© 2015. Published by The Company of Biologists Ltd | The Journal of Experimental Biology (2015) 218, 336-339 doi:10.1242/jeb.113266

METHODS & TECHNIQUES

The Yerkes–Dodson law and appropriate stimuli for conditioned taste aversion in *Lymnaea*

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ABSTRACT

The pond snail *Lymnaea stagnalis* can learn conditioned taste aversion and then consolidate it into long-term memory (LTM). A high-voltage electric shock was used as the unconditioned stimulus, where we have previously used KCl. We varied the strength of both the conditioned and unconditioned stimuli to determine whether the so-called Yerkes–Dodson law prevailed. This is an empirical relationship between the state of arousal and LTM formation, showing that there is an optimal level of arousal leading to memory formation. However, too little or too much arousal results in poorer LTM. We found here that the most appropriate stimuli to use in taste aversion training in *Lymnaea* were a 10 mmol l^{-1} sucrose solution as the conditioned stimulus and a 3 s electric shock as the unconditioned stimulus.

KEY WORDS: Conditioned taste aversion, Electric shock, *Lymnaea*, Yerkes–Dodson law

INTRODUCTION

The pond snail *Lymnaea stagnalis* is an excellent model system to elucidate the causal neuronal mechanisms underlying learning and the formation of memory (Ito et al., 2013; Otsuka et al., 2013; Takahashi et al., 2013; Lukowiak et al., 2014; Takigami et al., 2014). Here, we focus our attention on aversive conditioning of feeding behavior known as conditioned taste aversion. In conditioned taste aversion, a conditioned stimulus (CS) (sucrose, which elicits a feeding response) is paired with an unconditioned stimulus (US) such as KCl, which causes the termination of feeding (Kojima et al., 1996). With the appropriate pairing of the CS and US, the CS no longer elicits feeding.

Recently, the Sakakibara group successfully developed a new procedure for conditioned taste aversion (Takigami et al., 2013). They used a high-voltage electric shock with minimal current (~80 µA) that was easily applied as the US. This US elicits the whole-body withdrawal response, whereby the snail pulls itself into its shell and feeding is terminated. This new US has an important advantage over other USs used previously. This advantage is stimulus consistency over the course of training. With other USs used, this is not the case. For example, with using KCl as the US (Kojima et al., 1996), different experimenters aim the squirts differently and often during an experiment the same experimenter aims the squirts differently. We wished to compare conditioned taste aversion memory obtained using different durations of the electrical shock as well as different concentrations of sucrose as the CS.

In addition to the use of the novel US, the Takigami study (Takigami, 2013) used a 100 mmol l^{-1} sucrose solution as the CS whereas in our previous studies a 10 mmol l^{-1} sucrose solution was used (Mita et al., 2014a). The method of application of the CS also differed. The Takigami study (Takigami, 2013) used a perfusion system to deliver the CS, whilst we used a pipette to change the solutions (Mita et al., 2014a). With these differences in mind, we set out to compare conditioned taste aversion produced by the novel US with conditioned taste aversion produced using the KCl US.

We also tested the so-called Yerkes–Dodson law (Yerkes and Dodson, 1908) regarding the strength of the stimuli used in training. In the 1908 paper, the relationship between stimulus strength and rapidity of learning was studied. Their basic finding was ‘an easily acquired habit may be readily formed under strong stimulation, whereas a difficult habit may be acquired only under relatively weak stimulation’. Typically, however, this ‘law’ is shown as an inverted-U function. This formulation arises from Hebb’s paper (Hebb, 1955) examining the relationship between neuronal arousal and the nervous system. He characterized this relationship as what can be best described as inverted-U that states that there will be an optimal level of arousal for learning. We attempted to clarify this point in conditioned taste aversion of *Lymnaea*.

