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Chemical neurostimulation using pulse code modulation (PCM) microfluidic chips

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ABSTRACT

We report the implementation of a chemical neurostimulation technique using microfluidic devices. The microfluidic chip in this research is used for the *in vitro* study of the nervous system of *Aplysia californica* under localized chemical stimulation. The polydimethylsiloxane (PDMS) device is a one-bit pulse code modulator that digitally controls the concentration of the non-hydrolysable cholinergic agonist carbachol injected directly above a ganglion. The chip was successful in repeatedly and controllably inducing bursts of ingestive-like patterns. The ability of the chip to induce rhythmic activity through the sheath of the ganglion suggests that it could serve as the basis for an implantable, *in vivo* device to control neural activity and motor behavior using chemical stimulation.

1. Introduction

Both electrical and chemical signals have important roles in the function of the nervous system. Because electrical signals can be detected at some distance from their origin, many techniques and interfacing microsystems (Ziaie et al., 1997; Ghovanloo and Najafi, 2007; Rousche and Normann, 1999; Harrison et al., 2007; Kim et al., 2009) have been developed to stimulate and record the electrical activity of neurons. More recently, with the development of new technologies for precisely controlling small volumes of fluid (microfluidics), it has become feasible to affect neural activity by the application of controlled chemo-stimulation (Chung, 2008; Abgrall, 2007; Metz, 2004; Lee, 2004; Safadi, 2003; Zibek, 2007; Mehenti et al., 2007; Petterman et al., 2004; Santini et al., 1999; Vastag, 2002).

The Michigan fluid delivery (MFD) system, a highly cited device, is fabricated using the conventional microfabrication processes (Mehenti et al., 2007). The input flow channel is 4 μm wide, 4 mm long and is capable of delivering neurostimulant drugs at flow rates of 10 pL/s. Fishman and co-workers introduced another method (Petterman et al., 2004; Santini et al., 1999; Vastag, 2002) for chemical stimulation using PDMS chip technology (Dertinger et al., 2001). In this system neurons, which are placed on top of the microchannel, are stimulated by flowing the stimulant through the microchannel underneath at a flow rate of 16 μL/s. In these two chemical neurostimulation chips, the control of the flow was external to the chip, and thus the stimulation dose- and time-dependent concentration profiles are susceptible to errors originated from the large dead volume associated with external flow control components. In this paper we present a microfluidic device for chemical stimulation with on-chip valves. The chip operation is based on a 1-bit pulse code modulation (PCM) scheme. Using on-chip valves enables the device to provide fine control over dosage and temporal concentration. The device was tested at the flow rate of 43 nL/s.

2. PCM microfluidic chip

The schematic of the PCM chip is shown in Fig. 1(A). The chip principle is based on the concept of pulse-encoded modulation. In this technique, the chip output consists of a series of rapidly switched digitally encoded neurostimulant (solute) plugs embedded in a buffer (solvent) carrier stream. As the stream of encoded neurostimulant flows through a dispersive element such as a long microchannel, the plugs broaden and intermix with each other producing a smooth averaged output concentration signal $C(t)$. The chip consists of two main sections: a multiplexer unit (MUX)

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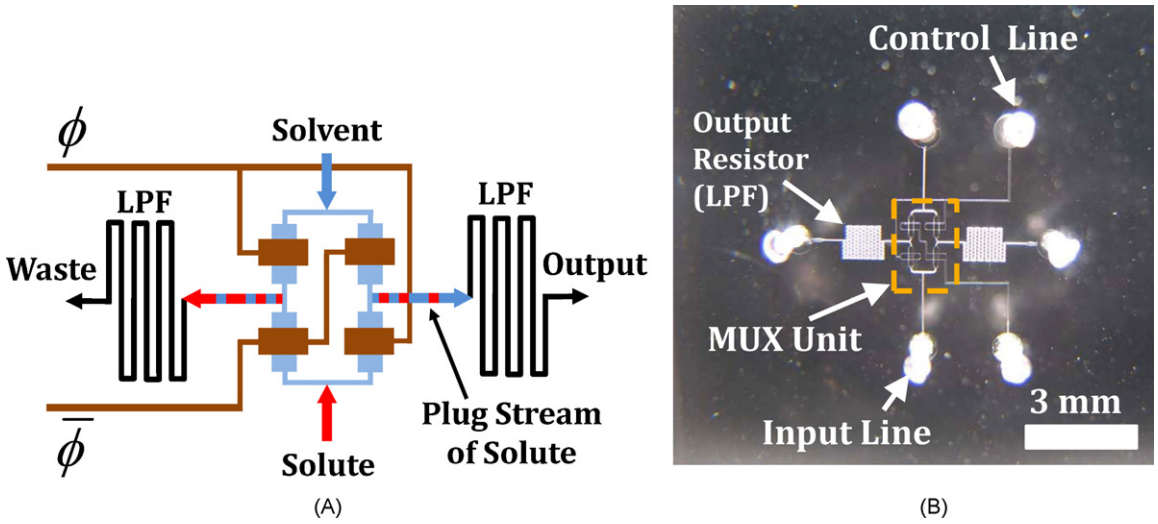

