Research

Extending In Vitro Conditioning in *Aplysia* to Analyze Operant and Classical Processes in the Same Preparation

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Operant and classical conditioning are major processes shaping behavioral responses in all animals. Although the understanding of the mechanisms of classical conditioning has expanded significantly, the understanding of the mechanisms of operant conditioning is more limited. Recent developments in *Aplysia* are helping to narrow the gap in the level of understanding between operant and classical conditioning, and have raised the possibility of studying the neuronal processes underlying the interaction of operant and classical components in a relatively complex learning task. In the present study, we describe a first step toward realizing this goal, by developing a single in vitro preparation in which both operant and classical conditioning can be studied concurrently. The new paradigm reproduced previously published results, even under more conservative and homogenous selection criteria and tonic stimulation regime. Moreover, the observed learning was resistant to delay, shortening, and signaling of reinforcement.

Ambulatory animals continuously face changing environmental situations. However, not all events are random occurrences. Some events are direct consequences either of the behavior of the animal or of some other events in the environment. If the nonrandom events are significant, animals that can predict them will have a strong adaptive advantage. Some of the most regular predictive relationships are inborn (e.g., reflexes), but many others are learned. Operant or instrumental conditioning is a form of learning in which an animal learns the predictive relationship between behaviors and the environment (Thorndike 1911; Skinner 1938), whereas classical or Pavlovian conditioning is a form of learning in which an animal learns the relationship between two environmental events (Pavlov 1927). In freely moving animals in the wild, it can be difficult to distinguish between the two, because a feedback loop exists between the behavior of the animal and the environment. For example, a frog may discover a small moving object while foraging for prey, extend its tongue toward the object, find that the object is striped and produces a noxious sting and hence in the future avoid striped insects. This well-known example of aversive conditioning illustrates the feedback loop between behavior and stimuli. The foraging behavior led to the perception of the moving object, which in turn elicited the extension of the tongue, which in turn had the noxious sting as a consequence, which in turn led to the avoidance of striped insects by the frog. It is not clear a priori which events have been remembered by the frog. Clearly, the stripes were somehow associated with the sting (a classical association between two stimuli), but was the extension of the tongue instrumental in this association? To understand such interacting events, it is necessary to first reduce them to their operant and classical components and then join them again under controlled conditions.

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Laboratory studies of classical conditioning have successfully interrupted the operant–classical feedback loop such that the behavior of the animal is irrelevant and the two environmental events (the conditioned stimulus, CS, which predicts the unconditioned stimulus, US) can be traced from their sensory afferents to the brain and, finally, to the point where they converge and the learning occurs (e.g., Walters and Byrne 1983; Bao et al. 1998; Hawkins et al. 1998; Kim et al. 1998; Lechner et al. 2000a,b; Schafe et al. 2001; Medina et al. 2002; Paschall and Davis 2002; Ressler et al. 2002; Antonov et al. 2003; Crow and Tian 2003; Davis et al. 2003; Epstein et al. 2003; Flynn et al. 2003; Mozzachiodi et al. 2003; Nader 2003). An analogous convergence point between operant behavior and the unconditioned stimulus (or reinforcer in the operant nomenclature) has recently been described in *Aplysia* (Nargeot et al. 1999a,b; Brembs et al. 2002).

The carefully controlled operant and classical conditioning protocols used in laboratory studies are somewhat artificial learning situations, because the closed feedback loop between behavioral outputs and sensory inputs in a freely moving animal inevitably leads to many sensory stimuli eliciting behavioral responses and many behavioral actions causing the perception of sensory stimuli, all at or near the same time. One would expect that evolutionary selection pressures would form around the natural situation in which both operant and classical predictors play their parts simultaneously, so that this situation may be more easily learned than in the separate, experimental cases (i.e., composite conditioning; Brembs 2000; Brembs and Heisenberg 2000; Heisenberg et al. 2001). On the other hand, studies from vertebrates suggest that such a combination can have various effects, depending on subtle details (Williams 1975; Williams and Heyneman 1982; Williams 1989; Williams et al. 1990; Hammerl 1993; Reed 1996, 1999, 2003; Williams 1999). Therefore, as a first step toward studying the neurobiological underpinnings of operant and classical interactions, we have designed an experimental system in which operant and classical conditioning can be investigated separately, concurrently, or sequentially and which is amenable to cellular and network analysis. We took advantage of the recent advances in operant and classical conditioning of *Aplysia* feeding behavior (Susswein and Schwarz 1983;

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Schwarz and Susswein 1986; Colwill et al. 1997; Nargeot et al. 1997, 1999a,b,c; Lechner et al. 2000a,b; Mozzachiodi et al. 2003) and developed a computer-supported, single *Aplysia* preparation in which operant and classical experiments can be conducted both separately and in combination.

The feeding behavior of *Aplysia* (Fig. 1) offers a useful system in which to investigate classical and operant conditioning. Recently, substantial progress has been made toward understanding the neurobiology of operant conditioning of feeding behavior in *Aplysia* (Nargeot et al. 1997, 1999a,b,c; Brembs et al. 2002; Katzoff et al. 2002) as well as toward understanding the neurobiology of classical conditioning (Lechner et al. 2000a,b; Mozzachiodi et al. 2003).

