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Electric Shock-Induced Associative Olfactory Learning in *Drosophila* Larvae

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Abstract

Associative plasticity is a basic essential attribute of nervous systems. As shown by numerous reports, *Drosophila* is able to establish simple forms of appetitive and aversive olfactory associations at both larval and adult stages. Whereas most adult studies on aversive learning employed electric shock as a negative reinforcer, larval paradigms essentially utilized gustatory stimuli to create negative associations, a discrepancy that limits the comparison of data. To overcome this drawback, we critically revisited larval odor–electric shock conditioning. First, we show that lithium chloride (LiCl), which was used in all previous larval electric shock paradigms, is not required per se in larval odor–electric shock learning. This is of considerable practical advantage because beside its peculiar effects LiCl is attractive to larvae at low concentration that renders comparative learning studies on genetically manipulated larvae complicated. Second, we confirm that in both a 2-odor reciprocal and a 1-odor nonreciprocal conditioning regimen, larvae are able to associate an odor with electric shock. In the latter experiments, initial learning scores reach an asymptote after 5 training trials, and aversive memory is still detectable after 60 min. Our experiments provide a comprehensive basis for future comparisons of larval olfactory conditioning reinforced by different modalities, for studies aimed at analyzing odor–electric shock learning in the larva and the adult, and for investigations of the cellular and molecular substrate of aversive olfactory learning in the simple *Drosophila* model.

Key words: *Drosophila* larvae, electric shock, learning and memory, lithium chloride, synaptic plasticity

Introduction

Learning can be defined as a lasting alteration in behavior or in the behavioral potential due to experience (Bower and Hilgard 1981; Dudai 2004). By using an extensive set of experimental approaches in different model organisms, memory scientists try to understand how behavioral changes are represented in terms of molecular and neuronal architecture in the brain (Milner et al. 1998; Heisenberg 2003; Keene and Waddell 2007). To analyze how associative plasticity on the synaptic level relates to associative changes in behavior, it is critical to choose a simple and experimentally accessible system (Martin et al. 2000). The *Drosophila* larva has turned out to satisfy these demands due to its elementary organization of the nervous system comprising 3–6 orders of magnitude fewer neurons compared with mammals, the availability of robust behavioral assays, and the existence of transgenic techniques that allow one to interfere with small sets of neurons (Duffy 2002; Gerber and Stocker 2007; Elliott and Brand 2008; Gerber et al. 2009). Therefore, the *Drosophila* larva allows for a multilevel approach toward understanding associative plasticity.

Drosophila larvae can form associations between odors and aversive gustatory reinforcement like quinine or high salt treatment (Gerber and Hendel 2006; Honjo and Furukubo-Tokunaga 2009; Selcho et al. 2009). As studies on adult aversive odor learning have mainly used electric shock as a negative reinforcer, larval and adult data are hard to compare (McGuire et al. 2005; Thum et al. 2007; Gerber et al. 2009). Furthermore, comparisons among the few existing studies on larval odor–electric shock learning are also limited because they vary in technical details and even differ in their basic design (Aceves-Pina and Quinn 1979; Heisenberg et al. 1985; Tully et al. 1994; Khurana et al. 2009). Thus, larvae were trained either in a 2-odor reciprocal assay (Michels et al. 2005; Neuser et al. 2005; Selcho et al. 2009) or in a single-odor nonreciprocal setup (Honjo and Furukubo-Tokunaga 2005, 2009; Khurana et al. 2009).

In the 2-odor, reciprocal training design, a first group of larvae is confronted with an odor A together with an electric shock and subsequently with an odor B without shock (Aceves-Pina and Quinn 1979; Heisenberg et al. 1985; Tully et al. 1994). A second group of larvae receives reciprocal training. Both groups are then tested for their preference between the 2 odors. Relatively higher preferences for the unpunished odor reflect associative learning and can be expressed as a performance index. The conclusion regarding the associative nature of the performance index is compelling because, apart from the reciprocal training, other parameters, such as odor and punishment exposure, passage of time, and handling do not differ between the 2 groups (Gerber and Stocker 2007).

In contrast, the 1-odor nonreciprocal design (Honjo and Furukubo-Tokunaga 2005, 2009) exposes the larvae to a single or several sequential odor–electric shock pairings before testing them for their odor preference (Khurana et al. 2009). To quantify associative learning, a normalized odor preference can be calculated by subtracting odor preferences before and after training. Importantly, differences in odor preference might simply be due to handling, odor exposure, or shock exposure itself. Hence, because these parameters have to be carefully controlled, data interpretation from a 1-odor nonreciprocal design is more complex (Gerber and Stocker 2007).

Given the conceptual differences between these studies, we carefully revisited associative odor–electric shock learning in *Drosophila* larvae. We show that larvae are able to form aversive associations after reciprocal 2-odor training as well as after 1-odor nonreciprocal training. Surprisingly, we observe learning even without adding lithium chloride (LiCl) to the substrate containing the electrode, a procedure which was reported to be essential for electrical conductivity (Aceves-Pina and Quinn 1979; Heisenberg et al. 1985; Tully et al. 1994; Khurana et al. 2009). The stability of memory depends on the number of training trials and, therefore, allows one to establish training protocols for testing selectively short-term memory or longer lasting memories. The systematic character of our study will simplify a comparative analysis of aversive reinforcement by different sensory modalities and electric shock. This will provide a crucial basis for investigating the cellular and molecular mechanisms underlying associative learning in this elementary model organism.

Materials and methods

Flies

Wild-type Canton-S flies were kept on standardized cornmeal medium at about 25 °C under a 14:10 h light:dark cycle. Adult flies were transferred to fresh food vials every second day allowing them to lay eggs for 48 h. All experiments were performed in normal light conditions (Yarali et al. 2006) under a fume hood at room temperature. Third-instar feeding-stage larvae aged 96–144 h were taken from a vial to collect groups of 30 animals that were briefly washed in tap water to remove food residues.

Assay plates, electrodes, and odors

As assay plates, we used Petri dishes (85 mm diameter; Greiner) filled with an about 5-mm layer of agarose gel. The agarose solution (2.5%; Sigma-Aldrich, A5093-500G) was boiled in a microwave oven before it was poured in Petri dishes and left to solidify. Depending on the experiment, we added 0.01 M lithium chloride (LiCl; Fluka, cat. 62486) to the heated solution. Assay plates were stored at room temperature and used on the following day latest. To apply the electric shock, we used 2 semicircular copper electrodes (conventional copper wire, 1 mm diameter, 70 mm length), adjusted to the dimension of the Petri dish. The 2 electrodes were arranged opposite to each other in the plate with a distance of 5 cm (at their ends) to 7.5 cm (in the middle) completely immersed in the agarose solution. Electric shocks were applied by a standard transformator (Autotransformateur Type FW10HMT3; Variac; 0–220 V; 50 Hz). For olfactory stimulation, 10 µl amyl acetate (AM, Fluka cat. 46022; diluted 1:250 in paraffin oil, Fluka cat. 76235) or benzaldehyde (BA, undiluted; Fluka cat. 12010) were loaded into custom-made Teflon containers (4.5 mm diameter) with perforated lids (0.5 mm hole diameter) similar as described in Gerber and Stocker (2007).

