The distribution of non-synaptic intercellular junctions during neurone differentiation in the developing spinal cord of the clawed toad

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SUMMARY

The distribution of intercellular junctions, other than synapses and their precursors, has been described in the developing spinal cord of Xenopus laevis between the neurula and free swimming tadpole stages. At the neurocoel, ventricular cells are joined in the apical contact zone by a sequence of junctions which usually has one or more intermediate junctions but often also includes close appositions, gap junctions and desmosomes. This apical complex is more diverse than that reported in other vertebrate embryos and between ependymal cells in the adult central nervous system. Gap junctions are also found between ventricular cells and their processes near the external cord surface. However, no other special junctions occur in this location under the basement lamella which surrounds the cord. Punctate intermediate junctions are generally distributed between undifferentiated and differentiating cells and their processes but were not found in neuropil after stage 28. These results are discussed in relation to cell movements during neural differentiation, possible effects on the freedom of movement of ions and molecules through extracellular pathways in the embryo, and possible intercytoplasmic pathways via gap junctions which may be responsible for the physiologically observed electrical coupling between neural tube cells.

INTRODUCTION

Unlike cells in many tissues (see, for example, Loewenstein, 1969) neurones are usually not in electrical contact with their neighbours. They are wrapped by glial processes and bathed by an extracellular medium which is carefully controlled, often separately from the extracellular space in the rest of the body. In contrast to this, the embryonic ectodermal cells which give rise to the nervous system are coupled to one another electrically and lie in an extracellular medium common to the whole embryo (Sheridan, 1968; Slack & Warner, 1973). Consequently, some important changes must occur in the contact relationships between cells in the nervous system during development. In the first place there must be changes in the pathways from the inside of one

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cell to the insides of its neighbours (the intercytoplasmic pathways). In the second place there may be changes in the freedom of movement in the extracellular pathways between the cells.

We have investigated these changes by looking at the junctions that are made by cells in the spinal cord of *Xenopus* embryos during the first differentiation of functional neurones. Neural differentiation starts as the neural tube is forming at stage 19 of Nieuwkoop & Faber (1956). By the time the animal is hatched and can swim (stage 35) we can be sure that some neurones are well differentiated. We have therefore examined this period of development paying particular attention to any differences which exist between the junctions made by undifferentiated ventricular cells and cells and processes showing signs of differentiation. [Junctional nomenclature is reviewed at length by McNutt & Weinstein (1973). We have used the following terms: tight junction = occludens junction, gap junction = small subunit gap junction or nexus, intermediate junction = zonula and fascia adherens and desmosome = macula adherens. Where membranes are close but it is not clear if the junction is tight or gap, we have used 'close apposition'.] The distribution of close membrane junctions has been most carefully examined. This is because zonula tight junctions (zonula occludentes) are thought to limit extracellular pathways (Bennett & Trinkaus, 1970; McNutt & Weinstein, 1973) and gap junctions have been closely correlated with intercytoplasmic pathways for ion movement (e.g. Bennett, Spira & Pappas, 1972; Joseph, Slack & Gould, 1973; McNutt & Weinstein, 1973; Payton, Bennett & Pappas, 1969; Sheridan, 1971). Also, it has been suggested that in *Xenopus* retina gap junctions disappear when neural elements differentiate (Dixon & Cronly-Dillon, 1972).

Our results show that differentiated neurones and the ventricular cells that give rise to them share the ubiquitous punctate intermediate or adherens junction. However, neurones may lack the gap junctions found between ventricular cells. The distribution of junctions is discussed in relation to pathways for ion movement, cell movement and differentiation.

**MATERIALS AND METHODS**

Eggs were obtained from breeding pairs of adult *Xenopus laevis* by priming and injecting with chorionic gonadotrophin; they were allowed to develop in aerated tap water at temperatures between 18 and 22 °C. Embryos were staged using the normal tables of Nieuwkoop & Faber (1956) and animals at stages 15, 16, 17, 19, 21, 22, 23, 25, 27, 28, 31, 33, 35 and 48 were prepared for electron microscopy as follows.