Given the greater accessibility for neurobiological research, we chose to work in vitro, with reduced preparations of the *Aplysia* CNS, similar to the two previously developed in our laboratory. One in vitro preparation has been developed to study operant conditioning and another to study classical conditioning (Nargeot et al. 1997; Mozzachiodi et al. 2003). These preparations are rather similar. For example, in both, patterned motor outputs (buccal motor patterns, BMPs) are recorded extracellularly from the peripheral nerves of the buccal ganglia. This patterned activity can be interpreted as the commands for the movements of the radula/odontophore (a tongue-like organ), which lead to ingestion (or rejection) behavior (i.e., fictive feeding behavior, Fig. 1). Ingestion behavior can be classically and operantly conditioned in vivo (Susswein et al. 1983; Susswein et al. 1986; Lechner et al. 2000b; Brembs et al. 2002). The esophageal nerve (En_2) conveys the US (Schwarz and Susswein 1986; Nargeot et al. 1997; Lechner et al. 2000b; Brembs et al. 2002; Mozzachiodi et al. 2003) and the anterior tentacle nerve (AT_4) conveys the CS (Lechner et al. 2000a,b; Mozzachiodi et al. 2003). In the analog of classical conditioning the CS and US are delivered as electrical stimulation of these nerves. Thus, in behavioral terms, the BMPs constitute the operant behavior (ingestion or rejection; Morton and Chiel 1993a,b; Nargeot et al. 1997) and extracellular stimulations of the aforementioned nerves constitute the environmental feedback (i.e., stimulation of AT_4 simulates tactile stimulation of the lips; Lechner et al. 2000a,b; Mozzachiodi et al. 2003; stimulation of En_2 simulates food reward, Brembs et al. 2002).

However, besides the training protocol (operant vs. classical), there is one major difference between the two preparations. The preparation for classical conditioning included the cerebral ganglion, because it mediates the CS pathway (Lechner et al. 2000a,b; Mozzachiodi et al. 2003), whereas the operant procedure did not (Nargeot et al. 1997, 1999a,b,c).

Thus, to be able to study the interaction of operant and classical conditioning, we developed a single buccal/cerebral preparation in which classical and operant conditioning experiments can be conducted and the results compared. Moreover, this preparation will allow for the concurrent presentation of classical and operant predictors, and thereby provide a preparation that is suitable for cellular analyses of composite learning. As part of this study, we also developed a computer-assisted neuronal pattern recognition system to identify the BMPs. Most stimulation parameters were entirely computer controlled. The new preparation reproduced the previously published operant learning. Various parameter modifications indicated that the in vitro conditioning was rather robust.

RESULTS

The first step toward developing a preparation in which the interaction of classical conditioning and operant conditioning can be analyzed was to determine whether in vitro operant conditioning is expressed in the preparation originally developed to study classical conditioning (Mozzachiodi et al. 2003). Specifically, we subjected a preparation consisting of the isolated cerebral ganglion and buccal ganglion to the in vitro protocol of Nargeot et al. (1997) and investigated the extent to which the preparation reproduced the previous results. The cerebral ganglion contains higher-order neurons that can trigger the occurrence of BMPs in the buccal ganglia (Rosen et al. 1991; Jing and Weiss 2001, 2002; Hurwitz et al. 2003). It is unknown whether it

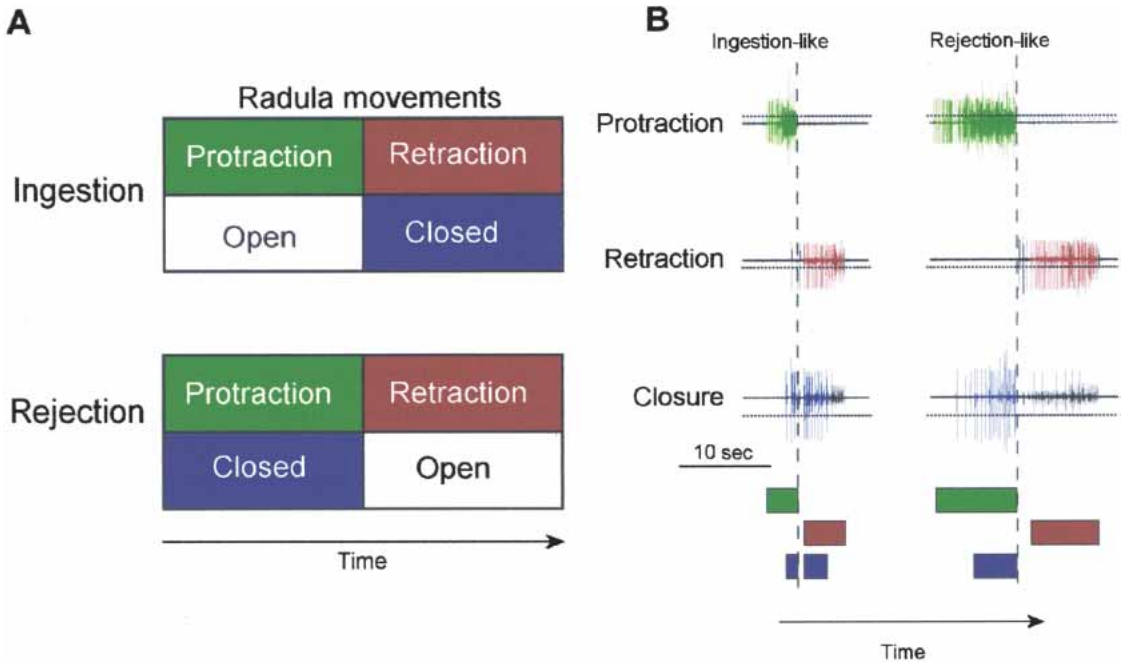

Figure 1 Pattern classification. (*A*) Schematic representation of the radula movements during ingestion and rejection. (*B*) Pattern classification deduced from the radula movements depicted in *A*. Note that only closure activity is counted that overlaps with radula movement (pro- or retraction; see Materials and Methods). Dotted lines–activity detection thresholds.

also contains cells that can silence neural activity in the buccal ganglia. Although preliminary experiments, in which we recorded from the cerebral-to-buccal connective (CBC) during spontaneous BMPs, did not reveal any evidence that spontaneous BMPs are either elicited or suppressed by signals originating in the cerebral ganglion (data not shown), the presence of either type of cell could disrupt either the occurrence of spontaneous BMPs, the ability of BMPs to be conditioned, or both.

