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EFFECT OF NON-NEURONAL CELLS ON PEPTIDE CONTENT OF CULTURED SENSORY NEURONES

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SUMMARY

The peptide substances P (SP) and somatostatin (SOM) are present in small-diameter neurones of dorsal root ganglia (DRG) and in small-diameter fibres that project to the spinal cord dorsal horn. It is not known whether SP or SOM coexist with other transmitter molecules but, since both peptides can be released from sensory neurones and both can alter neuronal firing rates in the dorsal horn, it seems likely that they are involved in some way in synaptic transmission in the spinal cord. SP has generally excitatory effects in the spinal cord whereas SOM exerts inhibitory effects, and it is not at all clear which subclass of nociceptive afferents contain SP or SOM.

Mudge, Leeman & Fischbach (1979) have studied sensory neurones derived from chick DRG grown in dissociated cell culture. When the neurones are grown in the absence of non-neuronal cells, they contain SP but relatively little SOM. The amount of SOM produced by these neurones is greatly increased (*c.* 50-fold) when the neurones are grown together with ganglionic non-neuronal cells or with medium 'conditioned' by incubation with such cells. The increase in SOM content is not accompanied by an increase in SP content or a detectable change in neuronal survival.

The DRG non-neuronal cells consist of two major cell types – glial cells and fibroblasts. Indirect evidence suggests that glial cells rather than fibroblasts are responsible for the increased production of SOM by the neurones. Raff and his colleagues (Brockes, Fields & Raff, 1979) have used immunological techniques to obtain purified cultures of these two cell types. Work currently in progress is aimed at extending the above observations to the rat and firmly establishing whether glial cells can indeed influence SOM production in sensory neurones.

INTRODUCTION

Neurones and glial cells of the vertebrate peripheral nervous system are derived predominantly from the neural crest (Weston, 1970). The neural crest is a transitory embryonic structure which forms as the neural tube closes and soon after the crest cells start to migrate widely throughout the embryo. Although at the time they begin their migration the crest cells seem to be a homogeneous group of cells, they give rise to melanocytes, peripheral glial cells (Schwann cells), sympathetic and parasympathetic neurones and sensory neurones. The question of when neural crest cells become

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committed to these various differentiated cell types and whether environmental factors can influence this commitment has been extensively studied in the autonomic nervous system both *in vivo* and *in vitro* (for reviews see Le Douarin *et al.* 1981; Le Douarin, 1980; Patterson, 1978; Varon & Bunge, 1978).

Environmental influences on the development of neural crest cells in vivo

Le Douarin and her colleagues have shown that the developmental fate of presumptive autonomic ganglion cells can be changed if their migration path and final localization site are experimentally altered. To follow the migration of neural crest cells this group uses the technique of transplanting crest cells from quail into a chicken host. The quail cells can be distinguished from the chicken host cells by the different staining characteristics of their nuclei, thereby providing a stable marker which is not diluted as the cells divide (Le Douarin, 1974). They transplanted quail cells, from the region of the neural crest which normally gives rise to 'adrenergic' adrenal medullary cells, into a chicken at the axial level of the neural crest, which normally gives rise to the 'cholinergic' enteric ganglia. They found that the enteric ganglia consisted entirely of quail cells. These cells did not contain detectable levels of catecholamines and were functionally cholinergic. Thus the neuroblasts did not differentiate according to their presumptive fate (adrenergic medullary cells) but according to the fate of cells normally in that environment (cholinergic neurones) (Le Douarin *et al.* 1975). Moreover, the ability of the tissue environment to influence the type of transmitter made by autonomic neurones persists late in development. For example, when the ciliary ganglion was transplanted back to the adrenomedullary region of the crest the ganglionic cells, which had already been expressing cholinergic properties, underwent a second migration and gave rise to adrenergic cells (Le Douarin *et al.* 1978).

The chemical environment determines whether individual sympathetic neurones in culture are 'cholinergic' or 'adrenergic'

The superior cervical ganglion of rats contains principal neurones that are predominantly 'adrenergic', with a small percentage of 'cholinergic' principal neurones. When these neurones are grown in culture in the absence of non-neuronal cells they synthesize and store norepinephrine (NE) from tyrosine in large amounts; only small amounts of acetylcholine (AcCh) are synthesized from choline (Mains & Patterson, 1973). However, when the neurones are cultured together with certain non-neuronal cells such as ganglionic non-neuronal cells, cardiac muscle, or skeletal muscle, these cultures produce up to 1000 times as much AcCh as 'neurones-alone' cultures (Patterson & Chun, 1974).