Learning experiments

In the 2-odor assay larvae were trained as follows: A first group of 30 animals was exposed to benzaldehyde (BA) for 1 min. During the last 30 s, the odor was paired with an electric shock exposing larvae to odor and electric shock simultaneously; 100 V alternating current (AC) output voltage was used as this leads to an optimum for odor–electric shock learning (see supplementary data). After a resting phase of 5 min on a neutral plate, the animals were exposed to AM for 1 min in the absence of electric shock, followed by another resting phase of 5 min on a neutral plate. A second group of larvae received the reciprocal training. Moreover, electric shock was alternately applied either together with the first-odor stimulus (CS1) or with the second-odor stimulus (CS2), which will be referred to as CS1+ training and CS2+ training hereafter. Depending on the experiment, the assay plates were filled either with pure agarose or agarose containing 0.01 M LiCl. Neutral plates always contained pure agarose and no odors. Immediately after the training, larvae were transferred onto test plates on which AM and BA were presented on opposite sides. After 5 min, individuals were counted on the AM side (#AM), the BA side (#BA), and in a neutral zone similar as described in Gerber and Stocker (2007). By subtracting the number of larvae on the BA side from the number of larvae on the AM

side divided by the total number of all counted individuals (#TOTAL), we calculated an odor preference index,

$$\text{PREF AM} = \frac{(\#\text{AM} - \#\text{BA})}{\#\text{TOTAL}}.$$

From alternate and reciprocal trainings, we then compiled a performance index,

$$\text{PI} = \frac{(\text{PREF AM+ BA}-) - (\text{PREF AM} - \text{BA}+)}{2}.$$

Negative Performance Index represent an aversive memory, whereas positive Performance Index indicate an appetitive memory.

To ease comparison between the 2-odor– and 1-odor–trained larvae, we used for the latter the same experimental setup as described above concerning Petri dishes, electrodes, voltage, BA concentration, and BA application. However, for the 1-odor assay, we initially measured the naive response of 30 larvae to BA, by subtracting the number of larvae on the empty side of the dish (#AIR) from the number of larvae on the BA side (#BA), divided by the total number of larvae (#TOTAL) in all 3 zones. We then calculated an odor preference index,

$$\text{PREF pretest} = \frac{(\#\text{BA} - \#\text{AIR})}{\#\text{TOTAL}}.$$

Afterward the same group of larvae was exposed to BA for 1 min paired with an electric shock (100 V AC output) during the last 30 s. After a resting phase of 5 min on a neutral plate, training either ended (1 trial) or was repeated several times yielding training sessions of 5, 8, or 10 trials. Finally, animals were tested again for their BA preference. By subtracting the number of larvae on the empty side (#AIR) from the number of larvae on the BA side (#BA) divided by the total number of individuals in all 3 zones (#TOTAL), we calculated an odor preference index after training,

$$\text{PREF test} = \frac{(\#\text{BA} - \#\text{AIR})}{\#\text{TOTAL}}.$$

To quantify associative learning, a normalized odor preference ΔPREF was then calculated by subtracting odor preferences before and after training.

$$\Delta\text{PREF} = \text{PREFpretest} - \text{PREF test}.$$

To exclude nonassociative effects in the 2-odor nonreciprocal assays, we performed 3 control experiments in which we omitted electric shock, BA exposure, or both during training. Only an unaltered BA odor preference for each of these control experiments shows explicitly the associative character of ΔPREF. In assays designed to investigate memory retention, the last resting phase of the training was extended to 15, 25, or 60 min.

Electricity and temperature analysis

For investigating the electrical properties of the agarose medium, we designed a virtual grid, which divided the Petri dish area into 43 fields. We applied AC of 100 V output voltage and measured the voltage per centimeter (V/cm) on the surface of the substrate for each field with a digital multimeter (Metex). Experiments were repeated 10 times. We ran this experiment on both pure and LiCl-containing agarose substrate to test for possible differences in conductivity (resistance of LiCl-containing plate about 2.6k Ω; resistance of the pure plate about 35k Ω).

The virtual grid was also used to perform thermometry. Temperature (°C) was analyzed for each field using a digital multimeter combined with a digital Celsius thermometer (both from Fluke). Experiments were repeated 10 times and ran on both pure and LiCl-containing agarose plates.

Electric shock sensory preference

For investigating larval electric shock avoidance, groups of 30 animals were placed on assay plates in which the electrodes were arranged to restrict the electric field to one-half of the plate. One semicircular electrode matched the shape of the Petri dish on one side and a second straight electrode ran longitudinally across the medium in the middle of the neutral zone. By subtracting the number of larvae on the side lacking an electric field (#nS) from the number of individuals on the shock side (#S) divided by the total number of larvae, a preference index for electric shock was calculated based on the distribution of larvae after 1, 3, and 5 min.

$$\text{PREFShock} = \frac{(\#\text{S} - \#\text{nS})}{\#\text{TOTAL}}.$$

Negative PREFShock values indicate shock-avoiding behavior, whereas positive PREFShock values would indicate attraction toward the electric shock. Electric shock was applied according to 3 different stimulus regimes: (i) 1-s shock to 9-s rest, (ii) 3-s shock to 7-s rest, or (iii) 5-s shock to 5-s rest.

LiCl gustatory sensory preference

To test for the ability of the larvae to detect LiCl, groups of 30 larvae were placed on agarose plates that contained LiCL only in one-half at different concentrations from 0.001 to 4.0 M. Larvae were allowed to crawl on the medium for 5 min and were then counted on the left side, right side, and the neutral zone. By subtracting the number of larvae on pure agarose side (#nL) from the number of larvae on the LiCl-containing side (#L) divided by the total number of counted larvae (#TOTAL), a preference index for LiCl was calculated:

$$\text{PREF LiCl} = \frac{(\#\text{L} - \#\text{nL})}{\#\text{TOTAL}}.$$

Negative PREF LiCl values indicate LiCl avoidance, whereas positive PREF LiCl values display LiCl attractiveness.