As part of the study, we also developed a computer program (see Materials and Methods) that allowed for the control of the stimulation schedule and parameters, and to assist in distinguishing between the different types of patterns and therefore eliminate the need for a blind observer. A final aspect of the study was to vary the stimulation parameters to investigate the feasibility of experiments in which operant and classical predictors are combined.

All preparations were treated identically up until the start of the experiment, where each preparation was randomly assigned to one of six groups (Fig. 2A,B,C). These groups were designed as two triplets, the difference between the two being that one received contingent reinforcement via stimulation of the esophageal nerve and the other did not (see Materials and Methods for details; Fig. 2A,B,C). Note that some of the noncontingent groups received contingent CSs, but never contingent USs. The groups were all operant in nature and received tonic $Bn_{2,3}$ stimulation throughout the experiment. This nerve provides afferent input to the buccal ganglia. Stimulation of $Bn_{2,3}$ at a constant rate with weak intensity stimuli increases the likelihood of generating spontaneous BMPs (Nargeot et al. 1997, 1999a,b,c; Fig. 2A,B,C). Only ingestion-like BMPs (iBMPs) were reinforced.

Experimental Groups

The respective first groups in each triplet (Fig. 2A) can be seen as forming a pair designed to replicate previous studies of in vitro operant conditioning (Nargeot et al. 1997, 1999a,b), with minor parameter variations. It included a group that received a contingent US (Fig. 2A1, UScon) and a yoked control group (Fig. 2A2, USyoke). The expected outcome was an elevated number of iBMPs in the contingently reinforced compared to the yoked control group.

The respective second groups (Fig. 2B) were designed to test the effect of a delay and shortening of the reinforcing stimulus (US), as well as the effect of adding a contingent CS without a US. The contingently reinforced group (USdcon, Fig. 2B1) received a contingent US as the "UScon" group. But compared to the UScon group, the US was shortened from 6 to 4 sec and delayed by 2 sec (USdcon, Fig. 2B1). The other group (CS, Fig. 2B2) received only contingent CS presentations and no US presentations. This group was included to control for possible effects of contingent CSs alone (Fig. 2B2). The expected outcome was an elevated number of iBMPs in the USdcon group versus any of the noncontingent groups, and an unaffected number of BMPs in the group that only received a CS, compared to the other two noncontingent groups (i.e., Figs. 2A2, 1C2). Potentially, the USdcon group could have shown a lower number of BMPs than either the UScon (Fig. 2A1) or the CS+USdcon (Fig. 2C1) group.

The respective last groups in each triplet (Fig. 2C) were designed to investigate the effect of combining the shortened and delayed US with a contingent CS to "signal" the occurrence of the US (Fig. 2C). Both groups received contingent CS presentations after every iBMP, throughout the experiment. The contingently reinforced group (CS+USdcon) received contingent US presentations after each iBMP/CS combination (Fig. 2C1), whereas the control group (CS+USyoke) received the same sequence of US presentations as the contingently reinforced group, but independent of its behavior (yoked control; Fig. 2C2). In an intact *Aplysia*, the protocol of CS+USdcon would be analogous to a bite (iBMP) leading to a tactile stimulation of the lips (AT_4 stimulation) followed by food (En_2 stimulation).

Thus, in the contingently reinforced group (Fig. 2C1; CS+USdcon), during training the CS signaled the occurrence of reinforcement (US), whereas in the yoked control group (Fig. 2C2; CS+USyoke) it did not. The expected outcome is a higher number of BMPs in the contingently reinforced as compared to the yoked control. In vertebrates, such signaling can increase or decrease the amount of operant responding, depending on the choice of parameters (Williams 1975; Williams and Heyneman 1982; Williams 1989; Williams et al. 1990; Hammerl 1993; Reed 1996, 1999, 2003; Williams 1999). If a signaling effect of the CS is present in the preparation, the number of iBMPs in the CS+USdcon group is expected to be higher or lower than the number of BMPs in either the UScon or the USdcon group.

BMP Analysis

In order to assess the effects of the different treatments on the buccal Central Pattern Generator, three levels of analysis were used. First, we analyzed the total number of BMPs, irrespective of BMP-type. To gather more detailed information, we then ana-

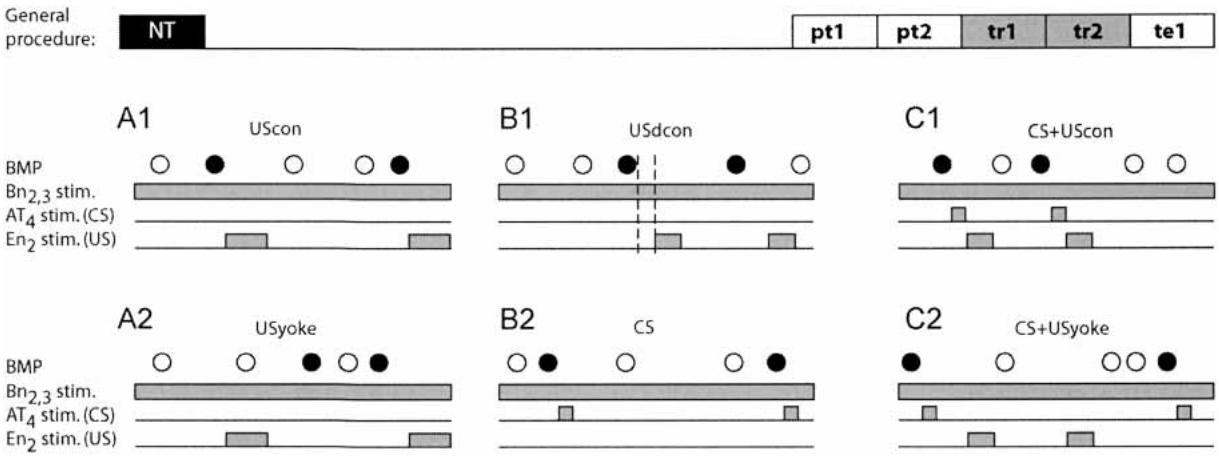

Figure 2 Schematic representation of the general procedure and the training protocols. The *top* trace illustrates the general training procedure. NT, nerve test done to establish proper conductivity of the electrodes to and from the nerves; Pt, pre-test; tr, training; te, test. The training regime for the different groups is presented schematically in *A,B,C.* Filled circles denote ingestion-like BMPs, open circles any other type of BMP.