It is not necessary for the neurones to be in contact with the non-neuronal cells for this increase in ability to synthesize AcCh to occur. Growth medium which has been 'conditioned' by incubation with the appropriate cells can also produce this effect if added to 'neurones-alone' cultures. Patterson & Chun (1977*a*, *b*) followed the change in the ratio of AcCh/CA synthesis with time in culture using 'neurones-alone' cultures fed with conditioned medium (CM). They found that the change in ratio was due to both an increase in AcCh synthesis and a decrease in CA synthesis. The maximum effect was not seen until after 3 weeks in culture. In those experiments the number of neurones present in the cultures was constant. Reichardt & Patterson (1977) analys-

transmitter synthesis by single neurones grown in isolation from other cells. They showed that almost all neurones participated in either AcCh or CA synthesis; that is, there was no significant populations which were silent. Taken together with the constancy of neuronal survival they conclude that the soluble factor produced by the non-neuronal cells affects the choice of transmitter made by individual neurones, rather than influencing the expression of transmitter by a selected population of neurones.

Furshpan and colleagues also demonstrated that individual sympathetic neurones grown in culture can exhibit either 'adrenergic' or 'cholinergic' properties. They studied the types of synapses made by single neurones grown in a restricted area of cardiac cells. They found that some neurones excited the myocytes and were pharmacologically adrenergic; others inhibited the myocytes and were pharmacologically cholinergic; some neurones were first inhibitory and then excitatory and apparently released both types of transmitter (Furshpan *et al.* 1976).

Sensory neurone transmitters?

Sensory neurones, whose cell bodies lie in the dorsal root ganglia (DRGs), also derive from the neural crest. Therefore, it is of interest to know if environmental influences are also exerted on developing sensory ganglia. Work in this area has been limited because the types of transmitters used by sensory neurones is not known. There are three major classes of sensory neurones: (i) large myelinated proprioceptive ones; (ii) large myelinated fibres carrying information about touch; (iii) small myelinated and unmyelinated fibres carrying information concerning pain and temperature.

A number of small biologically active peptides have been shown to be present in the latter group of neurones, namely substance P (SP), somatostatin (SOM), cholecystokinin (CCK) and vasoactive intestinal peptide (VIP) (Hökfelt *et al.* 1980). These peptides are thought to play a role in synaptic transmission in the pain/temperature pathway although their precise role is not clear at this time.

In this paper I show that non-neuronal cells derived from the DRG produce a soluble factor which is capable of increasing the amount of the peptide somatostatin contained in cultured sensory neurones. Some evidence is also presented which suggests that the cell type responsible for this effect may be the glia rather than the fibroblasts.

METHODS

(A) *Cultures*

Dorsal root ganglia (DRGs) were dissected from the thoracic and lumbar region of 9- to 10-day-old chicken embryos. The ganglia were dissociated to single cells with collagenase and the cells plated on to collagen-coated tissue culture dishes; usually cells from 5–6 ganglia were plated into each 35 mm dish (Mudge, Leeman & Fischbach, 1979; Mudge, 1981). Three different types of DRG cultures were grown.

(*a*) *Neurones-alone cultures.* These cultures were obtained by plating dissociated DRG cells on to collagen-coated dishes; cytosine arabinoside (5×10^{-6} M) was added to the medium for the period 12 h to 4 days after plating, to kill dividing non-neuronal cells. In some cases irradiation by a ^{60}Co source was used to halt cell division instead of cytosine arabinoside.

(*b*) *Ganglionic non-neuronal cell alone cultures.* These cultures were obtained by plating the DRG cells as above but allowing the non-neuronal cells to proliferate for 4 days. The neurones by this time had developed extensive processes. Trypsin was then added in Ca^{2+}-, Mg^{2+}-free salt solution for 15 min. The cells which detached from the substratum were then centrifuged, resuspended in growth medium, triturated, filtered through lens paper and replaced on to fresh collagen-coated tissue culture dishes. When the cells were confluent further cell division was prevented by γ irradiation using a ^{60}Co source. There were no neurones present in such cultures (using morphological criteria). These cultures were used (i) as a substratum on which to plate freshly dissociated neurones to yield neurone plus non-neuronal cell cultures; or (ii) as a source of cells with which to 'condition' medium.

