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Special Review

The developmental biology of neural connectivity

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«... we cannot say why an egg can turn into a chicken any more than we can say why a bear could turn into a fairy prince. As ideas, *the egg and the chicken are further off each other than the bear and the prince; for no egg in itself suggests a chicken, whereas some princes do suggest bears. (...) When we are asked why eggs turn to birds or fruits fall in autumn, we must answer exactly as the fairy godmother would answer if Cinderella asked her why mice turned to horses or her clothes fell from her at twelve o'clock. We must answer that it is* magic.*»*

(Chesterton, 1908)

Introduction

The magic of development is at its greatest in the formation of the nervous system. That billions of fibers can unerringly, or at least reproducibly, find their targets amidst a jungle of neurites and other cells defies the imagination, and remains one of the most perplexing questions of biology. Put in a few words, the question is twofold: how does each fiber know where to go, and how does it manage to get there?

In the past this dual question has often been addressed in the case of large populations of neurons, for example, the retinal axons of vertebrates (e.g., Hankin and Lund, 1991). Dealing with the collective response of large numbers of fibers makes it difficult to analyze the process at the cellular level. In order to understand what directs the growth of the individual axon *in vivo*, one may have to analyze the development of the individual axon *in vivo*. To a large extent this can be achieved in the case of the nervous system of some invertebrates (Bate, 1978).

One major advantage of the peripheral nervous system (PNS) of insects, which saves us the trouble of having to deal with populations, is that most neurons can be unambiguously identified. This is because each sensory neuron is located right under the sense organ it innervates, and many sense organs occupy reproducible positions in the body so that they can be uniquely recognized. Furthermore, the different cells that form the sense organ, including the sensory neuron, are derived from a common mother cell which is singled out from the epidermis at the position where the sense organ will be formed. Thus in insects we can afford to consider each sense organ as unique, to follow its particular development, to analyze the growth of the axon towards and into the central nervous system (CNS), and only then to see if general features can be extracted from the analysis of many independent cases. Further advantages which turned out to be essential are the accessibility of the early stages of axonal development during the embryogenesis of the grasshopper, and the availability of powerful genetic tools in the case of *Drosophila.*

The analysis of individual peripheral neurons in insects led, in the late 1970s, to the recognition of two important aspects of the establishment of neural connectivity: first, the role of individual pioneer neurons in laying down the foundation of the future nerve tracts (Bate, 1976), and second, the ability of growing axons to recognize and follow specific trails in the central nervous system (Ghysen, 1978). The combination of these two factors results in the setting up of a stereotyped network of trails which can be discriminated one from another by newly developing axons (Bastiani *et al.*, 1985).

Obviously the existence of such a network makes it easy to imagine how a given neuron could be programmed to follow a particular pathway, and thereby be brought in close vicinity to its prospective target, much as the existence of a subway network allows one to get wherever one chooses provided one knows which line to take in order to get there. At the same time, the idea that the establishment of defined connections is based on the exploitation of a preexisting network suggests that this network should be subject to extreme structural constraints during evolution, for any change in the network would disrupt the pattern of connections.

In the next two sections I will summarize our current knowledge about the ontogeny of pioneering and of pathway selection in insect nervous systems. This will be followed by an essay on the phylogenetic aspects of these processes, the rationale of which is as follows.

The reason why development resembles a fairy tale is that it follows its own logic, which is not our standard day-time logic. The logic used in fairy tales is the logic of the unconscious, the logic of dreams. In the case of development, the logic at work is that of evolution: an unpredictable mixture of chance and opportunism (Jacob, 1981; Gould, 1989). An overview of how the nervous system evolved may therefore provide a better perspective for understanding present-day nervous systems including our own, in line with Dobzhansky's famous aphorism that «nothing in Biology makes sense except in the light of evolution». Hopefully this evolutionary perspective might help us reach the «Clairvoyance» that Magritte so strikingly depicted (see cover picture) and allow us to see the bird in the egg, Chesterton notwithstanding.

Some of the ideas about neural development and evolution that are presented here are speculative, some are provocative, many are both. If this review could trigger new trains of thoughts in some reader, whether because some ideas are new to her/him or because she/he disapproves violently, I will be content.

Pioneering by sensory neurons in the periphery

The work of M. Bate brought into sharp focus the ability of growing axons to orient their course in uncharted territory, and stimulated a large amount of experimental work aimed at understanding in detail how individual axons can reproducibly pioneer a defined pathway. Our present view of this question is that oriented axonal growth is achieved by two mechanisms. First, the axon uses orienting cues laid down either on the basal surface of the epidermis along which the growth cone progresses, or on the basal lamina formed by this epidermis. These cues may take the form of a proximo-distal gradient of adhesiveness (Nardi, 1983) or of preferred stripes, as in the case of axons following the presumptive veins of

Abbreviations used in this paper: CNS, Central nervous system; PNS, Peripheral nervous system; HLH, Helix-Loop-Helix.

