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6. The birds were taken from their nests at 5 to 7 days of age, transferred to the Vogelwarte Radolfzell in southern Germany (47°46'N, 09°00'E), and hand-raised under simulated local light conditions standardized according to an arbitrary birth date of 10 May for all birds. Beginning at 50 days of age the birds were maintained under constant conditions [a cycle of 12.5 hours of light (400 lux) and 11.5 hours of darkness (0.01 lux); 20° ± 1.5°C] until the end of the season of migratory restlessness. Then they were transferred to natural southern German light conditions to allow them to achieve their normal spring breeding condition.
7. The German, F_1 hybrid, and African birds averaged 74, 53, and 35 nights of restlessness, respectively, and displayed restlessness during 0 to 2009, 0 to 991, and 0 to 534 total half-hours. Respective coefficients of variation: 145, 112, and 98 percent.
8. P. Berthold and U. Querner, in preparation.

9. We thank G. J. Kenagy for his especially extensive review of our report and the Finnish, French, Spanish, and German governments for permission to collect the birds.

4 December 1980

Selection of a Novel Connection by Adult Molluscan Neurons

Abstract. *Predictable changes in neuronal connectivity can be induced in the buccal ganglia of adult* Helisoma *snails when neuritic growth is evoked by axotomy. Both transient and stable novel electrical connections are established between identified neurons. The breaking of inappropriate, normally transient connections is contingent on the formation of an appropriate connection.*

The establishment and maintenance of specific connections in the nervous system is the culmination of a sequence of as yet poorly defined processes. In principle, the selection of such connections may depend on the initial formation of contacts with a range of possible targets, appropriate connections being maintained and inappropriate ones broken. We report here the establishment of both transient ("inappropriate") and stable ("appropriate") novel electrical connections between identified, adult neurons of the snail *Helisoma*. The mechanism accounting for the transient nature of some connections is of fundamental importance. In this system, the breaking of certain inappropriate connections requires the formation of an appropriate connection. Thus, specific patterns of connectivity are determined by the availability of potential targets.

Our initial studies (*1*) of the dynamic abilities of adult molluscan neurons demonstrated that neuritic growth is initiated within a few hours after axotomy. We now report that such outgrowth can result in the selective formation of a new, stable electrical connection between a pair of homologous buccal ganglion neurons, L5 and R5. This pair of neurons, in contrast to many homologues of gastropod buccal ganglia (*2*), are not normally electrically coupled (Fig. 1A). Furthermore, the normal neurites of each neuron 5 are restricted to the ipsilateral ganglion of the paired buccal ganglia (Fig. 1B).

Isolated, paired buccal ganglia of the pulmonate mollusk *Helisoma trivolvis* were cultured in host snails (*1*). Both esophageal trunks were crushed within 100 μm of their origins in order to evoke neuritic growth in the neuropil (*1*). Intracellular stimulation was performed with a balanced bridge circuit and intracellular recordings were made with 2*M* potassium acetate-filled electrodes. The neurons were stained with Lucifer Yellow CH and viewed as whole mounts (*1*, *3*).

The onset of novel coupling between the homologues of neuron 5 (Fig. 1C) was both rapid and predictable; the new connection has characteristics of a direct electrotonic synapse (*4*). Coupling between the homologues of neuron 5 was detected in eight of nine preparations after 2 days of culture and in all 38 after 5 to 30 days (Fig. 2A). The magnitude of coupling was maintained for at least 1 month; coupling coefficients at 7 days and 25 to 30 days were 0.17 ± 0.05 (N = 10) and 0.16 ± 0.04 (N = 7), respectively (*5*). In addition, although recordings become more difficult in older preparations (*6*), coupling of neurons L5 and R5 was observed in all three ganglia successfully recorded after 3 months of culture. That the 5-5 connection is direct and does not involve intervening neurons was further supported by morphological studies demonstrating that neurites from each neuron 5 rapidly traverse the commissure and project toward the contralateral homologue (Fig. 1D). Such newly formed neurites were observed in each of 13 preparations in which coupling was detected and subsequent dye fills made. This absolute correlation of morphology with function supports the role of the newly formed neurites as the substrate of the novel connection and mitigates against the possibility that axotomy is merely revealing a latent, weak connection. In addition, neuritic sprouting across the buccal commissure was observed in two of seven preparations maintained for 24 hours in physiological saline. The speed and inevitability with which such changes occur in *Helisoma* provide a general caution regarding the use of isolated neural preparations for physiological analysis.