Data analysis and statistics

Statistical computing and graphic representation of the results was generated with R version 2.7.0 and Photoshop CS2. For statistical analyses, Wilcoxon signed-rank test was used for comparison of single data points to zero; Wilcoxon rank sum test was used for comparison between 2 groups. Significance levels represent $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

Results

Larval electric shock learning: 2-odor reciprocal conditioning

To allow for a direct comparison between odor–electric shock learning and odor learning reinforced by aversive gustatory cues (Gerber et al. 2009; Honjo and Furukubo-Tokunaga 2009; Selcho et al. 2009), we created a modified 2-odor reciprocal conditioning assay which integrated technical aspects published in previous reports on larval shock learning (Aceves-Pina and Quinn 1979; Heisenberg et al. 1985; Tully et al. 1994; Khurana et al. 2009). In line with these publications, our training regime included initially lithium chloride (LiCl) in the plates. In detail, one group of larvae was alternately exposed to AM in the presence of electric shock and BA in its absence (AM+/BA), whereas another group was trained reciprocally (AM/BA+) (Figure 1A). In subsequent choice tests, relatively lower preferences for AM after punishing AM or for BA after punishing BA reflected associative learning (Gerber and Stocker 2007).

Wild-type larvae showed performance scores significantly different from zero after 1 ($P = 0.0381$), 3 ($P = 0.0175$), and 5 training trials ($P = 0.0001$) (data not shown); 1 and 3 training trials resulted in comparable performance ($P = 0.655$), whereas significantly higher scores after 5 training trials (compared with 3 training trials $P = 0.0068$) suggested increased learning with increased number of training trials.

A closer analysis of the performance scores shown before showed differences depending on whether the first odor (CS1+) or the second odor (CS2+) had been shocked during training (Figure 1B); this will be referred to as "sequence effect" and suggests to use CS2+ training only for odor–electric shock conditioning. Larvae conditioned for CS2+ showed significantly higher performance scores than those that had received CS1+ training, independent of the number of training trials (1 trial $P = 0.0009$; 3 trials $P = 0.0045$; 5 trials = 0.0024). Furthermore, CS2+-trained larvae yielded significant learning after 1 ($P = 0.0059$), 3 ($P = 0.0059$), and 5 training trials ($P = 0.0019$), whereas only 5 trials of CS1+ training gave rise to odor–electric shock learning ($P = 0.0097$).

The effects of LiCl on electric shock learning

LiCl was used in all former published larval paradigms to analyze odor–electric shock memory (Aceves-Pina and Quinn 1979; Heisenberg et al. 1985; Tully et al. 1994; Khurana et al. 2009). However, we noticed that larvae are able to form odor–electric shock associations even when omitting LiCl from the plates (Figure 2B). In addition, as LiCl is not used in adult odor–electric shock learning and because data demonstrating its necessity in larval electric shock learning are lacking (Aceves-Pina and Quinn 1979; Heisenberg et al. 1985; Tully et al. 1994; Khurana et al. 2009), we investigated the effect of LiCl in more detail. We analyzed (i) if LiCl is

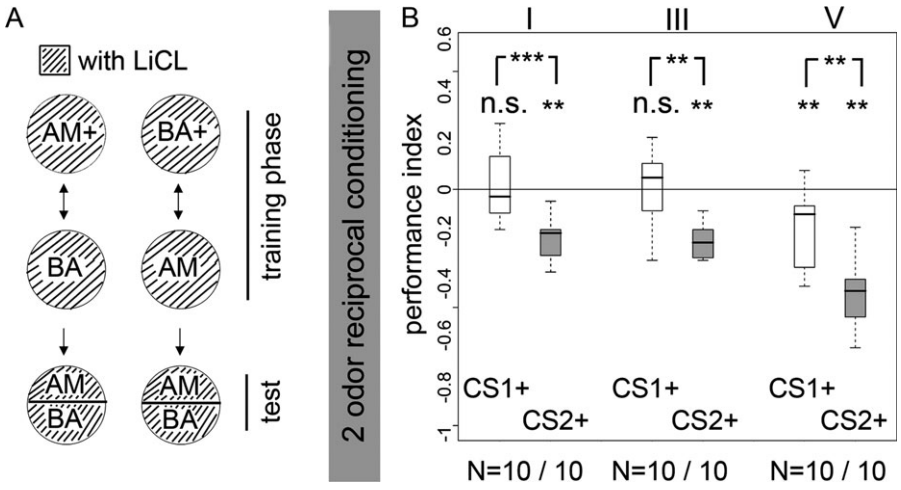

Figure 1 Two-odor reciprocal training protocol reinforced by electric shock. **(A)** Diagram of the procedure for the conditioning experiment on assay plates containing LiCl. Larvae received either AM paired with electric shock and BA without electric shock or vice versa. During test, a preference for AM against BA was measured. **(B)** Larvae were able to associate electric shock with a given odor after 1 (I), 3 (III), or 5 (V) training trials. Training protocols in which the first odor presented was paired with electric shock (CS1+) showed significantly reduced performance indices compared with protocols in which the second odor was punished (CS2+; sequence effect). Significant performance indices were achieved by CS2+ training after 1, 3, and 5 training trials and by CS1+ training only after 5 training trials. Significance levels represent $P < 0.01$ (**), and $P < 0.001$ (***).

necessary for odor–electric shock learning per se and (ii) whether it is required specifically during training or test. Furthermore, we asked (iii) if LiCl affects the physical properties of the electric shock like voltage and temperature and (iv) whether the sensory perception of electric shock in larvae differs in the presence or absence of LiCl. (v) Finally, we tested, if LiCl itself is able to induce larval gustatory preference behavior.

(i) LiCl during training and test is not required per se for odor–electric shock learning but significantly improves performance

To test for the necessity of LiCl for larval odor–electric shock learning, we studied larvae in the 5-trial 2-odor reciprocal design (see above) in the presence (+/+) and absence of LiCl (–/–) (Figures 2A,B). Unexpectedly, larvae that had received LiCl in both training and test as well as larvae that had not received LiCl showed significant performance scores (in the latter case only for CS2+ training; Figure 2B). However, larvae trained and tested on LiCl performed significantly better which was particularly obvious when focusing for the CS2+-trained larvae ($P = 0.0038$). Notably, with LiCl application, the performance score was significantly higher in CS2+ animals than in CS1+ animals ($P = 0.0024$; Figure 2B); this sequence effect could not be observed in the experiment lacking LiCl ($P = 0.1117$).