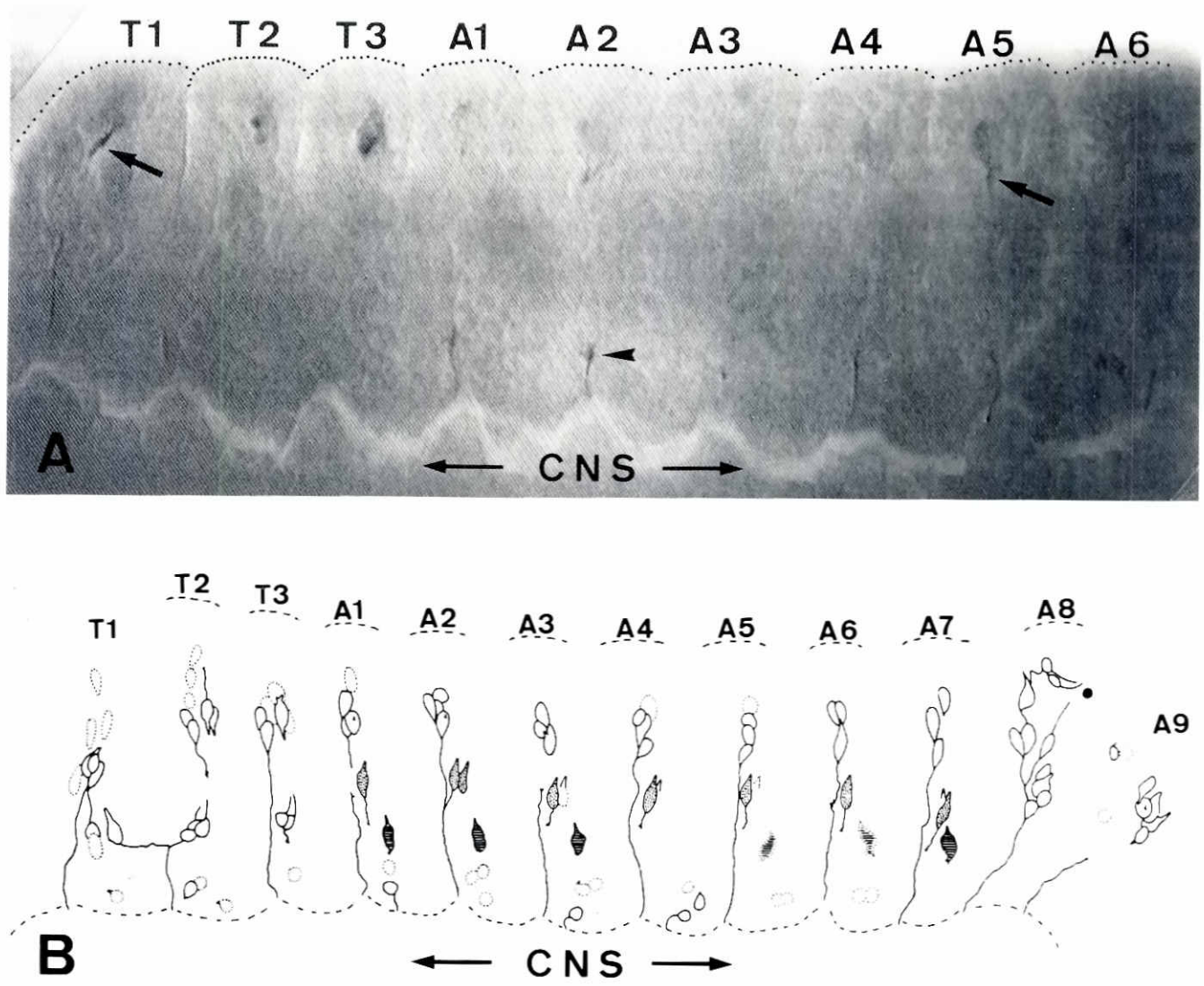


Fig. 1. Pioneering of the peripheral nerves during *Drosophila* embryogenesis (from Ghysen *et al.*, 1986). (A) *Before dorsal closure of the embryo has begun, one or two sensory neurons have appeared in all segments close to the dorsal edge of the epidermis (dotted line). Most of these early neurons are just beginning axonogenesis; the growing axons are in focus in segments T1 and A5 (arrows). At this time a fiber is leaving the CNS more or less synchronously in all segments; its growth cone can be seen in A2 (arrowhead).* **(B)** *Map of neurons halfway through the process of dorsal closure of the embryo, based on camera lucida drawings of two embryos with different orientations. The anterior fascicles have formed in nearly all segments by the convergence of the first sensory and motor axons shown in panel A. Additional neurons have begun to differentiate; the most ventral of these will pioneer another peripheral nerve, the posterior fascicle (see text), which innervates the leg rudiments in the thoracic segments of the fly larva (Keilin, 1911, 1915). While the role of the motor axon in guiding the sensory pioneer into the CNS has not been demonstrated, there is circumstantial evidence that this guidance is important. For example, the absence of AS-C genes has no obvious effect on either the determination or the differentiation of the chordotonal organs (Dambly-Chaudière and Ghysen, 1987), yet the axons of the chordotonal neurons (shown here as stippled cells) show substantial misroutings in AS-C mutant embryos (unpublished observations), presumably because of the defective motor output from the disorganized CNS. Likewise the sensory neurons of the vestigial 10th abdominal segment are the only ones not to have their own segmental nerve and to join the 9th abdominal segmental nerve (Campos-Ortega and Hartenstein, 1985), presumably because there is no segmental ganglion, and therefore no motor output, in the 10th abdominal segment.*

the developing wing (Blair *et al.*, 1987). Second, the axon may sometimes take advantage of «stepping stones», cells that are located along its prospective path, to hop towards the CNS (Bate, 1976). These stepping stones are neural or glial cells that the growing axon recognizes as «landmarks» (Ho and Goodman, 1982) or «guideposts» (Keshishian and Bentley, 1983a). In their absence, the growing axon becomes very confused (Bentley and Caudy, 1983).

The determinants of oriented axonal growth have been primarily studied in the case of pioneer neurons in appendages such as the leg (Bate, 1976; Keshishian, 1980) the wing (Nardi, 1983; Blair and Palka, 1985), the antenna (Bate, 1976; Berlot and Goodman,

1984) or the cercus (Edwards and Chen, 1979; Shankland and Bentley, 1983). Indeed the long lonesome journey of such pioneers is particularly suited for the analysis of guiding cues. It must be realized, however, that most if not all peripheral neurons display a similar ability, even if their performance is normally not as striking as that of their predecessors. In one case, it has been shown that «followers» are capable of establishing a fairly normal pathway in the absence of the normal pioneers (Keshishian and Bentley, 1983b)

Once a peripheral pathway has been pioneered, it may be used as a guide by other axons: a process known as contact guidance and first demonstrated by Wigglesworth (1953). An interesting case of contact guidance occurs when one pathway is pioneered from both ends by two axons growing towards each other: after the growth cones meet midway they follow each other's axon, resulting in the establishment of a very precise connection between the two pioneer cells. A system of mutual guidance is observed, for example, in the development of the first peripheral nerves in the fly embryo. Within each body segment, the first motor axon leaves the CNS and extends dorsalwards into the periphery at the same time as the first sensory axon is growing ventrally towards the CNS (Fig. 1). The two growth cones will meet somewhere on their way and thereby establish the first peripheral nerve — the anterior fascicle (Campos-Ortega and Hartenstein, 1985), or intersegmental nerve (Doe and Goodman, 1985). Since both sensory and motor neurons are segmentally repeated, one fascicle will be formed in each segment. Another set of peripheral and central pioneers will together establish a second peripheral nerve, the posterior fascicle or segmental nerve. Later developing peripheral neurons send axons along either of these pioneer tracts to form the larval peripheral nerves. During metamorphosis, the guides for the developing adult axons are the larval peripheral nerves themselves (Ghysen and Deak, 1978).

One unsolved question of contact guidance is that the growing axon needs to recognize the polarity of the guiding axon in order to follow it towards the CNS. Thus motor guides must be followed towards the cell body, while sensory guides must be followed away from it. How this discrimination is achieved is not known.

