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**State-of-Science Review: SR-E3 I
The Cellular and Molecular Logic of Neural Circuit Assembly:
Progress and Challenges**

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Summary

The main goal of developmental neuroscience is to define the principles and mechanisms that endow neurons with the ability to form precise and selective connections with their synaptic partners – a selectivity that underlies the normal function of these circuits in the mature brain. Attempts to explain how neuronal circuits are assembled have focused on four sequential developmental steps: the specification of distinct neuronal classes; the directed outgrowth of developing axons; the selection of appropriate synaptic partners; and the refinement of connections through the elimination of certain neurons, axons, and synapses. In recent years, the study of these processes has seen considerable progress, and to some extent, each step has emerged as an experimental discipline in its own right. This brief overview describes some of the major advances that have occurred in our understanding of the events that direct the development of neuronal connections, focusing primarily on the cellular and molecular discoveries of the past decade. Despite evident progress, a formidable gap still separates studies of neuronal circuitry at the developmental and functional levels, prompting the question of whether efforts to unravel the mechanisms that control the development of neuronal connections have told us much about the functions of the mature brain. And do developmental studies offer any prospect of providing such insight in the foreseeable future? In discussing the progress of studies on the development of the nervous system, this review indicates why such a gap exists and describes how technical advances in the ability to manipulate gene expression in developing neurons may provide new experimental strategies for studying the function of circuits embedded in the mature brain.

1. The control of neuronal identity

The generation of neuronal diversity represents an extreme example of the more general problem of how the fates of embryonic cells are specified. Extreme in the sense that the diversity of neuronal cell types, estimated to be in the range of many hundreds, far exceeds that for other tissues and organs. Nevertheless, as with other cell types, neural cell fate is now known to be specified through the interplay of two major classes of factors. The first class constitutes cell surface or secreted signalling molecules that, typically, are provided by localised embryonic cell groups that function as organising centres. These secreted signals influence the pathway of differentiation of neighbouring cells by activating the expression of cell-intrinsic determinants. In turn, these determinants direct the expression of downstream effector genes, which define the later functional properties of neurons, in essence their identity. Tracing the pathways that link the action of secreted factors to the expression and function of cell-intrinsic determinants thus lies at the core of attempts to discover how neuronal diversity is established.

1.1. *Neural induction*

The first contribution to studies of neural cell fate specification was the organiser grafting experiment of Spemann and Mangold, which showed that naive ectodermal cells could be directed to generate neural cells in response to signals secreted by cells in a specialised region of the gastrula stage embryo, termed the 'organiser'. Transplanted organiser cells were shown to maintain their normal mesodermal fates but were able to produce a dramatic change in the fate of neighbouring host cells, inducing the formation of a second body axis that included a well-developed and duplicated nervous system. Spemann and Mangold's findings prompted an intense, protracted, and initially unsuccessful search for the identity of relevant neural inducing factors.

Within the past two decades, however, significant progress has been made in defining the identity of such inductive factors. The prevailing view of the mechanism of neural induction currently centres on the ability

of several factors secreted from the organiser region to inhibit a signalling pathway mediated by members of the TGF β family of peptide growth factors. The function of TGF β proteins, when not constrained by organiser-derived signals, appears to be to promote epidermal fates at the expense of neural differentiation. The constraint on TGF β -related protein signalling appears to be achieved in part by proteins produced by the organiser such as noggin and chordin that bind to, and inhibit the function of, secreted TGF β -like proteins.

Although the TGF β protein inhibitor model is attractive in its simplicity, several apparently inconsistent results surfaced near the end of the 1990s. Mouse mutants lacking genes encoding candidate neural inducers expressed by the organiser region (the node in mouse) still exhibited neural differentiation. Moreover, genetic elimination of the mouse node in its entirety similarly failed to block neural differentiation. How can these findings be reconciled with the simple mid-1990s version of events?

It now appears that neural induction begins prior to the formation of the organiser region and, thus, must be initiated by signals derived from other cell types. In addition, members of other families of signalling molecules, notably the fibroblast growth factors (FGFs) have now been proposed as early-acting factors that initiate neural induction. So the suppression of TGF β signalling may maintain rather than initiate the process of neural differentiation. These new findings extend, rather than overturn, the TGF β -inhibition hypothesis. FGFs have been shown to function, at least in part, by repressing the expression of TGF β genes at a transcriptional level, in contrast to the organiser-derived factors such as noggin and chordin, which act extracellularly to block TGF β activity. Thus, the new data are not inconsistent with the idea that suppression of TGF β signalling lies at the heart of neural induction. In retrospect then, we began the 1990s knowing nearly nothing about the cellular or molecular bases of neural induction, passed through a brief period when the process appeared to be simple and straightforward, and left the decade with a richer, more complex, and hopefully more realistic view. We now appreciate neural induction as a multi-step process, with distinct signalling factor requirements at successive steps. Although current views remain somewhat fragmentary, we expect they will soon blend into a more harmonious account of this fundamental aspect of neural development.

