Extending *in vitro* conditioning in *Aplysia* to analyze operant and classical processes in the same preparation

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I. Introduction

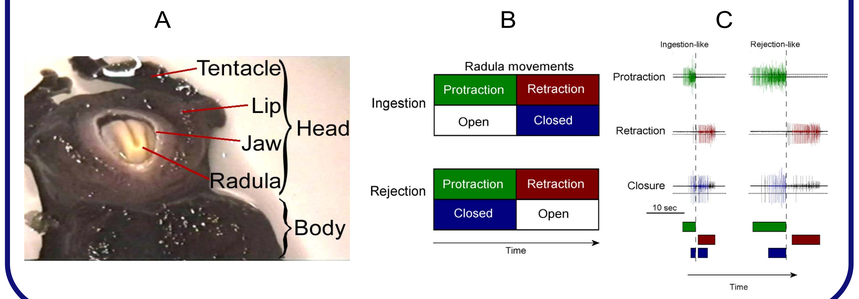
For most of the 20th century there has been a debate over the equivalence of classical and operant learning processes. Due in part to studies of learning in *Aplysia*, a great deal is known about the cellular basis of classical conditioning. In comparison, relatively little is known about the mechanisms underlying operant conditioning. This deficit results, in part, from the lack of a suitably traceable model system that manifests operant conditioning and that is amenable to cellular and molecular studies. If such a preparation existed, the old psychological problem could be developed into a biological experiment.

Ideally, both operant and classical processes should be studied in the same model system. Therefore, as a first step towards studying the neurobiological underpinnings of operant and classical interactions, we have designed an experimental system in which operant and classical conditioning can be investigated simultaneously or sequentially and which is amenable to cellular and network analysis. Moreover, this preparation will allow for the concurrent presentation of classical and operant predictors, and thereby provide a system 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.

We took advantage of the recent advances in operant and classical conditioning of *Aplysia* feeding behavior and developed a computer supported, single *Aplysia* preparation in which operant and classical experiments can be conducted both separately and in combination.

II. The model system: *Aplysia* feeding behavior

A. Photograph of the head and mouth of *Aplysia* during a bite. *Aplysia* bite spontaneously when hungry. B. Schematic representation of the radula movements during ingestion and rejection. C. Extracellular recordings from buccal motor nerves exemplifying Buccal Motor Pattern (BMP) classification deduced from the radula movements depicted in B. Note that only closure activity is counted that overlaps with radula movement (pro- or retraction). Dotted lines: activity detection thresholds. The computer binned all activity exceeding the thresholds and within a user determined timeframe to periods of activity. Patterns were then classified by means of the temporal relationships between these periods of activity, as indicated below the recorded traces.



III. One preparation for both operant and classical conditioning

The previous *in vitro* analogue of operant conditioning consisted of only the isolated buccal ganglia. 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. Therefore it was possible that the features of *in vitro* operant conditioning may be fundamentally different with the cerebral ganglion attached.

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). On the other hand, studies from vertebrates suggest that such a combination can have various effects, depending on subtle details. Therefore, we developed a preparation in which we could insert a conditioned stimulus (CS) between the behavior (BMP) and the contingent reinforcement (unconditioned stimulus; US). This arrangement is reminiscent of a natural situation in which a behavior leads to the perception of a sensory stimulus (CS) signalling reward (US).

Thus the experimental design reflects the need for a validation of the new preparation for operant conditioning and for an investigation of the various effects of inserting a CS, as well as delaying and shortening the US (necessary to accomodate the CS).