Pathfinding by sensory neurons in the CNS

Many insect sensory neurons establish long projections that extend in several consecutive segmental ganglia and occasionally up to the brain. The analysis of the central projection of ectopic or misrouted neurons in *Drosophila* led to the conclusion that sensory axons recognize and follow preexisting pathways in the fly CNS (Ghysen, 1978). A displaced or misrouted axon does in general manage to establish the appropriate projection, even if it has to follow part of its prospective pathway in the direction opposite to the normal one (Fig. 2). This observation seems difficult to reconcile with anything but the guidance along a labeled trail, much like ants recognize the specific tag that marks their trail and can walk the same trail in either direction. The analysis of different neurons shows that their axons will specifically recognize different trails even though they may enter the CNS through the same nerve root. Some of the pathways may partly overlap, that is, have some stretch in common, suggesting that the complete pathway traveled by a given axon is actually made up of different stretches, each with its distinct tag. Thus a virtually infinite number of different pathways could in theory be programmed by controlling the recognition of a relatively limited set of tags, in a typically combinatorial process (Ghysen and Janson, 1980).

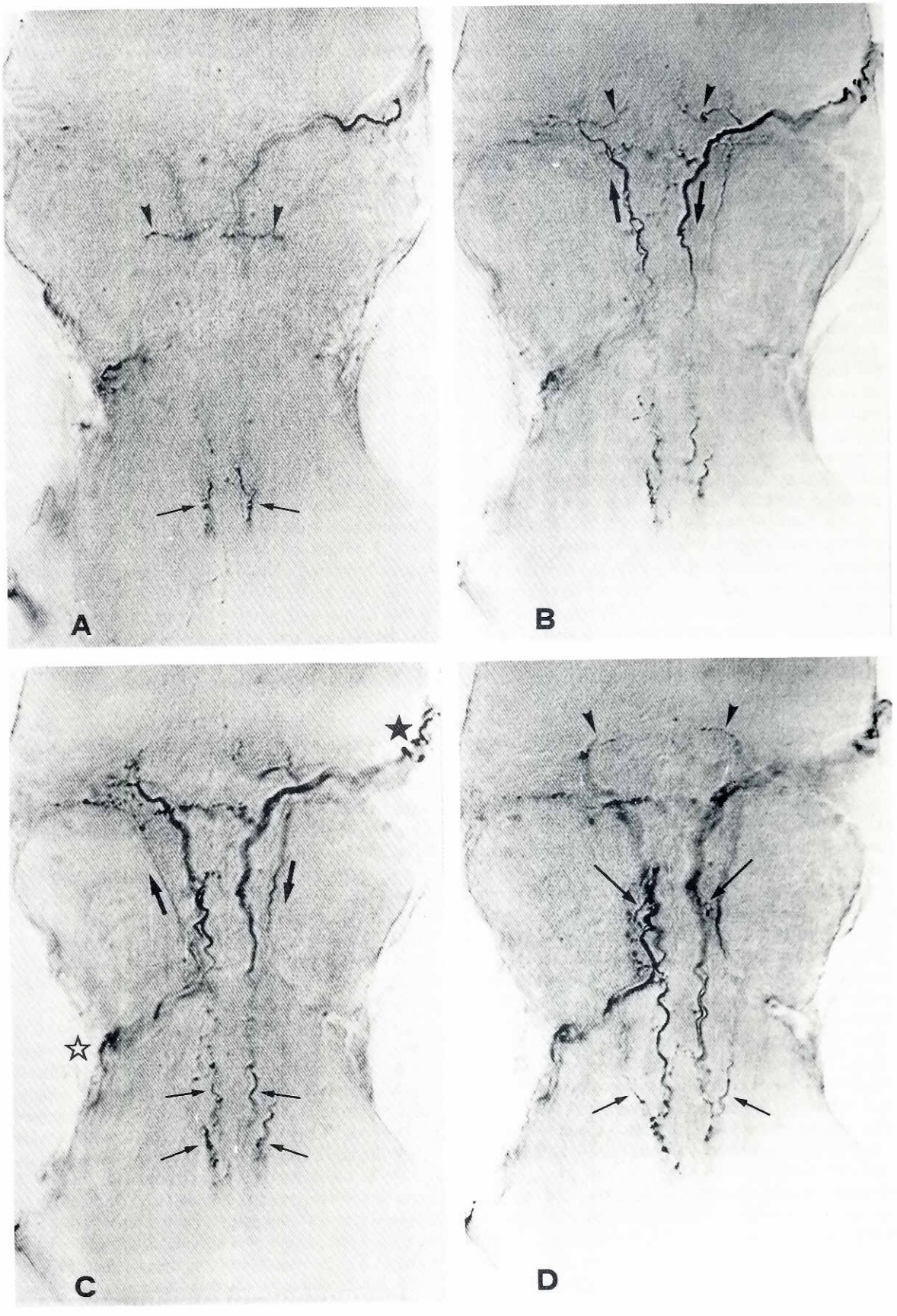
These results indicate that there is one major difference between peripheral and central guidance: within the CNS each sensory axon is able to recognize and follow one or a few among the many existing fiber tracts, implying that the different tracts within the CNS must be differentially tagged (hence the names of «substrate» pathways, Katz and Lasek 1980, or «labeled» pathways, Raper *et al.*, 1983). In the periphery, on the other hand, there is no indication that such specificity exists (Palka and Ghysen, 1982). This difference need not reflect basically different properties: it may be that the growing axon has a higher affinity for nerve fibers than for any other cell type; only when challenged with several potential guides will it demonstrate its specific preferences.

The analysis of identifiable central neurons showed that they too make reproducible choices so that their axons will specifically follow one among several possible guides (Raper *et al.*, 1983; Bastiani *et al.*, 1984), and will be confused if their appropriate guide is missing (Raper *et al.*, 1984). Thus central axons establish stereotyped pathways, and they do so by recognizing and following specific preexisting trails, much as in the case of the adult sensory axons. This work further demonstrated that the preexisting trails are themselves nerve fibers extended by earlier neurons.