Embryos were transferred to 10 % Holtfreter's solution where, if necessary, egg membranes were removed using fine forceps. The embryos were then immediately immersed in buffered fixative. Large areas of yolky endoderm were removed from the embryos to aid fixative penetration.
Fixative and post-fixation solutions were made up in 0.05 M sodium cacodylate buffer at pH 7.3 containing 1-1.5% glucose and 0.05% \( \text{CaCl}_2 \cdot 6\text{H}_2\text{O} \). Embryos were fixed in a solution of 4% glutaraldehyde and 1% formaldehyde prepared from paraformaldehyde for 2 h at room temperature, washed in buffer and postfixed in buffered 2% osmium tetroxide for 2-4 h. Several stage-27 embryos were stained 'in the block' with a 2% solution of uranyl acetate buffered with sodium hydrogen maleate (Brightman & Reese, 1969). Lanthanum hydroxide was introduced into the extracellular space of a single stage-27 embryo using fixative, buffer and osmium tetroxide containing 1% lanthanum nitrate at pH 7.8. All embryos were dehydrated in ethanol, cleared in propylene oxide and embedded in Araldite.

The spinal cord was sectioned transversely using a Cambridge ultramicrotome to give ribbons of silver-silver grey thin sections. Cross-sections were taken: of whole spinal cord at stages 15, 16, 17, 19, 22, 23, 25 and 28, in the cervical and mid-trunk regions; throughout the cord at stage 27; and in the cervical tract neuropil at stages 31, 33, 35 and 48. Sections were picked up on celloidion/carbon-coated copper grids, stained with solutions of 10% uranyl acetate in 50% ethanol and alkaline lead citrate (Reynolds, 1963). Sections stained 'in the block' with uranyl acetate or lanthanum hydroxide were stained with alkaline lead citrate alone. Sections were examined in an AEI EM 6B electron microscope. Micrographs of cell junctions were usually made at 30000 magnification. The position of each junction and characteristics of the cellular elements on either side were noted. In some cases junctions were examined through series of sections making descriptions of their shape possible.

Measurements of junctional length (to 0.1 \( \mu \text{m} \)) and cleft width (to 1 nm) were made from photographs at 105000 magnification.

RESULTS

Around the neurocoel of the embryonic spinal cord lie the apices of the ventricular cells of the neural epithelium. These ventricular cells are the ultimate progenitors of the neurones and macroglial cells in the CNS (see Boulder Committee, 1970, for nomenclature) and form a complex of junctions like that in other epithelia. The most obvious and usual feature of this apical contact region in *Xenopus* was a variable length of intermediate or 'adherens' junction (0.03-1.0 \( \mu \text{m} \) long in transverse sections of the cord). In such junctions the cleft was moderately dense, and 5-20 nm wide, membranes were dense, and this density graded off into the cytoplasm on each side of the junction (Figs. 1, 2). Sometimes a single clearly defined junction was present but in other cases a series of rather ill-defined junctions was found (cf. Figs. 1, 2). Occasionally, desmosomes with marked cytoplasmic density and associated fibrils occurred with the intermediate junctions and rarely, as in Fig. 3, a desmosome was found in place of any intermediate junction. Between the intermediate junction and
the neurocoel there was usually a short region of close membrane apposition with some membrane thickening and cytoplasmic density (see Figs. 1–3). A few similar close appositions occurred in the region of intermediate junctions in some cases. On the neurocoel side of the intermediate junctions the extracellular space was often expanded. Our survey has revealed no clear changes in the junctions of the apical contact region at the neurocoel during the period of development we have examined. The detailed arrangement of junctions was very variable, tending to be most diverse when the ventricular cell profiles were narrow and contained longitudinally orientated microtubules (Fig. 1).