Stimulation and recording regime:

Recording:

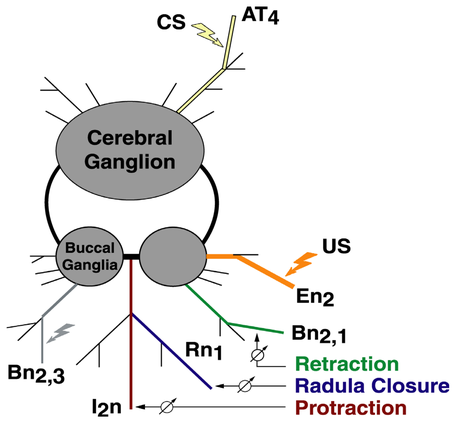
Previous *in vivo* recordings indicate that bursts of large-unit activity in nerves I2n, Rn1 and Bn2,1 are associated with the protraction, closure and retraction, respectively, of the radula/odontophore during feeding. Thus, fictive feeding (i.e., BMPs) was monitored by placing silver electrodes on nerves I2n, Rn1, and Bn2,1 of the right buccal ganglion.

Stimulation:

1. Electrical stimulation (4-6 sec, 10 Hz, 0.5-msec pulses, 7V) of the right En2, which innervates the buccal mass was used to mimic food reward (US). The duration and frequency of the stimulus resembled bursts of activity recorded *in vivo* from En2 during feeding.

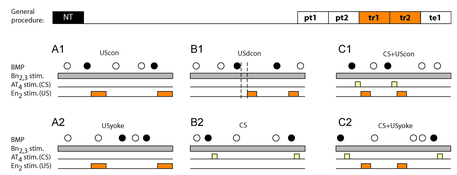
2. Electrical stimulation of AT4 (2 sec, 5 Hz, 0.5-msec pulses) was used to mimic the CS that was used in classical conditioning *in vivo* and *in vitro*. The frequency of AT4 stimulation used in the present study was similar to that recorded *in vivo* in during mechanical stimulation of the tentacles.

3. Tonic stimulation of the ventral branch of buccal nerve Bn2,3 (2Hz, 0.5-msec pulses, 7V) was used to non-specifically elevate the number of spontaneous BMPs produced by the preparation.



Conditioning procedure

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-C. Filled circles denote ingestion-like BMPs, open circles any other type of BMP.



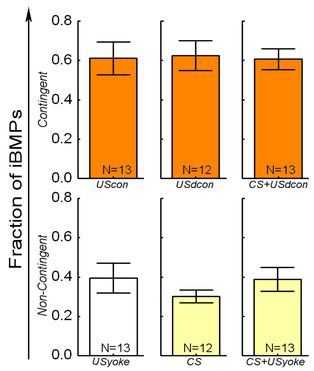
IV. Robust conditioning under varying parameters

A. Increased iBMP frequency in all contingently reinforced groups

The frequencies of ingestion-like BMPs (iBMPs) increased in all three contingently reinforced groups (upper bars), compared with the three groups inwhich the US was either delivered non-contingently or omitted (lower bars).

The variation between the other treatment conditions (i.e. comparing horizontally) was not significant.

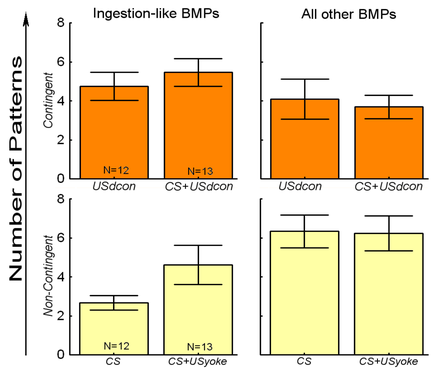
Thus, we found that shortening and delaying the reinforcement did not disrupt the operant learning. We further found that adding a CS between the ingestion-like BMPs and the reinforcement (US) also neither increased nor decreased the operant behavior.



B. All BMP types are modulated by conditioning

Evaluating the number of BMPs, we found that contingent reinforcement increases the number of iBMPs and reduces the number of other BMPs.

The conspicuously high number of iBMPs in the CS+USyoke group indicates a possible non-asociative effect of combined CS and US presentations.



V. Conclusion

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 parameter 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.

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 or whether classical and operant conditioning are merely two aspects of the same conditioning processes

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