1.2. Regionalisation of the neural tube

Cells in the neural tube acquire their regional identity by virtue of the position that they occupy along two primary neural axes: dorsoventral and anteroposterior. Inherent in this model is the idea that the position of a cell defines its identity, through exposure to regionally restricted signalling factors that operate over these two neural axes. With these advances, neural cell groups that previously represented anatomical curiosities of obscure nomenclature and function – the floor plate, the roof plate, the isthmus and the zona limitans intrathalamica, to name but four – have come to be appreciated as key sources of secreted factors that establish regional pattern. In addition, it has become apparent that a relatively small number of signalling factor families – notably, TGF- β s (including BMPs), hedgehogs, FGFs, Wnts and retinoids – can account for many features of regional cell specialisation within the neural tube. Along the dorsoventral axis, the two primary signalling factors appear to belong to the hedgehog and BMP families. A useful oversimplification is that the hedgehogs mark the ventral and BMPs the dorsal limit of the axis along the anteroposterior axis. In reality, the situation is somewhat more complex, with retinoids, FGFs, hedgehogs, Wnts and BMPs all proposed to function in different locations or developmental windows. In some instances, the combinatorial actions of several factors acting on a single region or cell appear to establish regional pattern and neuronal diversity. In other cases, inductive factors, most notably Sonic hedgehog, can act as gradient signals, or morphogens – inducing distinct neuronal subtypes at different concentration thresholds. In parallel with the definition of extrinsic signalling molecules, came advances in the biochemical characterisation of transcriptional regulatory mechanisms in mammalian cells, combined with the molecular cloning of genes responsible for homeotic transformations of body parts in *Drosophila melanogaster* in the late 1980s. These propelled sequence-specific DNA-binding proteins – loosely, transcription factors – into the forefront of studies on cell fate determination in the nervous system.

Current estimates indicate that there are over a thousand transcription factor genes in the mammalian genome. The little we know about the subset of these factors that are expressed in the developing nervous system indicates that different categories of transcriptional regulators, many containing homeodomains, play pivotal roles at several successive steps in neural induction (e.g. Sox genes), regional patterning (e.g. Pax and Nkx genes) and cell fate determination (e.g. LIM and POU genes). The sheer diversity of transcription factors recognised to exhibit cell-type-specific patterns of expression in the nervous system has posed the additional question of whether some of them act in a dedicated manner to direct specific cell fates. Nonetheless, a critical 'systems-level' question remains largely unresolved: how are dorsoventral and anteroposterior patterning mechanisms integrated within individual cells to specify their unique identities? The realisation that many different neuronal cell types can be generated in response to the actions of a single inductive factor has placed added emphasis on the idea that the specification of cell identity depends on distinct profiles of gene expression in target cells. Such specificity in gene expression may be achieved in part through differences in the initial signal transduction pathways activated by a given inductive signal. But the major contribution to specificity appears to be the selective expression of different target genes in cell types with diverse developmental histories and, thus, different responses to the same inductive factor. And genetic studies in mice and zebrafish have demonstrated that a high proportion of these genes have critical function in establishing the identity of the neural cell within which they are expressed. In many cases, the classes of embryonic neurons defined on the basis of differential transcription factor expressions have also been shown to be relevant to the later patterns of connectivity of these neurons.

Because of these advances, the problem of defining the mechanisms of cell fate specification in the developing nervous system can now largely be reduced to the issue of tracing the pathway that links an early inductive signal to the profile of transcription factor expression in a specific class of postmitotic neuron: a still daunting, but no longer unthinkable, task.

1.3. Neurogenesis

Over the past decades, descriptive studies of neuronal lineages had provided evidence that the developmental potential of neural progenitors is restricted progressively with time and advancing cell division, and that cells become committed to particular fates only late in their developmental history. Related studies had also raised the possibility that the decision of a newborn cell to become either a new progenitor or a neuron is related to the way in which the progenitor divides. We now have a richer understanding of these processes, and can begin to define a core programme of neurogenesis.

Once again, key insights into this issue have come from studies in *Drosophila*. Here, earlier work had identified a set of neurogenic genes, the loss of which resulted in the generation of excess neurons at the expense of supporting cells. These studies also led to the idea that the selection of the neural fate depended on a lateral, intercellular signalling process that operated between neighbours, directing one to become a neuronal precursor and the other to acquire a non-neural fate. Molecular analysis revealed that key neurogenic genes encoded components of this signalling pathway, including the membrane-associated ligands, Delta and Serrate, and their transmembrane receptor, Notch. Activation of Notch signalling biases cells towards non-neural fates, while inhibition of Notch signalling promotes neuronal differentiation. Moreover, it has become apparent that the Notch signalling pathway is highly conserved and also exerts a pivotal role in the control of neurogenesis in vertebrates.