Fig. 1. (A) Schematic of the microfluidic PCM chip and (B) photograph of the chip.

and two long microchannels. The MUX unit consists of four on-chip valves connected in a bridge configuration. The MUX unit multiplexes the flow of the two input fluids, either concentrated stimulant or solvent, into its output based on digital codes generated by a computer program. The long microchannel at the output of the MUX unit serves as a conventional Fourier low-pass filter (LPF) element for the incoming neurostimulant plug stream. The LPF produces a smooth time-averaged chemical concentration signal with an output concentration proportional to the plug density and duty cycle. Therefore, the output concentration can be controlled by adjusting the number of plugs per second and the ratio of plug lengths for solute and solvent flows. In order for the PCM chip to provide fast and smooth output signals it is necessary to use a high plug rate and a moderately fast LPF capillary. The details of the connection between dispersive capillary length, flow rate and plug switching frequencies and the selection of the PCM operating parameters are complex, but these have been discussed in some detail elsewhere (Azizi and Mastrangelo, 2008).

In order to integrate the on-chip valve components, the chip was fabricated using a two-layer PDMS (polydimethylsiloxane) technology (Unger et al., 2000). Fig. 1(B) shows a photograph of the PCM chip. Since the chip was to be placed on top of the *Aplysia ganglion*, one of the chip's outputs had to flow underneath the chip and through the support glass. Therefore, a thin glass microscope slide (150 μm thick) was drilled using electrochemical drilling (Li, 2006). Electrochemical drilling is done on the glass at a voltage of ~40 V in a 50% NaOH bath for ~20 s using a sharp needle. The orifice size was approximately 200 μm in diameter.