Ontogeny of the CNS scaffold

Given that an essential component in the establishment of precise connections within the CNS is the existence of a network of

Fig. 2. Pathway recognition in the central nervous system: projection of a particular type of external sense organ, the campaniform sensilla, from the normal (right) and ectopic (left) wing blades of a *bithorax* mutant fly. *The four panels show four focal planes of the same preparation (from Ghysen, 1978). The right projection is indistinguishable from the projection established in a wild type fly: the axons enter the CNS through the anterior dorsal mesothoracic nerve (wing nerve, black star in panel C), an adult derivative of the anterior fascicle pioneered during embryogenesis. The axons then establish a stereotyped projection comprising two longitudinal branches, one more medial and one more lateral, each with its specific local arborizations, as illustrated on four focal planes in panels A-D. In a normal fly, the metathoracic appendage is reduced to a knob-like haltere which bears no sense organs homologous to the wing blade campaniform sensilla. In a bithorax mutant fly, the halteres are transformed into wings, and therefore campaniform sensilla develop ectopically in the metathorax. Their axons (left) enter the central nervous system through the metathoracic nerve (open star, panel C), yet they manage to establish a projection that is nearly identical to the projection from the neurons of the normal wing. In particular, the more medial branch is complete with its characteristic ramifications in the anterior region (arrowheads, panels B and D) and its slightly dorsal, perpendicular offshoot at the level of the mesothoracic neuropile (arrowhead, panel A). The more lateral branch shows the typical oblique offshoot (long arrows, panel D) and the posterior coalescence with the medial branch (arrows in panel A, C, D). Since the ectopic axons enter the CNS at a place much posterior to the normal site, the axons from normal and from ectopic neurons have to grow in the opposite direction over part of their course (thick arrows in panels B, for the medial branch, and C, for the lateral branch). Similar results are obtained in a bithorax mutant combination where the normal wings have been removed due to the presence of the wingless mutation, showing that the ectopic axons do not simply recognize and follow their normal mesothoracic counterparts, but must specifically recognize and follow a preexisting pathway, i.e., a trail that is present in the central nervous system prior to the arrival of these axons (Ghysen and Janson, 1980). The axons have been visualized by filling the neurons with horse-radish peroxidase.*


A
B
C
D

specific trails, how does this network develop? An extensive analysis of this question has brought to light several tricks that are used to lay down this lattice. Interestingly, several of these tricks are basically the same as those used to set up the peripheral nerves.

First, only two types of orienting cues need be discriminated, because the entire scaffold is built as an orthogonal net. In other words, all the early tracts run either longitudinally (and are then called connectives) or transversally (and are then called commissures). Thus all pioneers follow either the antero-posterior or the dorso-ventral axis (since the axons grow along the basal surface of the ectoderm, the medio-lateral direction is effectively dorso-ventral).

Second, several of the early connections are pioneered from both end points, as was the case of the very first peripheral nerves which have a mixed sensory and motor origin. If the distance between the two pioneers is reasonably small (which is the case for all the early connections, the only ones that are established in the absence of preexisting fibers), then a coarse directionality of the axonal growth will ensure that the two growth cones will get in filopodial reach of each other (a fly filopodium extends over about 15 μm, or about 50% of the length of a segment at that early stage, Jacobs and Goodman 1989b). This system has been most clearly documented in the case of the very first fibers which extend anteriorwards and posteriorwards from each segmental ganglion. About halfway between consecutive ganglia these axons meet and fasciculate with their anterior and posterior homologs (Jacobs and Goodman, 1989b), thereby establishing the very first connection of the CNS: the longitudinal connectives that extend from the anterior to the posterior end of the CNS (Bate and Grunewald, 1981).

Third, pioneer axons may rely on landmark cells, again as was the case in the establishment of peripheral pathways. Some of these landmarks are glial cells that help establish the very first longitudinal and transversal tracts (Jacobs and Goodman, 1989a), others may help motor axons out of the CNS (Bastiani and Goodman 1986).

The central scaffold is not simply an orthogonal net of transversal and longitudinal fibers, however: both the connectives and commissures actually comprise several distinct bundles which can be discriminated by growing axons (Raper *et al.*, 1983). Thus a second aspect of the ontogeny of the CNS connectivity, and one which does not seem to exist in the PNS, is the development of parallel, differentially labeled pathways. The simple mechanism which is used to set up this multiplicity of pathways is reiteration.

At least two ways of achieving reiteration have been documented. One relies on the fact that growth cones initially grow along the basement membrane of the epidermis, or along glial surfaces. As soon as an axon fasciculates with another axon, however, the growth cone loses its interest for other surfaces and therefore the newly formed bundle becomes detached from whatever membrane was used as a substrate (Bate, personal communication). The substrate membrane is then available for a new growing axon which will pioneer a new bundle, independent of the one already formed. In this way a set of parallel fascicles can be consecutively formed by different pioneers and may therefore express different markers.

Another mechanism for allowing reiteration is to provide axons with different tags that will make them ignore each other, so that each will unknowingly repeat what the other is doing. For example, in the case of the pioneers of the very first longitudinal tracts in the grasshopper: anteriorly and posteriorly directed pioneers ignore each other and fasciculate only with their own homologs of the next segment, so that two parallel fascicles are pioneered simultaneously (Bastiani *et al.*, 1985). Interestingly this discrimination is lost in *Drosophila*, where anterior and posterior pioneers recognize each other and therefore a single fascicle is formed (Jacobs and Goodman, 1989b). This difference between grasshopper and fly illustrates the flexibility of the system of reiteration-differentiation, as well as its constraints: only parallel fascicles will be formed; what may vary is their number and differential labeling.

The combination of mechanisms that lead to the formation and differentiation of parallel fascicles may explain the complexity of the longitudinal connectives — which comprise at least 20 anatomically distinct parallel bundles (Thomas *et al.*, 1984) — or of the anterior and posterior commissures, which are similarly diverse (Raper *et al.*, 1983; Teugels and Ghysen, 1985).

There is an obvious structural and developmental resemblance between the establishment of peripheral and central connections. In both cases the scaffold is pioneered step by step. Each pioneer does no more than establish a relatively simple connection, often a straight one, rarely a crooked one. In both cases also, each pioneer relies on either longitudinal (antero-posterior) or transversal (dorso-ventral) orienting cues. Structural complexity arises later, when the reiteration leads to multiplicity, when the addition of different stretches leads to branching points, when the establishment of orthogonal connections leads to the appearance of topological complexity. The major difference between PNS and CNS, of course, is the specific tagging of the central connections, which allows later axons to make specific choices at each branch point, and thereby leads to a programmable specificity of connections.

Structural constraints on CNS evolution

According to the preceding sections, the stereotyped connectivity of the nervous system reflects to a large extent the existence of a stereotyped network of differentially labeled guides. If this is true, one would certainly expect this network to be extremely stable during evolution, for any modification in this scaffold would have tremendous consequences on the connectivity, and hence on the function, of the nervous tissue.