The analysis of Notch signalling in *Drosophila* has also led to the identification of intracellular proteins that regulate Notch function. Prominent among these is the Numb protein. In many cells, Numb appears to bind to Notch and inhibit its signalling, thus promoting neuronal cell fates. This interaction appears to be a critical part of the elaborate cellular machinery used to endow sibling cells with distinct fates following asymmetric divisions. Numb and many other proteins are concentrated at one pole of proliferative neural

precursors, and so they are segregated to one or both daughters, depending on the mitotic plane. As a consequence, the symmetry (or asymmetry) of a cell division may influence the nature of the progeny via symmetrical (or asymmetrical) intracellular partitioning of the same Notch pathway components that are activated extracellularly by neighbouring cells. Many of the same proteins exist in developing vertebrate neural cells where the same mechanisms appear to operate.

Studies of neurogenesis in *Drosophila* have also revealed many of the upstream regulators and downstream effectors of Notch signalling. Most notably, transcription factors of the basic helix-loop-helix (bHLH) class have been shown to have central roles in defining groups of proneural cells – cells that have the capacity to assume neural fates under the control of Notch signalling. Members of the same gene families play independent roles in directing the progression of neuronal differentiation once fate has been determined. Close relatives of these genes have been identified in vertebrates and shown to have strikingly similar functions in controlling the decision of progenitor cells to remain proliferative or to acquire neuronal or glial fates.

With the definition of this core neurogenic programme, it may now be possible to address crucial mechanistic questions. For example, the decision of progenitor cells to express neuronal properties is often tightly linked to the decision to exit the cell cycle, yet little is known about the integration of these two programmes. Recent evidence suggests that certain neurogenic bHLH proteins can drive neural precursor cells out of the cell cycle. But it remains uncertain whether this is a general property of these proteins, and the biochemical basis of the intersection between bHLH transcription factors and cell cycle machinery is obscure. In addition, the concept of lateral signalling as a method of forcing binary cell fate decisions during neural development implies a high degree of feedback control during the period that cells make these decisions. As yet, though, few of the proteins that mediate such feedback interactions have been identified. Thus, despite many striking advances in understanding basic programmes of neurogenesis in vertebrates, details of the pathway of neuronal differentiation still remain sketchy.

1.4. *The migration of neural cells*

Early in the 20th century, it became clear that most central neurons arise in a transient layer, the ventricular zone, and then migrate to their definitive nuclei or laminae before or as they differentiate. Between 1960 and 1990, some of the major features of this migratory process were elucidated. One feature was that the neuroblasts migrate along the surfaces of radial glial cells – cells that themselves span the thickness of the neural tube, from ventricle to pia. *In vitro* studies later confirmed that neurons use radial glia as migratory guides. Second, in some regions – most notably the cerebral cortex – there is a systematic relationship between the time a neuron is ‘born’ (that is, exits the cell cycle) and its laminar fate. In the cortex, the earliest born neurons populate layer 6, then succeeding cohorts migrate past their older siblings to form layer 5, then layer 4 and so on.

These phenomena imply the existence of signals on or near radial glia that first promote migration of neuroblasts in appropriate directions, and then arrest movement at appropriate locations. A major advance in the past decade has been the identification of the first few components of this migratory machinery. Progress has been driven in large part by genetics: positional cloning of genes mutated in humans and mice with cortical defects, and unexpected phenotypes of knockout and transgenic mice generated for other purposes. A particularly gratifying historical note is that the first key molecular insights into migration came from the isolation of a new allele of a mouse mutant, *reeler*, that had been used earlier used to elucidate cellular features of neuron-glia interactions.

In *reeler* mice, late-migrating neurons fail to pass their older siblings, leading to a scrambling of the normal inside-out relationship between birthdate and laminar position. The *reeler* gene turns out to encode a