lyzed the fraction of BMPs that were ingestion-like in nature (i.e., iBMPs). This measure has the advantage in that it describes the propensity of a preparation to produce iBMPs, irrespective of the total number of patterns produced. Finally, we evaluated the absolute number of iBMPs versus all other BMPs, to gain insight into the absolute changes in the generation of BMPs.

A one-way ANOVA (see Materials and Methods) over the total number of BMPs in all six groups did not reveal any significant variations in the total number of BMPs produced, neither in the pretest period immediately preceding the training (SS = 41.5, DF = 5, MS = 8.3, F = 0.48, p = 0.8), nor in the test immediately after the training (SS = 38.1, DF = 5, MS = 7.6, F = 0.38, p = 0.9). Thus, groups did not differ in their propensity to produce BMPs, before or after the training (i.e., treatment did not have any effect on the total number of all BMPs produced by the preparations).

Next, the fraction of iBMPs was evaluated. A one-way ANOVA over the six groups in the pretest period immediately preceding the training, was not significant (SS = 0.18; DF = 5; MS = 0.036; F = 0.74; p = 0.6). Thus, the six different groups did not differ significantly in the fraction of iBMPs produced before the training. This result indicates that all preparations had the same propensity to produce ingestion-like BMPs and any difference after training can only be attributed to the parameters of the stimulations during training.

All Contingently Reinforced Groups Increased the Propensity to Produce iBMPs

A one way ANOVA over the fraction of iBMPs in the six groups in the five minutes immediately following training, was significant (SS = 1.26; DF = 5; MS = 0.25; F = 4.5; p = 0.001). Fisher LSD post-hoc tests reveal that this significance was due to only the contingently reinforced groups differing from all noncontingent groups (Table 1). Thus, none of the different variations in US timing and duration had any effect on the magnitude of learning: contingently reinforced (via stimulation of En_2) preparations produced on average a larger fraction of iBMPs than preparations that received either no US at all or noncontingent USs, irrespective of the US parameters (Fig. 3).

Stimulation of the AT_4 nerve (such as the CS used here) can also elicit iBMPs, either after classical conditioning (Lechner et al. 2000a; Mozzachiodi et al. 2003) or if the stimulation is sufficiently intense. The application of contingent CSs in our experiments seemed to decrease (albeit insignificantly) the number of iBMPs (see Fig. 3; CS). To assess whether there was any effect from the presence or absence of the inserted CS, signaling the US, which was not uncovered by evaluating the fraction of iBMPs, a two-way repeated measures ANOVA was carried out over the absolute number of iBMPs and all other BMPs (Fig. 4). The first factor tested between contingently reinforced and noncontingent groups, whereas the second tested between pairs, and the repeated measures factor tested for differences between iBMPs and all other BMPs (Fig. 4). Only the groups with comparable US duration (4 sec) were compared, because these groups differed only in the presence or absence of the CS. Only the interaction between the repeated measures factor and the experimental/control factor was significant (SS = 92.9, DF = 1, F = 15.0, p = 0.0003; Fig. 4), meaning the distinction between experimental and control groups (i.e., the training regime) had a significant effect on the distribution of iBMPs and other BMPs among the groups. This result indicates that the presence or absence of the CS did not, but only the presence or absence of a contingency between ingestion-like BMPs and the US did have a statistically verifiable effect on the types of BMPs that were produced in the different groups. Thus, with our stimulation parameters, AT_4 stimulation by itself had no direct operant effects. The result corroborates our conclusions from the analysis of the fraction of iBMPs, namely that contingent reinforcement increases the relative number of iBMPs. In addition, a Fisher's LSD post-hoc analysis revealed that in the control groups, less ingestion-like BMPs are produced than other BMPs ($p < 0.001$) and that the number of other BMPs in the experimental groups is reduced, compared to the control groups ($p < 0.01$). Presumably because of the high value in the CS+USyoke group, the comparison of ingestion-like BMPs in experimental versus control groups fails to reach statistical significance ($p < 0.12$: see Discussion).

The limited number of preparations precludes statistically significant post-hoc differentiation between USdcon, CS+USyoke and CS+USdcon.

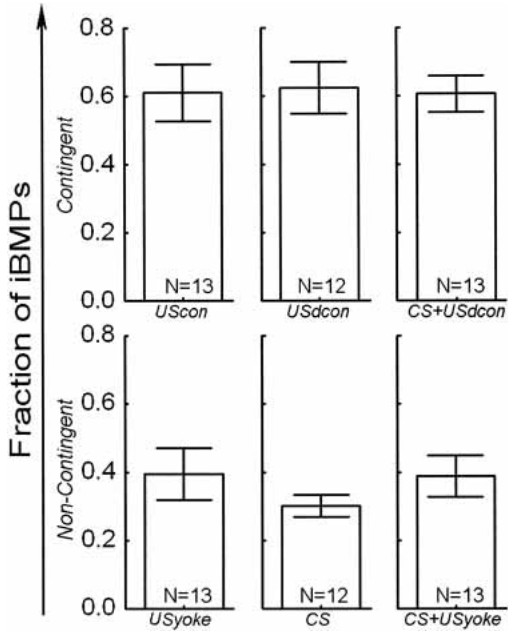

Figure 3 Frequency of ingestion-like BMPs in the six operant groups in the 5 min immediately following 10 min of training. The contingently reinforced groups showed an increased frequency of ingestion-like BMPs over the groups without contingent USs.

