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SHORT COMMUNICATION

SPROUTING BY UNDAMAGED ADULT MOLLUSCAN NEURONES: PUTATIVE ROLE FOR CHANGES IN HAEMOLYMPH OSMOREGULATION

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Sprouting of undamaged, adult neurones has been observed in a number of situations. For instance, in the peripheral nervous system of vertebrates, intact motoneurones exhibit terminal sprouting in response to axotomy of contralateral motoneurones (Rotshenker, 1979; Rotshenker & Tal, 1985) and in the central nervous system, neurones have been observed to sprout and occupy synapses vacated by lesioned fibres (Cotman, Nieto-Sampedro & Harris, 1981; Tsukahara, 1981). Furthermore, evidence also exists for dendritic sprouting and regression in the mature, human brain (Buell & Coleman, 1981). The plastic properties of the neurones of the freshwater pulmonate *Helisoma* have been examined in a number of regeneration and sprouting studies (e.g. Murphy & Kater, 1980; Bulloch & Kater, 1982; reviewed by Bulloch, 1985*a*). Recently, it was demonstrated that sprouting and retraction of a central neurite occurred from an undamaged adult neurone of *Helisoma* in response to stress (Bulloch, 1984). Specifically, sprouting occurred in a pair of buccal neurones, L5 and R5, and aestivation and body wall incision were the most effective treatments examined. Body wall incision results in haemolymph loss, and aestivation results in a loss of water and thus an increase in haemolymph osmolarity (Machin, 1975). These observations raise the possibility that the sprouting of neurones L5 and R5 may occur in response to changes in haemolymph osmoregulation. The current study directly tested the hypothesis that *Helisoma* neurones can sprout in response to a change in blood osmolarity. This was achieved by exposing snails to hyperosmotic and hyposmotic pond water which has known effects on blood osmolarity (Khan & Saleuddin, 1979).

Experimental animals (13–15 mm shell height) were placed in 250 ml beakers which contained either normal pond water (0·25 g Instant Ocean salts l^{-1}), hyperosmotic pond water, i.e. 20% sea water (7·6 g Instant Ocean salts l^{-1}) or hyposmotic (deionized) pond water, all of which were based on carbon-filtered Millipore 'Super Q' water. The two experimental conditions were chosen on the basis of previously reported salinity tolerance limits and osmotic regulation data of *Helisoma trivolvis*: compared to a normal haemolymph osmotic pressure of

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115 mosmol l^{-1}, *Helisoma* maintains a value of 88 mosmol l^{-1} in distilled water, whereas in hyperosmotic pond water (200 mosmol l^{-1}, i.e. 20 % sea water) the animal maintains a value of 215 mosmol l^{-1} (Khan & Saleuddin, 1979). After periods of up to 7 days the paired buccal ganglia were dissected and two paired neurones were injected with Lucifer Yellow CH, i.e. neurones L5 and R5, L4 and R4, or L19 and R19 were stained in any given preparation. Control animals were taken directly from holding tanks and dissected, and appropriate pairs of neurones were stained. Ganglia were fixed overnight in phosphate-buffered 4 % formaldehyde within 3 h of dissection. Specimens were dehydrated with a graded ethanol series, cleared in