RESULTS AND DISCUSSION

Aversiveness of various unconditioned stimuli

We tested the following USs: (1) 10 mmol l^{-1} KCl for 15 s, (2) 100 mmol l^{-1} KCl for 15 s, (3) 0.2 s electric shock, (4) 3 s electric shock and (5) 5 s electric shock. Each of these USs was tested on snails at day −1, day 1 and day 5. The food-deprivation status is defined in the following manner: the day when snails begin food deprivation is called day 0. Day −1 snails are thus ‘fed’ snails; day 1 snails are the snails deprived of food for 1 day; and day 5 snails are snails food deprived for 5 days (Mita et al., 2014a).

We found that the 100 mmol l^{-1} KCl solution was the most aversive stimulus in all three snail cohorts (Fig. 1A). In day −1 snails, the 100 mmol l^{-1} KCl solution caused snails to remain in their shells significantly longer than the 10 mmol l^{-1} KCl solution and both the 0.2 s or 3 s electric shock (Fig. 1A). Interestingly, the 10 mmol l^{-1} KCl solution was more aversive than either the 0.2 s or 3 s electric shock.

In day 1 snails, similar results were obtained. The duration of time in the shell was not significantly different for any of the stimuli compared with day −1 snails (Fig. 1A). Finally, the similar pattern of the recovery time was obtained in day 5 snails. That is, the 100 mmol l^{-1} KCl solution was again the most aversive stimulus in day 5 snails. However, the perception of aversiveness was altered in day 5 snails. While the 100 mmol l^{-1} KCl was still the most potent stimulus, the time that snails remained in their shells was significantly shorter than in the other cohorts. This was also the case

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Received 1 September 2014; Accepted 10 December 2014

