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Review

Molecular analysis of axonal target specificity and synapse formation

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Abstract. The development of neuronal connectivity requires the growth of axons to their target region and the formation of dendritic trees that extend into specific layers. Within the target region growth cones, the tips of extending axons are guided to finer target fields including specific subcellular compartments where they form synapses. In this article we highlight recent progress on molecular aspects of axonal subcellular target selection such as the axon initial segment or specific sublaminae of the vertebrate retina. We then discuss the very recent progress on the molecular analysis of synapse formation in the central nervous system, including the direction of differentiation into an inhibitory or excitatory synapse. Apparently, initial synaptic contacts are structurally and functionally modulated by neuronal activity, raising the question how neuronal activity can modify synaptic circuits. We therefore also focus on neural proteins that are up-regulated, secreted or converted by synaptic activity and, thus, might represent molecular candidates for experience-driven refinement or remodeling of synaptic connections.

Key words. Neurofascin; neuroligin/neurexin; SynCAM; thrombospondin; CPG15, FGF22; CALEB; tPA.

Introduction

The proper functioning of the nervous system requires the establishment of a precise and selective pattern of synaptic connectivity and an appropriate balance between excitatory and inhibitory synapses. To establish these contacts neurons must extend axons and dendrites, which are guided to their target region by a specialized structure, the growth cone, a highly motile structure at the tip of extending neurites. Growth cones respond to an array of molecular signals that are present in their local microenvironment. These signals activate axonal cell surface receptors and elicit specific signalling systems within growth cones, which might result in the reorganization of their cytoskeleton and consequently in an oriented extension towards specific directions.

A longstanding goal of many neuroscientists therefore is to define the molecular components involved in the guidance of axons to their target. Biochemical as well as genetic studies in the past 20 years have led to the identification of a large number of proteins that appear to be important for axonal extension and pathfinding. For a general overview by Goodman and Tessier-Lavigne, which has been widely accepted in the field of axonal guidance, these factors have been categorized as being attractive or repulsive to growth cones or being both – depending on which type of growth cone is being analyzed [1, 2]. These proteins belong to different structural protein families, including neural members of the immunologlobulin (Ig) superfamily, semaphorins, netrins, ephrins, neuropilins, plexins, Eph kinases and several extracellular matrix glycoproteins. Some of these proteins are associated with the plasma membrane and, thus, might serve as receptors for secreted ligands, extracellular matrix components, or, alternatively,

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they might interact with other proteins expressed on neighbouring cells. Some of the guidance factors are secreted from final or intermediate target regions and are therefore thought to act over longer distances, while others mediate direct cell-to-cell contact and therefore act only between neighbouring cells. Recently, this list of guidance components was expanded by demonstrating that morphogens such as WNT and SHH also steer growth cones of spinal cord neurons into specific directions [3–6]. Furthermore, guidance components appear to cooperate with factors normally acting in other cell functions. For example, the pathfinding of axons of olfactory sensory neurons to specific glomeruli of the olfactory bulb is regulated by odorant receptors in combination with the Eph kinases and ephrins [7].

In this article we concentrate on recent advances in the molecular analysis of subcellular targeting and synapse formation in vertebrates (fig. 1) – two developmental steps that are of immense importance in neuronal network formation and where substantial progress has been made recently. For a more detailed description of axonal guidance protein families, we refer to recent reviews in addition to those already given above [8–10]. For progress on synaptic target selection made in invertebrates, we refer to the article by Ackley and Jin [11]. Subcellular target selection and synapse formation can occur in the absence of neuronal activity and appear to be governed primarily by genetically determined mechanisms [12], however, it is generally assumed that neuronal activity modifies the initially established circuits [13, 14]. Therefore, we will also review articles on molecules that are modulated by neuronal activity and which might therefore refine neuronal circuits.