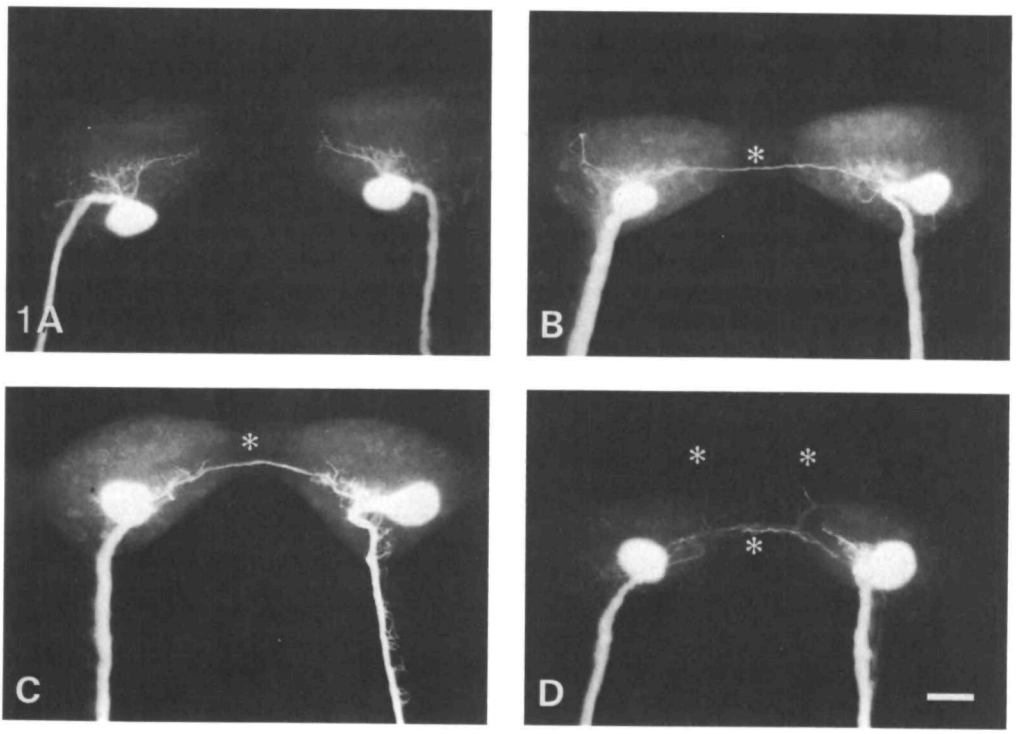

Fig. 1. Normal and sprouted morphology of intact, buccal neurones L5 and R5. In all examples, the oesophageal nerve trunks (which contain the axons of neurones 5) project towards the bottom of the page. (A) Control: the normal morphology of neurone 5 is characterized by a single axon which exits from the neuropile *via* the ipsilateral oesophageal nerve trunk. A dendritic arborization is present in the neuropile which is dominated by a single medial dendrite that projects towards, but does not cross the commissure (centre) between the paired ganglia. (B) Hypertonic: sprouted morphology of neurones 5 in a preparation from an animal exposed to 20 % sea water for 24 h. A new neurite, (*) whose origin is in the right-hand neurone 5, traverses the buccal commissure and appears to penetrate the dendritic arbor of the contralateral neurone. (C) Injury: in this example, the left-hand neurone 5 has produced a single sprout (*) in response to animal injury 3 days previously; note the similarity to that evoked by hypertonic saline. (D) Hypotonic: sprouted morphology of neurones 5 in a preparation from an animal exposed to deionized water for 24 h. In this example new neurites (*) are present in the commissure and in medial nerve trunks (centre, upper) from both neurones. Scale bar, 100 μm.

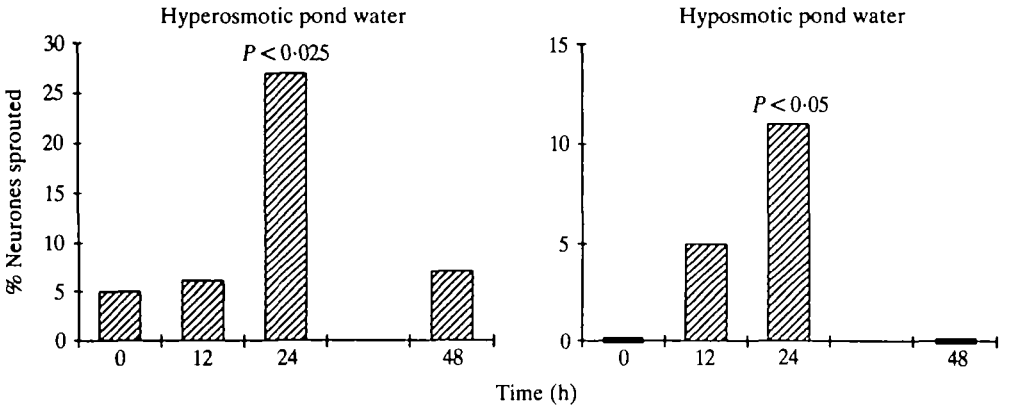

Fig. 2. Time course of sprouting of intact neurones 5 subsequent to exposure of animals either to hyperosmotic or hyposmotic pond water. The percentage of neurones sprouted was determined both in acutely dissected animals (0 h) and at three subsequent time periods. Each indicated time represents animals sampled during the following time periods: 12–14 h (12), 22–26 h (24) and 46–50 h (48). The data from hyperosmotic pond water represent neurones possessing single sprouts, whereas those from hyposmotic water represent neurones possessing multiple sprouts. In both cases the sprouting was statistically significant at 24 h. The percentage of sprouted neurones was reduced to statistically insignificant levels after both treatments by 48 h, and was similarly low in preparations sampled after 3 days and 1 week. For hyperosmotic pond water $N = 38$ (0 h), 32 (12 h), 26 (24 h) and 30 (48 h); for hyposmotic pond water $N = 38$ (0 h), 21 (12 h), 45 (24 h) and 21 (48 h).

methyl salicylate and viewed as whole mounts under fluorescence microscopy. Tests for significance between the percentage of neurones sprouted in control and experimental groups was by the Fisher's (2×2) exact test which examines the association between two variables.