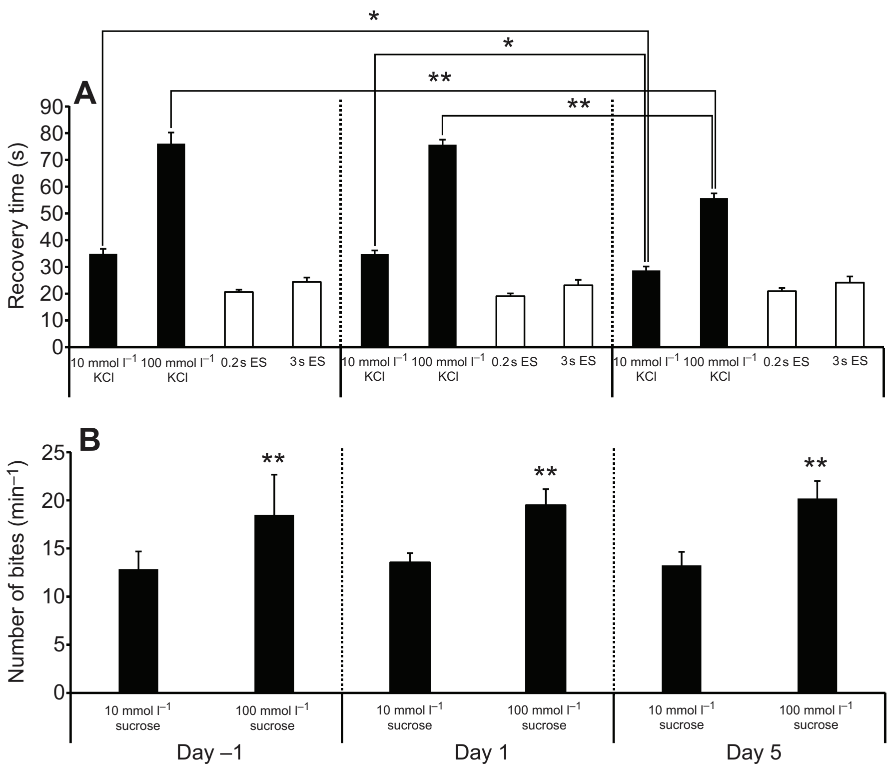

Fig. 1. Aversiveness of US and salience of CS in food-deprived *Lymnaea stagnalis* pond snails. (A) Aversiveness of US is indicated as the recovery time from withdrawal response induced by the US [KCl solution or electric stimulus (ES)] ($F_{11,228}$=111.7, P<0.01). In the severely food-deprived snails (day 5 snails), the aversiveness of both the 10 mmol l^{-1} KCl and the 100 mmol l^{-1} KCl US declined. The effectiveness of the US electric stimulus was not altered by the internal state of snails. (B) Salience of CS was indicated as number of bites per minute (i.e. rasps) in response to sucrose solution ($F_{5,114}$=14.44, P<0.01). The 10 mmol l^{-1} sucrose solution CS elicited similar numbers of biting irrespective of the internal state of the snails. *P<0.05, **P<0.01.

for 10 mmol l^{-1} KCl. However, the state of food deprivation did not alter the response to the electrical stimuli. Thus, although the internal state of the snail was changed by prolonged food deprivation as evidenced by differences in response to the KCl solution, the snails' responsiveness to the 0.2 s or 3 s electric shock was relatively unaltered. We conclude that the electric shock with a duration of 0.2 s or 3 s is an effective and reliable US in all three cohorts. The results of 5 s electric shock will be described below.

Salience of sucrose

We next tested the salience of the CSs in the three cohorts. The 100 mmol l^{-1} sucrose solution elicited a significantly larger feeding response (i.e. the number of bites per minute) in all three cohorts than the 10 mmol l^{-1} sucrose solution (Fig. 1B). We chose to use the lower concentration CS in our experiments. A 1 mmol l^{-1} sucrose solution did not elicit a strong enough response [for example, the number of bites per minute was 1.05±0.18 (mean ± s.e.m.; 20 day 5 snails)], it cannot confidently be used in our experiments.

Taste-aversion training in snails using 10 mmol l^{-1} sucrose CS and electric shock USs

The 0.2 s and the 3 s duration electric shocks were used as the US with a 15 s application of 10 mmol l^{-1} sucrose solution as the CS. In day 1 snails when a 0.2 s electric shock US was used, taste aversion learning was formed with 50 CS–US pairings (Fig. 2A) and was consolidated into long-term memory (LTM) that persisted for at least 1 week (Fig. 2B). When the 3 s electric shock was used as the US, with all other conditions the same, 20 CS–US pairings were sufficient to result in conditioned taste aversion and LTM persisted for at least 1 week (Fig. 2C,D). Thus, the stronger US resulted in more rapid learning. However, the 5 s electric shock was not suitable for a multi-trial taste aversion training (e.g. >10 CS–US pairings). When the snails were given 10 CS–US pairings or 10 US–CS pairings, 100% (i.e. 20 out of 20) of snails stayed retracted in their shells at the post-test sessions (data not shown).

However, the 5 sec US could be used in snails for one-trial learning. Using this US, a single pairing of the CS–US was sufficient to produce conditioned taste aversion (Fig. 2E). However, only a few snails were 'good performers' (i.e. snails that made 0–1 bites min^{-1} during the memory test session; Fig. 2F). This result indicates that only in certain snails could LTM result from a single paired presentation. In those snails that exhibited memory, it persisted for at least 1 week (Fig. 2G). Note that good and poor performers are defined in the Materials and methods section (Sugai et al., 2007; Mita et al., 2014a). When we used five pairings with this US, conditioned taste aversion was not formed because no difference was found in the data between the taste-aversion-trained snails and the backward-conditioned snails (Fig. 2E).

Electric shock as the appropriate US for conditioned taste aversion

Our data showed that the use of the new US (Takigami et al., 2013) resulted in good taste aversion learning and LTM (i.e. CTA-LTM), with the advantages being an easier learning curve for experimenters and far more stimulus consistency. Thus, conditioned taste aversion experiments will be more reproducible possibly allowing differences in memory forming ability to be seen between strains of *Lymnaea* (Orr et al., 2009; Dalesman et al., 2011). In the past, differences in both how quickly criteria for learning were met (i.e. the number of CS–US pairings) and for how long the memory persisted were not easily compared between studies because the manner in which the KCl stimulus (the US) was added may not have been similar between, and even within, the various studies. This new stimulation tool eliminates to a large extent this uncontrolled variable. In addition, the response elicited by this US was not altered by differences in duration of the food deprivation, unlike the situation with the KCl stimulus. This may also allow experimenters to be better able to study how differences in 'internal drive' alter learning acquisition and memory formation.