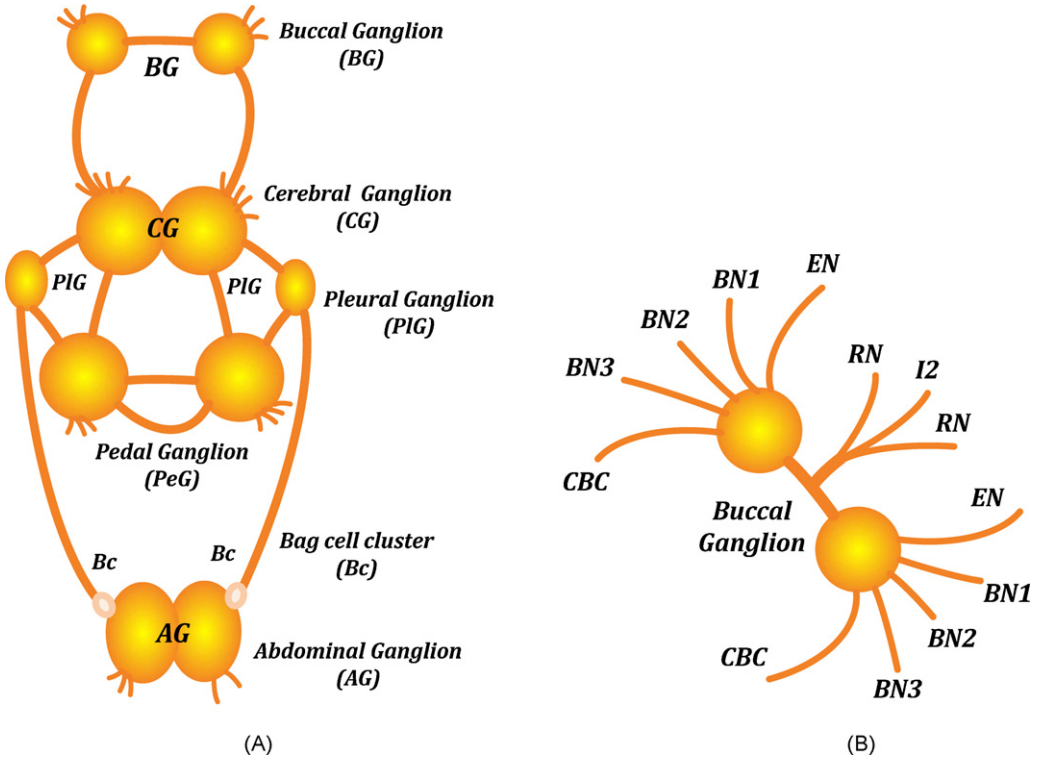

Fig. 2. Schematic of *Aplysia californica* ganglia. Only the buccal and cerebral ganglia were used in this experiment.

3. Experimental subject: *Aplysia californica*

Aplysia californica, a sea hare, has served as a useful model system for neurobiological studies for decades because its nervous system has a relatively small number of large, pigmented, and uniquely identifiable nerve cells. Moreover, extensive studies of the neural circuitry have made it possible to clarify details of the neural circuitry subserving a wide variety of complex behaviors at the level of individual identified sensory neurons, motor neurons, and interneurons. In turn, this has made it possible to clarify the biophysical and molecular mechanisms that underlie memory and learning, motivated behaviors, and complex motor control. Previous studies have shown that a specific motivated behavior such as feeding behavior can be triggered by the application of a cholinergic agonist to a collection of nerve cells known as the cerebral ganglion. Such stimulation can drive rhythmic patterned activity in the buccal ganglion, which generates the motor commands to the feeding apparatus of *Aplysia* (Susswein et al., 1996).

To study the effects of chemical stimulation with the PCM chip, we tested it by applying stimulants to the *Aplysia* cerebral ganglion (shown in the schematic of Fig. 2) while recording output activity from the buccal ganglia. Fig. 3 shows the experimental setup. The details of the experimental hardware used to drive the PCM chip are discussed in (Azizi and Mastrangelo, 2008). Average flow rate for the stimulant was 43 nL/s during the experiment.

4. Experimental methods

Aplysia californica weighing 200–400 g (Marinus Scientific, Garden Grove, CA) were maintained in an aerated aquarium containing artificial seawater (Instant Ocean; Aquarium Systems, Mentor, OH) kept at $18 \pm 1\,^{\circ}C$. Animals were anesthetized and their cerebral and buccal ganglia were dissected out. The ganglia were pinned caudal side up in *Aplysia* saline with cerebral–buccal-connectives (CBCs) intact. The nerves of interest that were studied to identify the nature of the feeding motor program were buccal nerve 2 (BN2), buccal nerve 3 (BN3), radular nerve (RN) and the I2 nerve on the same side of the buccal ganglia. Nerve recording suction electrodes were made from heated and pulled polyethylene tubing (catalogue #427421; Becton Dickinson, Sparks, MD; outer diameter 1.27 mm; inner diameter 0.86 mm). Electrodes were backfilled with *Aplysia* saline (460 mM NaCl, 10 mM KCl, 22 mM $MgCl_2$, 33 mM $MgSO_4$, 10 mM $CaCl_2$, 10 mM glucose, 10 mM MOPS, pH 7.4–7.5). Each nerve was suctioned into the tapered ends of recording electrodes and recorded extracellularly during and after the neurostimulant application. Nerve recording signals were amplified using an AC-coupled differential amplifier (model 1700; A-M Systems) and filtered using a 300 Hz high-pass filter and a 1 kHz low-pass filter. The microfluidic PCM chip was then placed directly onto the sheath of the cerebral ganglion to provide a good seal as shown in the experimental setup of Fig. 2(B). Two solutions were provided as inputs to the chip: *Aplysia* saline, or a solution of 2 mM carbachol (Sigma Chemical Company) in *Aplysia* saline.

