

High-NaCl Perception in *Drosophila melanogaster*

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Salt is a fundamental nutrient that is required for many physiological processes, including electrolyte homeostasis and neuronal activity. In mammals and *Drosophila*, the detection of NaCl induces two different behaviors: low-salt concentrations provide an attractive stimulus, whereas high-salt concentrations are avoided. We identified the gene called *serrano* (*sano*) as being expressed in the sensory organs of *Drosophila* larvae. A transgenic reporter line showed that *sano* was coexpressed with *Gr66a* in a subset of gustatory neurons in the terminal organ of third-instar larvae. The disruption of *sano* gene expression in gustatory neurons led to the specific loss of high-salt concentration avoidance in larvae, whereas the detection of other attractive or aversive substances was unaffected. Moreover, using a cellular marker sensitive to calcium levels, *Sano* function was shown to be required for neuronal activity in response to high-salt concentrations. In these neurons, the loss of the DEG/ENaC channel PPK19 function also eliminated the cellular response to high-salt concentrations. Our study revealed that PPK19 and *Sano* are required in the neurons of the larval gustatory organs for the detection of high-salt concentrations.

Key words: behavior; chemosensory system; *Drosophila melanogaster*; larva; salt; taste

Introduction

Gustatory information is considered to be important for the control of animal behaviors, such as searching for food or sexual partners. In *Drosophila*, larvae sense olfactory and gustatory cues with the three major chemosensory organs located on the head surface, dorsal organ (DO), terminal organ (TO) and ventral organ (VO), and three pharyngeal organs (Vosshall and Stocker, 2007). Olfactory receptor neurons located in the DO project into glomeruli of the antennal lobe, whereas gustatory receptor neurons (GRNs) project via four different nerves to the suboesophageal ganglion (Vosshall and Stocker, 2007).

Insects, like mammals, are able to detect and discriminate among different gustatory stimuli, such as sugars, bitter substances, and salts, that can induce an attractive or a repulsive response in behavioral tests. Electrophysiological studies performed on *Drosophila* adult taste sensilla have revealed that low- and high-NaCl concentrations are detected by two distinct gustatory neurons (Hiroi et al., 2004; Amrein and Thorne, 2005; Ishimoto and Tanimura, 2004). Larvae are also able to display an

attractive behavior in response to low-salt concentrations and an aversive response in the presence of high-salt concentrations (Heimbeck et al., 1999; Balakireva et al., 2000; Gerber and Stocker, 2007; Niewalda et al., 2008; Russell et al., 2011). The *dpr* locus (for defective proboscis extension response), a member of the Ig superfamily, is required for the aversive response to high-salt concentrations in adult flies (Nakamura et al., 2002). Two members of the *pickpocket* (*ppk*) gene family (DEG/ENaC channels, *ppk19* and *ppk11*) have been implicated in the responses to low- and high-NaCl concentrations (Liu et al., 2003). In mammals, the detection of NaCl also requires the expression of ENaC protein in a subset of the taste receptor cells in the taste buds (Chandrashekar et al., 2010). More recently, Zhang et al. (2013) suggested that NaCl perception in *Drosophila* adults is determined by a bimodal switch system operating in GRNs that allows for detecting separately low- and high-NaCl concentrations. Moreover, they demonstrated that the ionotropic channel IR76b is selectively involved in the attractive pathway.

In this study, we describe the *serrano* (*sano*) locus, which we identified by the specific loss of the aversive response to high-NaCl concentrations in the mutant larvae; the responses to other attractive (sucrose or low-NaCl concentrations) and aversive (caffeine) stimuli remained unaltered. The *sano* locus encodes a putative cytoplasmic protein of 778 aa, which is related to the Themis gene family (Chung et al., 2009; Johnson et al., 2009). In our study, we show that *Sano* function is specifically required in the larval peripheral nervous system for the detection of high-NaCl concentrations and the subsequent induction of aversive behavior. Moreover, we show that *Sano* is necessary for neuronal activity of GRNs present in the TO of the larvae in response to high salt. Thus, *Sano* plays a key role in the detection of high-NaCl concentrations through the gustatory neurons of the TO.

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Materials and Methods

Fly strains. The fly strains used in this study were the wild-type strain CantonS, the *sano*^{Gal4} strain (identified from lines generated in a previous screen and provided by Prof. Denise Busson, Université Pierre et Marie Curie, Paris, France), the *sano*^{GE12233} and *sano*^{GE15762} strains (purchased from Genexel), and the *UAS-TNT* (tetanic toxin light chain), *10XUAS-IVS-mCD8::GFP*, *10XUAS-IVS-mCD8::RFP*, *tubP-Gal80^{ts}*, and *UAS-Cameleon2.1 (Cam2.1)* strains (from the Bloomington Stock Center). The *UAS-ppk19^{dsRNA}* strain was kindly provided by Dr. Michael J. Welsh (Howard Hughes Medical Institute, University of Iowa, Iowa City, IA). The *Gr66a-GFP* and *Gr66a^{Gal4}* strains were kindly provided by Dr. Kristin Scott (Howard Hughes Medical Institute, University of California, Berkeley, Berkeley, CA) and Dr. Hubert Amrein (Texas A&M Health Science Center, College Station, TX), respectively. The strains used in behavioral tests have been outcrossed to a *w¹¹¹⁸* strain for five generations.

The generation of transgenic flies. The *sano* ORF was amplified from the cDNA clone RE56731 (Berkeley *Drosophila* Genome Project), which contained the full RB cDNA, and was cloned into the pGEM-T vector for sequencing and into the pUAST vector to generate the *UAS-sano* lines.

A fragment of 575 bp from the exon 7 sequence was amplified from the cDNA clone RE56731 with the primers 5'-tctagaactgcgactctggctgc and 5'-ctagacggaggtggctgctgg and cloned into the pGEM-T vector for sequencing. Then, the fragment was cloned into the pWIZ vector to generate the *UAS-sano^{dsRNA}* strain (protocol as in the study by Lee and Carthew, 2003). The construct was introduced into the *Drosophila* germ line by injection into *w¹¹¹⁸* embryos of either sex, as described previously (Rubin and Spradling, 1982).