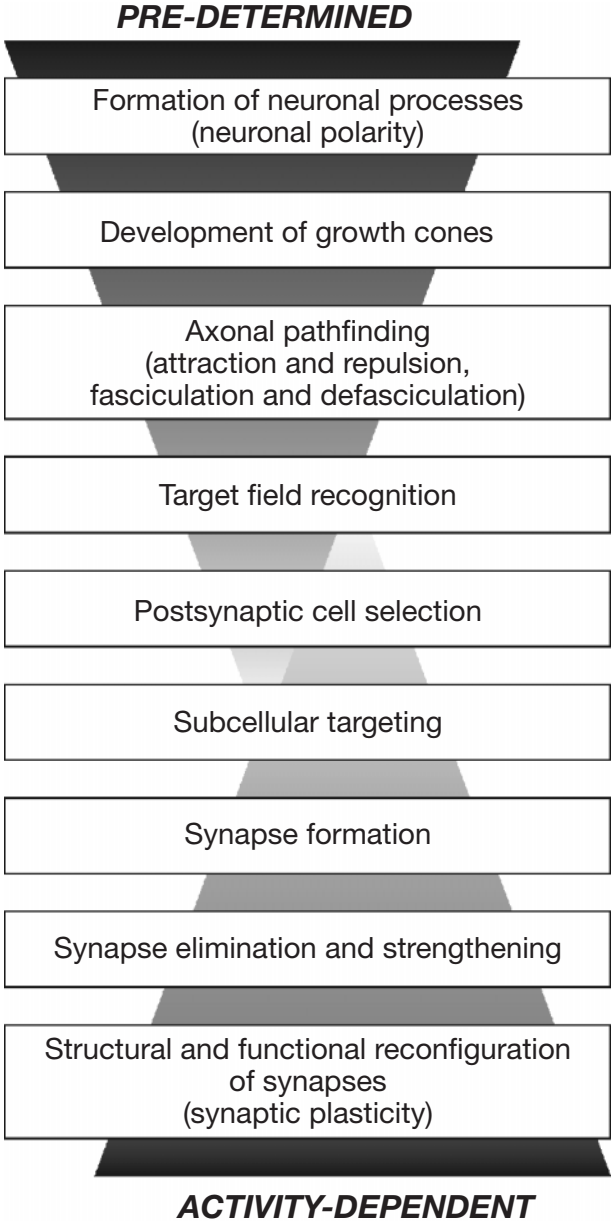

Figure 1. Development of neuronal circuits. The formation of neuronal circuits involves a series of steps. It starts with the formation of axonal and dendritic processes and ends with mechanisms of synaptic plasticity. As development proceeds, activity-dependent processes might be implicated in the formation or modulation of neuronal circuits. This article discusses recent developments regarding molecules involved in the generation of neuronal circuits with a focus on subcellular targeting, synapse formation and activity-dependent refinement.

I Molecular analysis of axonal subcellular and sublaminae-specific target selection

Once axons arrive at their target region and before synapse formation begins, growth cones are steered into finer target fields such as a selective lamina or a specific subcellular compartment on a specific postsynaptic cell. Many principal neurons consist of functionally distinct compartments, which increases the overall computational possibilities of single neurons. For example, axon initial segments (AISs) on large projection neurons are the sites of GABA (gamma-aminobutyric acid)ergic inhibition and initiation of action potentials.

A neurofascin gradient on Purkinje cells is important for GABAergic innervation at their axon initial segment

How precisely synapses are formed on subregions of large projections neurons in vertebrates is less understood. A recent analysis has shed some light on subcellular targeting mechanisms and emphasized the role of ankyrinG and neurofascin, a member of the L1 subfamily of Ig-like proteins. Purkinje cells, the principal neurons of the cerebellum, receive inhibitory GABAergic input from two different neurons within two different subcellular regions: stellate cells synapse onto dendrites of Purkinje cells in

the molecular layer, and basket cells contact Purkinje cells at their AIS where they form so-called pinceau synapses. To study the molecular mechanisms underlying the subcellular targeting of axons of basket cells on Purkinje cells, Huang and associates generated BAC transgenic mice that express green fluorescent protein (GFP) under the control of a GABAergic cell type-specific transcription promoter [15]. This technique allowed a detailed microscopical analysis of the path taken by basket cell axons when approaching and contacting the Purkinje cells. Basket cell axons were found to initially contact the soma of Purkinje cell at P8 before growing to and terminating on its AIS at P12. Basket cell axons then form exuberant terminal branches around the AIS to subsequently establish the full synaptic pattern of connectivity. These observations suggested the presence of local molecular cues on the plasma membrane or in the extracellular matrix of Purkinje cells. Previously published studies by Davis et al. had shown that a specific form of neurofascin (NF186) is concentrated at the AIS of Purkinje cells [16, 17] making this molecule a good candidate for directing the selection process. Consistent with this finding, Ango et al. have now observed a prominent subcellular gradient of neurofascin along the AIS-soma-dendritic axis with high concentrations at the AIS (fig. 2). This gradient of neurofascin was established before the GABAergic innervation and was disrupted if ankyrinG, an adaptor protein that binds to a specific intracellular segment of neurofascin, was genetically deleted in mice [18]. The consequence of the absence of this gradient was a loss of directionality of basket cell axons on Purkinje cells. Basket cell axons wandered around the Purkinje cell soma and followed the ectopically located neurofascin. Despite this mistargeting and the overall reduction in the number of pinceau synapses, several basket cell axons reached the AIS, suggesting that in addition to neurofascin other molecular guidance cues might be involved. However, NrCAM and CHL1, two proteins highly related to neurofascin (see below), are not involved in pinceau synapse formation as determined from the analysis of NrCAM- and CHL1-deficient tissue.

Neurofascin belongs to the L1 subgroup of the Ig superfamily, which in vertebrates consists of four members: L1 itself, neurofascin, NrCAM and CHL1 [19, 20]. They are type I transmembrane proteins that have been localized to growth cones and processes of postmitotic neurons where they mediate cell-cell contacts, axon outgrowth and bundling. All four members associate with the ankyrin-spectrin-based membrane skeleton via a specific stretch within their cytoplasmic segments, and this interaction localizes them to specific subcellular compartments. AnkyrinG has two neurofascin binding sites and therefore might form multimers of neurofascin. Together with the underlying βIV spectrin tetramer, which is also concentrated at the AIS and which has two binding sites for ankyrinG, a local adhesion compartment might be generated at the AIS [21, 22]. In contrast to L1, neurofascin is expressed as a complex population of isoforms during development that arise through alternative splicing [23]. This extensive alternative splicing in the extracellular region of neurofascin regulates binding to its ligands NrCAM, contactin1 (F11), axonin-1 (TAG-1) and tenascin-R [24, 25]. The alternatively spliced proline-alanine-threonine-rich segment (PAT segment) is of particular importance since it might extend neurofascin well above the cellular glycocalyx to allow it to interact with neighbouring structures. Which of the currently known or yet unknown ligands on basket cells interacts with neurofascin on Purkinje cells remains to be determined.