large, secreted molecule called reelin which is concentrated in the superficial cortical laminae and seems to promote dissociation of neuroblasts from the radial glial surface. Once *reeler* was identified, further progress required an analysis of its effects on neuroblasts. Here again, genetics has been critical: mouse mutants lacking a cytoplasmic adaptor protein, mdab-1, and members of the low-density lipoprotein receptor (VLDLR and ApoER2) family have phenotypes very similar to that of *reeler*, and mutants lacking integrin alpha 3 have a distinct but related phenotype. These discoveries motivated biochemical studies that now permit the assembly of a rudimentary genetic pathway in which reelin is a ligand, LDL receptors and integrins are reelin receptors, and mdab-1 is a critical component of the intracellular signal transduction apparatus that leads neurons to change course when they encounter reelin. Despite these advances in understanding radial migration, it has been apparent that some neural cells migrate non-radially along other guides, including axons and extracellular matrices. The extent of non-radial migration was clearly great in subcortical structures such as the tectum, but remained a matter of controversy in the cortex, where migration has traditionally been studied most intensively. In the late 1990s, it was found that a large fraction of cortical interneurons arise in subcortical areas rather than in the cortical ventricular zone, and migrate tangentially into the cortex. The structures and molecules that guide this migratory path – a much longer and more convoluted one than that guided by radial glia – remain to be discovered.

2. Axonal projections and the formation of selective connections

Attempts to unravel how selective neuronal connections are formed in the developing brain began in earnest with histological studies of the developing brain, applied most decisively by Ramón y Cajal but also by many others. This work provided a dramatic illustration of embryonic neurons captured in the process of extending dendrites and axons, apparently in a highly stereotyped fashion. These pioneering anatomical descriptions provided circumstantial but persuasive evidence that the assembly of neuronal connections is orchestrated in a very selective manner. Sperry's studies in the 1940s also emphasised the utility of combining embryological manipulation and neuroanatomical tracing methods to probe the specificity of neuronal connectivity. In the 1980s and 1990s, attempts to clarify further the cellular mechanisms of axonal growth and guidance focused on reducing the apparent complexity inherent in the development of axonal projections to a few basic modes of environmental signalling and growth cone response. As a first approximation, the multitude of cues thought to exist in the environment of a growing axon was proposed to act in one of two ways: (1) at long range, through the secretion of diffusible factors; or (2) at short range through cell surface-tethered or extracellular matrix-associated factors. In addition, such long- and short-range cues were argued to act either as attractants or local factors permissive for axonal growth or, in a complementary manner, as repellants or factors inhibiting axon extension. What remained unclear after this phase of conceptual reductionism and simplification was the molecular basis of selective axon growth.

2.1. *The molecular era of axon growth and guidance*

Today, there is no longer a paucity of molecules with convincing credentials as regulators of axonal growth and guidance. This molecular cornucopia is the product of two main experimental approaches: in vertebrate tissues, the biochemical purification of proteins that promote cell adhesion and axonal growth; and in *Drosophila* and *C. elegans*, the application of genetic screens to identify and characterise mutations that perturb axonal projection patterns. Over the past decade, these two complementary approaches have often supplied convergent information, resulting in the compilation of a rich catalogue of molecules with conserved functions in the control of axonal growth in insects, worms, and vertebrates. One early advance in the molecular characterisation of proteins that control axonal growth came with the biochemical dissection of two major adhesive forces that bind neural cells, one calcium independent and the other calcium dependent. Over a hundred Ig domain-containing neural adhesion and recognition proteins have been identified, although the function of most of these proteins *in vivo* remains unclear. The major calcium-

dependent adhesive force binding vertebrate cells are the cadherin proteins. Cadherins have been shown to have major roles in the calcium-dependent adhesive interaction of virtually all cells in the vertebrate embryo, and have also been identified in *Drosophila* and *C. elegans*. The calcium-dependence of cadherin function can be mapped to a critical, calcium-binding domain required for protein stability. Cadherins, like Ig domain proteins, are now known to represent a very large family.

A third general adhesive system is that involved in the interaction of cells with glycoproteins of the extracellular matrix. Biochemical studies by many groups identified collagens, fibronectins, and laminins as key adhesive glycoprotein components of the extracellular matrix. The search for cellular receptors for these structurally distinct glycoproteins converged with the identification of integrins, a large family of heterodimeric integral membrane proteins. Integrins have prominent roles in cell-matrix adhesion within the nervous system and in virtually all other tissue types.

Thus, three main classes of neuronal surface membrane proteins – Ig domain proteins, cadherins, and integrins – appear to provide neural cells with the major adhesive systems necessary for the growth of axons. These proteins may also contribute to more selective forms of neuronal recognition.

Many additional proteins that are expressed more selectively and appear to have selective roles in axonal growth have now been identified. Genetic screens in *C. elegans* and biochemical assays of axon growth regulatory factors in vertebrates converged with the characterisation of netrins, a small class of secreted proteins with cell context-dependent axonal attractant and repellent activities.

A similar convergence of biochemical and genetic assays led to the isolation of the semaphorin/collapsin class of growth cone collapse-inducing factors and to the characterisation of a slit signalling pathway that appear to function both to repel axons and to promote axon branching.