Larval behavioral assay. Petri dishes (9.5 cm diameter) were divided into two compartments. Each compartment was filled with either 2% agarose/water (control) or 2% agarose/test solution mixed in water (sucrose; S0389; Sigma-Aldrich), quinine (quinine hydrochloride; Q1125; Sigma-Aldrich), caffeine (C0750; Sigma-Aldrich), and sodium chloride (S9625; Sigma-Aldrich). At *t* = 0 min, 50 early-to-mid L3 larvae of either sex were placed on the dividing line between the two zones and allowed to move freely. The number of larvae found on the control (*N_c*) and test (*N_t*) areas was counted at 10 min. The larvae found at a distance of <0.5 cm on either side of the dividing line were not included in the index calculation. A gustatory preference index (GPI) was calculated using the following formula: GPI = (*N_t* - *N_c*)/(*N_t* + *N_c*). Positive preference indices indicate an attractive behavior, whereas negative ones indicate avoidance behavior toward tested substances. For each test, *n* = 10.

RT-PCR. RNA was extracted from various portions of the dissected larvae of either sex using the TRIzol reagent (Invitrogen) and treated with RNase-free DNase to eliminate contamination by genomic DNA. Total RNA (1 μg) was reverse transcribed using the iScript cDNA Synthesis kit (Bio-Rad).

PCR reactions were conducted using a thermocycler (Bio-Rad). PCR primers were designed for different exons of the *sano* coding region (see Fig. 2): forward 1 (exon 1), 5'-ctgctctcttgcgtgttgg; forward 2 (exon 2), 5'-gaagccggttcttctgtgtc; forward 3 (exons 5a and 5b), 5'-cgggtcgtgtgtccattgc; forward 4 (exon 5b), 5'-ggatcagcgtgtgttctgcac; forward 5 (exon 8), 5'-acaacaattgctctctgtgacg; reverse 1 (exon 6), 5'-ctcttctgattccttgcacg; reverse 2 (exon 8), 5'-atggcagctagaacgattgg; and reverse 3 (exon 9), 5'-cgattgcgagcagattgg.

Real-time PCR. RNA from the various genotypes was extracted from 80 anterior portions of the third-instar larvae of either sex and treated with DNase to eliminate contamination by genomic DNA. The standard protocol was used for real-time PCR (Applied Biosystems, Roche). PCR primers were designed for the region spanning from exon 6 to exon 7: *sano* exon 6 forward, 5'-ggtgtccacacgctcaaga; and *sano* exon 7 reverse, 5'-cactgacctggaagctgct.

Fluorescence and calcium imaging. For GFP and RFP expression imaging, the anterior part of the larvae was dissected and incubated in a glycerol/1 × PBS (50:50) mix for 1 h and mounted in Vectashield mounting medium (Kwon et al., 2011). Then direct observation of the fluorescence was performed using a confocal microscope (Leica TCS SP2).

For calcium imaging, living larvae of either sex were placed in 25 μl of distilled water between a cover slide and a perforated slide. The prepara-

tion was placed on an inverted confocal spectral Nikon C1Si microscope and observed with a 40× objective. Then, 25 μl of distilled water, 25 μl of 20 mM NaCl (to obtain a final concentration of 10 mM NaCl), or 25 μl of 600 mM NaCl (to obtain a final concentration of 300 mM NaCl) was injected through the hole in the slide. The fluorescence images (2 frames/s, 96 × 96 pixels) were acquired simultaneously using the Nikon EZ-C1 acquisition software and were analyzed using the Nikon EZ-C1 FreeViewer software. The FRET changes were measured as the ratio (*R*) of the donor over the acceptor emission intensities (427 nm/527 nm) and expressed as a percentage increase over the mean of the *R* values obtained during the measurement ($\Delta R/R_0\%$). Data are expressed as the mean ± SEM.

Statistical analysis. For each experiment, data were presented as mean ± SEM. All statistical analyses were performed with Prism 5 software for Mac OSX (GraphPad Software). Statistical analysis was generally made using either Student's *t* test or, for multiple comparisons, one-way ANOVA, followed by Bonferroni's *post hoc* test. *p* values < 0.05 were considered to be statistically significant.

Results

Identification of the *sano* gene

We identified the *sano* gene from a genetic screen of a P-Gal4 insertion collection based on both expression in larval chemosensory organs and the gustatory choice behavior defects observed in viable homozygous mutants. Among several selected lines, one P-Gal4 line (later called *sano*^{Gal4}), when combined with *10XUAS-IVS-mCD8::GFP*, showed expression in a few GRNs (5.5 ± 0.24; *n* = 10) in the TO and in three neurons in the ventral pharyngeal sense organ (VPS) of third-instar larvae (Fig. 1*Aa,Ab*). We also observed expression in a few neurons along the body. Then, we studied gustatory choice behavior on early third-instar *sano* mutant larvae with different attractive (100 mM sucrose) or aversive (10 mM caffeine and 10 mM quinine) substances and NaCl at different concentrations ranging from 10 to 500 mM. In a dose–response behavioral analysis toward NaCl, we observed that the homozygous larvae from the three mutant strains (*sano*^{Gal4}, *sano*^{GE12233}, and *sano*^{GE15762}) showed a response to low-salt concentrations (at 10 and 50 mM NaCl) similar to that observed for wild-type or *sano*^{Gal4/+} (Fig. 1*B*). At 100 mM NaCl, *sano* mutant larvae displayed an attractive response, whereas wild-type larvae showed no preference. For higher NaCl concentrations (200, 300, and 500 mM), mutant larvae showed strong defects in the aversion to NaCl. To verify that the behavioral defect was related to the reduction of *sano* expression, we generated a *UAS-sano* construct containing the cDNA of the RB transcript and introduced it into a *sano*^{Gal4} mutant background (Fig. 2*A*). The defects in aversion could then be rescued; therefore, these defects are likely attributable to *sano* misexpression (Fig. 1*B*). *sano* mutant larvae, such as wild-type larvae, were attracted by 100 mM sucrose and were repelled by 10 mM caffeine or 10 mM quinine, showing that the GRNs of the mutant larvae were functional and could detect other aversive and attractive substances (Fig. 1*C*).