Gap junctions were found between ventricular cells near the apical contact zone at the neurocoel and also between cells near the outer periphery of the spinal cord (see Table 1). They consisted of close appositions of parallel dense membranes with a narrow cleft. The total width of these junctions (i.e. the distance from cytoplasm of one cell to that of the other across the junction) was usually 14–16 nm, and the length from 0·1 to 0·9 μm in section-stained material. There were two types of cleft, either a moderately dense structureless region 7–9 nm wide (see Figs. 4, 6) or two longitudinal dense lines separated by 2–3 nm giving the junction a seven-layered appearance (see Figs. 2, 5). Junctions stained ‘in the block’ with uranyl acetate were of similar total width (see Table 1). They showed a well-defined seven-layered substructure with an electron-lucent ‘gap’ 2–3 nm wide (Figs. 7, 8). In oblique sections a polygonal network with an approximately 12 nm centre to centre spacing of subunits was apparent (Fig. 9). When lanthanum hydroxide was introduced into the intercellular cleft, the ‘gap’ of the junction appeared as a black line about 4 nm wide (Fig. 10). The ‘gap’ was crossed by more electron-lucent bars spaced about 10 nm apart. The total width of the junction was 16 nm, which is comparable with that found using the other staining methods. The examination of serial sections of these

**Figures 1–3**

Ventricular cell junctions near the neurocoel at stage 27.

Scale line 0·1 μm. \( a \) = apposition, \( d \) = desmosome, \( g \) = gap junction, \( i \) = intermediate junction, \( l \) = lipid yolk granule, \( mt \) = microtubules, \( nc \) = neurocoel.

Fig. 1. Two junction complexes between lateral ventricular cells in the cervical spinal cord. The junction sequence from the neurocoel is, on the left, an apposition, a desmosome, an intermediate junction and then further down the cell a second intermediate junction and, on the right, an apposition, a desmosome and a gap junction. The narrow apical profile in the centre contains longitudinal microtubules. 35000.

Fig. 2. Junctions between dorsolateral ventricular cells in the cervical spinal cord. The sequence from the neurocoel (top left) is two appositions, an intermediate junction and a gap junction 0·25 μm long and with a total width of about 15 nm. The seven-layered substructure of this junction can be seen in places. 210000.

Fig. 3. Junctions between ventricular cells in the trunk spinal cord. The sequence from the neurocoel is an apposition and a small desmosome. 105000.
Gap junctions between ventricular cells near the outer margin of the stage-27 spinal cord.

Scale line 0·1 μm. bl = basement lamella, g = gap junction.

Fig. 4. Gap junction, 0·3 μm long and with a total width of 15 nm between lateral ventricular cells. No other junctions are apparent. The loose basement lamella surrounding the cord can be seen (bottom left). 105000.

Fig. 5. Gap junction in the tail spinal cord. Only part of the junction (total length 0·75 μm) is shown to illustrate the seven-layered substructure where the total width is 15 nm. 210000.

Fig. 6. Gap junction between cells, one of which is wrapping a neuronal process (arrowed) containing microtubules. The basement lamella can be seen outside the cord and also the absence of special junctions between cells at the periphery. 25000.
## Table 1. Catalogue of gap junctions

<table>
<thead>
<tr>
<th>Embryo no.</th>
<th>Stage</th>
<th>Region</th>
<th>Level</th>
<th>Total width (nm)</th>
<th>Length (µm)</th>
<th>Substructure</th>
<th>Found between</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>—</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>2</td>
<td>15</td>
<td>—</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>—</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>22</td>
<td>—</td>
<td></td>
<td></td>
<td>15</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>27</td>
<td>Tail</td>
<td></td>
<td></td>
<td>15</td>
<td>0.25</td>
<td>7 layered</td>
</tr>
<tr>
<td>6</td>
<td>27</td>
<td>Tail</td>
<td></td>
<td></td>
<td>15</td>
<td>0.35</td>
<td>7 layered</td>
</tr>
<tr>
<td>7</td>
<td>27</td>
<td>Anterior trunk</td>
<td></td>
<td></td>
<td>15</td>
<td>0.3</td>
<td>7 layered</td>
</tr>
<tr>
<td>8</td>
<td>27</td>
<td>Tail</td>
<td></td>
<td></td>
<td>18</td>
<td>0.6</td>
<td>7 layered 2 nm gap</td>
</tr>
<tr>
<td>9</td>
<td>27</td>
<td>Tail</td>
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<td>17</td>
<td>0.6</td>
<td>7 layered 2.5 nm gap</td>
</tr>
<tr>
<td>10</td>
<td>27</td>
<td>Mid-trunk</td>
<td></td>
<td></td>
<td>16</td>
<td>0.8</td>
<td>4 nm gap</td>
</tr>
</tbody>
</table>