(*c*) *Neurones plus ganglionic non-neuronal cell cultures.* Two methods were used to obtain these cultures. Either (i) DRG cells were plated on to monolayer cultures of ganglionic non-neuronal cells and irradiated to halt division of additional non-neuronal cells; or (ii) DRG cells were plated on to collagen-coated dishes and the non-neuronal cells were allowed to proliferate until they reached confluency at which time the cultures were irradiated.

(*d*) *Muscle fibroblasts.* Pectoral muscle from 11-day chicken embryos was dissociated and plated on tissue culture dishes allowing only a brief time (15 min) for the cells to settle before changing the medium so as to increase the ratio of fibroblasts to myoblasts (Fischbach, 1972). After 3 days, when the myoblasts had fused, the cells were removed from the dish by trypsinization, centrifuged, re-suspended in growth medium, filtered through lens paper and replated. The myotubes do not survive this treatment. When confluent, the fibroblasts were irradiated. These cells were used either (i) as a substratum on which to plate freshly dissociated DRG cells or (ii) as a source of 'conditioned' medium.

(B) *Counting procedure*

Neuronal somata were counted using a microscope with phase-contrast optics (200 magnification). Usually 30 fields chosen at random were counted. In typical platings, there were roughly 15–20 neurones per field and the neurones were evenly distributed throughout the culture dish.

(C) *Time course experiments*

In the experiment shown in Figs. 1 and 2, DRG cells were plated on to a collagen substratum. One set of cultures was treated with cytosine arabinoside to yield 'neurones-alone' cultures; in the other set, the non-neuronal cells proliferated. Both sets were irradiated 6 days after plating. At particular times after plating the number of neurones per dish was counted, triplicate cultures of each set were then washed free of growth medium, extracted with 2 M acetic acid, boiled for 3 min, and the extracts lyophilized. Aliquots of these extracts were assayed for both immunoreactive substance P and mmunoreactive somatostatin.

(D) *Conditioned medium (CM) experiments*

'Neurones-alone' cultures were plated as described. Cultures were fed every day with medium which had been diluted with various concentrations of medium 'con-