Independently, *in vitro* assays to examine the molecular basis of the topographic mapping of retinotectal projections culminated in the identification and functional characterisation of ephrins: surface proteins that function as ligands for receptor tyrosine kinases of the Eph class. Ephrin-Eph kinase signalling is now thought to have a dominant role in the establishment of the molecular gradients used to form projection maps in the retinotectal system and in other regions of the CNS – apparently corresponding to some of the matching chemical labels postulated earlier by Sperry. Despite these advances, many aspects of the logic of axon guidance remain unclear. With the multitude of candidate cues now shown to possess repellent or attractant functions, we still need to understand why individual sets of molecules are used in particular cellular contexts. Are there unique, and as yet unappreciated, functions provided by one but not another class of guidance cue? Or is there simply ‘molecular opportunism’? That is, can similar steps in selective axon pathfinding be achieved by any one of a large and structurally unrelated group of guidance molecules?

One route to resolving such issues has been to define the signal transduction pathways triggered in growth cones by activation of receptors for guidance cues. Already, such studies have begun to lead to the molecular classification of biochemical signalling pathways and their modulators within the growth cone. They have also provided dramatic evidence *in vitro* that the ability of a growth cone to perceive an extrinsic signal as attractant or repellent can be modified by changing the ambient level of cyclic nucleotide activity. Further dissection of transduction mechanisms in the growth cone may thus help to clarify the logic that underlies the apparent selectivity of action of certain axonal growth and guidance factors.

Another critical but poorly resolved issue is that of determining which guidance factors genuinely have instructive roles in directing axon growth and which merely provide permissive signals that enable growth cones to respond to other, more critical, signals.

In sum, then we still do not have a clear picture of how even a single axonal guidance decision is determined. But, since many of the key molecular players have been discovered, it is now possible to ask biologically important questions about the strategies that axons use to reach their targets *in vivo*.

3. The formation of synapses

Axons can grow on their own, but it takes two cells to make a synapse. For this simple reason, our understanding of how axons connect with their targets has lagged behind knowledge of how they reach their targets in the first place. Nonetheless, during the 1970s and 1980s, many groups documented basic phenomena that underlie this crucial process: the organisation of presynaptic differentiation (especially the clustering of neurotransmitter-containing vesicles) by components of the postsynaptic apparatus; the organisation of postsynaptic differentiation (especially the clustering of neurotransmitter receptors) by the nerve terminal; and the localised synthesis of synaptic components at synaptic sites. Moreover, several candidate organising or inducing molecules were identified, based on their localisation or bioactivities. These studies provided a basis for rapid progress during the 1990s. Current views of this aspect of neuronal development derive largely from studies of one peripheral synapse, the neuromuscular junction. These studies have their origins in many classical physiological investigations of synaptic transmission at the neuromuscular junction. In particular, the ability to measure dynamic changes in the pattern of expression of acetylcholine receptors on the surface of muscle fibers as they become innervated provided many early insights into the cellular mechanisms by which the motor axon organises the elaborate programme of postsynaptic differentiation necessary for efficient synaptic transmission.

By the 1980s, powerful *in vivo* and *in vitro* assays to examine synaptic organisation under conditions of muscle denervation and reinnervation had been developed, and these facilitated biochemical efforts to purify neuronally derived factors with synaptic organising capacities. These efforts culminated in the identification of pre- to postsynaptic signalling pathways that appear to coordinate many aspects of the synaptic machinery in the postsynaptic muscle membrane. Pride of place goes to agrin, a nerve-derived organiser of postsynaptic differentiation. In the manner noted above for neuronal migration, related phenotypes of other mouse mutants led to the elucidation of a genetic pathway: in this case, one in which agrin is the signal, the tyrosine kinase MuSK is (a component of) the agrin receptor, the cytoplasmic protein rapsyn is an effector, and the dystrophin-glycoprotein complex (and especially its cytoplasmic component, α -dystrobrevin) modulates maturation and maintenance of the postsynaptic apparatus.

By similar means, synaptic laminins have been identified as retrograde messengers, though their precise roles remain poorly defined. A second set of nerve- and muscle-derived factors, the neuregulins which signal through ErbB class tyrosine kinase receptors, appear instead to control the local synthesis of acetylcholine receptor genes in muscle cells, and perhaps also to direct the local insertion of newly synthesised receptors at synaptic sites.

These molecular successes have provided the foundations of a comprehensive understanding of the steps involved in the formation and organisation of nerve-muscle synapses. Disappointingly, none of these molecules appears to play a major role at central synapses, but they do provide models of a molecular and cellular logic that may be generally applicable.