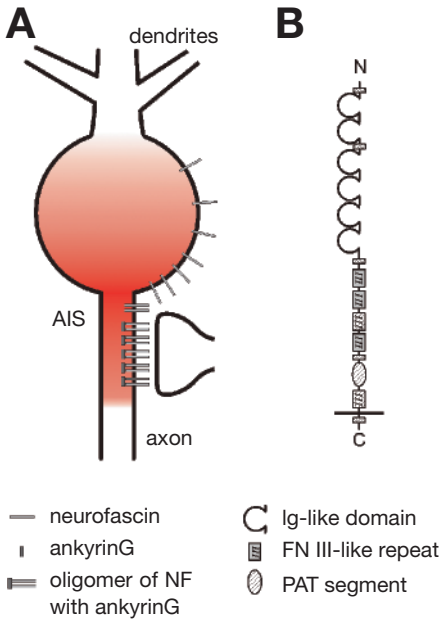

Figure 2. Neurofascin, an Ig superfamily member involved in subcellular targeting at the axon initial segment (AIS) of Purkinje cells. (A) A gradient of neurofascin and ankyrinG with an maximum at the AIS is expressed in Purkinje cells, which are essential for subcellular targeting of GABAergic synapses from basket cells at the AIS. Disruption of the ankyrinG gene results in loss of the neurofascin gradient (adopted from [15]). (B) Schematic representation of neurofascin, which belongs to the L1 subfamily of Ig-like proteins. Hatched areas indicate alternatively spliced small segments or domains. The N-terminal or C-terminal half of the fifth FNIII repeat may be spliced individually. The PAT segment (proline/alanine/threonine-rich) may be highly glycosylated, which may induce an extended structure within neurofascin (adopted from [19]).

Sidekick proteins are involved in determining sublamina-specific synapse formation within the inner plexiform layer of the vertebrate retina

Subcellular targeting also appears to be a result of a restricted growth of axons and dendrites to individual

laminae and sublaminae by which synaptic specificity might be determined. Such a lamination exists within the vertebrate retina, where the soma of the retinal ganglion cells are located to the innermost layer and their dendrites extend and arborize within the adjacent inner plexiform layer. This inner plexiform layer can be further subdivided into different sublaminae in which subclasses of retinal ganglion cells are specifically connected to processes from amacrine and bipolar cells. These neurons reside with their cell bodies in the inner nuclear layer, which is close to the inner plexiform layer. The correct wiring of synapses on subtypes of retinal ganglion cells within these sublaminae of the inner plexiform layer might be critical for processing visual information [26]. In searching for molecular determinants involved in the laminar-specific establishment of initial synaptic contacts, Yamagata et al. screened single-cell libraries from individual retinal ganglion cells to isolate genes differentially expressed in subsets of these neurons [27]. They identified two Ig superfamily members termed sidekick (Sdk)-1 and -2 which are orthologous to the product of the *Drosophila* sidekick gene. This gene controls photoreceptor differentiation in the fly eye [28]. Each sidekick protein in the vertebrate retina is expressed at synaptic sites, in non-overlapping subsets of postsynaptic retinal ganglion cells and the corresponding sets of presynaptic amacrine and bipolar cells. These sidekick-rich synapses are concentrated in narrow sublaminae of the inner plexiform layer. For example Sdk-2 is highly concentrated in sublaminae S2, with lower levels in S4, while Sdk-1 reveals an opposite expression pattern. Neither Sdk was found in sublaminae S1, S3 or S5 (fig. 3). These observations suggested that homophilic sidekick interactions may promote lamina-specific connectivity. Consistent with this interpretation, ectopic expression of sidekicks in the retina diverted neuronal processes from sidekick-negative to sidekick-positive synaptic layers [27].

The *sdk* genes code for proteins with six N-terminal Ig domains, 13 fibronectin type III repeats, a single transmembrane domain and a cytoplasmatic segment of about 200 residues with a putative PDZ domain binding stretch [27]. In vitro cell adhesion assays and bead-binding experiments indicated that sidekicks bind mainly or exclusively through homophilic interactions [27, 29]. Domain deletions and the use of synthetic peptides revealed that the first and second Ig domains of Sdk-1 and Sdk-2 and a specific peptide stretch in the second Ig domain are most important to mediate homophilic cell adhesion [29].

Conclusions

A precise selection of a subcellular compartment by the growth cone affects the distribution of inputs into a single neuron and therefore has a strong influence on the integration of synaptic inputs. Consequently, incorrect subcellular targeting might modulate circuit function and neuronal plasticity, including an imbalance of excitation and inhibition. The two studies discussed in the above paragraph emphasize the role of Ig superfamily members in target selection. Ango et al. demonstrated that neurofascin linked by ankyrinG at the AIS is a key determinant in the subcellular targeting of basket cell axons on Purkinje cells, while the expression of sidekicks in pre- and postsynaptic retinal neurons in a non-overlapping manner guide axon and dendritic terminals to specific sublaminae. Since sidekicks label only 2 out of more

