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Patterns of peanut agglutinin binding within the developing grasshopper central nervous system

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SUMMARY

The location of peanut agglutinin (PNA) binding was investigated in the segmental ganglia of the developing grasshopper embryo. Neuronal processes were stained but cell bodies were not. The first appearance of PNA binding in development was associated with the first neurons to initiate axon outgrowth, the progeny of the MP2 cells. In the early stages of development the location of PNA binding was congruent with that of antibodies against horseradish peroxidase (HRP), which bind to neurons. In more advanced ganglia only a subpopulation of those neuronal processes that bound auti-HRP also bound PNA. The results suggest that PNA-binding sites are present only on those neuronal processes which are still developing and raise the possibility that these molecules may play a rôle in neurite outgrowth and navigation.

INTRODUCTION

The developing segmental ganglia of the grasshopper embryo are a fruitful system in which to address questions in developmental neurobiology. The ganglia and their constituent cells are large and easily accessible to observation and manipulation, and there is a tractable number of cells in each ganglion. A substantial body of information exists about the course of neuronal development in these ganglia, including data on the behaviour of several identified cells as they send out their axons to establish a well-defined network of neuronal connections (Bate & Grünewald, 1981; Goodman, Raper, Ho & Chang, 1982).

Little, however, is known about the molecular mechanisms which mediate the precise and reproducible cellular behaviours which have been observed. One approach to this problem is to look for molecules whose temporal and spatial expression correlate with the occurrence of specific behaviours. Descriptions of the distribution of filopodia from identified growth cones have suggested that selective adhesion to cellular and extracellular surfaces may control the direction of axon outgrowth (Goodman *et al.* 1982). Likely candidates for selective cell adhesion molecules are the glycoconjugates on the surface of the growth cone. Two such glycoproteins mediating cell–cell adhesion have now been isolated in other systems; in *Dictyostelium* (Müller & Gerisch, 1978; Springer & Barondes, 1985), and in the chick (Rutishauser, 1983). Lectins, which bind to specific sugar

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residues, are a convenient means of probing the glycoconjugate constituents of cell surfaces and the intercellular environment (e.g. Oppenheimer, 1978; Pfenninger & Maylié-Pfenninger, 1981; Pfenninger, Maylié-Pfenninger, Friedman & Simkowitz, 1984). We screened a series of lectins (from *Arachis hypogaea, Concanavalia ensiformis, Dolichos biflorus, Glycine max, Ricinus communis, Triticum vulgaris, Ulex europaeus*, and *Helix pomatia*) for their capacity to bind to the embryonic nervous system and found that only peanut agglutinin (from *Arachis hypogaea*) showed strong and selective binding. Here we report on the occurrence of peanut agglutinin (PNA) binding during early stages of neuronal outgrowth in the segmental ganglia of grasshopper embryos.

MATERIALS AND METHODS

Grasshopper embryos (*Schistocerca gregaria*) were dissected out of their eggs at the 30–40 % stages of development (Bentley, Keshishian, Shankland & Toroian-Raymond, 1979) in a medium similar to that described by Shankland, Bentley & Goodman (1982) but with 39 mM-sucrose. Embryos were fixed for 1 h in 2 % paraformaldehyde in Millonig's phosphate buffer and washed overnight in PBS and 0·1M-glycine.

Fixed embryos were incubated for 2 days at 4°C in PBS containing 0·4 % Triton X-100 and PNA conjugated either to rhodamine (EY Laboratories) at a dilution of 1/200 (5 $\mu g\,ml^{-1}$) or to HRP (EY Laboratories) at a dilution of 1/600 (1·7 $\mu g\,ml^{-1}$).