(ii) LiCl is not specifically required during reinforcement signaling for larval odor–electric shock learning

To ask whether the presence of LiCl specifically affected the acquisition of the aversive memory or its retrieval, applying

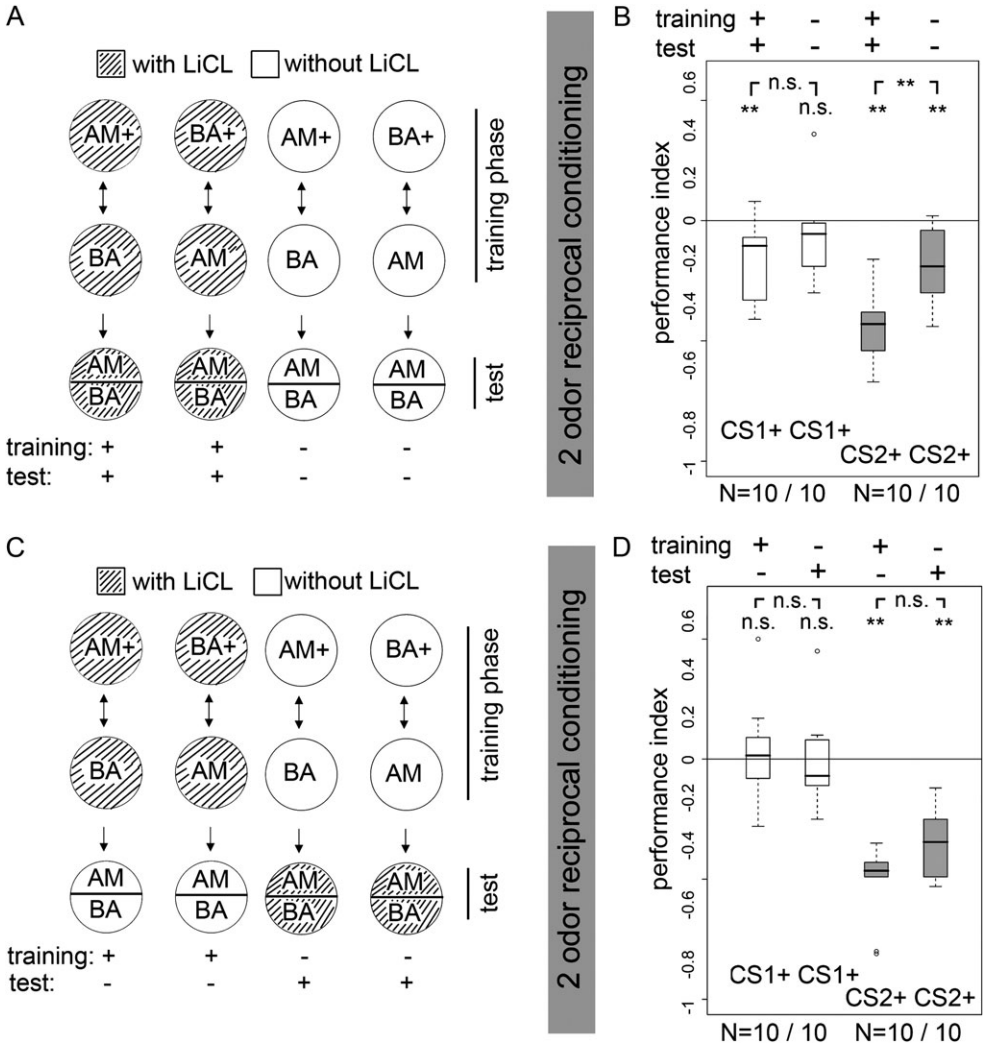

Figure 2 Odor–electric shock learning per se does not require LiCl. **(A)** Diagram of the procedure for a 2-odor reciprocal conditioning experiment reinforced by electric shock on LiCl plates during training and test (left) or without LiCl (right) using 5 training cycles. **(B)** Larvae trained and tested on plates lacking LiCl did exhibit odor–electric shock learning after CS2+ training, but they performed significantly worse than larvae trained and tested on LiCl. CS2+ training but not CS1+ led to a significantly increased performance index. **(C)** Diagram of the procedure for a 2-odor reciprocal conditioning experiment in which LiCl was restricted to training only (left) or test only (right) using 5 training cycles. **(D)** Larvae trained only on LiCl plates or tested only on LiCl plates did not significantly differ in performance, independent of whether the CS1+ values or the CS2+ values were considered. Significance levels represent $P < 0.01$ (**).

the 5-trial 2-odor reciprocal design, we compared groups of larvae that were either trained on LiCl plates and tested on pure plates (+/– experiment) or vice versa (–/+ experiment) (Figures 2C,D). When taking the different training regimes into account, both groups exhibited the same sequence effect (Figure 2D) as for both only CS2+ training, but not CS1+ training, gave rise to scores significantly different from zero (for +/–: $P = 0.0058$; for –/+: $P = 0.0058$). Both CS1+ and CS2+ scores were not different when comparing the 2 experimental groups (for CS1+: $P = 0.4955$; for CS2+: $P = 0.0751$). Thus, we conclude that LiCl is not specifically required during training for odor–electric shock learning.

(iii) LiCl has only a marginal effect on the physically measurable properties of the shock plate

To analyze the influence of LiCl on the physical properties of the assay plate during electric shock, we applied an output voltage of 100 V and measured both voltage per centimeter and temperature on the surface of 10 assay plates in the presence and absence of LiCl. Again surprisingly, we recorded significantly higher electric voltage per centimeter ($P = 0.00012$; Figure 3A) on plates lacking LiCl (mean: 16.75 V/cm) than on LiCl plates (mean: 16.21 V/cm). Furthermore, voltage per centimeter fluctuated within 30 s by 0.25 V on average in the presence of LiCl and by 0.28 V on average in its absence (Figure 3B).

After 100 V voltage application during 30 s, the mean surface temperature was 22.98 °C in LiCl plates and slightly lower, that is, 22.29 °C in plates lacking LiCl ($P = 0.0006$; Figure 3C). Additionally, we observed an increase in temperature by 0.64 °C on average in LiCl plates but only a small increase of 0.09 °C, and thus more stable temperatures, in plates devoid of LiCl ($P = 0.00017$; Figure 3D).