Using PCR, we located the P-Gal4 insertion 104 bp upstream of a transcription start site for *CG12758 (serrano)*. Five transcripts (RA, RB, RC, RD, and RE) were predicted, with at least two transcription start sites separated by 70 kb (Fig. 2*A*). All of the transcripts code for the same predicted protein of 778 aa. The *Sano* protein is a member of the uncharacterized Themis protein family (Chung et al., 2009; Johnson et al., 2009). Members of this family are present in animals from cnidarians to mammals and contain at least one cysteine-rich CABIT domain and a low-complexity proline-rich stretch (Chung et al., 2009; Johnson et al., 2009). In *Drosophila*, *Sano* interacts with *Dishevelled*, a planar cell polarity regulator, to control tracheal tube length during development (Chung et al., 2009).

The *sano* coding region spans over 80 kb and contains a second predicted transcription start site. To determine whether some of the putative transcripts were specifically present in the larval chemosensory organs, we examined *sano* expression by RT-PCR in several larval tissues. To detect the different transcripts, we selected specific primers located in exons spanning other introns (Fig. 2A). The combination of different primers allowed for the amplification of the cDNA fragments corresponding to each transcript. At least four *sano* transcripts (RA, RB, RC, and RD were detected, but RE was not) were expressed and detectable in several parts of the larvae, including the most anterior part of the larvae, which encompasses the chemosensory organs, the brain, and the salivary glands (Fig. 2B). This result indicated that both of the transcription start sites were activated in these different structures, resulting in the presence of all of the transcripts. To confirm that the P-Gal4 element insertion in the *sano* locus caused the gustatory choice behavior defects observed in the *sano*^{Gal4} larvae, we isolated two other transgenic lines containing P-UAS element insertions, which were located 147 bp downstream of the first transcription start site (*sano*^{GE12233}) and 352 bp upstream of the second transcription start site (*sano*^{GE15762}). Homozygous larvae for these P-UAS insertions were viable and showed salt perception defects similar to those observed for the *sano*^{Gal4} mutants (Fig. 1B). Because all of these insertions were located close to the *sano* gene or in the coding region, they should have disturbed and reduced *sano* transcription. Accordingly, we measured a significant 3.94-fold decrease of *sano* expression by qPCR in the *sano*^{Gal4} mutant compared with the wild-type *Drosophila* (Fig. 2C). When we introduced an UAS-*sano* construct in a *sano*^{Gal4} mutant background, we induced a significant 4.54-fold increase in the *sano* transcript levels (Fig. 2B). Therefore, the *sano*^{Gal4} mutant behavioral defects are attributable to the reduction of *sano* expression and can be rescued inducing *sano* expression in the *sano*^{Gal4}-expressing GRNs.

sano^{Gal4} is expressed in the gustatory neurons required for the aversive response to salt

Several chemosensory organs are located in the anterior part of the larval body (DO, TO, and VO) and along the larval pharynx (dorsal, ventral, and posterior pharyngeal sense organs; Colomb et al., 2007; Gerber