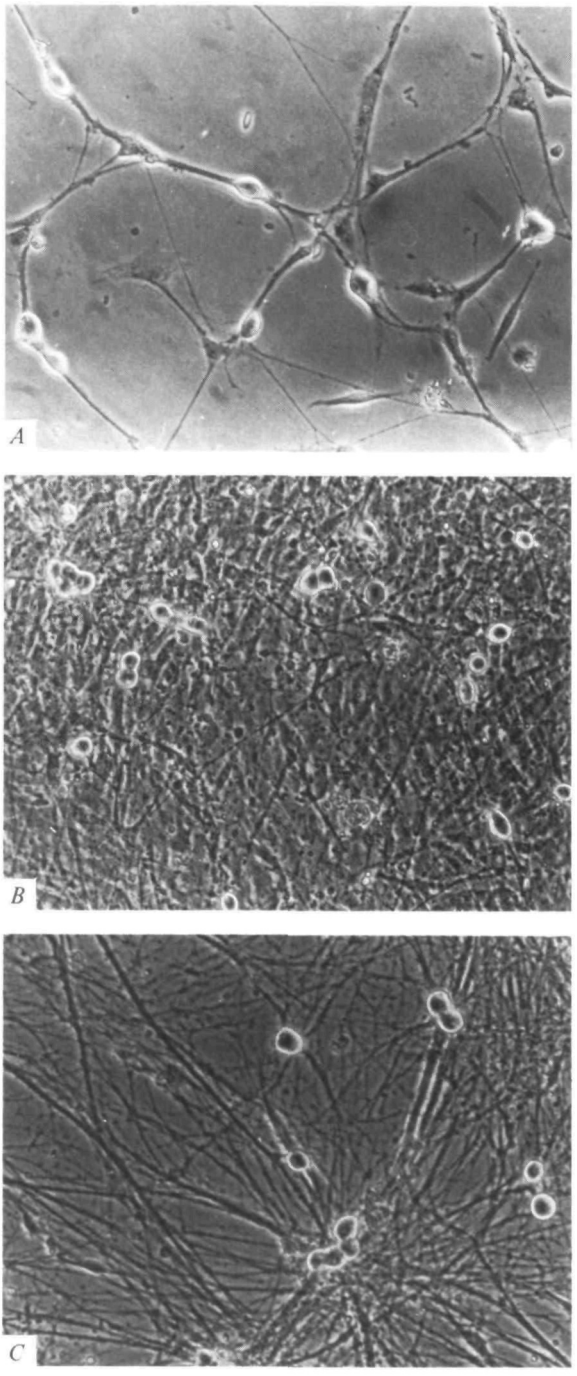

Fig. 1. Phase-contrast micrographs of chicken DRG neurones grown either in the presence or virtual absence of ganglionic non-neuronal cells. In B the non-neuronal cells grew to confluency. In C the cultures were treated with 5×10^{-6} M cytosine arabinoside for the period 12 h to 4 days after plating. Micrographs were taken of cultures 12 h after plating (A) and 15 days after plating (B, C). Calibration bar = 50 μm (A); 100 μm (B, C).

ditioned' by incubation with other cell types during the preceding day. Neurones were counted before extraction with 2 M acetic acid.

(E) *Radioimmunoassay (RIA)*

After lyophilization, samples were resuspended in assay buffer and centrifuged before assay. Substance P was assayed using antibody R6P according to the method of Mroz & Leeman (1979). Somatostatin was assayed using antibody M4 according to the method of Arnold & Fernstrom (1980).

(F) *Chromatography*

Samples of synthetic substance P and somatostatin and also an acetic acid extract of 'neurone plus non-neuronal cell' cultures were applied to a column of Sephadex G25–80 (Sigma) (1 × 20 cm). The column was developed with phosphate-buffered saline containing 0·2% bovine serum albumin (RIA buffer); 0·5 ml fractions were collected. Aliquots of samples were assayed for both substance P and somatostatin.

RESULTS

(A) *Time course*

As shown in Fig. 1 A both neurones and non-neuronal cells settle and attach to the collagen substratum within 24 h after plating. It can be seen that the phase-bright 'neuronal-like cells' have already elaborated a network of processes; growth cones are obvious at this time. The number of flat, phase-dark 'non-neuronal cells' present is roughly of the same order of magnitude as the 'neuronal-like cells'. Cultures in which non-neuronal cells were allowed to proliferate are shown in Fig. 1 B, the phase-bright neuronal cell bodies are sitting on a mat of non-neuronal cells. As shown in Fig. 1 C, cultures which were treated with cytosine arabinoside contain few non-neuronal cells and the processes are firmly attached to the collagen-coated culture dish. There is thus no gross difference between the number of neurones which survive in the two types of cultures: nor is there any obvious difference in the size or morphology of the neurones. Neurones from the same plating provided the micrographs of Fig. 1, and the data shown in Fig. 2.

The peptide content in the two types of developing cultures is shown in detail in Fig. 2. Fig. 2 C shows the mean number of neurones present in each culture at the time of harvesting. The number of neurones is constant, within the limits of the counting procedure, from the first day after plating through 3 weeks in culture; in the fourth week there was a 20% loss of neurones. It should be noted that there is no significant difference in the number of neurones which survive in either culture condition. (NGF was present in all cultures throughout this experiment.) Panel A illustrates that the level of substance P increases over the first 2 weeks of culture and plateaux during the next 2 weeks. In 'neurone plus non-neuronal cell' cultures (solid line) the amount of substance P is lower at all time points than in the 'neurones-alone' cultures (broken line). Fig. 2 B illustrates that in 'neurones-alone' cultures the level of somatostatin is comparatively low; in the mixed cultures (solid line) the amount of somatostatin increases from the second week onward. The ratio of somatostatin and substance P (SOM/SP) after 2 weeks in culture was 0·03 in 'neurones-alone' cultures (cytosine