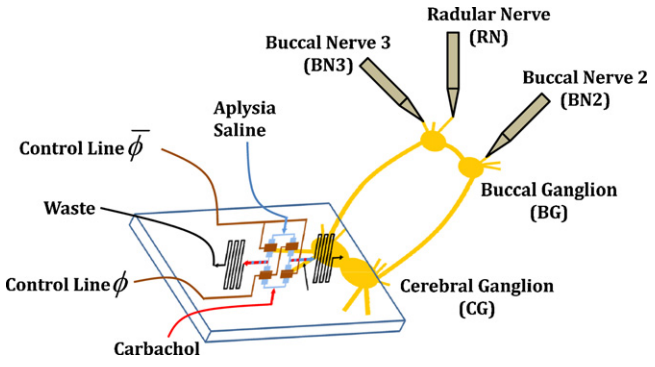

Fig. 3. Experimental setup. The PCM chip was placed on the cerebral ganglion such that one of the outputs led directly to the surface of the cerebral ganglion.

5. Experimental results

Using the setup shown in Fig. 3, we used the non-hydrolysable cholinergic agonist carbachol (2 mM dissolved in *Aplysia* saline) as a test (first fluid input) and used normal *Aplysia* saline as a control (second fluid input). Since the goal of this experiment was to study the behavior of the animal in the presence or absence of the chemical neurostimulation, the experimental design A–B–A–B was utilized. In this design, "A" stands for application of *Aplysia* saline to the cerebral ganglion for 1 min and recording the activity on the nerves of the buccal ganglion for 15 min beginning from the start of the application. In contrast, "B" stands for application of carbachol for 1 min to the cerebral ganglion and recording the activity on the nerves of the buccal ganglion for 15 min beginning from the start of the application. The flow rate for this experiment was 43 nL/s.

Neural recording was done extracellularly from buccal nerve 2 (BN2), buccal nerve 3 (BN3), the radular nerve (RN) and the I2 nerve on the same side of buccal ganglion. The large extracellular units on BN2 indicate the onset of retraction (Morton and Chiel, 1993). The large extracellular units on RN correspond to radular closure (Morton and Chiel, 1993).

Previous work demonstrated that applying the non-hydrolysable cholinergic agonist carbachol to *Aplysia* cerebral ganglion elicited sustained bursts of ingestive-like patterns (Susswein et al., 1996). We classified patterns as ingestive-like, egestive-like or intermediate according to the timing of RN (closure) and BN2 (retraction) large units, based on their occurrence in both *in vivo* and *in vitro* feeding motor patterns (Morton and Chiel, 1993). The patterns in which most of the large extracellular units in RN overlapped large units in BN2 represent ingestive-like patterns (Fig. 4(A)), i.e., radular closure associated with retraction; the patterns in which large units in RN proceeded large units in BN2 represent egestive-like patterns (Fig. 4(B)), i.e., radular closure associated with protraction; the patterns in which large RN units both proceeded and overlapped large BN2 units represent intermediate patterns (Fig. 4(C)) (Morton and Chiel, 1993). As shown in Fig. 4(D), during the first phase of the experiment, when *Aplysia* saline was applied to the cerebral ganglion, two spontaneous egestive-like patterns occurred during the recording.

In the second phase of the experiment, with 2 min delay from the start of the application of carbachol, 14 ingestive-like patterns, two egestive-like patterns and one intermediate pattern were recorded. During the third phase of the experiment, when *Aplysia* saline was again applied to the cerebral ganglion, the intensity of neural activity was greatly reduced and no feeding motor patterns occurred during the 15-min recording (Fig. 4(F)). Finally, in the fourth and final phase of the experiment, when carbachol was again applied to the ganglion, 14 ingestive-like patterns and one intermediate pattern were recorded. The delay to the onset of the ingestive-like patterns was again about 2 min. Multiple experiments demonstrated that carbachol application to the cerebral ganglion using the microfluidic chip was associated with ingestive-like activity, whereas application of *Aplysia* saline alone was associated with no activity, or egestive-like motor patterns (Fig. 4, $n = 3$). There were a few intermediate and egestive-like patterns in the 15-min recordings during the first and third phase of each experiment, as *Aplysia* saline was applied to the cerebral ganglion, but no ingestive-like patterns (Fig. 5(A)). During the second and fourth phase of each experiment, when carbachol was applied to the cerebral ganglion, the number of ingestive-like patterns per minute was