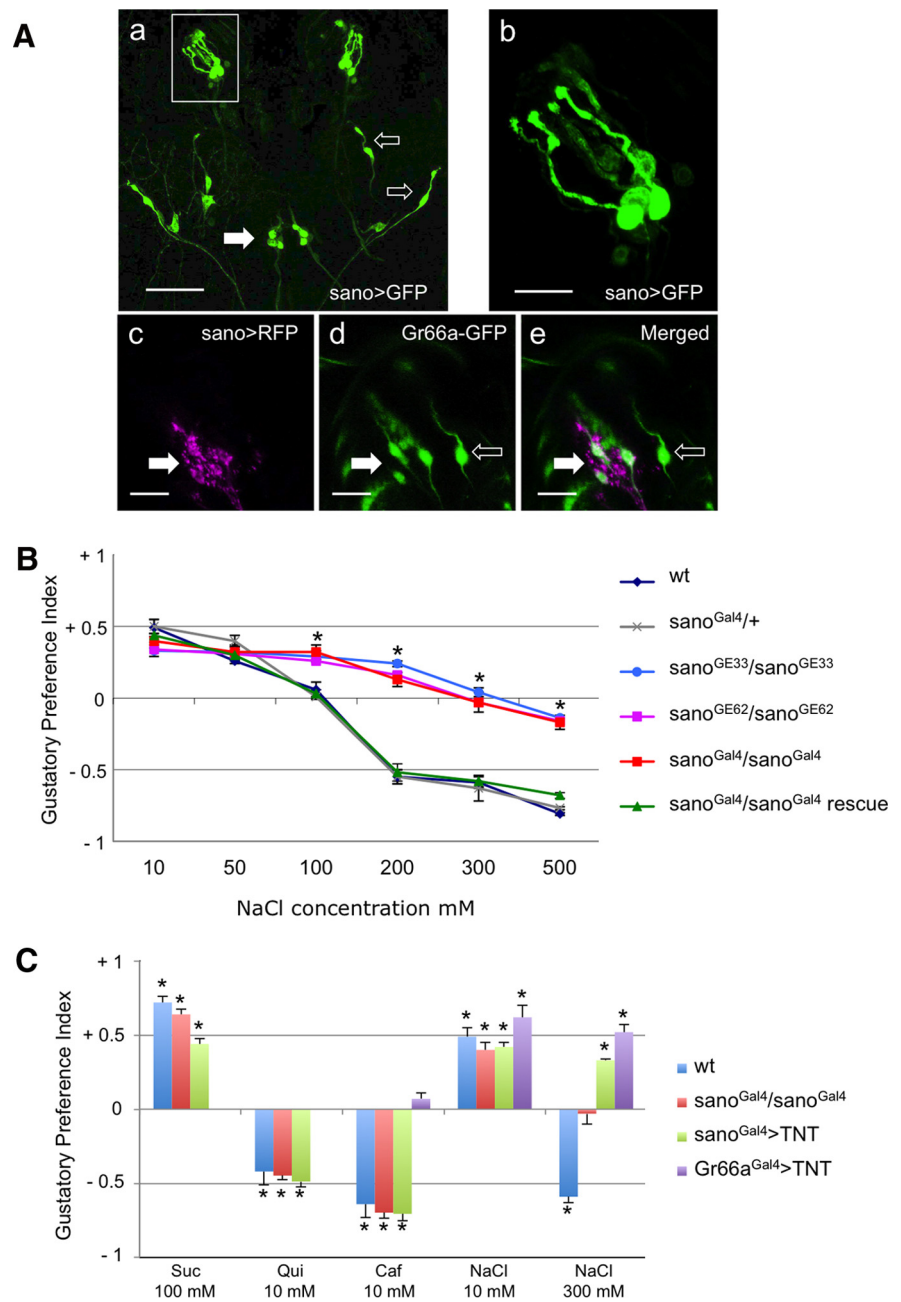


Figure 1. Role of *sano* in taste perception. **Aa, Ab**, Ventral view of the anterior part of a third-instar *sano*^{Gal4/sano}^{Gal4};10XUAS-IVS-mCD8::GFP/10XUAS-IVS-mCD8::GFP larva. **Aa**, Expression is observed in gustatory neurons: five neurons in the TO (box) and three neurons in the ventral pharyngeal sense organ (filled arrow). Expression is also present in few neurons along the body (open arrows). Scale bar, 50 μ m. **Ab**, Detailed view of the expression in five neurons of the TO. Scale bar, 10 μ m. **Ac–Ae**, Ventral view of the anterior part of a third-instar *sano*^{Gal4/+};10XUAS-IVS-mCD8::RFP/Gr66a-GFP larva. **Ac**, *sano*^{Gal4} drives expression of RFP in six neurons of the TO ganglion. **Ad**, *Gr66a*-GFP drives expression of GFP in four neurons of the TO ganglion (filled arrow) and in two adjacent neurons of the TO dorsolateral group (open arrow). **Ae**, *sano*^{Gal4} and *Gr66a*-GFP are coexpressed in four neurons of the TO ganglion (filled arrow). **B**, Behavioral responses of third-instar larvae to NaCl concentrations. The GPIs for indicated NaCl concentrations versus water were calculated. For each condition, 10 trials with 50 larvae were performed. Control lines (wild-type; *sano*^{Gal4/+}), *sano* mutants (*sano*^{Gal4/sano}^{Gal4}, *sano*^{GE12233/sano}^{GE12233}, *sano*^{GE15762/sano}^{GE15762}), and the *sano* rescue line (*sano*^{Gal4/sano}^{Gal4};UAS-*sano*/+) were used in this experiment. Each bar represents a mean \pm SEM of GPI ($n = 10$). Asterisks above error bars indicate that results were significantly different (ANOVA, $p < 0.05$). **C**, Behavioral responses of third-instar larvae to attractive [100 mM sucrose (Suc) or 10 mM NaCl] or repulsive [10 mM caffeine (Caf), 10 mM quinine (Qui), or 300 mM NaCl] tastants. Control larvae (wild-type), *sano* mutant larvae (*sano*^{Gal4/sano}^{Gal4}), and larvae with impaired gustatory neurons (*sano*^{Gal4>TNT} or *Gr66a*^{Gal4>TNT}) were tested. Each point represents the mean \pm SEM of GPI obtained from 10 trials of 50 larvae. A Student's *t* test comparing with the theoretical value 0 was performed. Asterisks above error bars indicate that results were significantly different ($p < 0.05$).