Alternatively, they were incubated for 2 days at 4°C in PBS containing 1 % BSA and 0·4 % Triton X-100 and FITC-conjugated goat antibody to HRP (Cappel) at a dilution of 1/200 (50 $\mu g\,ml^{-1}$). They were then washed in PBS and incubated for 2 more days in FITC-conjugated swine anti-goat secondary antibody (Medac) at 1/400 dilution (25 $\mu g\,ml^{-1}$). Double-labelled preparations had rhodamine-conjugated PNA added simultaneously with the secondary antibody. Preparations to be labelled with HRP for viewing with transmitted light underwent a third 2-day incubation with peroxidase–anti-peroxidase complex (Boehringer) at 1/20 dilution (0·5 $mg\,ml^{-1}$).

Fluorescent preparations were washed, dehydrated, embedded in Spurr's medium, and sectioned at 2·5 or 5 μm on an LKB 2128 ultramicrotome. HRP-labelled preparations were washed, incubated for 30 min in 0·5 % cobalt chloride, washed again, incubated in 3,3-diaminobenzidine at 0·4 $mg\,ml^{-1}$, then reacted in a 3,3-diaminobenzidine solution with hydrogen peroxide added to a final concentration of 0·002 % until the reaction product was judged to be appropriately intense by visual inspection. They were then embedded in Araldite and sectioned as above.

Fluorescent observations were carried out on a Zeiss Photomicroscope with ×25 or ×40 plan neofluar objectives under oil immersion. Photography of the HRP-labelled preparations was carried out on a Zeiss Axiomat.

RESULTS

PNA binds to outgrowing neural processes

PNA stains discrete regions of the core of each embryonic ganglion. Fig. 1A shows a frontal section through the dorsal part of the ganglion chain in a grasshopper embryo at about 34 % of development stained with HRP-conjugated PNA. The dark reaction product stains a well-defined and localized region in each hemiganglion. Simultaneous incubation with 0·1M-galactose, the sugar hapten of PNA, abolishes this specific labelling.

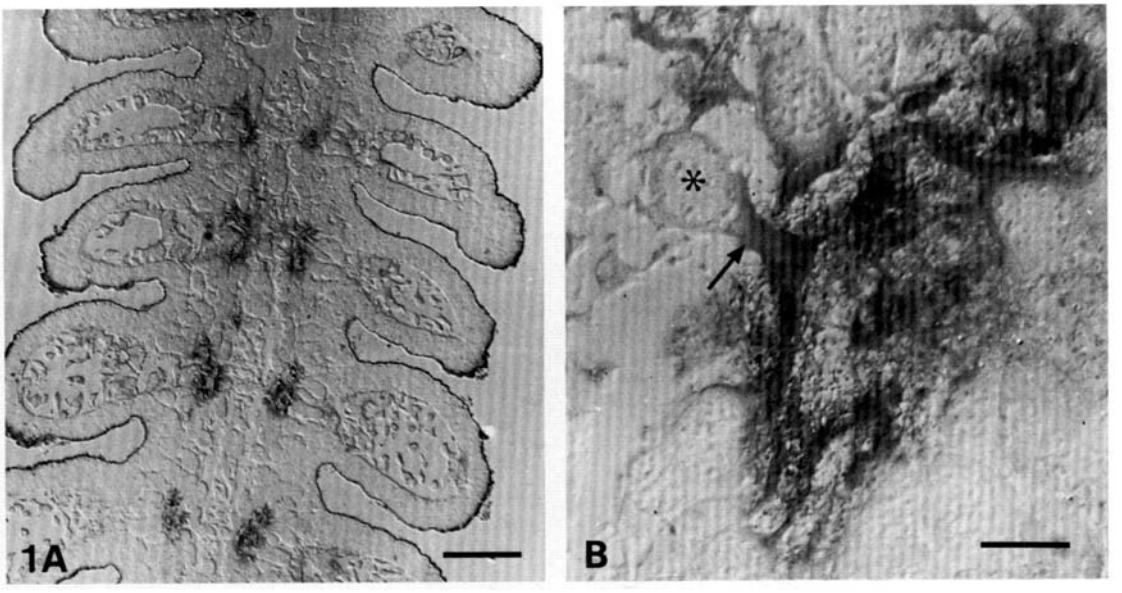

Fig. 1. (A) Frontal section through a 34% grasshopper embryo labelled with HRP-conjugated PNA. Five pairs of appendages and five developing ganglia can be seen. Anterior is up, scale bar is 100 μm. (B) High magnification view of one stained region, showing an example of an unstained cell body (asterisk) and its stained axon (arrow). Anterior is up, scale bar is 10 μm.