A first example of impressive structural conservation has been documented in the case of several pioneer neurons in the CNS of a fly, a moth, a grasshopper and even of a crayfish (Thomas *et al.*, 1984). That the morphology and connectivity of even a few neurons should be so strongly conserved over so long an evolutionary distance certainly suggests that the evolution of the early axonal scaffold has indeed been severely constrained.

A second example of striking structural constraints was demonstrated recently in the case of the PNS of flies and grasshoppers. As mentioned above the pioneering of the segmental nerves during fly embryogenesis depends on both central and peripheral neurons. The peripheral neurons themselves form a stereotyped pattern in the body segments of the larva (Campos-Ortega and Hartenstein, 1985; Ghysen *et al.*, 1986). Very early during embryogenesis this pattern is the same in all body segments, but by the time the neurons differentiate the thoracic and abdominal patterns have become quite different (Ghysen and O'Kane, 1989). A comparative analysis of the pattern of peripheral neurons in fly and grasshopper embryos (Fig. 3) reveals that the thoracic and abdominal patterns of the two species are unmistakably similar, even though in the first case the larva is a worm-like, featureless creature, while in the

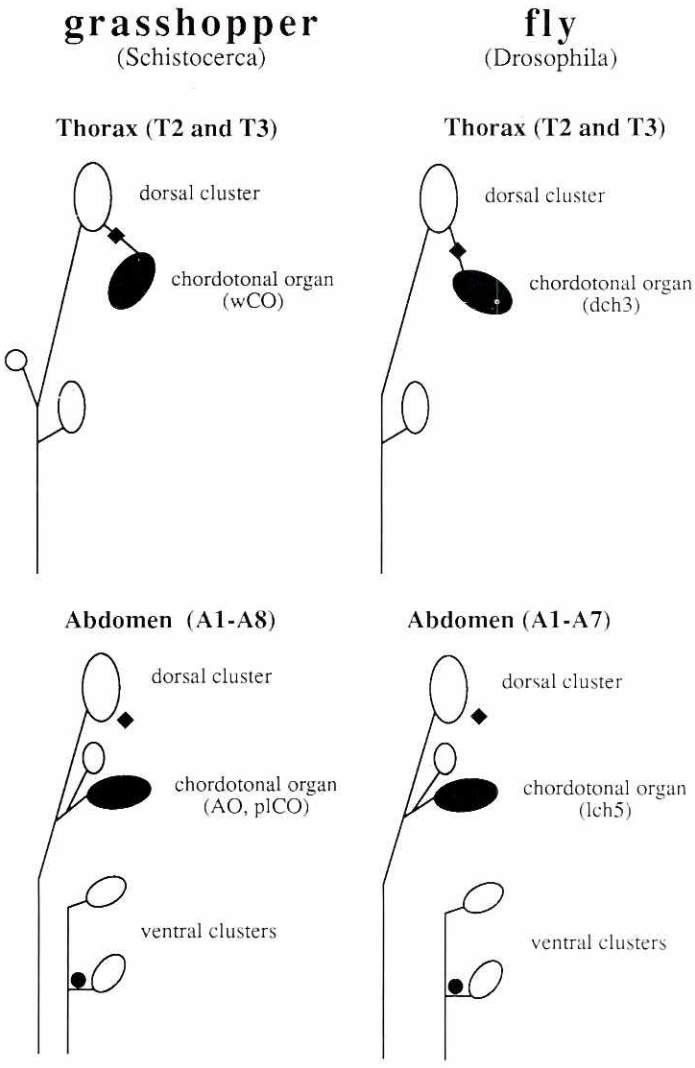


Fig. 3. Early stages of PNS development in the grasshopper and the fly *(redrawn from Meier* et al., *1991, with permission of the Company of Biologists Ltd). Ovals represent clusters of cells, lines represent peripheral nerves. For the abdominal segments all clusters of sensory neurons have been represented; in the thoracic segments the ventral clusters corresponding to the leg in the grasshopper embryo, and to the leg remnant in the fly embryo, have not been represented. Black ovals correspond to the precursors of a particular class of sense organs, the internal proprioceptive organs (chordotonal organs). The overall similarity between the fly and grasshopper patterns is impressive; the serial homology between thoracic and abdominal segments is already less obvious at this developmental stage, but has been amply documented elsewhere (Ghysen and Dambly-Chaudière, 1990).*

second case the larva is a fully equipped miniature adult. Furthermore a detailed analysis of later developmental stages revealed that a particular set of neurons located in the posterior compartment of each segment, and which early on is without any doubt homologous in all segments (Ghysen and O'Kane, 1989), ends up in the locust as being a stretch-responsive organ at the base of the wing in segment T3, an auditory organ in segment A1, and a spiracular receptor in the more posterior abdominal segments (Meier *et al.*, 1991).

In summary, then, it appears that many structural elements of the nervous system may have been conserved over long evolutionary distances, without any obvious functional reason other than their role in establishing a stereotyped pattern of connections.

Phylogeny of the nervous system

The appearance of specialized excitable cells and its molecular sequel

The first step in the definition of a nervous system was undoubtedly the specialization of some cells as excitable cells. The crucial point about neurons is that they are elongated, which enables them to transmit beyond their immediate neighbors without exciting all the intervening cells en route. This is the essence of the definition of neurons (Horridge, 1968). There are several theories about the origin of the neuron, either as a variant of a secretory cell, as a specialized conducting cell, or as a divergent effector cell (Lentz, 1968). The responses of a single excitable cell in primitive ancestral metazoans are clearly unknowable, but whatever they were, they would certainly have been trivial if they had not been propagated. Thus it seems likely that the primitive excitable cells were at the same time effectors and conductors, in effect forming a functional network: thanks to this primitive connectivity, the real effector was not a single cell but a whole field of interconnected contractile cells (Pantin, 1956).

Is there a molecular remnant of this early step in neural phylogeny? As surprising as it may seem, the answer might be yes. Recent work on the development of the PNS in *Drosophila* has revealed that the decision of an ectodermal cell to become a neural precursor is controlled by a battery of genes, the proneural genes (reviewed in Ghysen and Dambly-Chaudière, 1989), which all share a protein dimerization and DNA binding motif called HLH for Helix-Loop-Helix (Murre *et al.*, 1989a,b). A slightly different motif had previously been identified in a gene, MyoD1, involved in the decision to form myoblasts in mice (Davis *et al.*, 1990), and was subsequently found to be shared by a family of genes involved in muscle determination and differentiation in different vertebrates. *A priori* the difference between the two types of HLH motif, one found in the fly proneural genes and the other in the vertebrate MyoD1 family of genes, could be related to divergence between flies and mice, or between nerves and muscles. Further work revealed that the rat genome contains (at least) two genes with a HLH motif very similar to that of the fly proneural genes; these rat genes are expressed in neural precursors or related lineages (Johnson *et al.*, 1990). Conversely the fly genome contains (at least) one gene with a HLH motif very similar to that of the mouse MyoD1-like genes; this fly gene is expressed in muscle precursors (Thisse *et al.*, 1988).