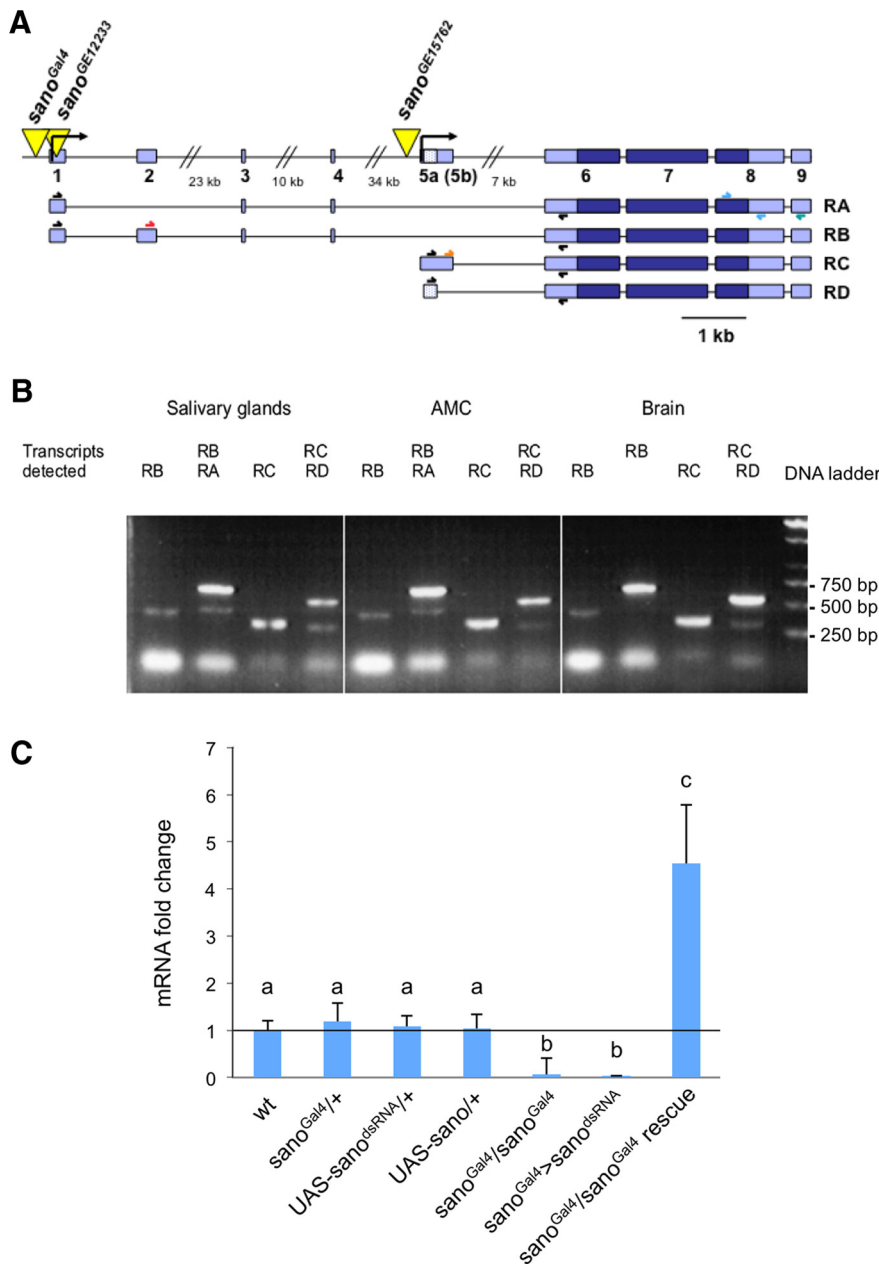


Figure 2. Map and transcript analysis of *sano* gene. **A**, Structure of the *sano* gene. Yellow triangles represent the P-element insertions [*sano^{Gal4}* is a P[GawB] line, and *sano^{GE12233}* and *sano^{GE15762}* are P[UAS] lines]. Exons are shown in blue. Light blue boxes represent the 5' and 3' untranslated regions. Scale bar, 1 kb. The arrows above the transcripts indicate the primers used for the RT-PCR analysis. **B**, *sano* expression analyzed by RT-PCR. RNAs were extracted from the dissected structures of the CantonS larvae. A PCR with a primer specific to the *sano* sequence was performed to obtain cDNAs by reverse transcription. All of the four transcripts were present in the different organs tested. AMC, Antenna-maxillary complex. **C**, The data represent the fold change in the amount of *sano* transcripts determined by qPCR. The CantonS strain was used as the wild-type reference, and the level of wild-type transcripts was defined as 1.0. The error bars represent the SEM, $n = 3-10$. The same letter over error bars indicates values that are not significantly different (ANOVA, $p < 0.05$).

et al., 2009). Previous studies have associated salt detection with the TO (Heimbeck et al., 1999; Liu et al., 2003). When combined with a *10XUAS-IVS-mCD8::GFP* transgene, *sano^{Gal4}* expression was detectable in 5.5 neurons of the TO and 3 neurons in the VPS (Fig. 1Aa,Ab). To better characterize *sano*-expressing neurons in the TO, we compared *sano^{Gal4}>RFP* and *Gr66a-GFP* expression in larvae. We observed that *sano* and *Gr66a* are coexpressed in four neurons, and *sano* is expressed alone in two neurons in the TO ganglion. *Gr66a* is expressed alone in two neurons that proj-

ect their dendrites to the terminal dome but located in the DO ganglion (TO dorso-lateral group; Kwon et al., 2011; Fig. 1Ac-Ae).

To confirm that *sano^{Gal4}* is specifically expressed in gustatory neurons involved in high-salt detection, we used an *UAS-TNT* transgene to block synaptic transmission and investigate taste perception defects in the presence of attractive or repellent substances (Sweeney et al., 1995; Heimbeck et al., 1999). We tested the behavioral responses of larvae from the *sano^{Gal4} × UAS-TNT* cross for 100 mM sucrose, bitter compounds (10 mM caffeine and 10 mM quinine), and two NaCl concentrations (10 and 300 mM). Mutant larvae showed responses similar to those observed with wild-type larvae toward sucrose, caffeine, quinine, and 10 mM NaCl (Fig. 1C). However, mutant larvae showed an avoidance defects toward 300 mM NaCl (Fig. 1C). More intriguingly, *sano^{Gal4} × UAS-TNT* larvae showed the same response toward low- and high-NaCl concentrations; they were also attracted by high-salt concentration. We also tested the behavioral responses of larvae from the *Gr66a^{Gal4} × UAS-TNT* cross for 10 mM caffeine and low- and high-NaCl concentrations. Mutant larvae show no preference for caffeine as expected and attraction to low-salt concentration. Surprisingly, they also showed an attraction to high-NaCl concentration (Fig. 1C). Our results showed that the inactivation of neural transmission in the *Gr66a^{Gal4}*-expressing neurons can block the avoidance to caffeine and high-salt concentrations, whereas blocking neural transmission in the *sano^{Gal4}*-expressing neurons specifically blocked the avoidance toward high-NaCl concentration.

sano is involved in high-salt perception

To confirm the role of *sano* in salt perception, we generated transgenic lines containing a dsRNA construct that included part of exon 7. This construct was intended to induce the degradation of all the *sano* transcripts. When we activated the expression of this *sano^{dsRNA}* construct using *sano^{Gal4}* as a driver, we observed a five-fold decrease of *sano* mRNA levels

detected by a qPCR analysis on dissected anterior parts of larvae (Fig. 2C). We then tested the ability of larvae expressing *sano^{dsRNA}* to discriminate between attractive or repellent compounds. As predicted, larvae from the *sano^{Gal4} × UAS-sano^{dsRNA}* cross showed responses similar to the wild-type larvae toward 10 mM NaCl, 10 mM sucrose, and 10 mM caffeine (Fig. 3A; data not shown). Similarly, larvae from the *elav^{Gal4} × UAS-sano^{dsRNA}* cross and *Gr66a^{Gal4} × UAS-sano^{dsRNA}* cross also showed responses similar to the wild-type larvae toward 10 mM NaCl, 10

mM sucrose, and 10 mM caffeine (Fig. 3A; data not shown). In the presence of 300 mM NaCl, larvae expressing *UAS-sano^d-sRNA* in *sano^{Gal4}* or *Gr66a^{Gal4}* neurons or all the neurons showed an attractive response, whereas wild-type larvae showed an aversive response (Fig. 3A). These results are similar to the response obtained when neural transmission is blocked by expressing TNT in *sano^{Gal4}*- or *Gr66a^{Gal4}*-expressing neurons.