**Section-stained**
- Deep median neural plate cells
- Deep median neural plate cell and a process
- Ventral cells
- Ventral processes
- Lateral ventricular cells
- Dorsal ventricular cells
- Dorsal undifferentiated processes
- Lateral undifferentiated processes

**‘In block’ stained**
- Ventricular cells
- Dorsolateral ventricular cells
- Dorsal undifferentiated processes

**Lanthanum-stained**
- Lateral ventricular cells
- Dorsal ventricular cells
- Dorsolateral ventricular cells
- Dorsal undifferentiated processes

- Deep median neural plate cells
- Deep median neural plate cells
- Deep median neural plate cells
- Ventral cells
- Ventral processes
- Lateral ventricular cells
- Dorsolateral ventricular cells
- Lateral undifferentiated processes

- Found between
- Lateral ventricular cells
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junctions showed that they were punctate (i.e. discreet patches in face view) rather than zones around the cells. Fig. 1 shows the usual position of gap junctions found between ventricular cells near the apical contact zone. Elsewhere they were found between cell bodies and processes, and between processes. Only 24 were found altogether. Three of these were between ventricular cells near the neurocoel. The rest were between cellular elements which contained closely spaced cytoplasmic dense granules (probably ribosomes), a few scattered rough endoplasmic reticulum cisternae, and poorly developed Golgi bodies. Such contents are typical of ventricular cells and in a number of cases the elements making gap junctions could be identified as such (Figs. 4, 6), either by tracing them to the neurocoel or by their contents. Near the periphery of the spinal cord the ventricular cells contained many dense granules, mitochondria, smooth-surface tubules and vesicles, and patches of microfilaments and granular material, like the external processes of ventricular cells in mouse cerebral vesicle (Hinds & Ruffett, 1971). During neurulation (stages 15 and 16) gap junctions were found between deep neural plate cells (see Schroeder, 1970) and their processes. When the neural tube had closed (stage 22) they occurred between ventrolateral ventricular cells and processes in the cervical spinal cord (see fig. 8 in Hayes & Roberts, 1973). At stage 27 they were most numerous in the tail region, although one was found in the anterior trunk and one at the cervical level of the cord (see Figs. 4, 6). None were found in the tract and neuropil areas around the margin of the cervical spinal cord after stage 27 although they may have persisted in other parts of the spinal cord which were not examined. Gap junctions were longest in the tail region at stage 27.

Intermediate or 'adherens' junctions (adherentia of Farquhar & Palade, 1963), recognized by a moderately dense cleft and dense membranes blending into amorphous dense cytoplasm on both sides of the junction, were not confined to

F I G U R E S 7–10

Gap junctions in the stage-27 spinal cord.

Figs. 7–8. Stained ‘in the block’ with uranyl acetate.

Fig. 7. Gap junction in the tail between dorsal ventricular cells near neurocoel. The total width is 17 nm, the ‘gap’ is 2 nm wide and the junction is 0.6 μm long. 240000.

Fig. 8. Gap junction in the mid-trunk between dorsal undifferentiated processes near the outer margin of the cord. The total width is 17 nm, the ‘gap’ is 2.5 nm and the length 0.35 nm. 240000.

Fig. 9. Gap junction sectioned obliquely showing network of polygonal subunits with 10–12 nm spacing. This junction is between ventricular cells near outer cord margin and is 0.8 μm long. 150000.

Fig. 10. Gap junction, infiltrated with lanthanum hydroxide, between ventricular cells near the neurocoel in the mid-trunk. Lanthanum hydroxide fills the ‘gap’ and is interrupted periodically by more electron-lucent bands crossing the junction. The ‘gap’ is 4 nm wide and the total width is 16 nm. 240000.
FIGURES 11, 12. Intermediate junctions in the stage-27 spinal cord.

Fig. 11. Intermediate junction between a ventricular cell and an undifferentiated process in the tract neuropil area of the trunk. The junction is 0.2 μm long and the intercellular cleft is 10–14 nm wide. 105,000. Scale line, 0.1 μm.