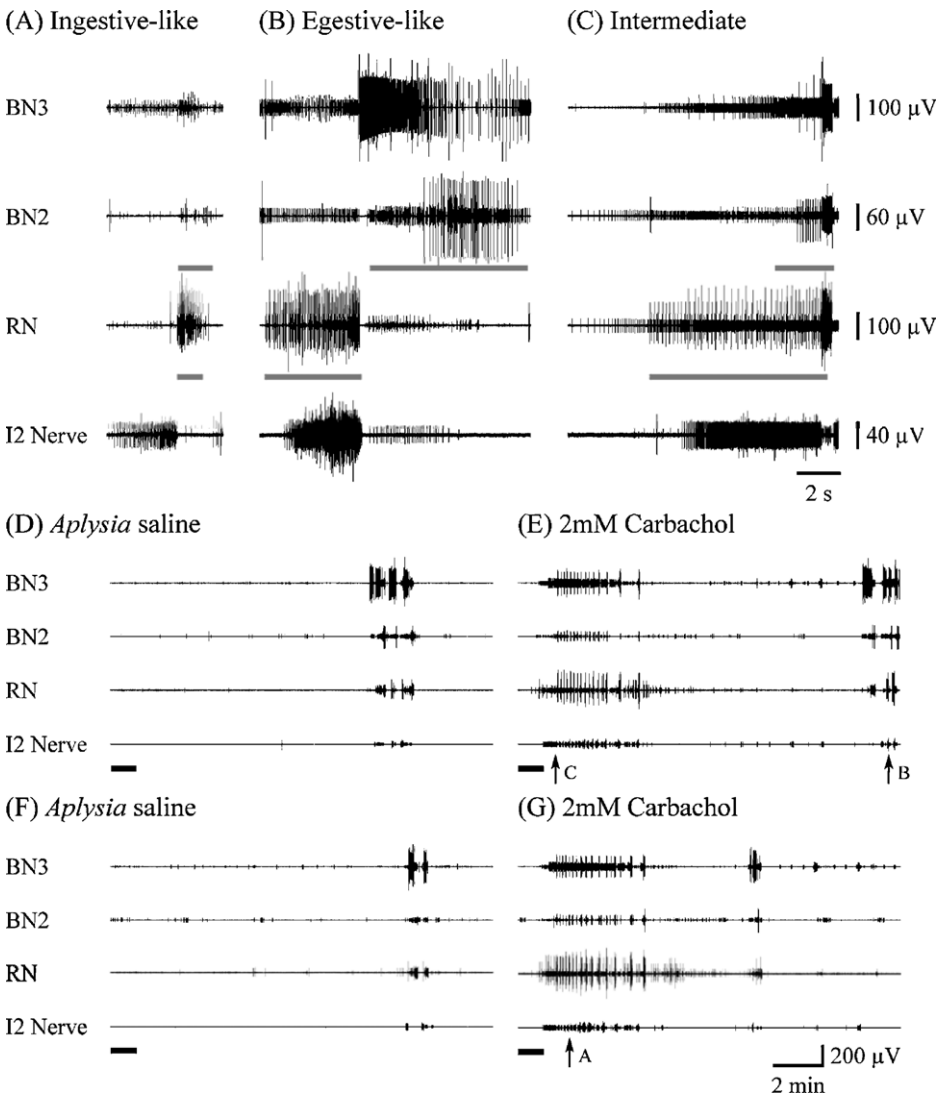

Fig. 4. Neural activity of the buccal ganglion in response to applications of *Aplysia* saline and carbachol. The nerves of interest, buccal nerve 3 (BN3), buccal nerve 2 (BN2), radular nerve (RN) and the I2 nerve on the same side of buccal ganglia were recorded extracellularly for 15 min during and after each chemical stimulation. Gray bars underneath BN2 and RN recordings in Fig. 1A–C show the duration of large units in BN2 and RN activity. (A) An example of an ingestive-like pattern, in which most of large units in RN overlapped large units in BN2 (Morton and Chiel, 1993). (B) An example of an egestive-like pattern, in which large units in RN precede large units in BN2 (Morton and Chiel, 1993). (C) An example of an intermediate pattern, in which large RN units both preceded and overlapped large BN2 units (Morton and Chiel, 1993). (D) First, *Aplysia* saline was applied for 1 min by the chip. Two spontaneous egestive-like patterns were observed during this 15-min recording. (E) Then, 2 mM carbachol in *Aplysia* saline was applied for 1 min. 14 ingestive-like patterns, 2 egestive-like patterns and 1 intermediate pattern occurred during this recording period. The burst of ingestive-like patterns began with a 2-min delay after the beginning of carbachol application. (F) After that, *Aplysia* saline was applied again for 1 min. The intensity of neural activity was greatly reduced compared to (E) and no ingestive-like motor patterns occurred during the 15-min recording. (G) Lastly, 2 mM carbachol was applied in *Aplysia* saline again. During this final recording period, 14 ingestive-like patterns and 1 intermediate pattern occurred. The delay of the bursting of ingestive-like patterns was again about 2 min. The arrows labeled "A", "B" and "C" point to typical ingestive-like, egestive-like and intermediate patterns that are shown in (A–C). Thus, the application of carbachol significantly affected the cerebral ganglion, initiating ingestive-like patterns that were not observed during spontaneous activity in response to applications of regular *Aplysia* saline.