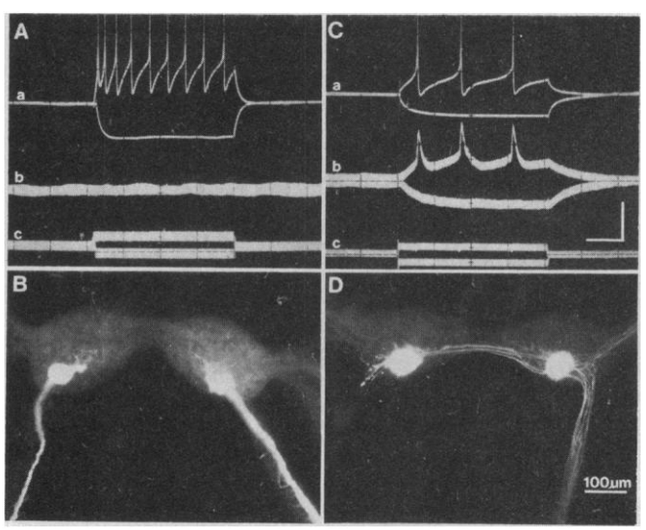

Fig. 1. Physiology and morphology of the electrical coupling between the homologues of neuron 5. (A) Lack of electrical coupling between the homologues of neuron 5 in a normal pair of buccal ganglia. Passage of depolarizing and hyperpolarizing currents (*c*) into L5 (*a*) does not elicit voltage deflections in R5 (*b*); two sweeps are superimposed. (B) Normal morphology of neurons L5 and R5 as shown by staining another preparation with Lucifer Yellow CH. The axon of each neuron 5 exits via an ipsilateral nerve (the esophageal trunk) and the dendrites do not cross the commissure between the paired buccal ganglia. (C) Electrical coupling between the neuron 5 homologues in a preparation cultured for 5 days. In this example the coupling coefficient is 0.22 and action potentials evoked in L5 (*a*) evoke electrotonic excitatory postsynaptic potentials in R5 (*b*). (D) Morphology of neurons L5 and R5 in a preparation cultured for 7 days. The axons of both neurons have been resorbed and newly formed neurites are visible traversing the buccal commissure and in a number of nerve trunks. Calibration for (A) and (C) is 200 msec (horizontal), 25 mV (upper traces), 5 mV (middle traces), and 2 nA (lower traces).

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The stability and reliability of 5-5 coupling strongly imply that its occurrence is not a random process but is derived from a latent, preprogrammed capability. However, it is important to determine the degree of specificity with which the novel 5-5 connection is established. Since recording from all the buccal neurons (400) is impractical, a survey was made which included identified protractor motoneurons 17, 18, 19, and 20 (*2*) and neuron 4. No evidence was found for aberrant coupling of neuron 5 with any of the protractor motoneurons (ten preparations were tested after 1 to 3 days and four were tested after 7 days). However, an interesting transient electrical interaction occurred between neurons 5 and 4 (*7*). The percentage of preparations with electrical coupling between these neurons was maximal (90 percent) after 2 days of culture and decreased thereafter (Fig. 2A). Physiologically, this interaction closely resembles that between the neuron 5 homologues, again indicating a direct connection, but in this case the coupling coefficient peaked (0.07 ± 0.03, $N = 12$) after 1 day and then decreased. A few (2 of 11) older preparations, tested after 15 to 30 days, also exhibited 5-4 coupling. This may indicate that the signal responsible for breaking this connection is not infallible. Alternatively, some remodeling may occur in older preparations.