To show that the gustatory choice behavior defects observed for the *sano* mutant could be attributable to a late disruption of *sano* expression during the third-larval instar, we investigated the effect of a temporal controlled inactivation of *sano* expression on NaCl perception. We performed temperature-shifting experiments on larvae bearing *sano^{Gal4}*, a *sano^{dsRNA}* construct, and a *tubP-Gal80^{ts}* transgene. The Gal80^{ts} protein can interact with and block Gal4 at low temperatures (19°C) but is inactivated at a higher temperature (30°C), allowing the Gal4 protein to activate *sano^{dsRNA}* expression (McGuire et al., 2003). When the larvae were reared at 19°C, *sano^{dsRNA}* was not expressed, and we observed an aversive response of the larvae to 300 mM NaCl similar to the wild-type response (Fig. 3B). When the larvae were transferred to 30°C overnight before the tests, *sano^{dsRNA}* expression is activated and larvae showed behavioral defects toward high-salt concentrations (Fig. 3B). Larvae showed an attractive response similar to that observed for the larvae from the *sano^{Gal4} × UAS-sano^{dsRNA}* cross. These results suggest that *sano* is expressed during the third-larval instar and is required for the activity of the gustatory neurons that detect high-salt concentrations.

Thus, the reduction of *sano* expression in *sano^{Gal4}*- or *Gr66a^{Gal4}*-expressing gustatory neurons or in the whole nervous system led to a similar disruption of high-NaCl perception in larvae.

ppk19 is involved in low- and high-salt perception

ppk19 has been implicated in the perception of both low- and high-NaCl concentration at larval instar (Liu et al., 2003). In our behavioral tests, we showed that expression of a *ppk19^{dsRNA}* construct in all the neurons using an *elav^{Gal4}* driver led to the absence of a preference toward low- or high-NaCl concentrations (Fig. 3A). The expression of *ppk19^{dsRNA}* using *sano^{Gal4}* or *Gr66a^{Gal4}* did not affect the attraction toward 10 mM NaCl (Fig. 3A). However, disruption of *ppk19* in *sano^{Gal4}* neurons induced an attractive response to 300 mM NaCl (Fig. 3A). Thus, loss of *ppk19* in *sano^{Gal4}* or *Gr66a^{Gal4}* neurons only impaired avoidance to high-NaCl concentration but not attraction attributable to low-salt perception.

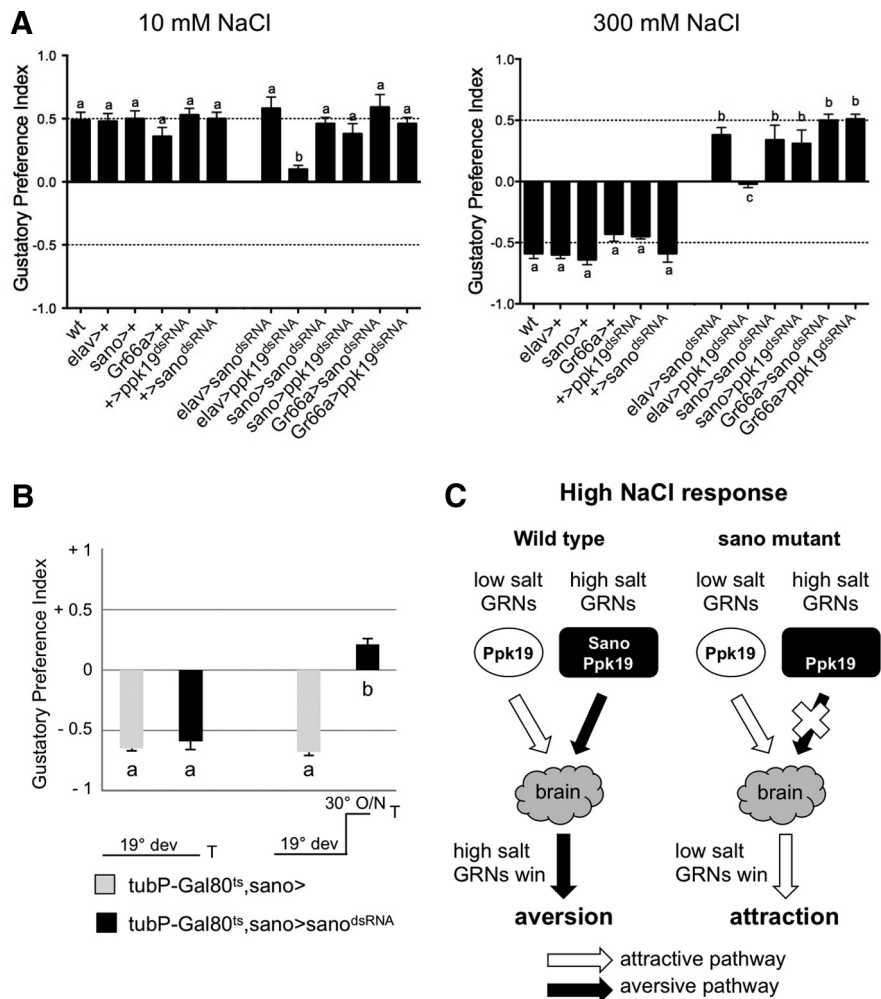


Figure 3. Disruption of *sano* and *ppk19* by dsRNA. **A**, Behavioral assay for 10 mM NaCl and 300 mM NaCl. The effects of *sano* or *ppk19* disruption in all larval neurons (*elav^{Gal4}* crosses), *sano*-expressing neurons (*sano^{Gal4}* crosses), or *Gr66a*-expressing neurons (*Gr66a^{Gal4}* crosses) were tested in these experiments. Each histogram (mean \pm SEM of GPI) was calculated from 10 trials. The same letter over error bars indicates values that were not significantly different (ANOVA, $p < 0.05$). **B**, The effects of the temporal disruption of Sano on aversive behavior at 300 mM NaCl. Two temperature conditions were used. The offspring from the crosses were raised at 19°C (19° dev). At 19°C, GAL4 activity is repressed by GAL80^{ts} such that *sano* transcripts are not impaired in *tubP-Gal80^{ts},sano>sano^{dsRNA}* larvae. When *tubP-Gal80^{ts},sano>sano^{dsRNA}* larvae are transferred to a permissive temperature of 30°C overnight (O/N), *sano^{Gal4}* drives expression of *sano^{dsRNA}*. Each histogram (mean \pm SEM of GPI) was calculated from 10 trials. The same letter over the error bars indicates values that were not significantly different (ANOVA, $p < 0.05$). **C**, Schematic model showing that the loss of Sano selectively impairs the aversive salt taste pathway. *ppk19* is required in both low- and high-salt GRNs to detect NaCl. *sano* is required in high-salt GRNs to detect high-NaCl concentrations. In wild-type context, both low- and high-salt GRNs are activated by high-NaCl concentration, leading to aversion. Loss of *sano* selectively disrupts the aversive pathway, leading to attraction in the presence of high-NaCl concentration.