PNA does not bind to neuronal cell bodies. Fig. 1B is a higher magnification view of such a stained region in which an axon growing out from an unmarked cell body can be seen to be stained. Numerous other stout axons are also stained. In addition, a mesh of numerous finer fibrous structures is stained. We propose, on the basis of their position and appearance, that these represent the filopodia which extend some distance from the growth cones of developing neuronal processes in the grasshopper (Taghert, Bastiani, Ho & Goodman, 1982). Such a designation is supported by observations such as Fig. 2, which we interpret as a single stained growth cone accompanied by radiating filopodia, found in mid-anterior ganglion before the establishment of commissures.

PNA binding can also be seen at other positions in the embryo, most notably as halos around the neuroblasts and in the lumen of segmental appendages in the early stages of development. Peripheral neurons in the developing appendages do not show PNA binding.

PNA binding changes in the course of development

In the early stages of development, when ganglia contain few outgrowing axons, all axons appear to be stained. A typical staining pattern is shown in Fig. 1B. No unstained axon is observed in such preparations. However, some early axon outgrowth does occur in regions of the ganglion where the cell bodies are packed densely together and unstained axons would be difficult to identify here. To

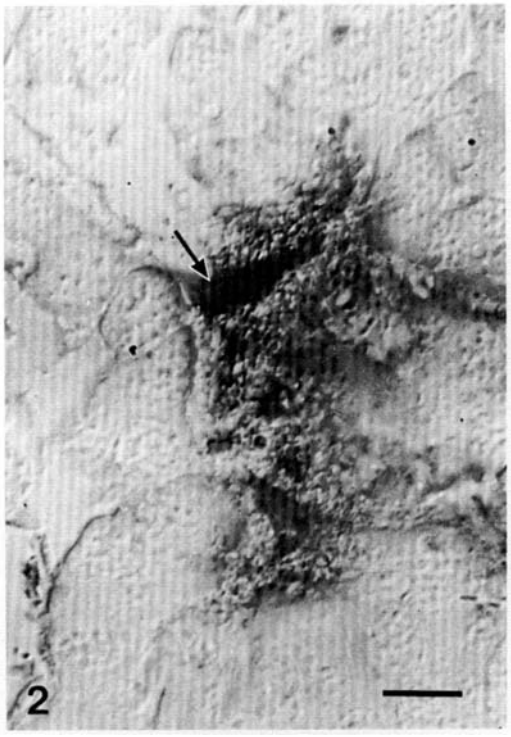

Fig. 2. A presumptive growth cone (arrow) and associated filopodia labelled with HRP-conjugated PNA. Scale bar is 10 μm.

overcome this problem, neurons were visualized with antibodies against horseradish peroxidase (anti-HRP), which bind to neurons (Jan & Jan, 1982), and a comparison was made between the location of PNA binding and that of anti-HRP binding.

Anti-HRP binding first appears in association with the progeny of the midline precursor 2 (MP2) cells (Fig. 3A). These are the first neurons to initiate axon outgrowth in the grasshopper segmental ganglia (Bate & Grünewald, 1981), and are also the first neurons to bind PNA. The binding appears as a spray of fine fibres that radiate laterally from the position of the MP2 progeny (Fig. 3B). The patterns of PNA binding and anti-HRP binding differ only in that anti-HRP binds not only to the neuronal processes but also to the neuron cell bodies.