Table 1. p-Values for the Fisher LSD Post-hoc Tests Revealing That all Contingently Reinforced Groups Differ From All Noncontingent Groups in the Fraction if iBMPs During the Final Test Immediately Following Training

	USyoke	UScon	CS	USdcon	CS + USyoke	CS + USdcon
USyoke		0.02	0.33	0.02	0.94	0.03
UScon	0.02		0.002	0.88	0.02	0.96
CS	0.33	0.002		0.001	0.37	0.002
USdcon	0.02	0.88	0.001		0.02	0.85
CS + USyoke	0.94	0.02	0.37	0.02		0.02
CS + USdcon	0.03	0.96	0.002	0.85	0.02	

Shaded cells mark $p < 0.05$, Error: Between MS = 0.06, DF = 70.0.

DISCUSSION

We developed a computer-assisted paradigm for in vitro operant and classical conditioning in *Aplysia* that included the isolated cerebral and buccal ganglia. As a first step we investigated whether the new preparation could exhibit operant conditioning and the robustness of the operant conditioning protocol to pa-

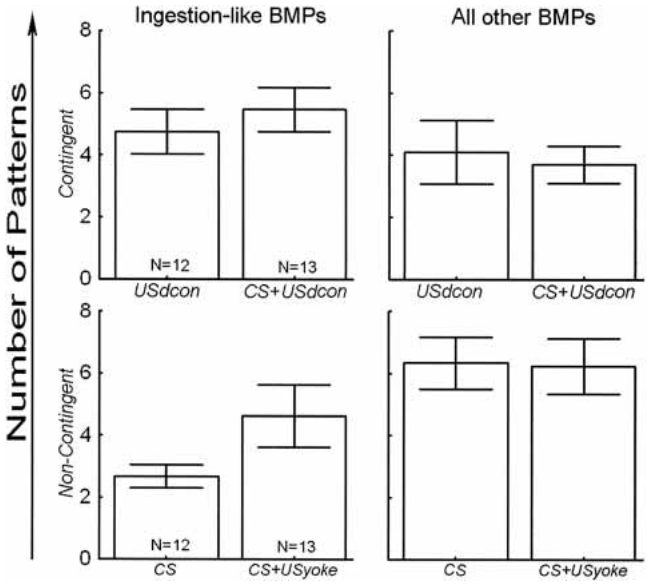

Figure 4 Absolute number of BMPs in the two pairs with a 4-sec US. The number of unrewarded patterns (i.e., noningestion-like BMPs) is reduced in the contingently reinforced groups, whereas the number of rewarded (ingestion-like) BMPs is elevated, compared to the groups that did not receive contingent USs. The high value for the CS+USyoke group may have prevented the difference in the ingestion-like BMPs from reaching statistical significance.

rameter variations including the presence of a CS signaling the reinforcer. The new paradigm reproduced previously published results, even under more conservative and homogenous selection criteria and tonic stimulation regime. Moreover, the observed learning was resistant to delay, shortening and signaling of reinforcement.

In Vitro Operant Conditioning Is Expressed in the Presence of the Cerebral Ganglion

The previous in vitro analog of operant conditioning consisted of only the isolated buccal ganglia (Nargeot et al. 1997, 1999a,b,c). It was therefore necessary to replicate the finding in a more physiological system that included the cerebral ganglion. The cerebral ganglion sends many projections to the buccal ganglion and vice versa (Rosen et al. 1991; Jing and Weiss 2001, 2002; Hurwitz et al. 2003). Therefore it was possible that the features of in vitro operant conditioning may be fundamentally different with the cerebral ganglion attached. With one exception (see below), we found that the features of operant conditioning were remarkably similar to that obtained with only the buccal ganglion. Indeed, after the six different training procedures, each contingently reinforced group produced a larger percentage of iBMPs than each group that did not receive contingent USs (Table 1; Fig. 3). Thus, we have successfully extended the in vitro operant conditioning procedure developed by Nargeot et al. (Nargeot et al., 1997, 1999a,b,c) to include the connected cerebral ganglion.

In Vitro Operant Conditioning With the Cerebral Ganglion is a Robust Phenomenon

We found that shortening and delaying the reinforcement by 2 sec did not disrupt the operant learning. We further found that adding a 2-sec CS between the ingestion-like BMPs and the reinforcement (US) also neither increased nor decreased the operant behavior.

Interestingly, delayed reinforcement is known from vertebrates to generally decrease the rate at which the operant behavior controlling the reinforcement is produced (e.g., Williams et al. 1990; Reed 1992a,b). In the case of in vitro operant conditioning of *Aplysia* feeding behavior this decrement due to delayed reinforcement apparently does not occur within the range of parameters used in the present study. Clearly, a sufficient delay of the US will eventually decrease the operant conditioning effect, as will a further shortening of the US. Thus, our paradigm has sufficient robustness to enable the study of US parameter variations: Slight variations in the reinforcement schedule do not completely disrupt learning.