Nevertheless, several classes of proteins have been implicated in the formation of selective connections at central synapses. The cadherins represent one class of cell surface recognition protein that exists in large numbers. Diversity in cadherin structure can be enhanced dramatically through a process in which one of a chromosomally arrayed cluster of variable cadherin domain gene sequences is appended to a nearby constant region sequence. The molecular mechanism used to assemble such modularly-constructed cadherin proteins remains unclear, but the number of these variable domains is high, bringing the total

number of predicted cadherins to well over 100. The vast majority of cadherins are known to be expressed by neural cells. Studies of the patterns of expression of the classical cadherins have revealed a striking segregation of individual cadherins within functionally interconnected regions of the brain. In addition, cadherins are concentrated at apposing pre- and postsynaptic membranes at central synapses. Although intriguing, the link between selective cadherin expression and the specificity of synaptic connections remains to be demonstrated functionally. A second class of proteins with the potential for considerable structural variation is the neurexins. These are surface proteins identified originally by virtue of their interaction with the neurotoxin α -latrotoxin. Analysis of the potential for alternative splicing of the *neurexin* genes suggests, in principle, that 1000 protein isoforms can be generated, and at least some of these potential isoforms are known to be expressed by central neurons. In addition, a class of neurexin receptors termed neuroligins have been identified.

Recent studies have indicated a role for neurexin-neuroligin interactions in the formation of synapses. A third, highly-diverse class of neuronal surface proteins are the seven-pass odorant receptors expressed on primary sensory neurons in the olfactory epithelium. Several major classes of odorant (or pheromone) receptors have now been identified in vertebrates, and in total this class of receptors is thought to be encoded by over 1,000 distinct genes. This genetic diversity is likely to underlie the remarkable discriminatory capacity of the mammalian olfactory sensory system. The creative manipulation of odorant receptor gene regulatory sequences to map the central projections of olfactory sensory axons through reporter gene expression in transgenic mice has also revealed a precise anatomical convergence of sensory axons linked by common receptor gene expression to individual target glomeruli in the olfactory bulb. This finding poses the additional question of the mechanisms directing sensory axon targeting to individual glomeruli. Strikingly, manipulation of the pattern of expression of individual odorant receptor genes in transgenic mice results in a predictable change in the central projection pattern of olfactory sensory axons.

An intriguing implication of these findings is that olfactory sensory receptors function not only in peripheral odour discrimination but also in axon targeting, potentially providing a direct link between the sensory receptive properties of a neuron and its central pattern of connectivity.

4. The refinement of neuronal connections

Some simple behaviours, and even some moderately complex ones, are mediated by circuits that are 'hard-wired' – that is, they form in embryos without obvious need for activity or experience. These include reflexes, motor programmes, and even some social behaviours that ethologists have shown to be 'innate'. In contrast, more complex behaviours, including those that seem to be most deeply human, are shaped by experience. Indeed, at least in vertebrates, experience, once transduced into action potentials, shapes each nervous system to the unique needs of its owner. At the intersection of developmental neurobiology and psychology is the fascinating question of how epigenetic influences such as neural activity interact with genetic instructions to form and modify circuits. A modern consensus view holds that both genetic predetermination and use-dependent refinement of connections are important contributors to the organisation of mature circuits. The relative contribution of these two sets of factors are, however, likely to vary considerably with the particular neural circuit under study. One possibility is that circuits constructed early in evolution or at early stages in the development of an organism, as, for example, the spinal monosynaptic stretch reflex circuit, are established in a predominantly activity-independent manner. In contrast, the more sophisticated cortical circuits associated with the processing of cognitive information, which emerge later in evolution and development, may require functional validation for the establishment of final patterns of connectivity.

At the beginning of this decade, thinking about experience-dependent rearrangement was deeply influenced by two sets of data. The first was the evidence that target-derived neurotrophic factors affect the growth

and survival of neural inputs. It was natural to extend this neurotrophic hypothesis to synapses, and to suggest that activity-dependent release of related or even identical factors might provide a mechanism by which activity could affect synaptic strength. Indeed, many results were viewed as consistent with such a 'synaptotrophic' mechanism.

The second data set was largely that of Hubel and Wiesel and their students on ocular dominance, visual deprivation, and critical periods in cats and monkeys. Their results provided seemingly irrefutable evidence that the relative level and synchrony of activity exerted a powerful influence on brain circuitry. The implication that 'neurons that fire together wire together' was later supported by several independent lines of evidence. Amongst this evidence was the discovery of patterned waves of electrical activity in the retina – even before eye-opening – revealing that neurons that wire together do, indeed, begin by firing together.