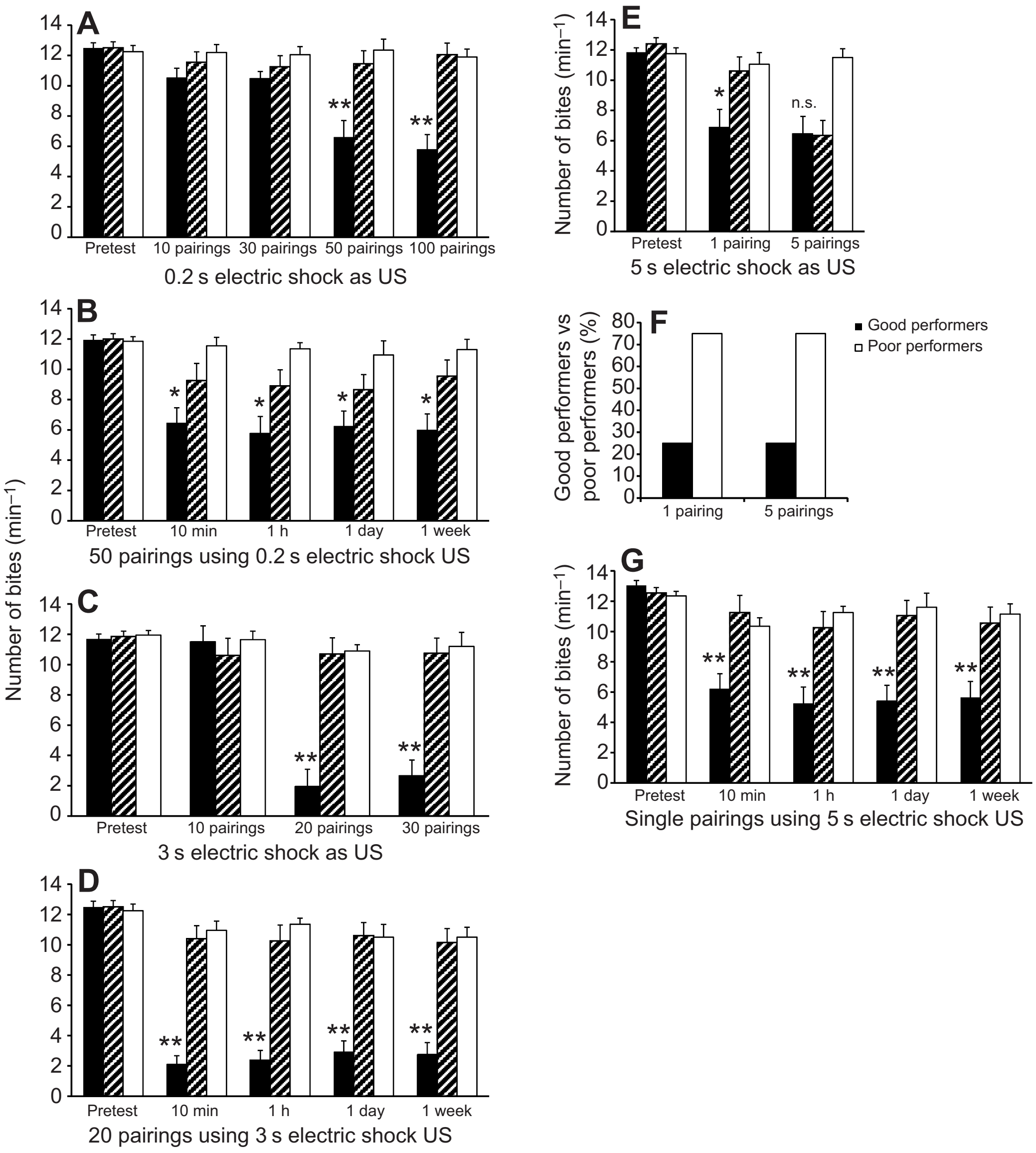

Fig. 2. Conditioned taste aversion scores using 10 mmol l^{-1} sucrose solution as the CS and electric shock as the US in day −1 snails. (A) We tested taste-aversion trained snails (black bars), backward-conditioned snails (hatched bars) and naive control snails (white bars) ($F_{14,285}$=9.381, P<0.01). When we used 0.2 s duration of electric shock as the US, CTA was acquired after 50 pairings of CS–US. (B) After 50 pairings of this CS and the 0.2 s electric shock US, CTA memory persisted for at least one week ($F_{14,285}$=7.566, P<0.01). (C) When we used the 3 s duration of electric shock as the US, CTA was acquired after only 20 pairings ($F_{11,228}$=21.43, P<0.01). (D) After 20 pairings of the CS and the 3 s US, CTA memory persisted for at least 1 week ($F_{14,285}$=32.27, P<0.01). (E) The 5 s duration of electric shock US caused one-trial learning in some snails ($F_{9,190}$=8.765, P<0.01). With five pairings, no significant difference was found between taste-aversion trained snails and backward-conditioned snails. (F) Only a few snails were good performers after a single pairing and five parings of CS-US. (G) After a single pairing of 10 mmol l^{-1} sucrose solution CS and 5 s electric shock US, taste aversion leaning was acquired and LTM (i.e. CTA-LTM) persisted for at least 1 week ($F_{14,285}$=10.90, P<0.01). *P<0.05, **P<0.01.

The US must be noxious enough to inhibit feeding but not too noxious otherwise the snail will remain in its shell (Kojima et al., 1996). A weak US does not result in conditioned taste aversion, whereas a too-strong US cannot be used as the snails can no longer receive paired CS–US presentations. Thus, both the 0.2 s and the 3 s US can be used but not the 5 s US for repeated CS–US pairings. In a similar manner, we found previously that too sweet a CS did not result in conditioned taste aversion. Because a