The novel connection between buccal neurons 5 and 4 is physiologically inappropriate since it tends to synchronize the activity of neurons that are driven by opposite synaptic inputs and that innervate different peripheral targets, namely the esophagus and the salivary glands. In contrast, since the homologues of neuron 5 are coactivated and innervate a common target, the 5-5 connection is physiologically appropriate [indeed, many other buccal homologues are normally coupled (*2*)].

In principle, the signal to break inappropriate connections could depend on the establishment of an appropriate connection. This hypothesis was tested by culturing single ganglia prepared by cutting the commissure of paired ganglia. Under these conditions the homologue of neuron 5 is not available as a target. The single ganglia were assayed after 7 days in culture since 5-4 coupling was not observed in any paired ganglia at this age (Fig. 2A). Coupling between neurons 5 and 4 was maintained in 9 of 10 of the single ganglia. This is the same as the maximum number of transient 5-4 connections recorded in paired ganglia (Fig. 2A). Furthermore, the degree of 5-4 coupling in single ganglia (coupling coefficient, 0.12 ± 0.03) was not significantly different from the maximum coupling of these neurons in paired ganglia. We conclude that dissolution of the transient 5-4 connection in paired ganglia during the first week of culture is probably due to an interaction between the homologues of neuron 5.

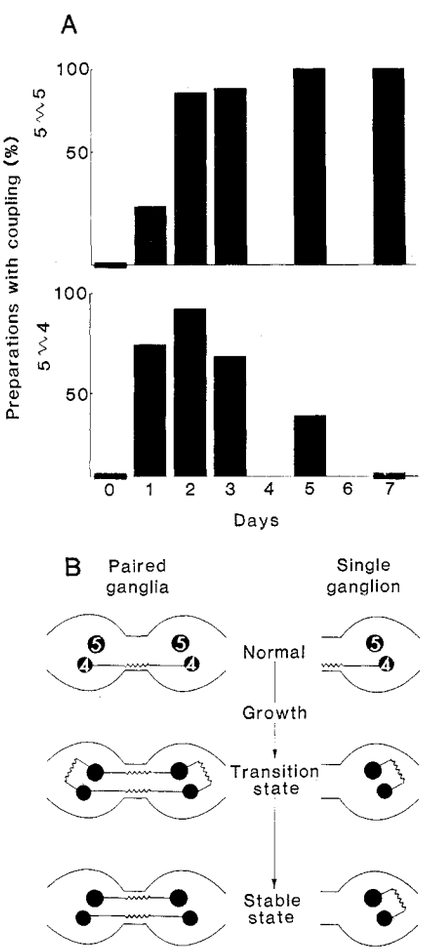

Fig. 2. (A) Time course of the transient electrical coupling of neurons 5 and 4 (lower histogram) and the onset of stable coupling between the homologues of neuron 5 (upper histogram) in paired buccal ganglia. In six control preparations (day 0), no coupling was detected between the homologues of neuron 5 or between neuron 4 and neuron 5. Neuron 5-5 coupling was tested in ten to twelve preparations on each day shown. Coupling between neurons 5 and 4 was sampled in ten to twelve neuron pairs from six to nine preparations on each day (*7*). In most cases the 5-5 connections were monosynaptic (that is, the relation of spike to excitatory postsynaptic potential was one-to-one at high frequency). However, when coupling is weak (as on day 1), an indirect pathway (as through neuron 4) could account for part or all of the interaction. The histograms represent data taken from over 80 preparations. (B) Schematic summary of the principal findings. In paired buccal ganglia, neuron 5, when induced to grow by axotomy, establishes novel electrical connections with both its contralateral homologue and neuron 4 (no specific neuron 5-4 pathways can be inferred). The 5-4 connections are transient, whereas the 5-5 connection is stable for at least 1 month. In single ganglia, the contralateral neuron 5 is not available as a target and the normally transient 5-4 connection is stabilized for at least 1 week. Thus, when the development of an appropriate (5-5) connection is prevented, an inappropriate connection (5-4) is maintained.