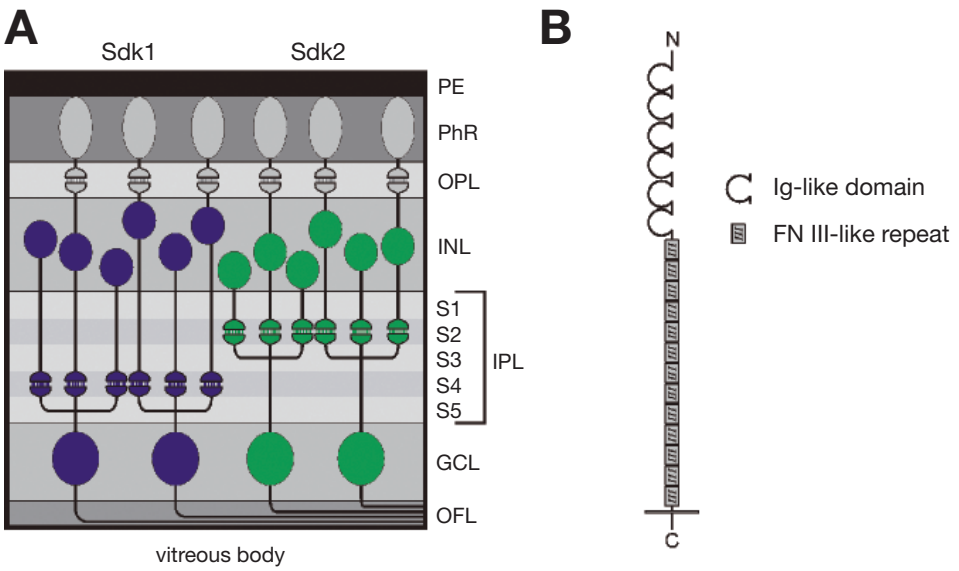

Figure 3. Sidekick proteins determine sublaminae specificity within the retina. (A) Expression of Sdk-1 and -2 in specific sublaminae of the inner plexiform layer of the vertebrate retina may contribute to the development or stabilization of sublaminae-specific connectivity (adopted from [27, 163]). OFL, optic fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; S1-S5, specific sublaminae of the IPL; INL, inner nuclear layer; OPL, outer plexiform layer; PhR, photoreceptor cells; PE, pigment epithelium. (B) Scheme of sidekick-1 or -2. The first and second Ig domains are important for homophilic binding.

than 10 sublaminae within the retinal inner plexiform layer, further molecules determining sublaminae specificity might be discovered in the future. These will most likely belong to a different protein family, since additional sidekick genes in mouse or human genomes do not exist. Although it is currently unknown whether neurofascin and sidekicks mediate the initial formation or stabilization of certain synapses, both studies suggest that the choice of the subcellular target appears to be independent of synaptic communication and primarily determined by activity-independent mechanisms.

II Molecular analysis of synaptogenesis in the CNS

Subcellular target recognition is followed by the assembly of the synapse between the presumptive pre- and postsynaptic neuron. Synapses are sites of unique cell-cell communications, which are asymmetrical and selectively formed between partner cells. Synaptogenesis is therefore a critical step in network generation, and molecules implicated in inducing synapses are of particular interest. Much of our current knowledge on synapse formation is based on investigations of the neuromuscular junction because of its relatively large size, accessibility and ability to regenerate. These studies have led to the identification of the extracellular matrix glycoprotein agrin, MuSK and intracellular signalling components within the myotube [30, 31]. Although agrin is expressed in the developing CNS its function remains to be determined at central synapses and is currently controversial [32]. While the acute suppression of agrin expression or function resulted in altered CNS synapse function [33,34], CNS neurons from mice carrying a targeted deletion of the agrin gene developed normal synaptic communication patterns [35, 36].

Synaptogenesis starts with the initial contact between a growth cone and the target cell; however, it is also conceivable that in the CNS, a substantial portion of synapses may develop from one of the many existing contacts a neuron has, while others remain normal cell-cell contact sites. Subsequent changes then appear to be regulated by reciprocal contact-mediated cell-cell communication. Although several of the structural components of the synaptic machinery are pre-assembled before synapses start to form [37, 38] contact-mediated processes play a more prominent role in synaptogenesis on CNS neurons than at the neuromuscular junction [39]. Molecules commonly classified as cell adhesion proteins are therefore considered to be crucial for the generation and maintenance of CNS synapses [40]. One of the most extensively studied groups of cell adhesion proteins in this context is the cadherin family, which also participate in the development of dendritic spines [41, 42] and which mediate their intracellular activities via β-catenin [43, 44].

Here we review very recent discoveries regarding factors that appear to be important for synapse induction on vertebrate central neurons but exclude the cadherins, which have received attention elsewhere [45]. For an overview of synaptogenesis at the neuromuscular junction, we refer to recent reviews [30, 31, 46].

Neuroligins on postsynaptic and β-neurexin on presynaptic neurons form trans-synaptic links and influence synaptic differentiation

Biochemical approaches led to the identification of the neurexin-neuroligin system

Neuroligins are postsynaptic transmembrane proteins that bind in a Ca^{2+}-dependent manner to β-neurexins on the presynaptic site (fig. 4). Originally, neurexin-Iα was isolated as binding protein of the black widow spider venom α-latrotoxin by Südhof and colleagues more than 10 years ago [47, 48]. Neurexins are transmembrane proteins with a short cytoplasmic region of 55 amino acid residues [47, 49]. Three genes (neurexins I–III) have been identified from which in principle two forms are always generated by two promoters (α and β) that differ only in their extracellular but not in their intracellular part: the longer α- and the shorter β-neurexin resulting in six neurexins (Iα, Iβ–IIIβ) [50, 51]. Additional diversity is generated by extensive alternative messenger RNA (mRNA) splicing with consequences on binding. For example, neuroligins bind to β-neurexin only if a specific insert (splice insertion 4) is lacking in neurexin-1β [52, 53]. A characteristic feature of neurexins is the LNS domain (laminin/neurexin/sex hormone binding globulin repeats; also termed laminin G-like domains, LG) from which six are found in α-neurexins and one in β-neurexins and that folds a β-sandwich structure [54]. The different neurexins are found throughout the brain but exhibit distinct differences in the intensity of expression. It is likely that neurons co-express multiple neurexins but in different combinations [55].

Neuroligins were discovered because they can be purified on an immobilized β- but not α-neurexin column [56]. Neuroligins contain a large extracellular segment that is homologous to the α/β-hydrolase fold domain of acetylcholinesterases and other serine hydrolases that dimerizes on the plasma membrane but is catalytically not active [57, 58]. In humans five and in rodents four neuroligins have been identified that are widely expressed in brain. Similar to the neurexins, the mRNAs of neuroligins are alternatively spliced, although to a lesser extent [59].

Binding between both type of proteins was demonstrated in biochemical as well as in typical cell-cell aggregation experiments from which a trans binding could be transduced [52, 57]. Only β-neurexins bind to

neuroligins in the biochemical assays, although α-neurexins contain the extracellular segments of β-neurexins required for binding. α-Neurexins serve as receptors for neurexophilins, a family of neuropeptide-like proteins [60, 61]. In contrast, both neurexin forms are found to interact with the cell surface protein dystroglycan (fig. 4) [62].