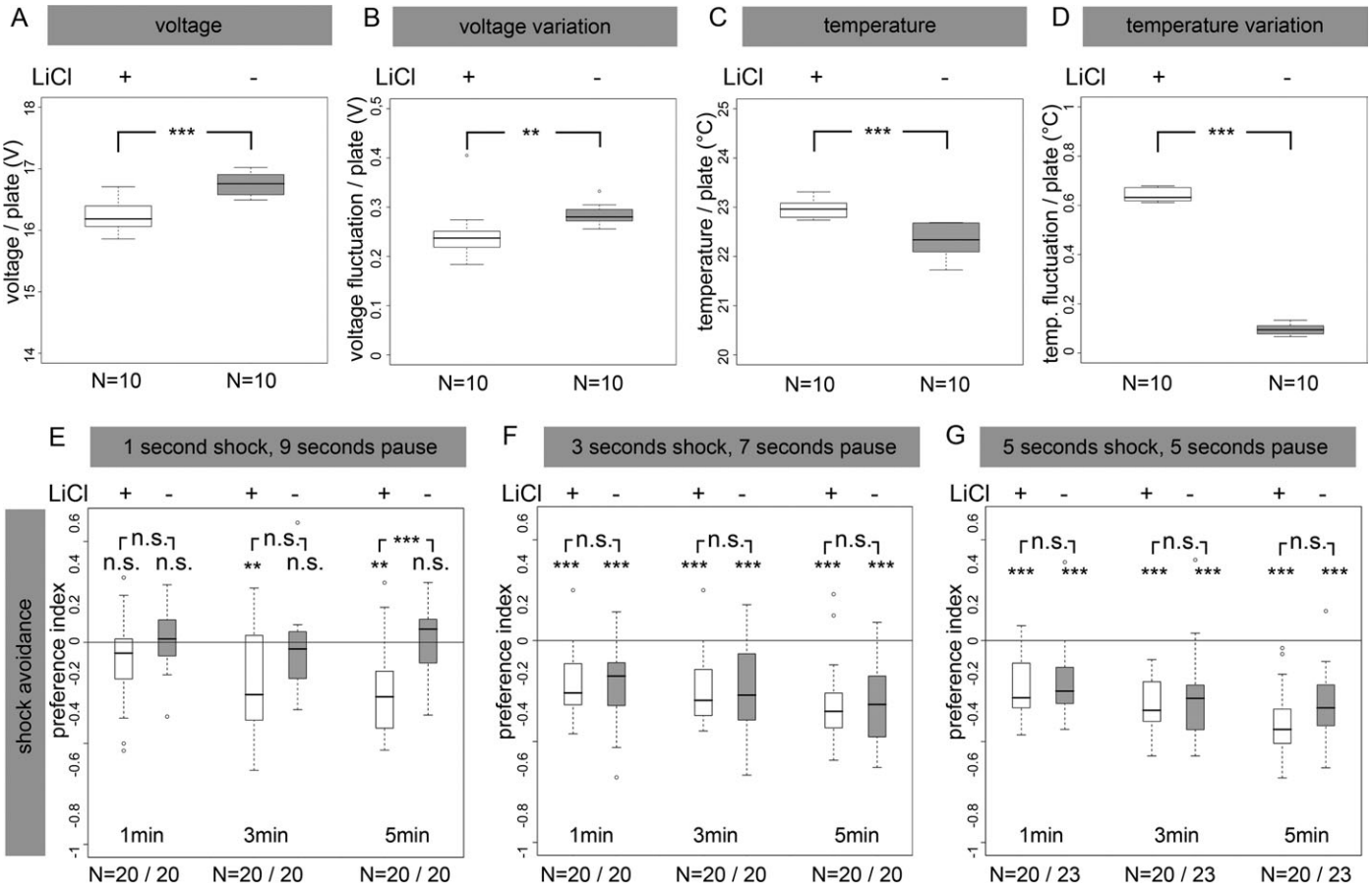

Figure 3 Effect of LiCl on voltage, temperature, and electric shock avoidance behavior. **(A)** Plates lacking LiCl (gray bars) showed a significant voltage increase compared with plates containing LiCl (white bars). **(B)** Voltage per centimeter varied significantly more on plates lacking LiCl than on plates containing LiCl. **(C)** After electric shock application for 30 s, temperature values of plates containing LiCl were slightly higher than in pure plates. **(D)** Temperature was more stable on plates without LiCl than on plates containing LiCl. **(E–G)** Naive electric shock avoidance behavior was measured by applying 1-s shock/9-s pause **(E)**, 3 s shock/7 s pause **(F)**, or 5 s shock/5 s pause intervals **(G)** on LiCl-containing plates and pure plates continuous over 5 min. When third-instar larvae were tested for their naive electric shock preference after 1, 3, and 5 min in general, no difference was detected. As an exception, after 5 min, a significant increase in avoidance was detectable for the 1-/9-s shock protocol **(E)**. Significance levels represent $P < 0.01$ (**) and $P < 0.001$ (***).

(iv) LiCl is not necessary for larvae to express electric shock avoidance behavior

To analyze the effect of LiCl on larval electric shock avoidance, groups of larvae were subjected to one of the following shock-rest regimes: 1-s shock and 9-s rest; 3-s shock and 7-s rest; 5-s shock and 5-s rest. For all 3 experiments, avoidance behavior after 1, 3, and 5 min was measured, both in the presence and absence of LiCl (Figures 3E–G). Remarkably, for the shock-rest protocols, we did not find any consistent difference in shock avoidance behavior with respect to the presence or absence of LiCl, neither after 1, 3, or 5 min ($P > 0.05$; Figures 3E–G). As an exception, larvae subjected to 1-s shock and 9-s rest showed significantly stronger avoidance after 5 min on LiCl plates than on pure plates ($P = 0.0009$; Figures 3E). We conclude that LiCl is not necessary for inducing larval electric shock avoidance behavior.

(v) LiCl per se induces appetitive and aversive gustatory preference behavior in a concentration-dependent manner

As LiCl was applied in all present larval odor–electric shock studies (Aceves-Pina and Quinn 1979; Heisenberg et al. 1985; Tully et al. 1994; Khurana et al. 2009), we tested whether LiCl per se can trigger gustatory preference behavior in larvae. We performed binary choice tests in which larvae were tested for their preference between pure agarose versus agarose containing different LiCl concentrations (from 0.001 up to 4.0 M; Figure 4). Similar to published NaCl salt preferences (Niewalda et al. 2008), larvae show attractive responses to low concentrations (0.001 to 0.075 M; $P < 0.01$); as concentration is further increased, these responses gradually turn into aversion for high concentrations of LiCl (0.15–4.0 M; $P < 0.001$); consequently, there is an intermediate concentration at which appetitive and aversive properties cancel out (0.125 M; $P = 0.183$). We conclude that LiCl per se in a concentration-dependent manner is sufficient to trigger gustatory attraction or aversion.

Electric shock learning: 1-odor nonreciprocal conditioning

A second paradigm to study larval olfactory learning used 1-odor nonreciprocal training reinforced by gustatory stimuli (Honjo and Furukubo-Tokunaga 2005, 2009). To allow for a better comparison with these data, we tested whether this type of training can also drive learning when applying electric shock (Figure 5A). Given our evidence about the dispensability of LiCl (Figures 2 and 3), we omitted this chemical in all these experiments.