These results suggest that both flies and mammals contain genes with a «proneural» type of HLH which seem to be related to a neural fate, and genes with a «myoD» type of HLH motif which are somehow related to muscle determination. This correspondence might be fortuitous, and indeed there exist HLH-containing genes which are not clearly related to either neuron or muscle, for example in the oncogene myc. Alternatively it may be that the nerve/muscle association reflects an ancient relation, and that the existence of HLH genes with unrelated functions is a recent development (like, for example, the recruitment of two of the fly proneural genes in a completely different process, sex determination). If true, this

suggests that the primitive HLH motif appeared in the primitive excitable cell before it specialized as either neuron or muscle cell, and that this primitive motif later diverged in the two cell types to produce respectively the proneural and myoD-like HLH motifs now found in arthropods and vertebrates alike. The identification of HLH-containing genes in sponges, which contain excitable/contractile cells but no true neurons or muscle cells, might provide crucial evidence on this question.

The formation of a nervous system and its molecular sequel

The reader is referred to the excellent description of Horridge (1968) for a comprehensive review on the origins of the nervous system, from which I extract the following paragraph: «The most primitive nerve net is one in which the neurons are scattered in an epithelium and make connections with any other neuron or with a muscle cell. The spatial pattern is irrelevant, the connectivity pattern has no restrictions. In such a net, considered as that typical of coelenterates since the work of Romaned (1876) and Schäfer (1879), any fiber is equivalent to any other in either growth or transmission. Collapsing such a net of equivalent neurons results in a tract of parallel fibers», and in the formation of the most primitive nerve cords.

The tendency towards the aggregation of nerve cells in well-organized cords at the expense of the primitive diffuse network is already manifest in some cnidarians and in the ctenarians and culminates in the most primitive flatworms, which possess several (often 8) well-defined longitudinal cords connected by an anterior ring: the CNS is now firmly established.

The idea that all extant CNS are derived from a primitive ctenarian- or flatworm-like design of radially symmetrical longitudinal cords may seem surprising in view of the common statement that the chordate/vertebrate and the annelid/arthropod nervous systems are very different, the first one being composed of one dorsal cord, while the second comprises two ventral cords. It should be emphasized that this apparent dichotomy is a gross oversimplification, and that many other types of nerve cord organization are known, which blurs the convenient dorsal cord/ventral cord opposition. This diversity, on the other hand, is easily explained if the present types are derived from a common radially symmetrical pattern by the progressive elision or fusion of some of the nerve cords. Of the primitive 8 longitudinal cords, nemertinean worms have retained two lateral ones, and occasionally a dorsal one as well; primitive molluscs have retained two ventral and two lateral cords, echinoderms have retained five, and prochordates have retained two: a dorsal and a ventral one (Bullock and Horridge, 1965). Thus the single dorsal cord of vertebrate is a relatively recent development. Remarkably the 8 cords typical of the radially organized primitive flatworms are still present in the annelid larva, even though the clearly bilateral adult retains only the two ventral cords. Even more surprisingly, the larva of *Drosophila* still contains two dorsolateral cords of unknown function, the «Seitenstrangen», in addition to the more usual ventral pair (Hertweck, 1931; Bodmer and Jan, 1987). This brief survey indicates that, in this case as in others (Murphey, 1986) the usual arthropod vs vertebrate dichotomy might be more akin to propaganda than to biology.

The «collapse» of a diffuse neuronal network into a discrete array of cords and nerve bundles involves the emergence of a most important property of nerve cells: the tendency for cell bodies to aggregate, and for axons to fasciculate. Fasciculation depends on the existence of specific cell adhesion molecules that allow axons to recognize and adhere to each other. In present-day organisms, there is a whole battery of neural cell adhesion molecules which are largely conserved across the major phyla. It seems likely that these result from the diversification of one or a few ancestral, neuron-specific factors.

Likely candidates for the ancestral neuro-adhesive factor are the homophilic proteins of the N-CAM type, which are part of the immunoglobulin superfamily. Although the N-CAM family was originally identified as neural cell adhesion molecules in vertebrates, several homologous genes have now been identified in insect neurons (reviewed in Grenningloh *et al.*, 1990). Harrelson and Goodman (1988) proposed that the proteins of this family originated as neural cell recognition molecules, and were later widely used in other cell types to mediate cell recognition. Although the major effect of an ancestral neuroadhesive molecule would have been its role in the condensation of the nervous system, it is possible that variants produced by early divergence played some role in cell or path discrimination by mediating selective fasciculation. To what extent the different proteins of the N-CAM family may confer some specificity to the neural recognition in present-day organisms, in addition to their contribution to general neural adhesiveness, is not clear (Elkins *et al.*, 1990).

Once a neuro-adhesive molecule has become available, its expression by non-neuronal cells (e.g., ectodermal cells) in specific regions of the organism may be used to orient the growth of axons and help pioneers establish reproducible connections, and may therefore provide a mechanism for the establishment of the early scaffold according to a specified, reproducible pattern. Thus the condensation of the diffuse net into a array of cords and axon in bundles, and the establishment of a patterned network of connections, may have been nearly concomitant.

The emergence of periodicity: no molecular sequel known?

Virtually all animals that have developed longitudinal cords also have transversal connections extending between the different cords. In their most primitive form, the transversal bundles join adjacent cords at random positions, presumably improving the general connectivity of the system. Starting from that point, two trends can be observed (Fig. 4). One trend has been to multiply the connections and develop a crisscross pattern of cords, as illustrated in Fig. 4 in the case of *Planocera*. This turned out to be an evolutionary dead end, presumably because it soon resulted in an unmanageably tangled network. Indeed this tendency is observed only in one particular group of flatworms, the polyclads, on which no other groups seem to be rooted.