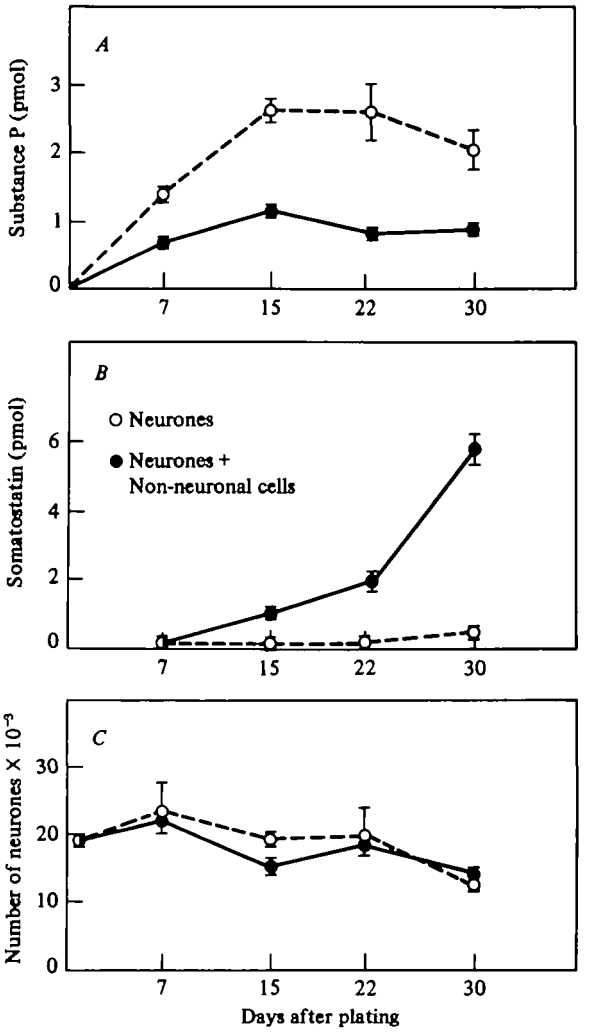

Fig. 2. DRG neurones were grown in the presence of ganglionic non-neuronal cells (solid lines) or in the virtual absence of non-neuronal cells (broken lines). Cultures were harvested at the time indicated. The number of neurones present at the time of harvesting is shown in panel C. The levels of substance P and somatostatin present in each culture is shown in panels A and B. Results are means ± S.E.M. of either triplicate or quadruplicate cultures.

arabinoside treated) and 0·90 in cultures in which the ganglionic non-neuronal cells proliferated. After 4 weeks in culture the difference in the ratio was ~ 50-fold. This change was a result of both a ~ 20-fold increase in the level of somatostatin and a ~ 2·5-fold decrease in substance P level.

The immunoreactive material present in the cultures was characterized using gel-exclusion chromatography. Fig. 3 shows that acetic acid extracts of the cultures contain immunoreactive SP and SOM which is indistinguishable from synthetic SP and SOM with respect to size.

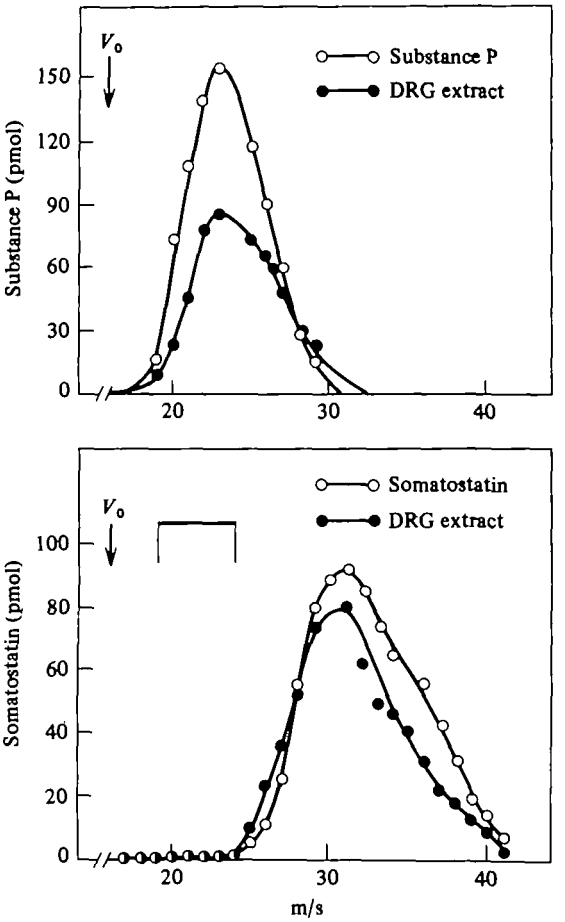

Fig. 3. Gel filtration chromatography on Sephadex G25. Elution profiles of synthetic SP, synthetic SOM (○) and acetic acid extracts of neurone plus non-neuronal cell cultures containing immunoreactive SP and immunoreactive SOM (●).