Sano and PPK19 regulate the neuronal activity of taste neurons

To provide physiological evidence showing that *sano* is required for NaCl detection, a calcium-sensitive protein, Cam2.1, was expressed in larval taste neurons to monitor Ca^{2+} levels in response to NaCl (Diegelmann et al., 2002). Expression of this protein was driven by the *sano^{Gal4}* line and was thus expressed in a subset of neurons in the TO (Fig. 1A). Fluorescence levels were analyzed in the cell bodies of these neurons in the TO that expressed the Cam2.1 protein. This allowed for the precise analysis of the calcium levels after stimulation with different concentrations of NaCl. In wild-type larvae, when a solution of 20 mM NaCl was applied, a variation of fluorescence was observed that was not significantly different from the variation observed in the controls without NaCl (Fig. 4). With the application of a 300 mM NaCl

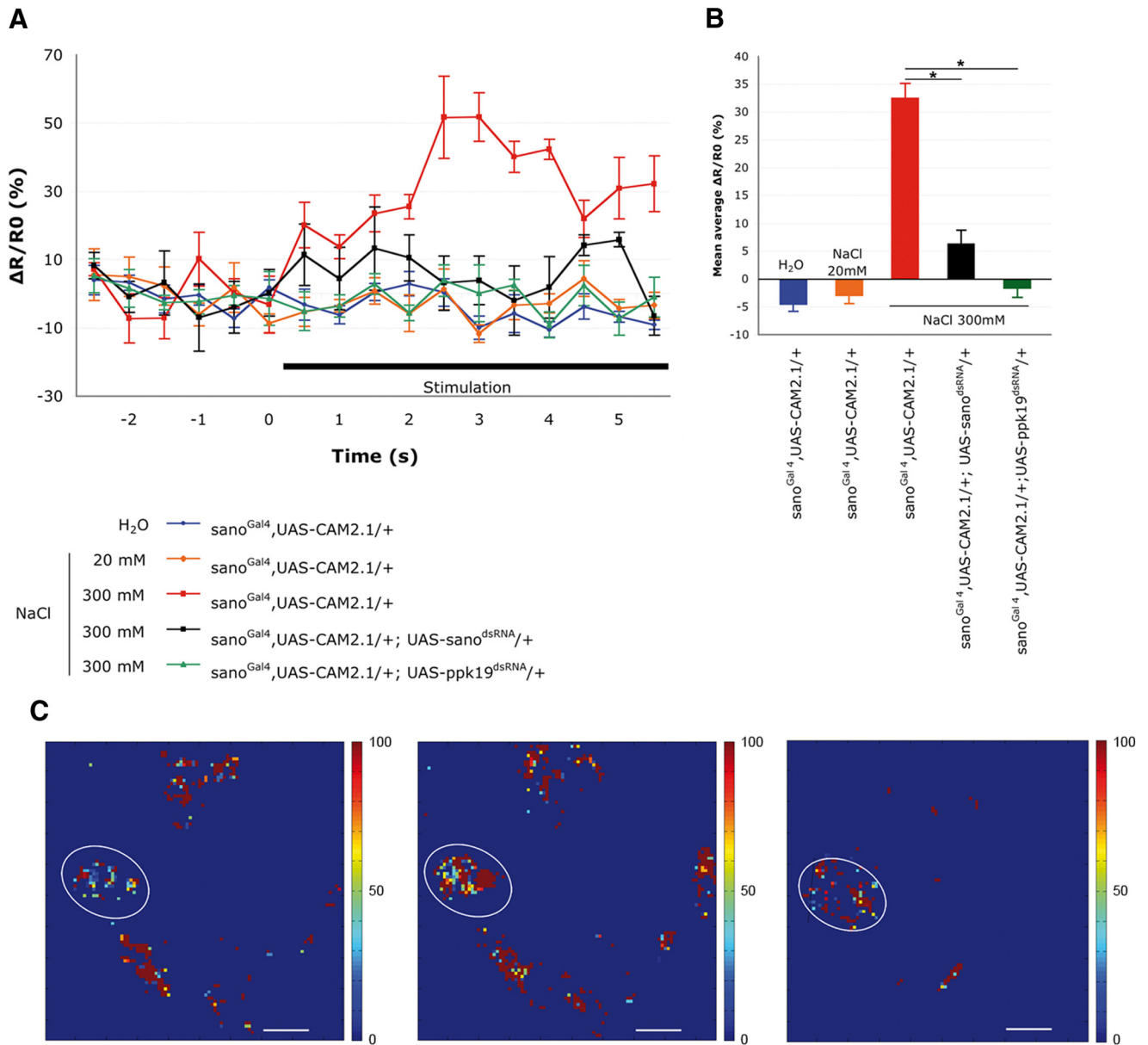


Figure 4. Sano and PPK19 are required for TO neuron activation by high-NaCl concentrations. **A**, Representative fluorescence change ($\Delta R/RO$) of CAM2.1 expressed in *sano*^{Gal4} neurons in control and mutant larvae. Neuronal activity was measured before and after the addition of different stimuli: distilled water, 20 mM NaCl, or 300 mM NaCl. Responses to 300 mM NaCl were measured in larvae expressing either *sano*^{dsRNA} or *ppk19*^{dsRNA} in *sano*^{Gal4} neurons ($n = 4 - 6$). The error bars represent the SEM. **B**, Mean FRET ratio changes. Changes were measured during the first 5 s of the stimulation of different control and mutant larvae ($n = 4 - 6$). The error bars represent the SEM. Asterisks indicate that results are significantly different (Student's *t* test, $p < 0.05$). **C**, Peak responses are shown in false-color scale ($\Delta R/RO\%$, right of images). Left, *sano*^{Gal4}, UAS-CAM2.1/+ before stimulation. Middle, *sano*^{Gal4}, UAS-CAM2.1/+ at 2 s after stimulation with 300 mM NaCl. Right, *sano*^{Gal4}, UAS-CAM2.1/+; UAS-sano^{dsRNA}/+ at 2 s after stimulation with 300 mM NaCl. The cell bodies of the neurons used for the FRET measurements are circled. Scale bar, 10 μ m.