Taken together, then, these results led to an intuitively satisfying model in which synchronous presynaptic and postsynaptic activity could couple release and uptake of synaptotrophins, thereby weakening or strengthening connections in rough accord with Hebb's postulate. Given this promising start, it is frustrating that the 'smoking synaptotrophic gun' remains undiscovered, or at least unrecognised. That is, despite clear evidence that neurotrophins modulate connectivity and synaptic efficacy, their precise role in linking activity to plasticity remains obscure. Likewise, despite clear evidence that activity modulates neural circuitry, there remains considerable controversy about which of the many effects of activity are permissive and which are instructive. This unfulfilled state of affairs, however, should not be grounds for discouragement: it is little wonder that such complex, 'systems-level' phenomena are slower to yield to cellular analysis than many of the unitary processes discussed above. Indeed, in our optimistic view, it should soon be possible to subject the synaptotrophic hypothesis to more critical tests, and there is every expectation that at least some of its main elements will receive direct support. Although the critical role of activity in the formation of neuronal circuits in the visual system and in many other regions of the CNS is well established, the precise nature of its contribution is less well defined. Information encoded by patterns of activity could be sufficient to direct certain connections. It remains possible, however, that for many neuronal circuits, a basal but unpatterned level of activity is all that is required. In this view, activity may simply permit neurons to respond to other signals that have more direct roles in the control of selective connections or may permit the maintenance of connections formed at earlier stages and through separate mechanisms. Evidence supportive of this latter view has come from studies on the role of visually-driven activity in the formation of orientation and ocular dominance columns in the developing visual cortex. Neural activity may therefore exert its influence in large part by consolidating connections that have been established earlier through mechanisms which have their basis in molecular recognition between afferent neurons and their cortical target cells.

5. Future studies of neural development and circuit assembly

Despite the dramatic advances of the past two decades, several important but unresolved issues cloud our view of the assembly of synaptic connections. These problems will need to be addressed before any satisfying understanding of neural circuit assembly can be claimed.

5.1. *Pathways of neuronal specification and the logic of neural circuit assembly*

One issue stems from the pursuit of mechanisms determining neuronal cell fate and controlling axonal pathfinding and connectivity as largely separate disciplines. With the many available details of cell fate specification and of the regulation of axonal growth and guidance, it is still not clear if and how the transcriptional codes that control neuronal identity intersect with the expression of the effector molecules that direct axonal connectivity. For example, in only a few cases have relevant genetic targets of the transcription factors that control early steps in neuronal identity been identified. Indeed, a superficial

survey of patterns of expression of transcription factors and axonal receptors for guidance cues reveals little obvious coincidence at the cellular level. Thus, the extent to which the regulated expression of genes that encode receptors for axon guidance cues depends on the sets of determinant factors implicated in earlier aspects of neuronal subtype identity remains unclear. Defining the full complement of transcription factors that specify the identity of an individual neuronal subtype and the molecular sequence of cell-cell interactions that guide the axon of the same neuron to its target is one obvious but laborious route to resolving this issue. Similarly, the relationship between transcription factor expression and other later aspects of neuronal phenotype, for example neurotransmitter synthesis and chemosensitivity, also remain unclear. In a few instances, cell-specific transcription factors have been linked to the expression of genes that control neurotransmitter synthesis. Nevertheless, the general logic linking transcriptional identity and the expression of the neuronal traits that confer specialised synaptic signalling properties and connectivity remains obscure.

Assuming, as seems likely, that these issues can be resolved in a relatively rapid fashion, what does the future hold for studies of neural development? Clearly, there will be interesting variation in the strategies used to establish selective connections in different regions of the developing brain and in different circuits. The documentation of these variations will provide a richer and more profound appreciation of the core principles of neuronal circuit assembly. But the reiteration of a few basic themes in different brain regions can sustain excitement in the field only briefly, and in any event will not provide an obvious intellectual bridge between studies of development and of the function of mature neuronal circuits.

5.2. *Genetically encoded sensors and modifiers of neuronal activity*

One additional contribution of neural development may emerge by taking advantage of the compendium of information now available on cell-specific gene expression in developing neurons and of the ease of genetic manipulation in mammals, notably the mouse. With these methods in hand, it may be possible to modify the function of highly-restricted classes of neurons in the adult animal and to assay resultant changes in the function of specific neuronal circuits.

One initial limitation in the application of information about neuronal subtype-specific gene expression during development is that the majority of such genes are transiently expressed. Thus, the normal temporal profile of gene expression does not permit direct tracing of the relationship between embryonic neuronal subtype identity and the physiological properties of the same neuronal subsets in the adult. This problem can now be overcome through the use of genetically-based lineage tracing methods. For example, genes encoding yeast- or bacterially-derived recombinase enzymes can be introduced into specific genetic loci by targeted recombination, to generate mouse strains. These can then be crossed with other genetically modified mice in which recombinase-driven DNA rearrangement results in the irreversible activation of reporter gene expression at all subsequent stages in the life of a neuron. This relatively simple methodology offers the immediate promise of providing a direct link between subsets of neurons defined at embryonic stages and the location, and functional identity, of these neurons within the mature CNS.