Importantly, the presentation of a sensory signal (or operant CS; the 2-sec AT_4 stimulation) of reinforcement in the delay after a BMP and before reinforcement does not disrupt or enhance the production of ingestion-like BMPs, compared to the situation in which the US is merely delayed. This paradigm would be analogous to a behavior controlling both a predictive neutral stimulus (the CS) and a biologically relevant one (the US) at the same time. Returning to the example of a frog trying to capture a bee, extending the tongue would lead to a sting (US) by the striped bee (CS). In an intact *Aplysia*, the protocol would be analogous to a bite (ingestion-like BMP) leading to a tactile stimulation of the lips (AT_4 stimulation) followed by food (En_2 stimulation). It is easy to assume that the tactile lip stimulus may be interpreted as the food item moving, caused by the biting and swallowing movements. In both cases, the operant (the tongue extension or the bite) and the classical (the stripes of the bee or the lip stimulation) predictors can be perceived as competitors in the animal's search for a predictor of the reinforcer (Rescorla 1994) and antagonism as well as synergism may result. The fact that our choice of parameters led to neither synergism nor antagonism opens the possibility for parameter variations that can generate these effects. For example, the delay between the BMP and the US can be increased, allowing for a number of different arrangements of the CS within that delay. Because AT_4 stimulation has been shown previously to be able to function as a predictive signal (Mozzachiodi et al. 2003), the optimal choice of parameters should be able to create increments and decrements in the operant effect. The conspicuously high number of iBMPs in the CS+USyoke group (Fig. 4) may be an indication of how such an effect may manifest itself. In some preparations of the CS+USyoke group, concatenations of ingestion-like BMPs were observed, caused by contingent CSs eliciting BMPs. Without the reduced number of other BMPs in the CS+USyoke group and only the iBMPs thus enhanced, it is tempting to interpret this as a nonassociative effect of a combination of contingent CSs and noncontingent USs, particularly, since the CS+USdcon group was the only other group where such a concatenation of BMP–CS–BMP was observed. Although with our choice of parameters such effects were too weak to reach statistical significance, it seems possible that a different set of stimulation parameters could lead to a significant classical component in the CS+USdcon group, which, in turn, would lead to all these preparations exhibiting these concatenations of BMPs, while the yoked control preparations would remain at the same level. The accessibility of the preparation allows for a detailed analysis of the neuronal underpinnings of any such effects.

Thus, the operant effect described by Nargeot and colleagues is a robust, reproducible case of operant conditioning with the potential to study an even wider variety of behavior–CS–US relationships than space permits to present here.

Differences Between Previous Work

One of the results in Nargeot et al. (1997) that could not be reproduced was an increase in the total number of BMPs pro-

duced by the contingently reinforced preparations. In our experiments the experimental groups still produced more ingestion-like BMPs than the control groups, even in absolute numbers (data not shown), but the most clear-cut results were obtained when the frequency of ingestion-like BMPs was evaluated. Although we would not exclude the possibility that this effect stems from the presence of the cerebral ganglion, it could also be due to the asymmetrical selection criteria that were used in Nargeot and colleagues' work. Nargeot and colleagues discarded experimental preparations that produced less than five ingestion-like BMPs during the 10-min training period. No such selection was used for the control groups. Such a procedure may have selected animals in the experimental group that showed an increase in general BMP activity, independent of the operant conditioning. In our experiments, the same selection criteria were used for both experimental and control groups (see Materials and Methods). Because Nargeot and colleagues reinforced the first ingestion-like BMP in each contingently reinforced preparation, there were no latent inhibition effects that could have possibly reduced the ability of the circuit to be conditioned. In our experiments, the amount of pretest was fixed and any occurring ingestion-like BMPs during this time remained unreinforced. Moreover, our selection regime required three ingestion-like BMPs from the control groups as well and thus may have selected for too high a number of ingestion-like BMPs in these groups, masking the effect of an increase in total BMPs. Thus, while Nargeot and colleagues used a proactive selection regime that may enhance any conditioning effects, our approach was more conservative. Therefore, even under our testing conditions, the associative conditioning effect found by Nargeot and colleagues could be reproduced, emphasizing the robustness of the paradigm.

Outlook

In the future, this in vitro operant/classical conditioning paradigm can be employed to examine such long-standing questions as whether there are any operant components even in purely classical conditioning (e.g., Gormezano and Tait 1976 and references therein) or whether classical and operant conditioning are merely two aspects of the same conditioning processes (Skinner 1935; Konorski and Miller 1937a,b; Skinner 1937; Rescorla and Solomon 1967; Trapold and Winokur 1967; Trapold et al. 1968; Trapold and Overmier 1972; Rescorla and Holland 1982; Rescorla 1990a,b, 1994; Brembs and Heisenberg 2000; Heisenberg et al. 2001; Corbit et al. 2003; Holland and Gallagher 2003; Phillips et al. 2003).

MATERIALS AND METHODS

General Methods

Aplysia californica (80–350 g) were obtained from Alacrity Marine Biological Specimens and Marinus and housed individually in perforated plastic cages, floating in aerated seawater tanks at 15°C. Animals were fed ~1 g of dried seaweed three times a week. To help ensure that all animals were in a similar motivational state, experimental animals were food deprived 3–5 d before the dissection.

Dissection

Prior to dissection, the motivational state of all animals was enhanced by first feeding them a small piece of dried seaweed (~1.5 cm^2) and 30 min later a larger (8-cm^2) piece. While the animal was feeding on the larger piece, it was anaesthetized by an injection of isotonic $MgCl_2$ equivalent to 50% of its body mass. The dissection follows the procedure described in Nargeot et al. (1997, 1999a,b,c): An incision was made along the midline of the foot to expose the buccal mass and the esophagus. The most medial-ventral branch (designated branch 4) of the right anterior tentacle nerve (AT, for nomenclature, see Jahan-Parwar and Fredman 1976), which terminates in the lip region of the animal, was retained. All other peripheral nerves of the cerebral ganglion were cut short. The esophagus and the buccal mass together with the cerebral and buccal ganglia were removed and transferred to a chamber containing artificial seawater with a high concentration of divalent cations (high divalent ASW) composed of (in mM): NaCl 210, KCl 10, $MgCl_2$ 145, $MgSO_4$ 20, $CaCl_2$ 33, and HEPES 10 (pH adjusted to 7.5 with NaOH). The high divalent ASW was used to decrease neural activity during further dissection (Byrne et al. 1978). Selected peripheral nerves of the buccal ganglion were retained for extracellular recording and stimulation. The cerebral and the buccal ganglia were then pinned to the bottom of a petri dish coated with silicone elastomer (Sylgard, Dow Corning). In all experiments, the connective tissue sheath that covers the ganglia was left intact. The temperature of the static bath was maintained at 15°C with a feedback-controlled Peltier cooling device (Model SE 5010, Marlow Industries). The high divalent ASW was exchanged for normal ASW for 30 min prior to the beginning of an experiment, once the extracellular electrodes for both stimulation and recording were in place and tested for connectivity (see below). The normal ASW was composed of (in mM): NaCl 450, KCl 10, $MgCl_2$ 30, $MgSO_4$ 20, $CaCl_2$ 10, and HEPES 10 (pH adjusted to 7.5 with NaOH).