Fig. 12. Intermediate junction between ventricular cells in the tail. There is a large coated vesicle on the right of the junction. The cleft is about 18 nm and the length 0.15 μm. Scale line, 0.1 μm.

the apical contact zone at the neurocoel. They were also found between cell bodies, processes and cells, and between processes in all parts of the spinal cord. Serial sections showed that these generally distributed junctions were punctate rather than zones. They were found in all regions of the cord examined at stages up to and including stage 28. However, they were not present in tract and neuropil areas of stages 31, 33, 35 and 48 cervical cord. The cellular elements on either side were sometimes undifferentiated ventricular cells (Figs. 10, 11) or showed signs of neural differentiation such as microtubules, microfilaments and vesicles of about 50 nm diameter. Junctional lengths were between 0.05 and 0.9 μm and the mean lengths for all stages were between 0.1 and 0.3 μm. There was no significant increase in the mean junctional length with age; at stage 15 the mean length was 0.24 ± 0.07 μm, at stage 27, 0.19 ± 0.02 μm. t tests between the two samples showed no significant difference at the 1% level with 18 degrees of freedom (t = 0.133). The width of the cleft at these
intermediate junctions was between 5 and 25 nm and varied by as much as 15 nm within an individual junction. No change was detected in cleft width with age.

At the outside margin of the spinal cord there was no evidence of any special cell junctions under the basement lamella which surrounds the cord (see, for example, Figs. 4 and 6). The basement lamella was first detected at stage 17 as sparse clumps of fibrillar material. By stage 20, as the neural tube was closing, it formed a continuous layer around the cord (20–100 nm thick) and by stage 23 distinct layers of fibrils were present, in places up to 0.5 μm thick. No further changes were noted up to stage 35. Along the stage-27 cord, steps in the formation of the basement lamella could be seen, from clumps of fibrils around the tail spinal cord, to a continuous layer of many striated collagen fibrils in the anterior trunk region.

**DISCUSSION**

In the apical contact zone between ventricular cells lining the neurocoel we have found a series of junctions similar to that in other epithelia. A typical sequence is shown in Fig. 13 but considerable variations exist in the details of
this sequence. No significant changes were noticed with age and in general the
junctions were very similar to those found between cells in *Xenopus* embryo
skin (Roberts & Stirling, 1971; Roberts & Hayes, unpublished observations),
*Fundulus* blastoderm (Lentz & Trinkaus, 1971), amphibian adult skin (Farquhar
& Palade, 1964; Kelly, 1966) and mouse cerebral vesicle, (Hinds & Ruffet,
1971). A somewhat simpler arrangement, lacking close membrane junctions, is
described in the neural tubes of *Xenopus* (Schroeder, 1970), chick (Fujita &
Fujita, 1963) and mouse (Herman & Kauffmann, 1966). However, in *Rana*
*pipiens* Decker & Friend (1974) have described a series of junctions between
ventricular cells at the neurocoel which is similar to that in *Xenopus*. A point
which still requires clarification in *Xenopus* is the nature of the close membrane
apposition next to the neurocoel.

Gap junctions in our section-stained material have two distinct appearances;
sometimes the cleft is a moderately dense structureless region 7–9 nm across as
in the junctions of embryonic *Xenopus* retina (Dixon, 1971; Dixon & Cronly-
Dillon, 1972), sometimes the cleft contains two dark lines with a 2–3 nm separa-
tion. In both cases the total width of the junctional membrane complex was
usually 14–16 nm. A 2–3 nm ‘gap’ has been reported in gap junctions elsewhere
(e.g. vertebrate brain (Brightman & Reese, 1969; Sotelo & Llinas, 1972), mouse
liver (Goodenough & Revel, 1970) and embryonic chick heart (Spira, 1971)).
We therefore checked the membrane spacing in *Xenopus* cord gap junctions
using ‘in the block’ uranyl acetate staining. The total width was the same and
the ‘gap’ was 2–3 nm. In a single junction infiltrated with lanthanum, the 4 nm
‘gap’ seen was probably a result of staining the outer membrane leaflets. The
gap junctions seen between ventricular cells in *Xenopus* embryonic spinal cord
therefore fit the criteria for gap junctions established by Brightman & Reese
(1969). They also have lighter bars across the lanthanum-filled ‘gap’ which
these authors report as typical of interneuronal junctions in adult vertebrates.