Prior to the conditioning phase, groups of larvae were pretested for their preference to BA. Subsequently, in the training phase, an electric shock was presented together with BA,

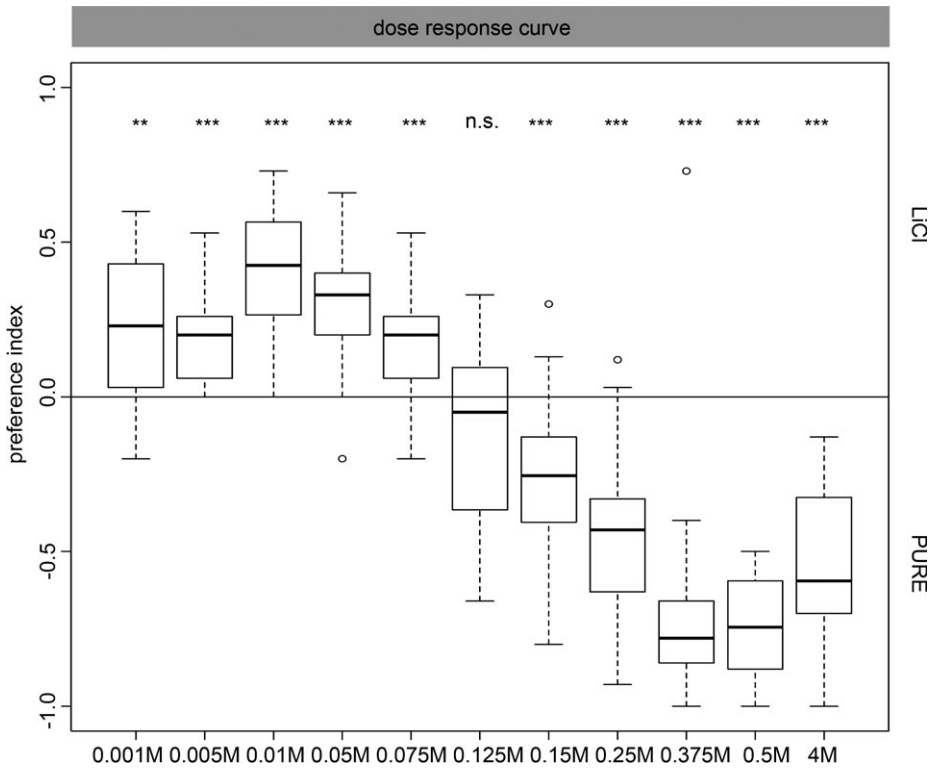

Figure 4 Dose-response curve of larval preference behavior to LiCl. Preferences between plain agarose (pure) versus various concentrations of LiCl; positive values indicate attraction and negative values repulsion. Behavior turns from appetitive to aversive as salt concentration are increased. Data are displayed as box plots with $n = 20$ in all experiments. Rank test was used for comparison of single data points to zero. Significance levels represent $P < 0.01$ (**) and $P < 0.001$ (***).

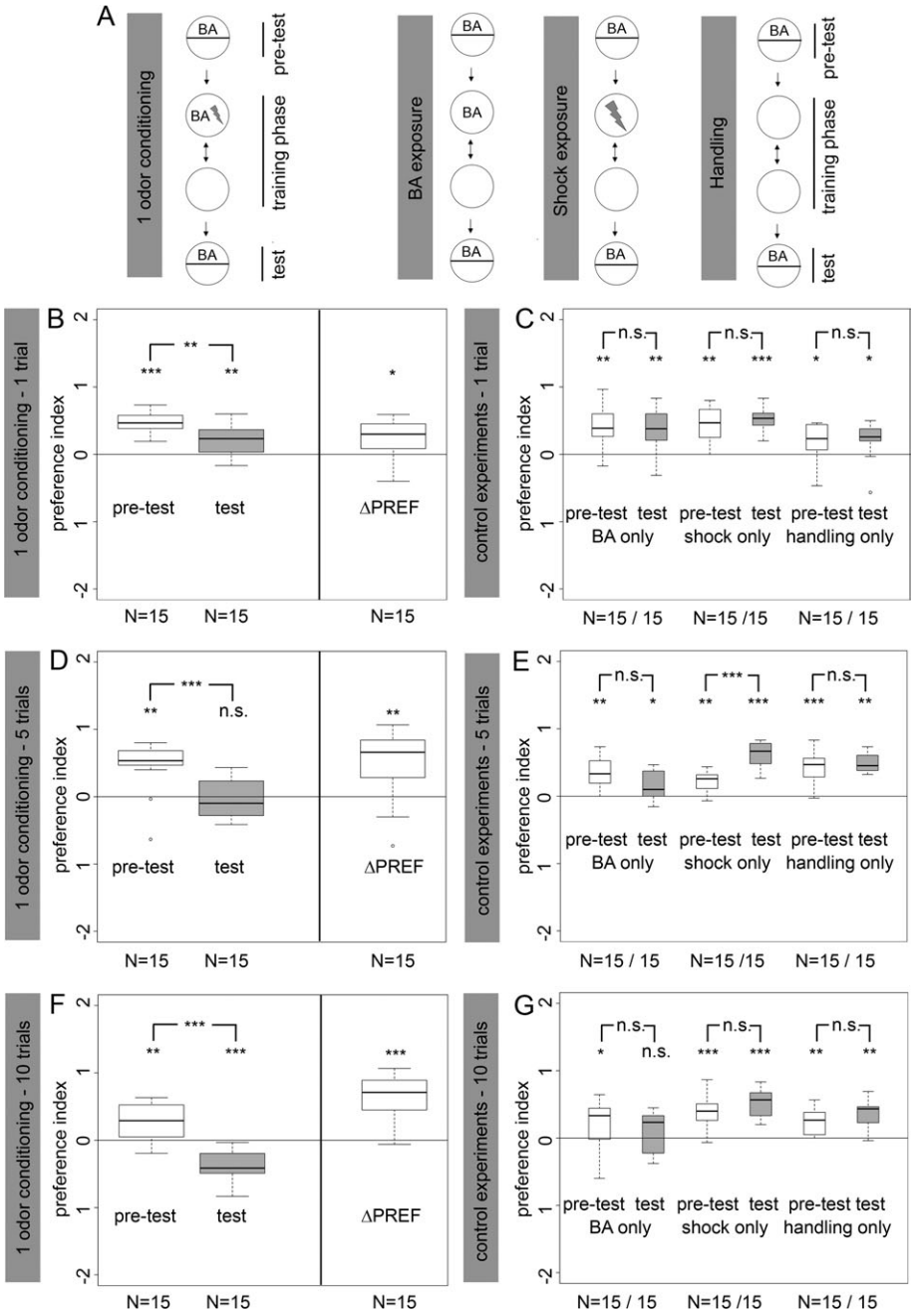

Figure 5 One-odor nonreciprocal training protocol reinforced by electric shock on assay plates lacking LiCl. **(A)** The left diagram shows the general procedure of the conditioning experiment. The remaining diagrams represent control experiments for analyzing selectively the effects of odor exposure, electric shock exposure, and handling, on BA odor preference. One-odor nonreciprocal training led to a significant decrease in BA odor preference when comparing values before (pre-test) and after training (test) for 1 **(B)**, 5 **(D)**, and 10 training trials **(F)**. The resulting differences (ΔPREF) that are shown as positive values indicate associative learning. Essentially all control experiments testing for BA odor preference before and after odor exposure, electric shock application, or handling alone led to similar values after 1 **(C)**, 5 **(E)**, and 10 training trials **(G)**, except for electric shock application only in 5 training trials (E). Significance levels represent $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