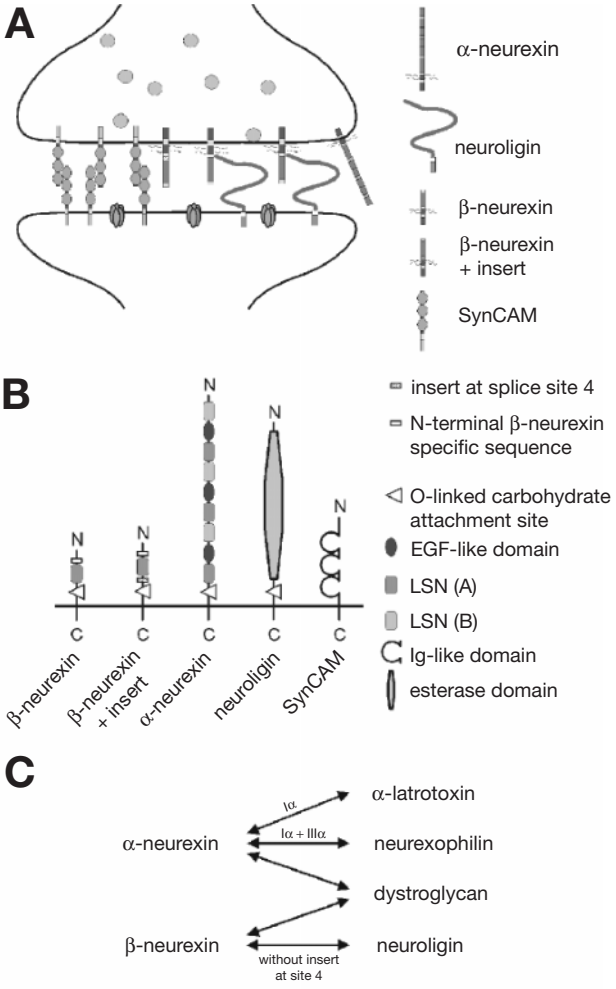

Figure 4. Diagram of a mature synapse and domain structures of synaptic adhesion proteins. (A) Neuroligin and β-neurexin form a heterophilic binding pair on synapses expressed on postsynaptic and presynaptic sides, respectively. SynCAM is expressed on both sides and binds homophilically. In rodents there are known four neuroligins. Neuroligin-2 is preferentially expressed on inhibitory synapses, while neuroligin-1 is found at excitatory synapses. (B) Scheme of neurexins, neuroligins and SynCAM. LNS, laminin A/neurexin/sex hormone-binding globulin repeats (also termed laminin G-like domains, LG). (C) Heterophilic binding profile of extracellular regions of neurexins. Bindings are indicated by arrows. It is not known whether specific neurexins have a preference for individual neuroligins. It is also not understood why α-neurexins do not bind to neuroligins. All α-neurexins may bind to neurexophilins; however, only Iα and IIIα have been tested so far. Intracellularly, neurexins bind to CASK, Mint1, Mint2 and synaptotagmin (not shown).

Neuroligins and neurexins induce synaptic specializations in cultivated neurons

To study the function of neuroligins, Scheiffele and Serafini developed an in vitro assay where non-neuronal cells such as HEK293 overexpressing neuroligin-1 or -2 by transfection were co-cultured with pontine neurons [63]. This co-cultivation resulted in the induction of presynaptic specializations in those neurites that grow over the surface of neuroligin-expressing cells as shown by staining with antibodies to presynaptic vesicle proteins. Other tested proteins, such as agrin, ephrinB1, TAG-1 (axonin-1) and N-cadherin or mutated forms of neuroligin-1, did not induce vesicle clustering [63–65].

One question that evolved from this pioneering work was whether the β-neurexin-neuroligin binding pair can also mediate postsynaptic differentiation. To address this point, in principle, the following in vitro approaches were used: application of soluble forms of neurexins, cocultivation of neurons with non-neuronal cells expressing β-neurexin, and overexpression or RNA interference knockdown of neuroligins in neurons. Clustering of postsynaptic proteins and recordings of postsynaptic currents served as read-out in these assays. The same group mentioned above then overexpressed neuroligin-1, -2 or -3 in cultivated neurons and observed stimulation in the formation of excitatory postsynaptic specializations including the intracellular scaffolding protein PSD-95 and neurotransmitter receptors such as for N-methyl-D-aspartate (NMDA) [66]. Consequently, RNA interference experiments resulting in a downregulation of neuroligin-1, -2 or -3 (single or in combination) diminished the numbers of excitatory presynaptic puncta. In line with these observations it was found that neurexin-1β expressed on non-neuronal cells or attached to beads induce clusters of neuroligin-1 or -2 and PSD-95 as well as other postsynaptic components, including the NR1 subunit of NMDA receptors in hippocampal neurons. Similar observations were made on inhibitory synapses by studying gephyrin, a postsynaptic scaffolding protein, and $GABA_A$ receptor subunits [67, 68]. Domain mapping experiments indicated that the LNS domain of neurexin-1β is essential for induction of postsynaptic clusters which could not be replaced by a related LNS domain from the extracellular matrix glycoprotein agrin.