With the compilation of such lineage information, variants of this same basic genetic strategy can be used to modify the function of neuronal subsets at predefined times. One drastic method for eliminating neuronal function involves the activation of toxins in a neuron-specific manner, under precise temporal control, thus permitting the physical ablation of predefined populations of CNS neurons with a specificity unattainable by conventional lesioning methods. More subtly, specific populations of neurons could, in principle, be activated or inactivated reversibly in the adult animal through temporally regulated expression of ion channels or ligand-gated receptors that change the threshold for neuronal excitability. In addition, the development of transgenic mice methods for anterograde or retrograde transsynaptic transport of virally encoded marker proteins may provide information on neuronal connectivity in the CNS that cannot easily be extracted by

other anatomical tracing methods. In this way, the increasingly detailed molecular information that derives from attempts to examine the principles of neural circuit assembly during development should have clear application to the major problems of systems neuroscience discussed in the following sections of this review. At present, the routine application of these genetic methodologies is feasible only in the mouse, and thus the issue of linking studies obtained in lower mammals with information obtained in primates, and ideally in humans, still needs to be addressed.

Nevertheless, with advances in the resolution of functional imaging methods, and in the application of these methods to small mammals, the link between studies in mouse and primates can be strengthened. When this is achieved, the information that emerges from studies of the development of neural circuits may assume a more prominent place in the repertoire of experimental strategies that aim to decipher how such circuits function in the adult brain.

5.3. *Neural development and neurological and psychiatric disorders*

One additional area in which studies of neural development are likely to have significant impact is in the application of fundamental information on the specification of cell fate and the guidance of axons to problems posed by neurodegenerative diseases and traumatic injury to the nervous system.

As discussed above, we are beginning to obtain a rather detailed outline of the relationship between inductive signalling and the expression of cell-specific transcription factors that define cell fate. In some cases, details of these pathways have progressed to the point that certain transcription factors expressed by single classes of CNS neurons have been shown to be sufficient to direct neuronal subtype fate in a manner that is largely independent of the prior developmental history of the progenitor cell. If this is the case for the few classes of neurons in which inductive signalling pathways have been particularly well studied, it seems likely that similar dedicated determinant factors will exist for many other classes of neurons in the CNS. The identification of such factors may be of significance in the context of the many ongoing attempts to identify neural progenitor cells and then to drive them along specific pathways of neurogenesis. One outcome of such developmental studies may therefore be to rationalise strategies for reintroduction of fate-restricted neural progenitor cells into the CNS *in vivo*. In principle, these advances could offer the potential of more efficient cell replacement therapies in a wide variety of neurological degenerative disorders.

Similarly, the wealth of information on molecules that promote or inhibit axonal growth is likely to be of relevance for studies of axonal regeneration and repair. The pioneering studies of Aguayo and colleagues of the regenerative capacity of central neurons in a cellular environment composed of peripheral, rather than CNS, nerve cells revealed the potential of central neurons to regenerate. These studies prompted the search for molecules expressed by cells of the mature central nervous system that inhibit the growth of axons and for molecules expressed in early development that have the capacity to promote the growth of axons of CNS neurons. Progress in identifying axon growth-promoting and inhibitory factors may, therefore, eventually permit rational changes to be made in the environment through which regenerating axons in the mature CNS are required to project.

Of equal promise are studies to clarify the signal transduction pathways by which axons respond to these environmental cues. The elucidation of these pathways may permit a more general manipulation of axonal responses, for example, rendering axons insensitive to broad classes of inhibitory factors, or supersensitive to many distinct axonal growth-promoting factors. It may also be worth considering whether there is a common molecular basis for the marked differences in the regenerative capacity of different vertebrate species, evident in studies of both nerve and limb regeneration.

As noted above, the past decade has witnessed the identification of genes that control cortical migration – for example, the genes responsible for heterotopias and lissencephalies. Likewise, a few congenital neurological diseases have now been found to result from mutations of genes that encode axon guidance molecules. Defining the function of these genes is likely to require a more profound appreciation of the normal mechanisms of cortical development.

If we achieve this level of understanding, there is the possibility of providing satisfying cellular and molecular explanations of the role of developmental defects in cognitive disorders – Williams-Beuren and Rett syndromes, for example, or, conceivably, even schizophrenia or autism spectrum disorders.

These are distant and daring goals, certainly, but goals that are clearly visible and thus worth striving for.

Author's note

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