Ganglia at more advanced stages of development, and containing many developing neurons, also showed congruent patterns of PNA binding and anti-HRP binding (Fig. 4A,B). These observations were made in double-labelled fluorescent preparations in which rhodamine-conjugated PNA binding could be viewed in the same preparation as anti-HRP binding made visible by an FITC-conjugated secondary antibody. Whilst this method allows direct comparison of binding patterns in a single preparation, it does not generally allow the resolution of all single fibres because of the inherent limitations of fluorescence microscopy.

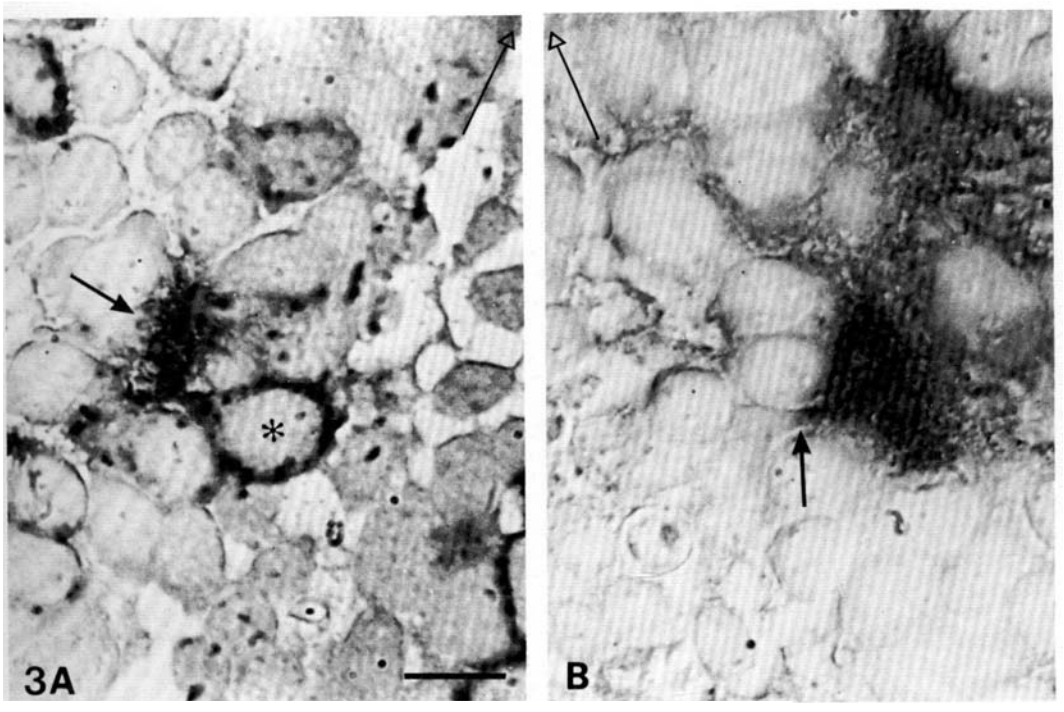

Fig. 3. (A) The first cell to initiate axon outgrowth, one of the progeny of the MP2 cells, treated with antibody against HRP and visualized with an HRP label. Note the laterally directed tuft of fibres (closed arrow), and the labelled cell body (asterisk). (B) The first location of PNA binding in the contralateral half of a ganglion, similar in position to the MP2 progeny outgrowth indicated in A. The neck (closed arrow) of a cell from which the PNA-labelled filopodia are radiating is visible at centre. For both photomicrographs the midline is indicated by the open arrow which points anteriorly, and the scale bar is 10 μm.

Ganglia at still later stages of development showed a different pattern of binding. By the time a ganglion shows all three commissures to be well developed (38–40 % in the mesothoracic ganglion) PNA binding was observed only as isolated patches within the nerve tracts (Fig. 5A,B). Isolated single patches of PNA staining were also observed in preparations treated only with PNA so the phenomenon is not likely to be a result of competition with anti-HRP for the same binding sites.