successful taste aversion training requires snails to be in a food-deprived state (Mita et al., 2014a), the too-sweet CS elicits a stronger drive to eat that overcomes the learned behavior. The more salient CS must cause changes in activity of neurons in the central pattern generator that drives feeding behavior that overcomes the suppression of feeding caused by taste aversion training. Sunada et al. (Sunada et al., 2014) showed that RPeD11, whose activity triggers withdrawal behavior, inhibits neurons that influence feeding behavior (i.e. the cerebral giant cells). This inhibitory synaptic effect increases with the CS–US pairings and in part underlies conditioned taste aversion (Ito et al., 2013). It is unclear presently whether a CS with a high salience counteracts this inhibition.

Yerkes–Dodson law

In addition to demonstrating the usefulness of this new US, our data allow us the opportunity to better examine whether our findings are consistent with the so-called Yerkes–Dodson law. This ‘law’, as it is most often stated, is not what Yerkes and Dodson in their 1908 paper actually reported. What is typically presented as the ‘law’ is actually a figure adapted from Donald Hebb’s 1954 presidential address to the American Psychological Association (Hebb, 1955). Hebb was championing the notion that the psychologists of that time must be cognizant of the ‘best brand of neurology we can find’ in order to understand how arousal and motivation affect learning. Thus, Hebb hypothesized that there is an optimal level of learning and that with too little or too much ‘arousal’ learning is not optimal (i.e. the inverted-U function). The data presented here with regard to both the strength of the US and CS are consistent with Hebb’s 1955 hypothesis. Thus, neither the 1 mmol l^{-1} nor the 1000 mmol l^{-1} sucrose solution could reliably serve as a CS in taste aversion training and testing for CTA-LTM.