solution, a significant increase (50%) of the fluorescence was observed after 2 s of exposure. This result indicated that the observed neurons were sensitive to a high concentration of NaCl and respond to an increase of the Ca^{2+} level. To determine the role of *sano* and *ppk19* in the activation of these neurons, a UAS-*sano*^{dsRNA} or a UAS-*ppk19*^{dsRNA} construct was then introduced into the *sano*^{Gal4}; UAS-Cam2.1 background. In these two genetic contexts, the signal-to-noise level remained low after the addition of a 300 mM NaCl solution and close to the control levels in the neurons stimulated by high-NaCl concentrations. These data showed that PPK19 and Sano are required in *sano*^{Gal4} neurons to induce an increase of the intracellular calcium levels in response to high-NaCl concentrations. Therefore, PPK19 and Sano are both required for

the activity of gustatory neurons in the TO that are involved in the detection of high-salt concentrations.

Discussion

sano is expressed in the neurons that detect high-salt concentrations

Our behavioral results on larvae showed that *sano*^{Gal4} is expressed in a group of GRNs in the TO that are specifically involved in the detection of high-NaCl concentrations. Because *sano* and *Gr66a* are coexpressed in four neurons in the TO, we propose that these neurons are specifically required for high-salt detection. The attractive behavioral response of larvae observed when these neurons are inactivated showed that other gustatory neurons are still

able to detect NaCl. This result strongly suggests that two distinct types of neurons respond simultaneously to either low- or high-salt concentrations to generate an attractive or aversive response (Fig. 3C). Recently, Zhang et al. (2013) proposed a model in which competition between low-salt GRNs and high-salt GRNs in S- and L-type sensilla results in the bidirectional behavioral responses to salt in adults. They showed that loss of Ir76b selectively disrupted the attractive salt pathway and left the aversive salt pathway intact, leading to an aversive response to low-salt concentrations. In our experiments, we observed that inactivation of *sano*-expressing GRNs induced an attraction to high-salt concentrations, suggesting that the aversive salt pathway is disrupted and the attractive salt pathway remained unaffected. Thus, we conclude that, in larvae as in adults, a bimodal switch system determines the opposing behavioral responses to low- and high-salt; *sano* is only expressed in the neurons of the aversive salt pathway.

Previous results have demonstrated that the TO shows only weak electrophysiological responses to 100 mM NaCl (Oppiger et al., 2000). Here, using the FRET technique, we showed that the *sano*-expressing neurons are strongly stimulated by 300 mM NaCl but not by a low concentration of NaCl. Therefore, *sano*^{Gal4} neurons are involved in the detection of high-NaCl concentrations in larvae. Thus, the *sano*^{Gal4} line is characteristic of larval sensory neurons required for the perception of high-NaCl concentrations and provides an excellent genetic tool to specifically target these neurons for additional cellular and molecular study.

Disruption of *sano* impairs the aversive response to high-NaCl concentrations

Larvae with impaired *sano* function display behavioral defects in the presence of high-salt concentrations. Tests performed with caffeine and quinine provided evidence that the *sano* mutant larvae were able to detect and avoid other aversive molecules, suggesting that Sano does not play a role in the detection of aversive molecules in general. Attractive behaviors to sucrose or low-salt concentrations were also not affected. Similarly, the disruption of Sano function using *UAS-sano*^{dsRNA} constructs under the control of *sano*^{Gal4}, *Gr66a*^{Gal4}, or *elav*^{Gal4} only caused the loss of high-salt detection in larvae. Thus, the disruption of Sano throughout the whole nervous system or in a subset of GRNs (including bitter GRNs) only affects the perception of high-NaCl concentrations.

Studies on the *ppk* gene family have shown that PPK19 is required for the detection of low- and high-salt concentrations (Liu et al., 2003). Our results highlight the particular role of Sano in the detection of high-NaCl concentrations; thus, the *sano* gene is the first characterized gene specifically required for an aversive response to salt.

Sano is involved in the activity of high-salt larval GRNs

Little is known about the cellular mechanisms of salt detection by GRNs. Here, we identified a protein that appears to be cytoplasmic and is part of the cellular mechanism required in high-salt detecting neurons. In mammals, intracellular calcium levels are modulated in response to chemosensory stimuli, such as salt, in taste-receptor cells (Chandrashekar et al., 2010). In *Drosophila*, calcium imaging has been used in adults to measure neuronal activity in response to sugars, bitter substances, and NaCl (Marella et al., 2006). Our results show that calcium levels are increased in *sano*-expressing neurons in the TO in response to

high-salt concentrations. This increase in GRN activity requires PPK19 and Sano. Then Sano is involved in the detection of high salt by GRNs in the TO.

Previous studies have shown that Sano is a cytoplasmic protein that interacts with other proteins, such as Grb2 (a protein involved in the signaling pathways of tracheal and wing development; Chung et al., 2009). Moreover, a molecular interaction with Epac (exchange protein directly activated by cAMP) has been detected in a two-hybrid study (Giot et al., 2003). Epac is a member of the Rap1 signal transduction pathway and is involved in cell adhesion and differentiation, as well as in neuronal activity by regulating calcium levels or neurotransmitter release (Gloerich and Bos, 2010). The genetic and molecular interactions between Sano and Epac remain to be fully demonstrated by future *in vitro* and *in vivo* studies and may provide new insights into the cellular mechanisms taking place downstream of the DEG/ENaC channels in salt-detecting neurons.

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