Extracellular Nerve Recordings

Previous in vivo recordings indicate that bursts of large-unit activity in nerves I_2n,Rn_1 and $Bn_{2,1}$ are associated with the protraction, closure, and retraction, respectively, of the radula/odontophore during feeding (Morton and Chiel 1993b; Hurwitz et al. 1996). Moreover, in vitro recordings indicate that BMPs, which represent fictive feeding, can be recorded from I_2n, Rn_1, and $Bn_{2,1}$ (Morton and Chiel 1993a; Nargeot et al. 1997; Lechner et al. 2000a). Thus, fictive feeding (i.e., BMPs) was monitored by placing silver electrodes on nerves I_2n,Rn_1, and $Bn_{2,1}$ (Nargeot et al. 1997) of the right buccal ganglion (see below). All extracellular electrodes were isolated from the surrounding bath using petroleum jelly (Vaseline, Sherwood Medical). Signals were amplified with a differential AC amplifier (Model 1700, A-M Systems). The amplified signals were displayed on a computer screen and saved on the hard drive using a PCI 9112 A/D converter card (Adlink Technology, Inc.) and custom-written software.

Extracellular Nerve Stimulation

Similar to our previous studies (Nargeot et al. 1997, 1999a,b,c; Brembs et al. 2002), electrical stimulation (4–6 sec, 10 Hz, 0.5-msec pulses, 7 V) of the right En_2, which innervates the buccal mass (Schwarz and Susswein, 1986) was used to mimic food reward. The duration and frequency of the stimulus resembled bursts of activity recorded in vivo from En_2 during feeding (Brembs et al. 2002). En_2 mediates several aspects of feeding behavior such as conveying efferent activity that controls peristaltic movements of the gut (Lloyd et al. 1988) and conveying afferent activity that encodes information related to feeding arousal (Susswein et al. 1984) and satiety (Kuslansky et al. 1978, 1987). Stimulation of En_2 has been used as a reinforcer to modify behavior and neural activity in a training paradigm used for operant conditioning of *Aplysia* feeding behavior both in vivo (Brembs et al. 2002) and in vitro (Nargeot et al. 1997) and in classical conditioning (Mozzachiodi et al. 2003). Moreover, En_2 is necessary for classical conditioning of feeding behavior in vivo (Lechner et al. 2000b). Finally, En_2 is necessary in an operant paradigm for learning that food is inedible (Susswein and Schwarz 1983; Schwarz and Susswein 1986). Thus, En_2 appears to be part of the reinforcement pathway that contributes to both classical and operant conditioning.

Electrical stimulation of AT_4 (2 sec, 5 Hz, 0.5-msec pulses) was used to mimic the CS that was used in classical conditioning in vivo (Lechner et al. 2000a,b) and in vitro (Mozzachiodi et al. 2003). The frequency of AT_4 stimulation used in the present study was similar to that recorded in vivo during mechanical

stimulation of the tentacles (Anderson 1967; Fredman and Jahan-Parwar 1980). The AT nerve mediates several aspects of feeding behavior. For example, AT conveys afferent activity that encodes information about both mechanical and chemical stimuli that signal the presence of food on the lips (Anderson 1967; Rosen et al. 1979; Xin et al. 1995). In addition, AT conveys efferent activity that controls the movement of the lips (Perrins and Weiss 1996). Several lines of evidence suggest that AT_4 also mediates aspects of the tactile CS that was used for in vivo classical conditioning (Lechner et al. 2000a,b). Finally, Lechner et al. (2000a) found that in vivo classical conditioning (1) increased the probability that a weak stimulation of AT_4 would elicit BMPs, and (2) enhanced the AT_4-elicited synaptic input to B31/32 in cerebral and buccal ganglia dissected from trained animals.

Following Nargeot et al. (1997), tonic stimulation of the ventral branch of buccal nerve $Bn_{2,3}$ (2 Hz, 0.5-msec pulses, 7 V) was used to nonspecifically elevate the number of spontaneous BMPs produced by the preparation.

Pulses for extracellular nerve stimulation were generated by a digital pulse generator (Pulsemaster A300, WPI) and applied, via a stimulus isolator (A360; WPI, Sarasota, FL), to bipolar silver electrodes that were placed on nerves $Bn_{2,3}$, AT_4, and En_2 and isolated from the bath with Vaseline.

Once the extracellular electrodes were in place, the high divalent ASW was exchanged for normal ASW. Preparations were washed with 50 ml ASW and then single stimulations were applied to each of the three nerves to verify electrode connectivity. Pilot studies showed that due to the high incidence of BMPs immediately after the tonic stimulation of $Bn_{2,3}$ was switched on, it was impossible to determine the appropriate sub-threshold AT_4 intensity during $Bn_{2,3}$ stimulation. Therefore, the intensity was empirically set to 3 V for all operant preparations, an intensity that on its own did not increase the number of BMPs in the pilot studies.