We have found gap junctions between ventricular cells in and near the apical
contact zone at the neurocoel, and between ventricular cell processes near the
outer margin of the spinal cord. As the total number of gap junctions was small,
we cannot be sure that they do not exist between other classes of cells. However,
the distribution we have found provides corroboration of previous observations
of *Xenopus* retina where gap junctions were similarly only found between non-
differentiated cells (Dixon, 1971; Dixon & Cronly-Dillon, 1972). In the spinal
cord and retina gap junctions have not been found so far on differentiating
neurones. This could be because they are even less frequent or that they are
absent altogether.

Small intermediate or adherens junctions have been found generally between
all types of cell and process, both undifferentiated and differentiated. They are
similar to small punctate junctions found in mature nervous tissue (Peters,
Palay & Webster, 1970) and in embryonic *Xenopus* retina (Dixon & Cronly-
Dillon, 1972), embryonic chick retina (Sheffield & Fischman, 1970; Sheffield,
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1971) and spinal ganglia (Pannese, 1968), rabbit dorsal root (Tennyson, 1970) and mouse cerebral vesicle (Hinds & Ruffet, 1971). Similar junctions in other animals are said to be temporary (see Pannese, 1968; Tennyson, 1970) and we have not found them in the tract and neuropil areas of Xenopus after stage 28. They are the only type of junction that we have found between differentiating neurones and their processes until synaptic contacts develop.

What then is the significance of the junction distribution in the early Xenopus spinal cord for movements of ions and molecules in the extracellular and intercytoplasmic pathways? We have found no signs of any junction which would limit movements across the outer margin of the spinal cord. Extracellular space within the spinal cord is therefore probably continuous with that in the rest of the animal at least up to stage 35, the latest we examined. If the close membrane apposition between ventricular cells at the neurocoel formed a zonula tight junction all round the necks of the cells, then a barrier could exist here in the extracellular pathway for movement to and from the neurocoel. Such a barrier probably exists in Xenopus skin (Roberts & Stirling, 1971). However, in both places further evidence would be needed to establish this conclusively.

The distribution of gap junctions suggests that intercytoplasmic pathways are open between ventricular cells, both near the neurocoel and also near the outer margin of the cord. Other undifferentiated processes may also be coupled to one another. Very similar gap junctions have been found occasionally between skin cells in Xenopus where it has also been shown physiologically that the cells are electrically coupled (Roberts & Stirling, 1971; Roberts & Hayes, unpublished). In the neural tube of Xenopus and the axolotl it has been shown similarly that unidentified cells are electrically coupled (Warner, 1970, 1973). Our fine structural evidence on the distribution of gap junctions in the neural tube and early spinal cord suggests that these coupled cells would be undifferentiated ventricular cells. Furthermore, the ventricular cells remain coupled by gap junctions until at least stage 27. As the ventricular cells can extend from the neurocoel to the outer margin of the spinal cord, physiological probing into any part of the cord may reveal electrical coupling between them or their processes.

We have not found gap junctions on cells and processes showing signs of differentiation into neurones. However, we do not regard this as evidence that such junctions are absent. Small macula gap junctions are difficult to detect by conventional transmission electron microscopy and this method can give little idea of their numbers. The same reservations apply to the suggestion by Dixon & Cronly-Dillon (1972) that gap junctions disappear from Xenopus central retina during neural differentiation. It seems more likely that as a general rule, classes of similar neurones may be interconnected by gap junctions and coupled to one another electrically, like the horizontal cells in dogfish retina (Kaneko, 1971; Witkovsky & Stell, 1973) or the motorneurones of frogs (Grinnell, 1966). Such open intercytoplasmic pathways between groups of similar neurones
would have interesting implications for the differentiation of neuronal cell types (see also Bennett et al. 1972; Furshpan & Potter, 1968).

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