first significantly increased during the early recordings and then declined. In addition a few egestive-like and intermediate patterns also occurred (Fig. 5(B)). Results from the first and third phase of each experiment, and from the second and fourth phase of each experiments were therefore combined, and a two-way ANOVA was performed on the data taken in the first 5 min after the application of either *Aplysia* saline or carbachol in multiple experiments ($n = 3$). Overall two-way ANOVA on these data were highly significant ($p < 0.001$); Bonferroni-corrected *post hoc* tests showed that the number of ingestive-like patterns was significantly different from the number of either egestive-like or intermediate patterns ($p < 0.001$) but that the numbers of egestive-like patterns and intermediate patterns were not significantly different from each other. These results strongly support the hypothesis that during the application of carbachol to the cerebral ganglion from the microfluidic chip, chemical stimulation induces a highly significant increase in ingestive-like motor patterns.

6. Discussion and technological relevance

Although patterns may occur spontaneously in the buccal ganglion, our data clearly demonstrate that in the absence of the application of the cholinergic antagonist carbachol, the ganglion only produced non-ingestive patterns (i.e., intermediate or egestive patterns: Figs. 4 and 5). It is interesting that the number of ingestive-like patterns declined after exposure to carbachol, but we have observed this phenomenon even when carbachol is applied directly to a desheathed cerebral ganglion, and thus the decline is not likely to be due to either the presence of the sheath, or the microflu-