Neurone 5 is characterized morphologically by a dendritic arborization in the neuropile which is dominated by a medial dendrite (Fig. 1A). No processes normally penetrate the contralateral buccal hemiganglion. Sprouts are defined as neuritic processes which enter the contralateral neuropile and/or foreign nerve trunks. In the present study the normal morphology of neurone 5 was reconfirmed ($N = 38$), but in a single preparation both neurones L5 and R5 exhibited a neurite that traversed the commissure which resembled that reported to be evoked by stress (Bulloch, 1984). This suggests that a baseline of sprouting (5 % in this study) exists in the 'normal' animal. However, these are the first control neurones 5 observed to sprout out of many (>100) used in a variety of studies (e.g. see Bulloch, 1984 for references).

Sprouting of neurone 5 was seen following placement of the animal in hyperosmotic pond water (Fig. 1B). The observed sprouts had a characteristic morphology: they projected across the buccal commissure and extended towards and/or into the dendritic arborization of the contralateral neurone 5. The sprout was never observed to leave the buccal ganglia, i.e. to penetrate a nerve trunk. Furthermore, only a single cell was observed to sprout in any given preparation. The sprout observed in response to animal placement in hyperosmotic pond water is

identical in morphology to the sprouts observed in response to body wall incision (Fig. 1C) and aestivation (Bulloch, 1984). The morphological similarities of the sprouts observed under these conditions suggest that the sprouting response may be triggered by the same factor in all three treatments.

The sprouting in response to animal immersion in hyperosmotic pond water was transient, being maximal (26 %) at 1 day (Fig. 2). Given the distance traversed by sprouts, the maximum rate of neurite elongation is about 0·5 mm day^{-1}. In contrast to these results, maximal sprouting has been observed at 3 days in response to body wall incision (Bulloch, 1984). This discrepancy may be due to the absence of direct injury in the present protocol. Although the time course of sprouting was not identical in the hyperosmotic *vs* injury response, the maximal observed sprouting was similar: 27 % for hyperosmotic water at 1 day and 25 % for body wall incision at 3 days (Table 1). The significance of the transient nature of the sprouting response by neurone 5 is unknown, and is especially paradoxical in the present study which involves exposure of animals to a constant environment.

To determine if the sprouting response in neurone 5 was due to non-specific stress (e.g. from handling), as opposed to an increase in haemolymph osmolarity, neurone 5 was examined for sprouting in animals immersed in normal pond water in beakers for 1 day. Although examples of sprouts by neurone 5 were observed under this condition, their occurrence was not statistically significant (Table 1). This control experiment reinforces the idea that the sprouting response may be due to an increase in haemolymph osmolarity.

In animals placed in hyposmotic pond water, a new type of sprouting response was observed that was characterized by multiple processes. Furthermore, 50 % of the sprouted neurones had processes which extended into peripheral nerve trunks (Fig. 1D). In common with the sprouting response in hyperosmotic pond water, the sprouting found in animals immersed in hyposmotic pond water was maximal (11 %) at 1 day and was transient (Fig. 2). A few examples of neurones with the single sprout described previously were observed, but the occurrence of this type of sprout was not above control values. Thus, the type of sprouting observed in response to body wall incision and aestivation (Bulloch, 1984) is found in animals placed in a hyperosmotic environment which increases haemolymph osmolarity (Khan & Saleuddin, 1979). In contrast, a qualitatively and quantitatively different type of

Table 1. *Neurone 5 sprouting* versus *animal treatment*

Treatment	% Neurones sprouted (*N*) Single sprouts	Multiple sprouts
Control 0 day	5 (38)	0 (38)
Control 1 day in beakers	8 (26) $P > 0{\cdot}05$	0 (26) $P > 0{\cdot}05$
Hyperosmotic pond water 1 day	27 (26) $P < 0{\cdot}025$	0 (26) $P > 0{\cdot}05$
Hyposmotic pond water 1 day	7 (27) $P > 0{\cdot}05$	11 (45) $P < 0{\cdot}05$
Body wall incision 3 day*	25 (20) $P < 0{\cdot}02$	0 (20) $P > 0{\cdot}05$

* From Bulloch (1984).

sprouting response occurs when animals are placed in a hyposmotic environment which slightly lowers haemolymph osmolarity (Khan & Saleuddin, 1979) and requires an increase in diuresis and salt retention (Van Aardt, 1968).