Pre- and postsynatic scaffolding proteins bind to the intracellular segments of neurexins and neuroligins

How binding of neuroligins to presynaptic β-neurexins initiates presynaptic differentiation is currently unknown. It is conceivable that neuroligins act by dimerizing β-neurexins or by immobilizing β-neurexins within the plane of the plasma membrane, leading to nucleation of presynaptic scaffolding components on the cytoplasmic segment of β-neurexins. These initial complexes may recruit additional β-neurexins, and subsequently

neuroligins generate a local adhesion domain that induces pre- and postsynaptic signalling [64]. Furthermore, neurexins are known to associate with the presynaptic scaffolding proteins CASK and Mints and also to interact with synaptotagmin. These observations might therefore indicate that the cytoplasmic segments of β-neurexins establish a direct link to presynaptic scaffold and vesicles [69–71]. Similarly, preformed microdomains of presynaptic β-neurexins could aggregate neuroligins which then cluster postsynaptic scaffolding proteins followed by associations with other components of the postsynaptic apparatus. The scaffolding protein PSD-95 could induce subcellular clustering of neuroligin-1 in addition to other structural elements within the cytoplasmic segment of neuroligin-1 [72–76]. Such a primarily cell-cell contact-based clustering mechanism would be distinct from the differentiation of the neuromuscular junction, where secreted agrin induces acetylcholine receptor clustering via the MuSK signalling system [30].

β-Neurexin-neuroligin interaction might influence the balance between inhibitory and excitatory synapses

Brain function appears to be critically dependent on the balance between excitation and inhibition, and an imbalance might result in several neurological diseases, such as epilepsy or autism [77, 78]. In this context observations on the subcellular localization of neuroligins are of special importance: while neuroligin-1 is primarily found at the postsynaptic side of excitatory synapses, neuroligin-2 is expressed predominantly in inhibitory synapses in vivo [79, 80] and in vitro [67]. Although all three neuroligins (number 4 has not been tested so far) are able to induce both inhibitory as well as excitatory synaptic spots [72, 81], the question arose whether specific neuroligins could influence differentiation into excitatory or inhibitory synapses. Although no conclusive answers are yet possible, some experiments, indicated that the manipulation of the expression of neuroligins in combination with PSD-95 influenced the balance between excitation and inhibition in vitro: transfection of neuroligin-2 was found to be more potent in induction of inhibitory terminals, and neuroligin (NL1,2,3) knockdown in neurons from the hippocampus by RNA interference resulted in reduced frequency of miniature inhibitory postsynaptic currents, while those of excitatory synapses were only slightly affected [66]. Another recent report also described alteration of the ratio of excitation and inhibition by overexpression of neuroligin-1 in combination with PSD-95 [72]. In particular, PSD-95 decreased the number and size of presynaptic inhibitory terminals that were induced by neuroligin-1. These immunocytochemical studies were supported by whole-cell voltage-clamp recordings, which revealed a decrease of miniature inhibitory postsynaptic current (IPSC) frequency, but not of miniature EPSC frequency if neuroligin-1 is co-expressed with PSD-95. In summary, the current studies suggest that neuroligins affect the direction of development into an inhibitory or excitatory synapse in vitro. An alternative but less interesting explanation of the studies described above might be that inhibitory synapses are more vulnerable to molecular changes during development than excitatory terminals.

The importance of the neuroligins in circuit formation and/or function is supported by the observations that neuroligin-3 and -4 are linked to rare forms of mental retardation and autism in humans [65, 82–85] (but see also: [86–88]), however the detailed analysis of their function during postnatal development in knockout mice as well as other models is essential to confirm the conclusions derived from the in vitro experiments described above. Preliminary information indicates that adult single knockouts and double knockouts of neuroligin-1 and -2 are behaviourally normal [79]. The absence of all three α-neurexins, which do not bind to neuroligins but share with β-neurexins an identical cytoplasmic segment, results in a reduction of GABAergic but not glutamatergic synapses in vivo. These findings suggest that the formation of GABAergic synapses is indeed dependent on α-neurexin signalling during development. However, as discussed by the authors, it is also conceivable that GABAergic synapses are more sensitive to genetic manipulation than glutamatergic synapses. In any event, the primary in vivo function of α-neurexin is assumed to establish a linkage between presynaptic Ca^{2+} channels and other components of the presynaptic machinery [89,90], but additional electrophysiological recordings suggest that the function of α-neurexins might be considered to be broader. Their absence also caused a decrease in NMDA receptor function [91].

SynCAM, a homophilic Ig superfamily member that is expressed on pre- and postsynaptic sites induces presynaptic differentiation in vitro

Although neuroligin/neurexin signalling is important for synapse formation in vitro other factors have recently been identified that appear to support synapse formation as well. One component that functionally overlaps with neuroligin/neurexin is SynCAM (also termed TLSC1, IGSF4 or SgIGSF), an Ig superfamily member expressed both pre- and postsynaptically (fig. 4) [92, 93]. In the in vitro bioassay for studying the formation of synapses described above, it induces presynaptic differentiation in hippocampal neurons. If expressed together with glutamate receptors, it also stimulates postsynaptic differentiation and promotes excitatory synaptic transmission. This induction depends on the cytosolic sequence of SynCAM that might bind to PDZ-containing scaffolding proteins. Although chemically distinct from each other, neuroligin-1 and SynCAM appear comparable in such

co-cultures which suggests that both proteins activate a conserved signalling pathway, while other adhesion proteins, such as L1, NCAM-140 and N-cadherin are unable to induce presynaptic differentiation in this system [94]. In contrast to the neuroligin/β-neurexin binding pair, SynCAM reveals homophilic binding via three extracellular Ig domains independent of Ca^{2+} and therefore lacks directionality. As for neuroligin/neurexin, the in vivo function of SynCAM is currently unclear.

