1*^{f03311}*/Tdc1*^{f03311}*;c42-gal4/UAS-Tdc1* and the other three genotypes (*P*<0.01). The response ratios for the other three genotypes were not significantly different from zero (one-way *t*-test). The response ratio of the *w*;*Tdc1*^{f03311}*/Tdc1*^{f03311}*;c42-gal4/UAS-Tdc1* tubules was not different from that of *w*;*Tdc1*^{f03311}*/CyO* tubules shown in Fig. 3 (unpaired *t*-test).

Discussion

I have shown that *Tdc1* expression is required for normal sensitivity of the *Drosophila* MT to tyrosine. Tubules isolated from *Tdc1*^{f03311} homozygotes, which contain less than 1% of the normal amount of *Tdc1* mRNA, show no depolarizing or diuretic responses to 1 mmol l^{-1} tyrosine. This concentration of tyrosine elicited robust responses from heterozygous tubules and is near the solubility limit for tyrosine. Disruption of *Tdc1* expression had no significant effect on either the depolarizations or the diuresis caused by TA application. These data, combined with my earlier demonstration that tyrosine responses could be blocked by antagonists of the TA receptor (Blumenthal, 2003), provide strong evidence that *Tdc1* is required for the synthesis of TA from applied tyrosine in the MT. This represents

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the first demonstration of a physiological function for the *Tdc1* gene in *Drosophila* and for a peripherally expressed TDC isoform in any invertebrate.

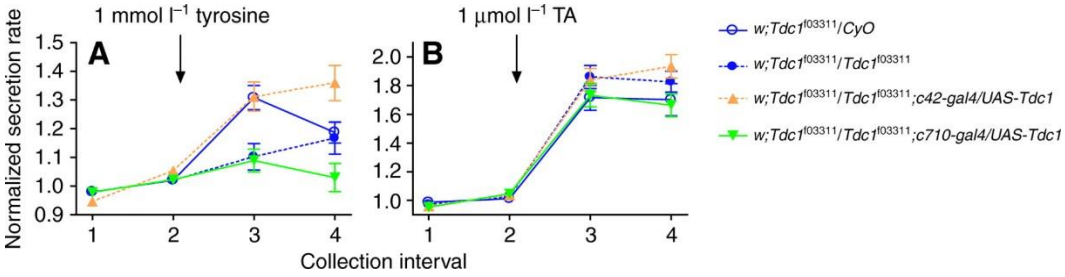

Fig. 7. Cell-specific rescue of the *Tdc1*^{f03311} urine secretion phenotype. At the end of the second collection interval, tubules were treated with either 1 mmol l^{-1} tyrosine (A) or 1 µmol l^{-1} tyramine (TA) (B). Data from Fig. 5 for Tdc1^{f03311} homozygotes and heterozygotes are included for comparison. *N*=10 tubules/condition. In A, the four genotypes were compared by nonparametric Kruskal—Wallis tests due to the non-Gaussian distribution of *w*;*Tdc1*^{f03311}/*CyO* intervals 3 and 4. Significant effects of genotype were seen in both intervals (*P*<0.005). Dunn's *post-hoc* test showed that in both intervals, *w*;*Tdc1*^{f03311}/*Tdc1*^{f03311};*c42-gal4/UAS-Tdc1* differed from *w*;*Tdc1*^{f03311}/*Tdc1*^{f03311};*c710-gal4/UAS-Tdc1*, and in interval 3, *w*;*Tdc1*^{f03311}/*Tdc1*^{f03311};*c42-gal4/UAS-Tdc1* also differed from *w*;*Tdc1*^{f03311}/*Tdc1*^{f03311}and *w*;*Tdc1*^{f03311}/*Tdc1*^{f03311};*c710-gal4/UAS-Tdc1* differed from *w*;*Tdc1*^{f03311}/*CyO*. In B, the four genotypes were again compared by Kruskal—Wallis tests due to the non-Gaussian distribution of *w*;*Tdc1*^{f03311}/*CyO* interval 3 and *w*;*Tdc1*^{f03311}/ *Tdc1*^{f03311};*c710-gal4/UAS-Tdc1* intervals 3 and 4. There were no significant effects of genotype at interval 3. At interval 4, there was a significant effect of genotype (*P*=0.04), but a Dunn's *post-hoc* test showed no differences between any two genotypes. Initial secretion rates (mean ± s.e.m. in nl min^{-1}) for the groups in A were: *w*;*Tdc1*^{f03311}/*Tdc1*^{f03311};*c42-gal4/UAS-Tdc1*, 0.77±0.04; *w*;*Tdc1*^{f03311}/*Tdc1*^{f03311};*c710-gal4/UAS-Tdc1*, 0.81±0.05 (see Fig. 5 legend for other rates). A one-way ANOVA of the rates showed no significant differences. Initial secretion rates for the groups in B were: *w*;*Tdc1*^{f03311}/*Tdc1*^{f03311};*c42-gal4/UAS-Tdc1*, 0.84±0.07; *w*;*Tdc1*^{f03311}/ *Tdc1*^{f03311};*c710-gal4/UAS-Tdc1*, 0.90±0.04 (see Fig. 5 legend for other rates). A one-way ANOVA showed no significant differences among these rates.