The other trend has been to bring the transversal cords in register, thereby laying down the essential foundation of periodicity along the antero-posterior axis. The orthogonal structure of longitudinal connectives and periodically arranged transversal commissures is thereby achieved, as illustrated in the case of *Bothrioplana* in Fig. 4, and has remained the structural basis on which all subsequent nervous systems are built. The appearance of a metamerically organized CNS was probably not ineluctable, and with some bad luck the world might still be inhabited by polyclads and sea anemones, but once serial reiteration was introduced as a developmental device it allowed a fast spreading of multiple variants, much like gene duplication is the key to the divergent evolution of gene families. Metamerization, therefore, was almost sure to be conserved through whatever next catastrophe lurked around.

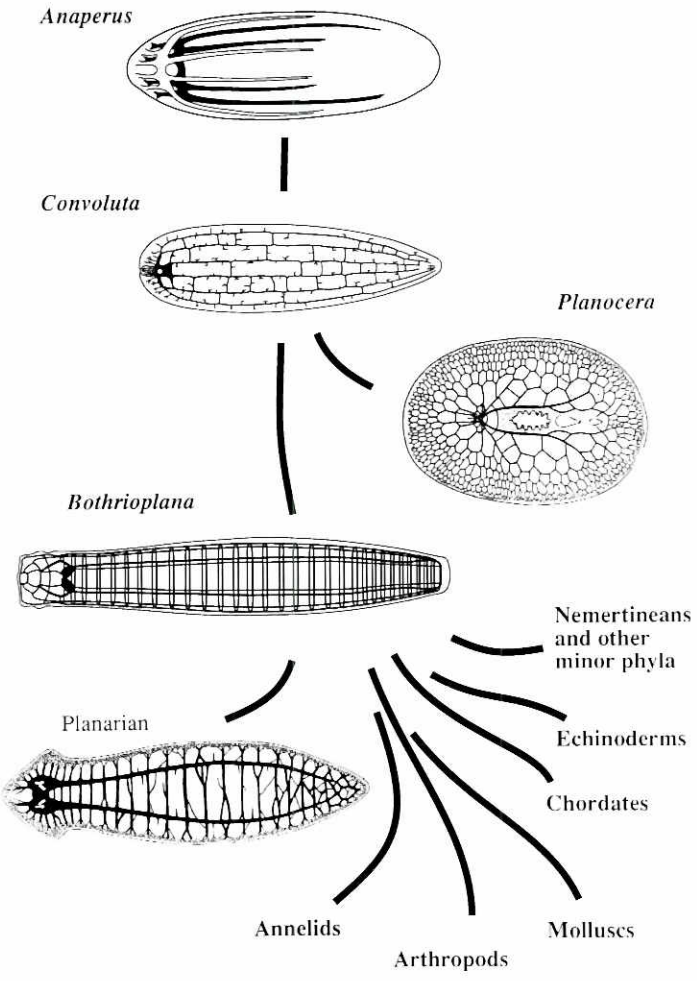


Fig. 4. The gross organization of the CNS in various species of flatworms, and a tentative scheme of how they may reflect the major stages in the appearance of a metamerized, orthogonal CNS. Anaperus *is a representative of the most primitive flatworms, the CNS of which is similar to that of ctenarians in having well-defined longitudinal nerve cords (connectives) but no transversal nerve cords (commissures).* Convoluta *has developed a set of irregularly spaced transversal cords. Two major trends can thereupon be observed: one leads to an increasingly complicated crisscross arrangement of cords as observed in* Planocera, *a typical polyclad. The other leads to the regular, metameric organization shown here in the case of* Bothrioplana. *All the extant types of CNS, including that of evolved flatworms like planarians, may have derived from the simple metameric pattern by elision or condensation of some of the longitudinal cords, and reduction of part or all of the commissures to peripheral nerves (see text). Note that at its early stages the appearance of a repeated organization does not necessarily involve an extremely precise reiteration mechanism, as shown here by the relatively rough metamerization of the planarian CNS. A similar roughness in metamery is found in the CNS of primitive molluscs (monoplacophorans and polyplacophorans). The nervous systems of* Anaperus *and* Bothrioplana *are redrawn from Bullock and Horridge (1965), those of* Convoluta *and* Planocera *are from Lameere (1933), and that of the planarian is from Lentz (1968).*

Metamerized patterns are often considered to have developed after the divergence between arthropods and chordates. Recent data, however, lend strong support to the idea that the major phyla derive from ancestors that were already metamerized, consistent with the observation that overt segmentation was widespread in the cambrian fauna (reviewed in Gould, 1989). For example a re-examination of sequence data has shown that annelids and molluscs are best considered as modifications of a segmented ancestral proto-arthropod (Lake, 1990). Another argument suggesting independent origins for chordate and arthropod segmentation, that the segmentation of the CNS in vertebrates is secondary to mesoderm segmentation, is now challenged by the observation that intrinsic segmentation can be detected in the vertebrate hindbrain (Lumsden and Keynes, 1989; reviewed in Lumsden, 1990). In the case of echinoderms, and in particular of ophiurids, the obvious periodic organization of the five cords has usually been considered as secondary to the distribution of feet or ossicles, rather than as a sign of intrinsic metamerization of the CNS. However this view may owe more to prejudice than to neurobiological research, and a reassessment of the ontogeny of periodicity in these animals might be rewarding. For a recent review of the ontogeny and phylogeny of segmentation, see Kimmel *et al.* (1991).

Is there a molecular trace of the establishment of a periodic organization in the CNS? We still know next to nothing about how segmentation is established, and nothing at all about its genetics and molecular biology, except in the case of *Drosophila.* The elucidation of the genetics and molecular biology of segmentation in this organism has been one of the great breakthroughs of developmental genetics (Nüsslein-Volhard and Wieschaus, 1980; reviewed in Ingham, 1988). Unfortunately, segmentation in the fly is rather peculiar, because it consists of the subdivision of about 3000 blastoderm cells into progressively smaller domains, eventually defining 14 consecutive repeats (segments), while in more primitive insects and most other organisms, segments are defined sequentially one after another. Thus the genetic system elucidated in the fly may well be a recent feature that evolved for the particular task of allocating a definite number of cells to a definite number of domains, and it is not clear at all which, if any, of the molecules that have been identified so far as essential for segmentation in *Drosophila* were already associated to the primitive metamerization mechanism.

Segment diversification and its molecular sequel

The appearance of periodicity turned out to be a major simplifying principle and as such may have made possible a vastly increased complexity of neural development, and therefore of behavior. Furthermore, the existence of a repetitive organization lends itself to a diversification of its repeated units. In the case of the antero-posterior repeats, this diversification was achieved by the deployment along the A-P axis of a battery of position-specifying genes, the homeotic genes (Lewis, 1978; reviewed in Akam, 1987). Here again these genes are characterized by their possession of a sequence, the homeobox, that encodes a particular DNA binding motif (reviewed in Gehring, 1987).