Factors secreted by astrocytes: thrombospondins and cholesterol promote synaptic differentiation

Protrusions of astrocytes are found to surround and ensheathe synapses, and in mammalian brains the bulk of astrocytes are generated when synapse formation occurs. Both observations suggest that astrocytes might play a role in the differentiation of synapses. To test this hypothesis, Pfrieger and Barres used highly enriched rat retinal ganglion cells and co-cultivated these with astrocytes. In the absence of astrocytes, retinal ganglion cells generated less and inefficient synapses in comparison to retinal cultures in the presence of secreted factor(s) from astrocytes [95–97]. To identify these secreted components, two-dimensional gel electrophoresis patterns of membranes from retinal ganglion cells grown in the presence or absence of glia-conditioned cell culture medium were compared. These studies resulted in the identification of apolipoprotein E that appeared upregulated in astrocyte-treated cultures and most likely is of glia origin [98, 99]. Apolipoprotein E-containing lipoproteins serve as cholesterol carriers, suggesting that cholesterol complexes with apolipoprotein E might be relevant or might be a limiting factor for the differentiation of retinal synapses (fig. 5). Indeed, application of cholesterol increased the frequency of spontaneous EPSCs, and drugs that blocked cholesterol synthesis in astrocytic cultures removed the effect of glia-conditioned medium on synapse differentiation. Furthermore, immunostaining of cholesterol-treated retinal cultures revealed an increase in the number of puncta of presynaptic vesicle proteins. Massive synaptogenesis therefore depends on cholesterol production by glial cells that is delivered to neurons by apolipoprotein E-containing lipoproteins [99]. Whether cholesterol also has an influence on the number of release sites as suggested by Mauch et al. is controversial [98, 100], but it may have an additional role in the differentiation of dendrites [101].

Independent of the studies described above, Christopherson et al. began to search for glia-derived factors enhancing synapse formation. The synaptogenic activity bound to heparin columns and appeared to be very large. These authors therefore tested a panel of large extracellular matrix glycoproteins to see whether they would increase synapse numbers in culture and observed that specifically thrombospondins 1 and 2 increased the numbers of synaptic puncta in a dose-dependent manner [100]. Thrombospondins are oligomeric extracellular matrix proteins composed of several protein domains that were initially identified in the context of platelet activation and cell adhesion [102]. They are known to be generated by astrocytes and promote neurite extension [103, 104].

Thrombospondin-induced synapses are ultrastructurally normal and express several pre- and postsynaptic marker proteins. These synapses are presynaptically active, as indicated by uptake of the styryl dye FM2-10 or of an antibody to the luminal domain of the vesicular protein synaptotagmin. But surprisingly electrophysiological recordings revealed no influence of thrombospondins on the frequency of spontaneous excitatory postsynaptic

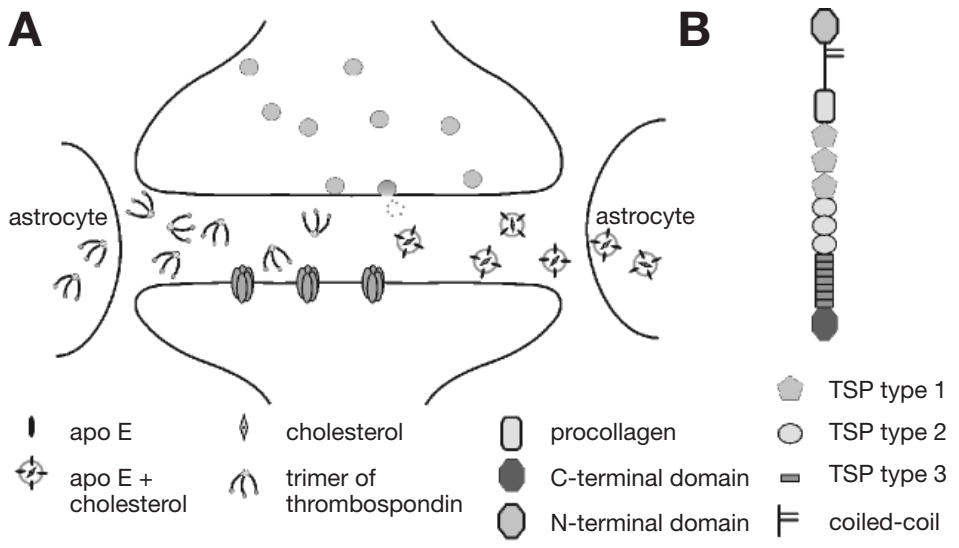

Figure 5. Factors released by astrocytes that influence synaptic differentiation. (A) Cholesterol bound to apolipoprotein E-containing lipoprotein particles is secreted by astrocytes and taken up by neurons. Similarly thrombospondin-1 and -2 are released from astrocytes and promote synapse formation. (B) Scheme of the thrombospondin polypeptide subgroup A, which forms trimers.

currents (EPSCs) due to a lack of α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA) receptor responsiveness. These newly formed synapses are therefore postsynaptically silent. One explanation for this unexpected result is that another signal needs to be secreted by astrocytes to activate glutamate receptors. Consistent with the in vitro observations is the finding that mice deficient for thrombospondin 1 and 2 reveal a decrease in the number of pre- and postsynaptic puncta, possibly reflecting a decrease in synapse numbers. Single knock-outs did not reveal a decrease in synaptic puncta, suggesting that thrombospondins can be replaced by each other. In summary, these findings revealed that astrocyte-secreted thrombospondins are sufficient to induce the formation of ultrastructurally normal CNS synapses, most likely by affecting the localization of synaptic proteins.