The neuronal specificity of the sprouting response to hyperosmotic pond water was determined by examining two other pairs of identified buccal neurones: R4 and L4, and R19 and L19. Neurones R4 and L4 are salivary effector neurones and R19 and L19 are protractor motoneurones. The morphology of these two pairs of buccal neurones has been described previously (see Bulloch, 1985*a* for references). Both pairs of neurones were examined for sprouts 1 day after animal placement in hyperosmotic pond water. No evidence of sprouting in either pair of identified neurones was observed ($N = 28$ for each neurone type). Thus the sprouting response in animals placed in hyperosmotic pond water is specific to particular identified neurones. In agreement with this conclusion is the evidence that neurones 4 and 19 do not sprout in response to body wall incision (A. G. M. Bulloch, unpublished observations). Since neurone 5 innervates the oesophagus, it would be of interest to determine the functional significance of sprouting under these various conditions. It is as yet unknown if the electrical properties of neurone 5 are affected by sprouting, but, in contrast to the consequences of axotomy (Bulloch & Kater, 1982), a new electrical synapse does not form between the two neurones 5 that sprout as a result of animal injury (Bulloch, 1985*b*).

Taken together, evidence presented previously (Bulloch, 1984) and in this report suggests that neurone 5 can sprout in response to altered blood osmoregulation. The evidence presented thus far does not, however, indicate if sprouting by neurone 5 under these conditions is due to changes in haemolymph osmolarity *per se* (or selected ion levels) or a change in the levels of hormones associated with osmoregulation. Two further observations support the possible involvement of hormones. First, the osmolarity of blood is unchanged after body wall incision (S. J. Rosser & A. G. M. Bulloch, unpublished observation), a condition which evokes sprouting by neurone 5 (Bulloch, 1984). Secondly, a new sprouting response has been observed in the current study from neurones of animals in a hyposmotic environment, i.e. under conditions in which the change of blood osmolarity is comparatively small. The fact that sprouting and retraction of neurites of specific identified neurones can be induced under conditions similar to those which could occur in nature (i.e. spring run-off dilutes pond water and late summer droughts increase its salinity) suggests that this morphological plasticity could be environmentally induced.

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REFERENCES

BUELL, S. J. & COLEMAN, P. D. (1981). Quantitative evidence for selective dendritic growth in normal ageing, but not in senile dementia. *Brain Res.* **214**, 23–43.

BULLOCH, A. G. M. (1984). Sprouting and retraction of neurites by undamaged adult molluscan neurons. *Brain Res.* **321**, 369–373.

BULLOCH, A. G. M. (1985*a*). Development and plasticity of the molluscan nervous system. In *The Mollusca, Neurobiology and Behavior Part 1,* vol. 8 (ed. A. O. D. Willows), pp. 335–410. New York: Academic Press.

BULLOCH, A. G. M. (1985*b*). Stress enhances electrical synapse formation between regenerating neurons. *Brain Res.* **343**, 370–373.

BULLOCH, A. G. M. & KATER, S. B. (1982). Neurite outgrowth and selection of new electrical connections by adult *Helisoma* neurons. *J. Neurophysiol.* **48**, 569–583.

COTMAN, C. W., NIETO-SAMPEDRO, M. & HARRIS, E. W. (1981). Synapse replacement in the nervous system of adult vertebrates. *Physiol. Rev.* **61**, 684–784.

KHAN, H. R. & SALEUDDIN, A. S. M. (1979). Osmotic regulation and osmotically induced changes in the neurosecretory cells of the pulmonate snail *Helisoma*. *Can. J. Zool.* **57**, 1371–1383.

MACHIN, J. (1975). Water relationships. In *Pulmonates* (ed. V. Fretter & J. Peake), pp. 105–163. London: Academic Press.

MURPHY, A. D. & KATER, S. B. (1980). Sprouting and functional regeneration of an identified neuron in *Helisoma*. *Brain Res.* **186**, 251–272.

ROTSHENKER, S. (1979). Synapse formation in intact innervated cutaneous-pectoris muscles of the frog following denervation of the opposite muscle. *J. Physiol., Lond.* **292**, 535–547.

ROTSHENKER, S. & TAL, M. (1985). The transneuronal induction of sprouting and synapse formation in intact mouse muscles. *J. Physiol., Lond.* **360**, 387–396.

TSUKAHARA, N. (1981). Synaptic plasticity in the mammalian central nervous system. *A. Rev. Neurosci.* **4**, 351–379.

VAN AARDT, W. J. (1968). Quantitative aspects of the water balance in *Lymnaea stagnalis*. *Neth. J. Zool.* **18**, 253–312.