The striking molecular conservation of the homeobox motif between mammalian and diptera, the fact that in both cases these genes are organized in clusters where the order of the genes within the cluster parallels their order of deployment along the antero-posterior body axis, and finally the observation that there is a gene-by-gene homology between fly and mammalian clusters (reviewed in Holland, 1990), argues in favor of the idea that the primitive cluster of homeobox gene (and presumably its spatial pattern of deployment in the nervous system) predated the separation between the two lineages. An analysis of the homeobox-containing genes in present-

day flatworms (García-Fernández *et al.*, 1991) may give interesting clues about the function and organization of this primitive cluster.

The function of homeotic genes in the development of neural connectivity has been assessed in the fly by the analysis of mutant phenotypes and of mutant phenocopies. It was shown that the homeotic genes control segmental differences in the pattern of projections of sensory neurons (Ghysen *et al.*, 1983) and in the organization of the CNS, both at the level of gross anatomy (Jiménez and Campos-Ortega, 1981), neuron position (Green, 1981), neuromeric structure (Teugels and Ghysen, 1983; Ghysen *et al.*, 1985) and of commissural bundles (Teugels and Ghysen, 1985).

The homeotic genes code for nuclear transcriptional regulators (Laughon and Scott, 1984) and therefore their effect on neural development and pathway tagging is almost certainly indirect, through the activation or repression of specific path recognition molecules. This regulatory function makes the universality of the homeobox gene set somewhat of a paradox. Indeed one of the most remarkable illustrations of the universality of the homeotic gene set is the recent discovery that a mammalian homeotic gene can effectively substitute for its fly homolog (Malicki *et al.*, 1991). Yet it seems highly unlikely that the pattern of connections is also conserved between flies and mice! What sense does it make, then, that a conserved set of genes act on a non-conserved set of targets? This brings us back to the observation that we made in the section on the role of structural constraints in evolution: it seems easier to adapt an existing pattern to generate seemingly very different results, than to generate a new pattern. In the case of the pattern of expression of the homeotic genes, this implies that the same gene, and therefore the same or very similar binding sites, control different batteries of target genes in different animals. That specific binding sites can move from one gene to another implies that rearrangements on a micro scale (involving small stretches of the order of a few tens or hundreds of base pairs) are relatively common. Remarkably, a detailed comparison of the regulatory regions of a particular neuron-specific gene in two *Drosophila* species that diverged 60 to 80 million years ago revealed a substantial amount of rearrangements and reshuffling of the putative regulatory binding sites (Taghert and Schneider, 1990), suggesting that these sites might indeed be relatively mobile, though the mechanism by which these micro-scale rearrangements are achieved is far from clear.

Pathway diversification: molecular sequel in sight?

What are the molecules that mediate selective fasciculation, pathway discrimination and target recognition? We do not know. The few tags that have been isolated to date from insect neurons do not show segment specificity in their distribution, and may be more related to general adhesiveness than to specific path labeling. In other systems, neuronal tags may take the form of membrane-bound repulsive factors (reviewed in Walter *et al.*, 1990). Axonal behaviors suggestive of chemotaxis have also been reported (reviewed in Tessier-Lavigne and Placzek, 1991). This type of guidance might possibly orient populations of axons in particular situations. It seems unlikely, however, that such a coarse mechanism could provide the exquisite specificity that is required for the establishment of the stereotyped scaffold of major tracts present in all but the most primitive nervous systems. The case of the nerve growth factor is certainly a good example that even the most compelling evidence in favor of chemotactic guidance should be considered with some caution.

So far, then, we are still in the dark about the nature of the signs that show axons their way. There is good hope that this situation will soon change, however, because more and more genes are known that specify the pathway an axon will follow (reviewed in Jan and Jan, 1990). Besides the homeotic genes themselves, which affect all cell types in different manners and therefore might act through a complicated cascade of subordinate regulatory genes, genes that seem directly responsible for specifying a particular type of sensory neuron have recently been identified (Blochlinger *et al.*, 1988; Dambly-Chaudière *et al.*, 1992). All those genes are transcriptional regulators that act by controlling the expression of a discrete set of target genes, among which must be the genes that effectively mediate path recognition. Methods of identifying the targets of a particular transcriptional regulator are becoming available. Therefore the nature of the path-specific neuronal tags may soon be discovered, at least in a few cases, and we will then know whether at this level also we can find molecular remnants of the earliest steps of neural diversification. We will also know how far into the past this quest will lead us. It is conceivable that some neural recognition molecules can be traced back to the early sorting out of the diffuse net of primitive coelenterates, even before the nets collapsed into a central nervous system — or, at the other extreme, that neural complexification involved the accumulation of all sorts of odd tricks by the different phyla, most of which may have no homologs in other phyla.

Conclusion

In summary, the first two steps in the evolution of the nervous system were the specialization of some cells to form an excitable network, and the condensation of this network in a discrete set of cords and connecting bundles of fibers. One way this pattern further evolved was by introducing periodicity in the transversal commissures, thereby establishing a reiterated pattern of connections. It seems likely that both this reiteration of a basic plan, and the tools that made it possible to introduce modifications of the detailed connectivity in the different repeats, predated the cambrian explosion. Together these two innovations were instrumental in allowing the establishment of an increasingly rich network of connections, resulting in the development of a much more complex nervous system than previously attainable, and opening the way to an unprecedented sophistication in connectivity and hence in behavior. This access to a higher order of neural and behavioral complexity, and the subsequent extension of these advances to other cell types, may have paved the way to the major phylogenetic explosion from which arose the separate lineages that would in due time produce the vertebrates and the insects, as well as many other now extinct groups (reviewed in Gould, 1989).

Summary

How can the development of an ordered array of neuronal connections be encoded in the genome? Results on the establishment of sensory connections in insects indicate that this programming is a multi-stepped process which begins as soon as the first axons develop. Because each step relies on the previous level of organization, the first steps of the process are subject to intense structural constraints, and therefore have been largely conserved through evolution. What is known of the molecular biology of some essential steps, like the differentiation of excitable

cells, their aggregation in nerve cords, and the diversification of a periodic structure, supports the idea that the basic organization of the CNS evolved before the divergence between the chordate and the arthropod/annelid lineage.

KEYWORDS: *peripheral nervous system, neural connectivity, Drosophila, sensory projections, evolution, flatworms, neurogenesis*

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