The different ganglia in a growing embryo at any time, are at different stages of development (Bentley *et al.* 1979). The ganglia posterior to the mesothorax are at a progressively less advanced stage. This allows the observation in one and the same embryo, at 38–39 % of development, that the less developed ganglia show congruence of PNA and anti-HRP binding while in the more advanced ganglia PNA binds to only a subpopulation of those neurons which bind anti-HRP. This makes it unlikely that the patchiness of PNA staining observed in more mature ganglia is merely the result of the vagaries of the staining procedures employed.

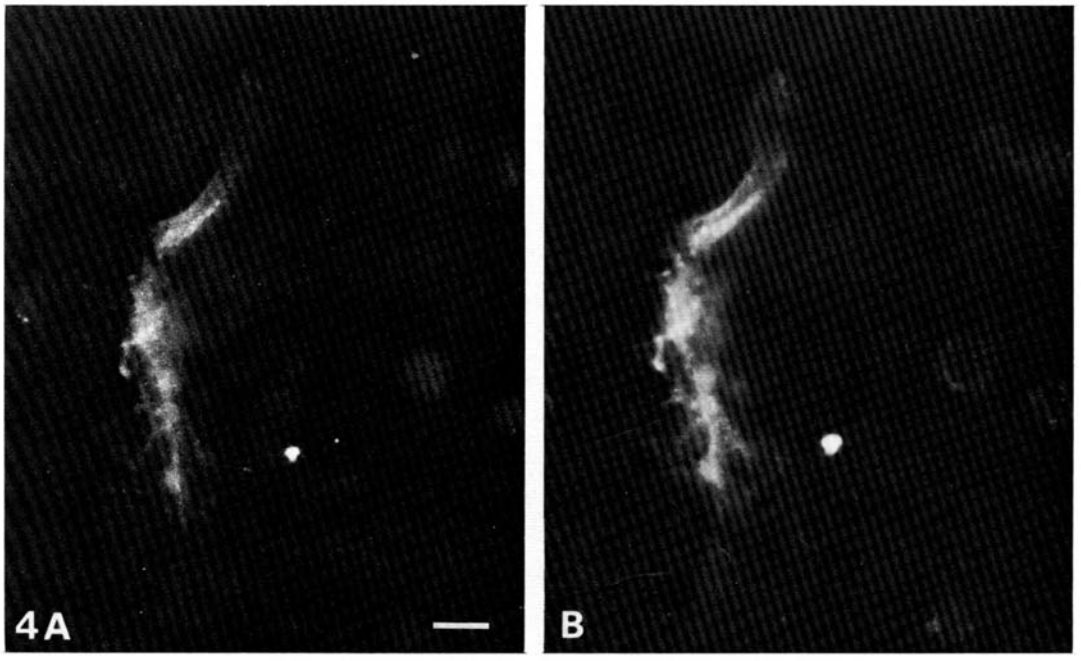

Fig. 4. (A) Pattern of binding, in a double-labelled 38% midabdominal ganglion, of anti-HRP visualized with FITC by indirect immunofluorescence. (B) The same field, showing the location of rhodamine-PNA binding. Details, such as the three branches in the lower part of the field or the posteriorly directed branch on the left in midfield, are present in both photomicrographs. Anterior is up, scale bar is 10 μm for both photomicrographs.

DISCUSSION

PNA binding is first observed in association with the first axon outgrowth in the grasshopper segmental ganglia. It appears to be specific for CNS neurons since it is not observed in the peripheral neurons of the developing appendages, although these do bind anti-HRP. At this and slightly later stages of development, regions of PNA binding are congruent with regions of binding to anti-HRP. We think it likely that both agents are binding to all outgrowing neural processes in the CNS.