The factors that dictate or modulate the establishment of specific neuronal connections are of fundamental importance. The precision afforded by the identified neurons of mollusks allows the selective formation of their connections to be examined (Fig. 2B). Neuron 5 selectively connects with neurons 4 and 5, but not the protractor motoneurons. The inappropriate connection (5-4) is then broken and the appropriate connection (5-5) is maintained. This selectivity may be hierarchical. The protractor neurons never connect with neuron 5, whereas the homologue of neuron 5 is a preferred target with which a stable connection is usually established. In the middle tier of the hierarchy is neuron 4, which, in the absence of the preferred target, can form a stable connection with neuron 5. The connections established by other neurons may be governed by similar hierarchies.

The novel connections reported here both provide direct evidence for selective stabilization of connections and support the working hypothesis that specific neuronal connectivity patterns can be determined by the availability of potential targets.

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4. Action potentials driven in either neuron evoked excitatory postsynaptic potentials in its homologue which arose without measurable delay and followed one for one at a constant peak latency at high frequency (10 Hz), indicating a direct connection. Furthermore, blocking the chemical synapses with 10 × normal $Mg^{2+}OCa^{2+}$ saline had no effect on the amplitude of the potentials.
5. Electrical coupling was measured after superfusing ganglia with 10 × normal $Mg^{2+}OCa^{2+}$ saline for 5 minutes to block chemical synapses. Neurons maintained constant resting potentials and input resistances for at least 30 minutes under these conditions. Electrodes were balanced with 1- to 3-msec pulses and coupling was measured by using currents of 0.2 to 1.0 nA. Pairs of neurons were classified as not coupled if a current of 4 to 5 nA in one neuron did not produce visible voltage deflection in the other. The lowest coupling coefficient that could be measured accurately was 0.02. However, we estimate that coupling as low as 0.003 could be detected with this technique.
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8. Supported by PHS research grant 1 PO1 NS15350 and a grant from the Whitehall Foundation. We thank D. L. Barker, A. D. Murphy, and C.-F. Wu for criticism, A. Bulloch and J. Kater for helping to prepare the manuscript, M. Jolly and J. Sedgewick for photographic assistance, and W. H. Stewart for providing Lucifer Yellow CH.

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Sustained Intracerebroventricular Infusion of Brain Fuels Reduces Body Weight and Food Intake in Rats

Abstract. *Long-term infusion of glucose, β-hydroxybutyrate, and glycerol into the third ventricle of the rat brain caused a stabilization of body weight at a lower than normal level. Among the glucose- and glycerol-treated animals this weight loss was caused in part by temporary hypophagia. Among the animals treated with β-hydroxybutyrate the weight loss was unaccompanied by a reduction in food intake. The results are consistent with the view that the systems controlling food intake and body weight are sensitive to the availability of brain fuels. They are not consistent, however, with the view that these control systems monitor calories independently of their source.*

The body weight and food intake of animals appear to be controlled within a fairly narrow range, but the mechanisms involved in their control are not well understood. Although ablation and stimulation studies indicate that an important part of the control system is located in the hypothalamus, they cannot elucidate the nature of the information that is processed. Such clarification can be obtained, however, by determining the effects of chemicals believed to participate in the central control of food intake and body weight. The food intake control system may directly monitor the concentrations of circulating nutrient metabolites and may alter food intake in response to either a surfeit or a deficit in these substances. Hypothalamic electrical activity can be altered by intravenous or iontophoretic glucose (*1*) and by insulin administered in sufficient amounts to significantly lower the concentration of blood glucose (*2*). Also, injections of glucose and amino acids into the hypothalamus decrease food intake (*3*) and injections of insulin antibodies (*4*) or 2-deoxy-D-glucose (*5*) into the same general region of the brain increase food intake. It has not been known whether these substances have parallel effects on body weight.