1, 5, 8 or 10 times, interrupted by 5-min intervals on a neutral plate. Finally, larvae were tested again in a choice assay for their preference for BA. Reduced responses against BA suggest a negative association between odor and electric shock, which can be quantified as ΔPREF (Figures 5B,D,F). Larvae were able to associate the 2 stimuli even after a single training trial ($P = 0.0105$; Figure 5B) as shown by a median ΔPREF value of 0.3 and did so at even higher levels when applying 5 ($P = 0.0033$; Figure 5D), 8 ($P = 8.9 \times 10^{-6}$; data not shown), or 10 training trials ($P = 0.00012$; Figure 5F).

As this type of conditioning regime lacks reciprocity and therefore defines learning as the difference in BA preference between pre-test and test after conditioning, we designed 3 control experiments to ensure whether during training odor exposure, electric shock, or the handling procedure per se change BA preference in the final test (Figure 5A). Only if these control assays fail to reveal any significant differences, ΔPREF scores can be interpreted as representing aversive odor–electric shock learning (Gerber and Stocker 2007; Honjo and Furukubo-Tokunaga 2005). After 1, 8, and 10 training trials, we did indeed not detect any significant difference in these experiments ($P > 0.05$; Figure 5C,G; data not shown). However, as an exception, after 5 training trials, larvae showed higher BA preference scores in the test after electric shock application only ($P = 8.1 \times 10^{-5}$; Figure 5E).

Memory stability after extended 1-odor nonreciprocal training

We finally addressed the question if extended training affects the retention time for 1-odor nonreciprocal electric shock learning (Honjo and Furukubo-Tokunaga 2005, 2009; Khurana et al. 2009). Therefore, we tested groups of larvae that had undergone 1, 5, and 10 training trials and measured their memory performance after 5, 15, 25, and 60 min (Figure 6). Larvae trained once showed significant performance scores when tested after 5 min ($P = 0.0105$), but their memory rapidly decayed reaching zero after 25 min ($P = 0.783$; Figure 6). By contrast, larvae that had undergone 5 ($P = 0.0033$) or 10 training trials ($P = 0.00012$) showed twice as high performance scores after 5 min compared with 1-trial assays. Their memory retention curves declined much slower and were still significantly different from zero when tested after 25 min (for 5 training trials: 6.1×10^{-5}; for 10 training trials: 0.0097; Figure 6). For 10 training cycles, memory persisted even up to 60 min ($P = 0.0057$).

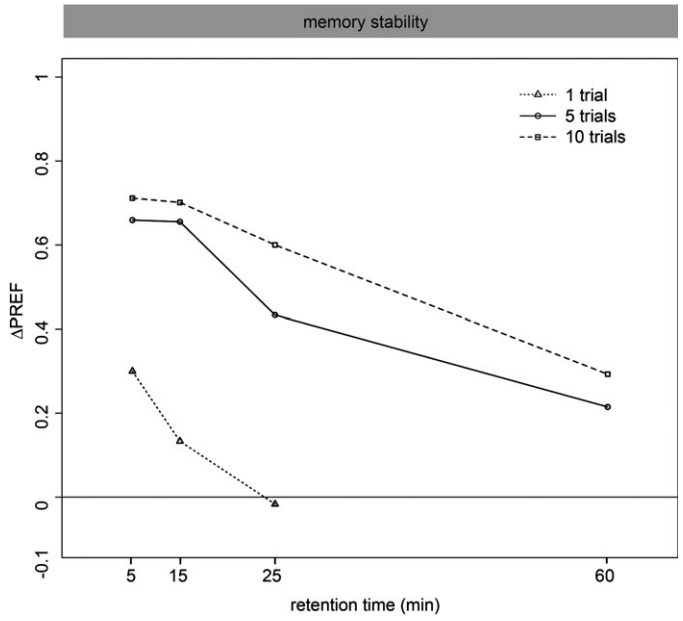

Figure 6 Memory stability after one-odor nonreciprocal training on plates lacking LiCl. After a single training trial significant, initial odor–electric shock learning was detectable that did not persist up to 20 min (triangle). Similarly, 5 (circle) and 10 training trials (square) led an increased performance score that decayed over time but still persisted up to 55 min. Each data point represents 15 experiments ($n = 15$).

Discussion

Electric shock learning in *Drosophila* larvae

To our knowledge, only 4 studies over the last 30 years have used *Drosophila* larvae to analyze olfactory learning reinforced by electric shock (Aceves-Pina and Quinn 1979; Heisenberg et al. 1985; Tully et al. 1994; Khurana et al. 2009). In their initial report, Aceves-Pina and Quinn (1979) showed that larvae are able to associate odor stimuli with electric shock using a 2-odor reciprocal training design. Furthermore, they demonstrated for 3 mutants that are impaired in adult learning and memory (*dunce*, *turnip*, and *cabbage*) a learning phenotype in third-instar larvae by using a similar approach; Heisenberg et al. (1985) revealed impaired odor–electric shock learning of a mushroom body structural mutant *mushroom body miniature* (*mbm*). Tully et al. (1994) described a long-lasting larval odor–electric shock memory throughout metamorphosis by applying 8 cycles of 2-odor, reciprocal training. Recently, Khurana et al. (2009) introduced an improved method for aversive electric shock conditioning. Their article includes a quantitative analysis of memory phases as a function of training cycles and reveals memory deficits for the classical learning mutants *dunce*, *amnesiac*, *rutabaga*, and *radish*. For their studies, they mainly employed a 1-odor nonreciprocal training design, called olfactory avoidance learning. Taken together, the published data and our own study convincingly demonstrate that larvae are able to associate olfactory stimuli with electric shock punishment after 2-odor reciprocal training (Figure 1) as well as after 1-odor nonreciprocal training (Figure 5). Depending on the number of training cycles, longer lasting forms of olfactory memory can be established (Figure 6; Khurana et al. 2009), potentially persisting through metamorphosis (Tully et al. 1994). On the molecular level, the current findings support the conclusion that cyclic adenosine monophosphate regulation is involved in the formation of the memory trace, similar to adult flies (Heisenberg 2003; McGuire et al. 2005; Keene and Waddell 2007; Tomchik and Davis 2009).