As development progresses PNA binding becomes restricted to certain regions within the nerve tracts. These may represent the regions in which active axonal outgrowth is still taking place. We find the coincidence of PNA binding with the time and location of axon outgrowth and navigation interesting and suggest the molecules binding PNA may play a rôle in these processes. Interestingly, many glycoproteins which, like PNA (Lotan, Skutelsky, Danon & Sharon, 1975), bind β-galactosides have been isolated from vertebrate extracellular matrix (Barondes, 1984). Should similar glycoproteins be present in locust extracellular matrix surrounding the growth cones, they would be prime candidates for regulating neuron development through the PNA-binding molecules on the growth cone surface. We propose to investigate further the possible importance of PNA-

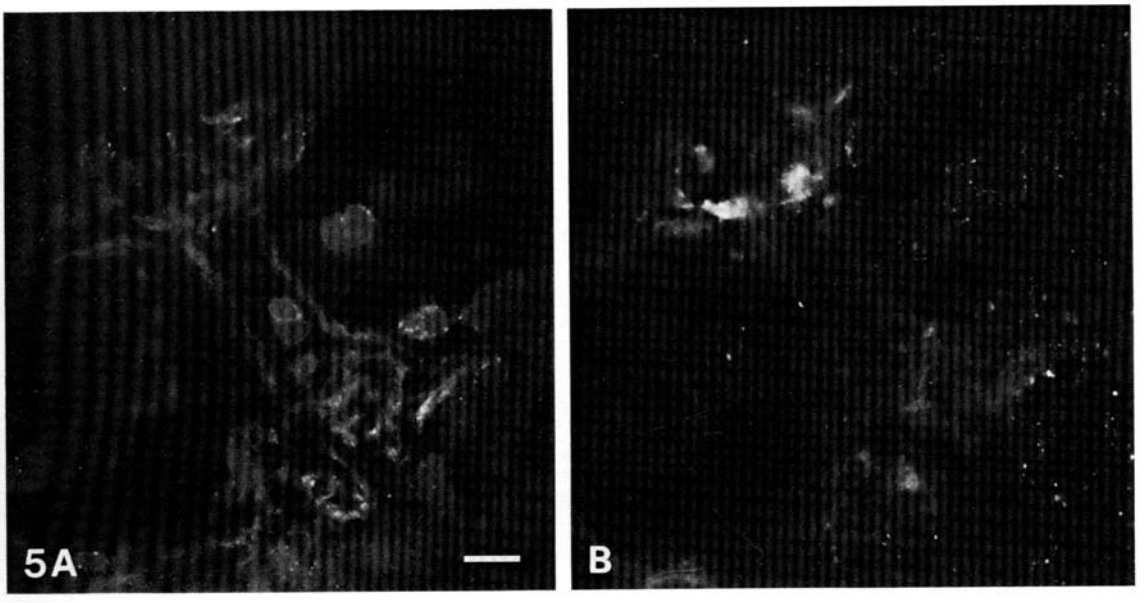

Fig. 5. (A) FITC anti-HRP binding in the metathorax of a 38–39 % embryo. (B) The same field viewed with optics revealing rhodamine–PNA binding. PNA binding is intense only on some of the structures which bind anti-HRP. All structures binding rhodamine–PNA can be found also showing FITC anti-HRP binding. Scale bar is 30 μm for both photomicrographs.

binding surface molecules if systematic perturbations in neuron growth occur when PNA is administered to embryos developing in culture.

The finding that PNA binding occurs in regions of neuron outgrowth is not confined to insects. Observations of PNA binding in the developing chick retina (Liu, Layer & Gierer, 1983), may be interpreted in a similar fashion, supporting our suggestion. They report that PNA binding occurs in the plexiform layers of the retina, which are regions containing a high density of neural processes, that staining is associated with fibres rather than cell bodies, and that PNA binding first appears at a time when neural processes should first be growing into those regions in substantial numbers.

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