This report describes the results of a study designed to determine the effects of long-term intracerebroventricular infusions of glucose, glycerol, and β-hydroxybutyrate on both food intake and body weight (*6*). Glucose and β-hydroxybutyrate were chosen because they are the major metabolites of carbohydrate and lipid metabolism which have been shown to be utilized by the brain as energy sources (*7*). Glycerol was selected for three reasons: (i) it can be metabolized by the brain (*8*), (ii) when administered peripherally by a variety of routes it is capable of suppressing food intake (*9–12*), and (iii) its long-term administration leads to a reduction in body weight (*12, 13*). Infusions were made into the third ventricle because the surrounding hypothalamic regions are involved in the control of food intake and body weight and because substances that cross the blood-brain barrier poorly, such as β-hydroxybutyrate, are taken up maximally in this region (*14*). It is obvious that weight regulation can only be investigated with long-term treatments, and it is likely that a system concerned with the long-term control of food intake would be more responsive to a sustained rather than short-term change in the availability or utilization of energy-producing metabolites.

The subjects were male albino rats (Charles River) weighing an average of 300 g at the beginning of the experiment. They were maintained in individual cages on a 12-hour light-dark cycle. A guide cannula made of 21-gauge stainless steel tubing was implanted, aimed at the third ventricle (*15*). The stereotaxic coordinates were 6.4 mm anterior to bregma, 2.6 mm below the surface of the brain, and on the midline. The animals were allowed to recover for at least 3 weeks, and were then given unrestricted access to a pellet diet (*16*). Food intake, water intake, and body weight were measured daily at about 8 a.m. and 6 p.m.

After at least 7 days, each animal was fitted with an Alzet minipump (*17*) filled with the test solution. The pump was connected by a length of polyethylene tubing (inner diameter, 0.015 inch), also filled with the test solution, to an insert cannula made of 27-gauge stainless steel tubing trimmed so that when it was inserted into the guide cannula it would terminate in the third ventricle. The insert cannula was cemented in place while the animal was under light ether anesthesia. The pump and tubing were then inserted into a subcutaneous channel opened under the skin on the animal's back. The pump rested just over the scapula when in place. The incision was closed with surgical silk and the animal was returned to its cage. The animals weighed between 350 and 420 g at the time of pump implantation.

The Alzet pump delivered its contents at a rate of 1 μl per hour for 7 days. On the seventh day the tubing emerging from the insert cannula was cut and the end was heat-sealed. Food and water intake and body weight were measured in the morning and evening during the entire time that the pump was connected and for at least 7 days after the tubing was cut.

The rats were infused with D-glucose, glycerol, or DL-β-hydroxybutyrate at a concentration of 0.15*M* and an osmolar concentration (adjusted with saline) of 300 milliosmoles per liter. The substances were delivered to the ventricle at a rate of 1.5×10^{-7} mole per hour. A 0.15*M* NaCl solution was infused into members of a control group to evaluate the effects of infusing the vehicle into the ventricle.

There was a small weight loss, probably due to surgical trauma, in the animals infused with saline, but they gained weight at a normal rate thereafter (Fig. 1). The other compounds caused sustained, significant changes in body weight [$F(3, 20) = 6.9$, $P < .005$]—the greatest loss occurring during the first few days of infusion. Post hoc comparisons showed that these three compounds

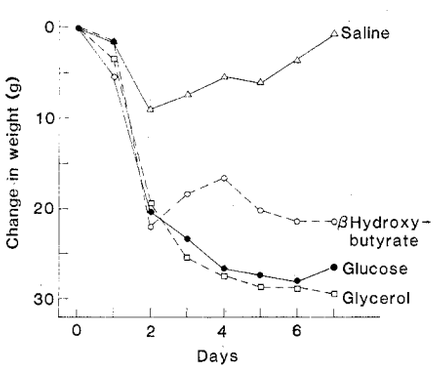

Fig. 1. Changes in body weight from initial weight. The label at the end of each curve indicates the substance infused.

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