(B) *Conditioned medium (CM) from ganglionic non-neuronal cells*

It was not necessary for the neurones to be in contact with the non-neuronal cells in order for the ratio of SOM/SP to increase. When the neurones were fed with medium 'conditioned' by incubation with ganglionic non-neuronal cells there was a 60-fold increase in somatostatin after 11 days in culture (Table 1). There was no significant difference in the number of neurones surviving in either case. This experiment also shows that it is neurones which increase their SOM content as neither the non-neuronal cells nor the conditioned medium contained detectable amounts of SOM or SP.

(C) *Conditioned medium (CM) from muscle fibroblasts*

When neurones were fed with medium conditioned by incubation with fibroblasts the ratio of SOM/SP did *not* increase; rather the ratio was somewhat decreased (Table 1).

Table 1. *Comparison of effects of medium conditioned by ganglionic non-neuronal cells or by fibroblasts*

Experiment	Conditions	SOM/SP	Substance P (f-mol)	Somatostatin (f-mol)	Number of neurones ($\times 10^{-3}$)
1	+NGF	0·07	1588 ± 46	107 ± 16	16 ± 1
	+NGF+CM (ganglionic non-neuronal cells)	3·31	1989 ± 136	6577 ± 553	15 ± 2
2	+NGF	0·13	1655 ± 185	217 ± 31	17 ± 2
	+NGF+CM (muscle fibroblasts)	0·02	4212 ± 366	85 ± 14	21 ± 1

DRG neurones were grown in the virtual absence of ganglionic non-neuronal cells. In the first experiment, groups of cultures were grown with or without the addition of medium 'conditioned' in the presence of ganglionic non-neuronal cells. In the second experiment, groups of cultures were grown with or without the addition of medium 'conditioned' by incubation with fibroblasts derived from muscle. NGF was added to both groups. The total amount of both substance P and somatostatin and the total number of neurones present in the cultures is expressed as the mean ± S.E.M. of triplicate cultures.

DISCUSSION

These results, which show that the peptide content of cultured sensory neurones can be influenced by a soluble factor from non-neuronal cells, are similar to the effect of non-neuronal cells on the expression of cholinergic properties by cultured autonomic neurones described by Patterson (1978). They suggest that the tissue environment may influence the development of sensory neurones as well as influencing the development of autonomic neurones.

However, the type of non-neuronal cell responsible for the increased content of somatostatin in cultured sensory neurones may be different to the type of non-neuronal cell responsible for inducing cholinergic properties in cultured sympathetic neurones. In the latter case, a variety of tissues, including cardiac muscle and fibroblasts, can produce a factor capable of inducing ACh synthesis. The effect on increased somatostatin content of sensory neurones could not be produced by medium conditioned by fibroblasts – the effect was only seen with non-neuronal cells derived from the DRG. These ganglionic non-neuronal cells contain two major cell types – fibroblasts and glial cells (Schwann cells). Although the results shown in Table 1 suggest that it may be the glial cells rather than the fibroblasts that signal the sensory neurones to increase their somatostatin content, this suggestion can only be confirmed by the use of purified cultures of glial cells and fibroblastic cells derived from the DRG. In the chick it has proved difficult to obtain such purified cultures, although Brockes, Fields & Raff (1979) and Wood (1976) have purified glial cells and fibroblasts from rat sciatic nerve. Experiments using rat glial cells are in progress to settle this point.

Although at this point it is not clear what are the consequences of increased levels of somatostatin in the sensory neurones, we think that this phenomenon reflects a major change in the metabolism of the neurones involved. We hope that studying this effect of non-neuronal cells on the ability of neurones to synthesize biologically active peptides will provide a model ofr other interactions between neurones and their supporting cells.

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