Similarly, too strong a US could not be used. This stimulus elicited a strong arousal response such that the majority of snails stay withdrawn in their shells and thus they could not receive

CS–US pairings. Are our data consistent with the empirical findings of the Yerkes–Dodson 1908 paper regarding stimulus strength and difficulty of the learning task? If we assume that conditioned taste aversion associative learning and its subsequent consolidation into LTM is an easy task, then yes, our data are consistent. Snails learned faster with the stronger US (20 pairings with the 3 s US versus 50 pairings with the 0.2 s US) and with the strongest US (5 s) learned in a single pairing. We did not test here as predicted by the Yerkes–Dodson 1908 paper whether a more difficult learning task can only be acquired under relative weak stimuli.

In summary, our data are consistent with the Yerkes–Dodson law as it is most often presented in text books and in many literature reviews on stress and memory (but which is really Hebb's 1955 inverted-U description of how the level of neuronal arousal affects learning). Interested readers are directed to the following articles regarding the 'evolution' of the Yerkes–Dodson law (Brown, 1965; Broadhurst, 1957; Berlyne, 1969; Staal, 2004; Diamond et al., 2007).

MATERIALS AND METHODS

Snails

Lymnaea stagnalis Linnaeus 1758 originally from Vrije Universiteit Amsterdam with a 18–23 mm shell were obtained from our snail-rearing facility. All snails were maintained in dechlorinated tap water (i.e. pond water) under a 12 h:12 h light:dark cycle at 20°C and fed *ad libitum* on turnip leaf (*Brassica rapa* var. *peruviridis*, known as Komatsuna) and Spiral Shell Food (Nisso, Saitama, Japan) every other day (Ito et al., 2012).

Taste aversion training procedure

All snails were given a pretest; in this observation period (1 min), the number of bites was counted in distilled water following a 15 sec application of the CS to the lips (Mita et al., 2014a). In the taste aversion training, we paired the CS with the US. Two different USs were used: KCl solution (Kojima et al., 1996) and a high-voltage electric shock with minimal current with distilled water (Takigami et al., 2013; Mita et al., 2014b). The electric shock was applied near the head in the distilled water. We did not touch the head with the stimulator. We applied the electric shock with 'high voltage' and 'minimal current' to snails in water. Thus, small variations in distance between the snails and the electrode had minimal effect on the current delivered to the snail. The stimuli were required enough to evoke the withdrawal response. The inter-stimulus interval between the onset of the CS and US was 15 s. A 10 min inter-trial interval was interposed between each pairing of the CS–US. Controls included a backward-conditioned (US–CS) group and a naive group (only received distilled water as both the CS and US). The first post-tests were always performed 10 min after the onset of the last CS application in the taste aversion training procedure or the onset of the last US application in the backward-conditioning procedure. In the post-test sessions, snails were challenged with the CS, and the number of bites was recorded. All tests were performed blind. The behavioral experiments were performed in the morning, because the learning scores are better in the morning than at other times.

We set a performance boundary in the learning and memory test sessions to distinguish between good and poor performers (Sugai et al., 2007). In good performers, the CS did not elicit a bite. Spontaneous bites occur at a rate of about 1 per minute. Thus, a good performer is a snail that made 0–1 bites min^{-1} during the pos-ttest session. Poor performers were defined as snails that made ≥2 bites min^{-1} in response to the CS during the post-test session.

Statistics

The data are expressed as the means ± s.e.m. In all cases, the number for each treatment was 20 snails. Significant differences among more than three groups were examined by one-way ANOVA and *post hoc* Tukey test. Significance was at least $P<0.05$.

Competing interests

The authors declare no competing or financial interests.

Author contributions

E.I., M.S. and K.L. designed the research; E.I., M.Y. and S.T. performed the experiments; E.I., S.T., M.S. and Y.F. analyzed the data; E.I., M.S., Y.F. and K.L. wrote the paper.

Funding

This work was supported by KAKENHI from JSPS (Nos. 24657055 and 25291074) to E.I. and a grant from NSERC to K.L.

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