Classifications of BMPs

The feeding CPG expresses BMPs, which can be associated with ingestion or rejection of food (Morton and Chiel 1993a,b). BMPs consist of specific patterns of neural activity, which correspond to cycles of protraction and retraction of the radula/odontophore. BMPs can be recorded from the buccal nerves I_2n,Rn_1, and $Bn_{2,1}$. Large-unit activity in I_2n (i.e., radula protraction) precedes large-unit activity in $Bn_{2,1}$ (i.e., radula retraction). Large-unit activity in Rn_1 (i.e., radula closure) overlaps to a varying extent with protraction and retraction activity (Cropper et al. 1990; Morton and Chiel 1993a,b; Nargeot et al. 1997; Kabotyanski et al. 2000). The large-unit activity in Rn_1 corresponds to action potentials in the radula closure motor neuron B8, which has an axon in Rn_1 (Morton and Chiel 1993b; Nargeot et al. 1999b).

As in previous studies (Morton and Chiel 1993a,b; Nargeot et al. 1997; Lechner et al. 2000a; Jing and Weiss 2001, 2002; Mozzachiodi et al. 2003), we classified BMPs as ingestion-like if $\geq$50% of radula closure (Rn_1) activity occurred after the termination of the protraction (I_2n) activity. The criterion for rejection-like BMPs was the occurrence of closure (Rn_1) activity during the protraction (I_2n) activity, but no overlap between closure (Rn_1) and retraction ($Bn_{2,1}$) activity. BMPs that did not meet either of these two criteria were classified as other BMPs (Nargeot et al. 1997; Lechner et al. 2000a).

In the present study, only patterns that consisted of activity in all three buccal nerves clustered in a complete protraction/retraction cycle were classified as BMPs. Patterns consisting of bursts of activity in only one or two of the three nerves were classified as incomplete patterns and were not included in the study.

Computer-Assisted BMP Recognition

The custom-written software provided computer-assisted pattern recognition (i.e., the computer attempted an online classification and suggested a pattern type at the end of each BMP). The software was written on a MS Windows based PC using C++ and the provided software development kit for the PCI 9112 converter card. The acquisition rate was limited by processor speed, in our case to ~8 kHz. The experimenter then determined whether to follow the suggested classification or not. In the 30-min rest period, spontaneous BMPs were used to individually adjust spike detection threshold and maximal inter-spike-interval for each nerve to the individual BMPs of the experimental animal. Using these two parameters, the computer then detected "activity" in the three nerves (i.e., more than two spikes over the threshold and within the given inter-spike-interval) and correlated the timing of activity in the nerves according to the rules above. A colored line along the baseline of the recordings denoted the detected pattern type. BMP classification is usually unequivocal (Nargeot et al. 1997), but in the few ambiguous cases where radula closure activity is divided almost equally between protraction and retraction, the computer can make the objective classification much faster than the human eye.

Procedures for In Vitro Training

The procedures were based on the in vitro operant conditioning experiment developed by Nargeot et al. (1997, 1999a,b,c) and on the in vitro classical conditioning procedure developed by Mozzachiodi et al. (Lechner et al. 2000a; Mozzachiodi et al. 2003). Unlike the cited operant experiments, our preparations were given a fixed 30-min rest period without any stimulation after the connectivity of all electrodes was determined. After the rest period, two 5-min pretest periods followed, which were followed immediately by two 5-min training periods, similar to the in vivo experiments in Brembs et al. (2002). The experiment concluded with a 5-min test period, which immediately followed training. USs were only delivered to the preparation during training periods. Tonic stimulation and, where applicable, CS delivery was performed throughout the experiment. The CS presentation regime was kept constant throughout the experiment, so that only the application of the US would differentiate between training and test.

Animals were divided randomly in six groups. Each group received tonic stimulation of $Bn_{2,3}$, which began after the 30-min rest period and continued uninterrupted until the experiment ended. The groups differed from each other by the application regime of CS and US applications.

The first two groups were designed to replicate previous findings (Nargeot et al. 1997) with the difference that the cerebral ganglion was attached to the preparation. During the training period, the UScon group received contingent reinforcement (operant US deliveries) consisting of a 6-sec stimulation of En_2 immediately following each ingestion-like BMP. The corresponding USyoke group received the same sequence of En_2 stimulations during training, but uncorrelated with the occurrence of any BMPs ("yoked" control).

The third group was designed to test for the effect of a delay and shortening of the US (USdcon). This group received a contingent 4-sec US with a 2-sec delay after each ingestion-like BMP produced during training.

The fourth group was designed to test the effect of introducing contingent CSs after each iBMP without a US. This group (CS) received contingent 2-sec AT_4 stimulations (operant CSs) immediately after each ingestion-like BMP throughout the experiment and no USs during the training period.

The last two groups were designed to test the effects of introducing a signal of the delayed US. Both groups received contingent 2-sec AT_4 stimulations (operant CSs) immediately after each ingestion-like BMP throughout the experiment, starting after the 30-min rest period. During training, the CS+USdcon group received contingent reinforcement (operant 4-sec USs) immediately upon cessation of the operant CS after each ingestion-like BMP. Thus, each ingestion-like BMP in this group was followed first by a CS and then by a US; both stimulations together yielded a total of 6 sec of stimulation after each ingestion-like BMP (the US in Nargeot and colleagues original experiment had been 6 sec as well). The CS+USyoke group received the same sequence of 4-sec En_2 stimulations during the training period as

the CS+USdcon group, but uncorrelated with either generated BMPs or received CSs (yoked control).

Preparations that did not produce at least one ingestion-like BMP during training and at least three ingestion-like BMPs in the entire experiment were discarded.

Statistics

One-way or multifactor Analyses of Variance (ANOVAs) were carried out to estimate the significance of within- and between-group differences. Fisher LSD Post-hoc tests were used to detect the significant contributions to the variance in the data.

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