In contrast to the phenotype observed upon disruption of *Tdc1*, there was no effect of mutating the neuronal isoform *Tdc2*. The *Tdc2*^{RO54} mutation has previously been shown to be a null allele (Cole et al., 2005), but no differences were detected between *Tdc2*^{RO54} heterozygotes and homozygotes in responses to either tyrosine or TA. This lack of a phenotype is consistent with the low level of *Tdc2* expression in the MT reported in the microarray database (Chintapalli et al., 2007) and the inability of the *Tdc2-gal4* transgene to drive detectable reporter gene expression in the MT. There is no evidence at present, therefore, for a functional role of *Tdc2* in the MT.

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Expression of *Tdc1* in the principal cells of the tubule is required for tyrosine sensitivity. Based on the observation that immunostaining with an anti-TA antibody selectively labels the principal cells, I had previously speculated that these cells were the site of TA synthesis (Blumenthal, 2003). While the current work does not identify the actual location of TA synthesis, which would require immunolocalization of TDC1 protein, it does show that expression of the *Tdc1* gene in the principal cells and not the stellate cells is sufficient to allow application of tyrosine to cause the activation of TA receptors. This finding is consistent with the localization of *Tdc1-gal4* driven reporter gene expression to the principal cells. In all but one respect, driving *Tdc1* expression in the principal cells resulted in tubules that behaved identically to $Tdc1^{f03311}$ heterozygotes; the exception was the duration of tyrosine-mediated diuresis, which was transient in the heterozygotes but appeared to be more sustained in the rescued tubules (Fig. 7). In this respect, the rescued tubules more closely resembled $Tdc2^{RO54}$ heterozygotes and homozygotes, which also showed a sustained tyrosine-mediated diuresis (Fig. 5). It is possible that this difference is related to the expression level of *Tdc1*; that disruption of one copy of the gene in the heterozygous mutants lowers the level of TDC1 protein below that required for a sustained diuresis and Gal4-mediated overexpression of the gene reverses this deficit.

Does tyrosine have any effect on the MT beyond being a substrate for TDC? In insects, tyrosine can also serve as a substrate for the synthesis of tyrosine glucosides (Wright, 1987), one of which has recently been identified as a humoral factor in the silkmoth *Bombyx mori* (Ohnishi et al., 2005). In addition, tyrosine can serve as a substrate for amino acid transporters, including some members of the iNAT family of electrogenic transporters (Meleshkevitch et al., 2006; Miller et al., 2008); several transporters in this gene family are known to be expressed at high levels in the *Drosophila* MT (Chintapalli et al., 2007; Mueller and Blumenthal, 2007; Thimgan et al., 2006). In the current work, however, tyrosine application caused no significant depolarization or diuresis in *Tdc1* mutant tubules; thus, it appears that the only route through which applied tyrosine can cause diuresis or rapid changes in the TEP is through the production of TA.

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Because *Drosophila* hemolymph contains a significant concentration of tyrosine (Pierce et al., 1999), it is likely that MTs *in vivo* are tonically stimulated by endogenously produced TA. The current work has shown that *Tdc1* expression is required for the tubule to respond to tyrosine; therefore, regulation of *Tdc1* expression and the activity of the encoded enzyme are potential mechanisms by which urine secretion could be modulated in the intact fly. At present, nothing is known about the factors that regulate either levels of *Tdc1* transcript or the enzymatic activity of its protein product, but such questions would now be interesting to study in the context of excretory function and osmoregulation.

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Footnotes

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Abbreviations

GFP green fluorescent protein
MT Malpighian tubule
OA octopamine
TA tyramine
TDC tyrosine decarboxylase
TEP transepithelial potential

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