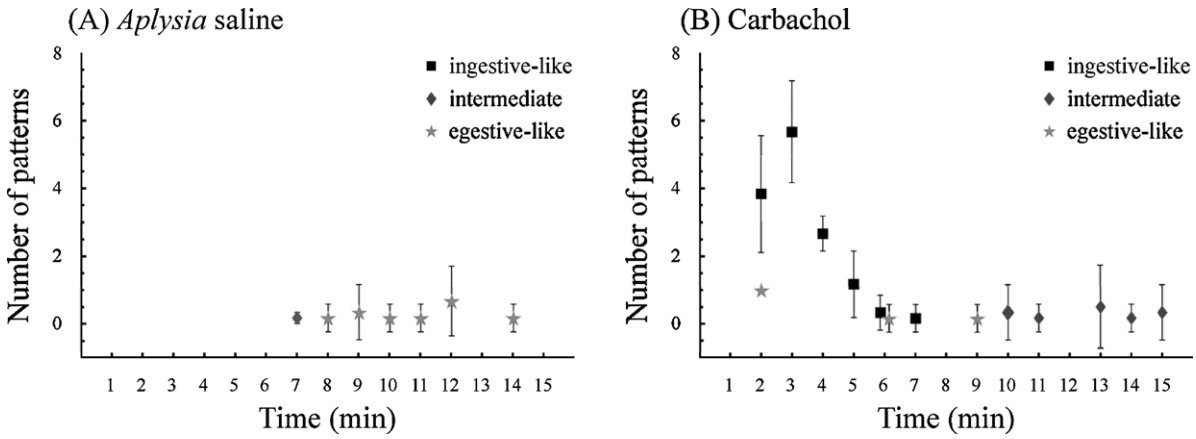

Fig. 5. Number of different feeding patterns during and after the applications of *Aplysia* saline and carbachol. *Aplysia* saline and 2 mM carbachol were both applied for 1 min from the beginning of the recording. The numbers of different feeding patterns per minute were counted at the end of each minute. Black square symbols represent ingestive-like patterns; dark gray diamond symbols represent intermediate patterns; light gray star symbols represent egestive-like patterns. (A) Number of ingestive-like, intermediate and egestive-like patterns per minute with the application of *Aplysia* saline ($n = 3$). There were one or two intermediate or egestive-like patterns per minute during the last half of the recordings. Note that there was no ingestive-like pattern at all. (B) Number of ingestive-like, intermediate and egestive-like patterns per minute with the application of 2 mM carbachol ($n = 3$). Note that the number of ingestive-like patterns per minute was significantly increased during the early recordings and then declined. There were also a few intermediate and egestive-like patterns throughout the recordings.

idic application of the agonist. It could be due to de-sensitization of cholinergic receptors in the ganglion, or to the absence of other sensory inputs indicating the presence of food, which would prolong ingestive-like responses. Indeed, in a suspended buccal mass preparation, we have observed that applying seaweed to the surface of the grasper can induce a vigorous increase in ingestive-like movements after they have begun to diminish (McManus and Chiel, unpublished observations). Thus, it appears that the results we present do not differ significantly than those obtained by direct application of carbachol to a desheathed ganglion.

Microfluidic devices have begun to be applied to a variety of neural systems, and hold great promise for creating novel brain–machine interfaces. For example, a PDMS chip was recently fabricated in combination with micro-molding in capillaries (MMIC) to explore the use of microfluidics and micropatterning on defining substrates that might allow spinal cord neurons to grow successfully across regions of injury (Vahidi et al., 2008). Microfluidic channels have been incorporated into implantable microelectrode arrays (Retterer et al., 2004), and the access of the device to surrounding tissue in response to constant pressure infusion has recently been characterized (Retterer et al., 2008), suggesting that incorporating these devices could help control tissue reactivity, which reduces the ability of conventional microelectrodes to record and stimulate neural tissue, and at the same time, may make it possible to chemically control the activity of neural tissue (Rohatgi et al., 2009; Kobayashi et al., 2009).

The work described in this report has significant implications for *in vivo* applications of microfluidic technology to the control of the neural activity in *Aplysia* and in other animals. For this purpose the whole electrical and fluidic system needs to be redesigned. For example, there are smaller solenoid valves that could be used along with a small refillable capsule to store the pressurized air necessary to operate the on-chip valves. The computer, which was used to control the solenoid valves carrying pressurized valves, also could be replaced by any commercially available microcontroller system.

In *Aplysia*, we have previously shown that the sheath covering the ganglion has a conductance similar to that of seawater (Lu et al., 2008). This study demonstrates that the sheath is permeable (at the very least) to small molecular weight compounds (carbachol has a molecular weight of 147), so that it may be possible to apply transmitters or other drugs from outside the sheath and affect neural activity. The permeability of the sheath also suggests that some transmitter compounds released within the nervous system could be recorded from the surface of the sheath. Thus, it may be possible to create closed loop devices for both electrical and chemical control of ganglion activity. These studies might serve as the basis for similar closed loop controllers in vertebrates and humans.

7. Conclusions

The results of this study have demonstrated that it is possible to use a microfluidic chip device to control neural activity through the controlled application of chemical substances in *A. californica*. Based on the experimental results, this device could be used as the basis for an implantable device to control neural activity and motor behavior using chemical stimulation.

Acknowledgements

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