Basic parameters of the different learning paradigms

Apart from these general conclusions, substantial differences in the basic parameters of the used protocols—in particular 2-odor reciprocal training versus 1-odor nonreciprocal training—render a comparison of these studies delicate. Whereas most of them applied during 30 s AC pulses of

about 10 V/cm (Aceves-Pina and Quinn 1979), 14 V/cm (Khurana et al. 2009), or 90 V total output current (Tully et al. 1994), comparable to our approach, another study used 200 V pulses of 100 ms duration (Heisenberg et al. 1985). Even the dimension of the apparatus varied. Four studies including our own (Aceves-Pina and Quinn 1979; Heisenberg et al. 1985; Khurana et al. 2009) used simple Petri dishes of different dimensions, whereas one study utilized a self-designed chamber (Tully et al. 1994). Other variables were the precise timing of odor and electric shock application, the duration of interstimulus interval and test as well as the number of larvae per group that differed from 80 to 100 (Aceves-Pina and Quinn 1979; Tully et al. 1994) to 400 (Khurana et al. 2009). Finally, the 4 published studies applied lithium chloride (LiCl) in concentrations that varied over the range of 2 potencies (0.15 M LiCl, Aceves-Pina and Quinn 1979; 0.02 M LiCl, Heisenberg et al. 1985; Khurana et al. 2009; 0.002 M LiCl, Tully et al. 1994).

Given these discrepancies and the recent revival of interest in larval learning (reviewed by Gerber and Stocker 2007), we established 2 paradigms for larval odor–electric shock learning that integrate previous experimental data on larval olfactory learning. These paradigms will allow in the future a rigorous comparison with other larval studies and simplify the relation between larval and countless adult studies on learning and memory.

LiCl in olfactory electric shock learning

Our data clearly show that LiCl is not required for proper electric shock signaling in the assay plate, in contrast to earlier assumptions (Aceves-Pina and Quinn 1979; Heisenberg et al. 1985; Tully et al. 1994; Khurana et al. 2009). We discuss this observation in the following context: (i) None of the previous investigations convincingly demonstrated the necessity of LiCl in electric shock conductivity. (ii) The concentrations of LiCl applied in these studies differed over 2 orders of magnitude (see above), which should seriously affect conductivity if based on LiCl. (iii) Larvae exhibit an increased performance in the 2-odor reciprocal assay when trained on plates containing LiCl but tested on plates lacking LiCl, but they show the same increase in the opposite experimental situation (Figure 2D). Thus, as the electric shock is not present during test, the effect of LiCl on learning performance is not correlated with the proper conductivity of the electric stimulus. (iv) When comparing the physical properties of LiCl-containing and pure plates, the mean temperature difference was less than 1 °C (Figure 3C); moreover, the mean voltage was slightly higher in the pure plates than in the LiCl plates (Figure 3A). (v) A difference in electric shock avoidance behavior between plates with and without LiCl was detectable only for electric shocks of 1-s duration but not for stimuli lasting 3 or 5 s (Figures 3E–G), which is far less than the 30 s of electric shock used during training. Therefore, improvement of larval odor–electric shock learning by LiCl (Figure 2B) cannot be due to increased conductivity of electric shock during reinforcement.

In addition omission of LiCl in future electric shock conditioning experiments would be of considerable relevance because its attractiveness to larvae at low concentrations complicates the interpretation of learning scores (Figure 4). Moreover, numerous studies have shown a variety of unpredictable interactions of this molecule with cellular functions apparently shared by *Drosophila* and vertebrates (Chen et al. 2000; Schou 2001; Mudher et al. 2004; Padiath et al. 2004; Berger et al. 2005; Dokucu et al. 2005; Iitaka et al. 2005; McBride et al. 2005; Min et al. 2009). Also *Drosophila* Affymetrix Genome Arrays after lithium induction showed changes in amino acid metabolic processes, genes implicated in detoxification, and potential candidate genes involved in psychiatric or neurological disorders (Kasuya et al. 2009a).

In conclusion, based on these unpredictable effects of LiCl (Xia et al. 1997) and on its tastant property, we suggest that further experiments on larval odor–electric shock learning should refrain from using this chemical. Moreover, it will be crucial to revisit larval conditioning in *dunce*, *turnip*, *cabbage* (Aceves-Pina and Quinn 1979), *mbm* (Heisenberg et al. 1985), *amnesiac*, *rutabaga*, and *radish* mutants (Khurana et al. 2009) to check whether the described phenotypes are not due to changes in LiCl perception or other neurobiological processes independent of learning. On the other hand, our finding that LiCl improves larval odor–electric shock learning (Figure 2B) may allow for a multilevel approach that can help to understand lithium-dependent neurobiological processes.

One-odor nonreciprocal versus two-odor reciprocal training design: assets and drawbacks

The 2-odor reciprocal design was widely used in larval olfactory learning reinforced by gustatory stimuli (Michels et al. 2005; Neuser et al. 2005; Gerber and Hendel 2006). Using a comparable experimental setup, we therefore established a protocol for larval 2-odor–electric shock learning (Figure 2B). In the future, this will allow for a comparison of larval aversive olfactory conditioning in general. Yet, apart from the methodological advantages mentioned before, the relatively low learning scores obtained (Figure 2B) may render comparative studies of genetically manipulated larvae difficult, especially when dealing with partial memory defects. This drawback may be partially overcome by increasing the number of training trials, although a prolongation in handling time might trigger starvation-dependent effects (Wu et al. 2003; Wu, Zhang, et al. 2005; Wu, Zhao, and Shen 2005; Krashes et al. 2009). Hence, for many tasks, a 1-odor nonreciprocal design may be preferable due to its shorter training cycles (Figure 5A) and relatively high differences in preference scores before and after training (Figure 5H) yet requiring additional controls (Figure 5C).

Outlook

Taken together, our study introduces 2 paradigms for analyzing larval olfactory learning reinforced by electric shock. These assays allow for a direct comparison with larval olfactory learning reinforced by gustatory stimuli and with adult odor–electric shock learning. Moreover, the comprehensive description of the larval olfactory pathway (Ramaekers et al. 2005; Masuda-Nakagawa et al. 2009) may provide a basis for interpreting associative learning on the single-cell level. Finally, future functional studies taking advantage of the versatile molecular and genetic tools in *Drosophila* may help to better understand lithium-dependent processes in the brain as well as aversive olfactory learning in general.

Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/

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