A subfamily of FGF members (FGF22, -7, -10) are target-derived factors involved in presynaptic organization via FGFR2

As soon as the axon arrives at its target region, the presynaptic neuron accumulates the machinery required for the calcium-dependent vesicular release of the neurotransmitter at the contact site of the postsynaptic neuron. This cascade of events might imply that factors released by the postsynaptic target cell regulate presynaptic development. These soluble factors might act in concert with the above-described cell-cell contact proteins but have the advantage of being able to act over longer distances. To identify such target-derived components with presynaptic differentiation activity, Sanes and associates used an assay that analyzes the redistribution of the presynaptic vesicle protein synapsin in cultivated chick motoneurons. High salt extracts from forebrains of early postnatal mice induced motoneuron branching and aggregation of presynaptic vesicles but did not affect neurite extension. Vesicle recycling experiments using the fluorescent styryl dye FM4-64 demonstrated that these newly formed vesicle clusters are functionally active. Purification of this activity by a combination of chromatographic steps resulted in the identification of FGF22 as a presynaptic organizer protein [105]. Together with FGF7 and -10 FGF22 forms a subfamily within the large family of FGF proteins, which consists of more than 20 members that can interact with four distinct FGF receptors [106, 107]. To assay whether the observed in vitro effect is specific for FGF22, several other members of the FGF family were tested for vesicle aggregation, neurite branching and elongation. FGF7 and FGF10, the closest relatives of FGF22, caused the same effects in the in vitro assay while other members had no effect at all or only caused branching but not vesicle aggregation or vesicle aggregation and neurite extension.

To relate these in vitro studies to the in vivo function, the distribution of FGF22 and its receptor FGFR2 were analyzed in the cerebellum. FGF22 was expressed by granular cells and its receptor (most likely its isoform FGF2b) by pontine and vestibular neurons that innervate granular cells. Cultivation of pontine neurons in the presence of a soluble extracellular fusion protein of FGFR2b diminished the ability of FGF22 to induce vesicle-rich varicosities, supporting the assumption that FGF22 mediates its effects via FGFR2b. Similarly, injection of this fusion protein into the lateral ventricle of the brain at P3 caused a reduction of synaptophysin-positive aggregates in the granular layer of the cerebellum at P8. Postnatal inactivation of FGFR2 by a conditional allele of FGFR2 and Cre recombination resulted in a significant inhibition of presynaptic differentiation within the granular layer of the cerebellum. In summary, these investigations provided evidence that FGF22 and its direct relatives are target-derived organizers of presynatic vesicles in cerebellar mossy fibers.

Conclusions

Impressive studies have been performed in recent years and have begun to characterize proteins involved in synapse formation in the CNS. Several proteins, secreted and membrane associated, appear to act in concert to form a CNS synapse. The transmembrane proteins neuroligin and neurexin constitute an asymmetric pair of the synaptic machinery, while SynCAM is symmetrical. They might work together with soluble proteins, including FGF22, Wnt7a [108] and astrocyte-derived signals such as cholesterol and thrombospondins.

Although the progress on these different factors is enormous, it is currently not possible to group these proteins in a hierarchy of events. An interesting question for future research therefore is how these multiple factors cooperate to induce presynaptic and postsynaptic differentiation or whether some of these factors act only on specific subtypes of neurons. The neuroligins and neurexins are clearly promising candidates for synapse induction in vitro, and results from patients with mutations in neuroligin genes and studies on α-neurexin knockouts suggest that this signalling system might be relevant for synaptic function and/or development of neuronal circuits. Furthermore, their molecular complexity due to alternative splicing makes the neurexin/neuroligin system an interesting candidates, and its further characterization in vivo will most likely improve our understanding of the mechanisms of synapse formation in the CNS.

III Molecular analysis of the modulation of circuits by neuronal activity

Synaptic connections that are formed during development might be subsequently refined and specified by

various forms of neuronal activity. Several studies have indicated that during development and throughout adulthood, synapses are continuously structurally and functionally reconfigured, a process that is described by the term 'synaptic plasticity'. Although the mechanisms by which membrane depolarization, modulation of intracellular calcium levels and action potential generation exert their influence on the number, distribution and properties of synapses is currently more or less unknown, it is widely accepted that the specification and refinement of synapses requires neuronal activity [109]. During embryonic development, neuronal activity might be initially spontaneous; later on, however, activity is influenced by sensory experience [110, 111]. In particular, neuronal circuits appear to be sensitive to sensory experience during specific early postnatal phases, termed critical periods, after which plasticity is then decreased.

Chondroitin sulfate proteoglycans are inhibitory for experience-dependent plasticity

Monocular deprivation is a model that is intensely investigated in the context of experience-dependent plasticity and critical periods [14, 112]. In critical periods monocular deprivation induces a shift of ocular dominance of neurons in the primary visual cortex in favour of the non-deprived eye. Several studies indicate that increased synaptic inhibition by maturation of GABAergic interneurons reduces the extraordinary degree of plasticity and causes termination of the critical period [113–115]. However, there might be additional components present at synapses in the adult brain that limit experience-dependent plasticity and stabilize synaptic connections. Pizzorusso et al. investigated whether chondroitin sulfate proteoglycans, which inhibit neurite extension in in vitro assays, might restrict plasticity [116]. In the adult several components, including chondroitin sulfate proteoglycans, are organized around the soma and dendrites in structures termed perineuronal nets [117], which develop continuously from P22 until P70 when the critical period ends in rats. Intracortical injection of chondroitinase ABC degrades chondroitin sulfate side chains within the primary visual cortex. Ocular dominance was then tested in these adult rats well after the end of the critical period. At these stages monocular deprivation is completely ineffective in shifting ocular dominance in normal animals. However, after treatment with chondroitinase ABC, a clear shift toward the nondeprived eye was induced. These findings indicate that the degradation of chondroitin sulfate chains by chondroitinase ABC restores ocular dominance columns and that extracellular as well as membrane-associated components containing such side chains are important in regulating visual system plasticity. Although several chondroitin sulfate-containing extracellular matrix as well as transmembrane proteins have been identified, the identity of such factors in the primary visual cortex remains to be determined.

Spine dynamics and loss are regulated by the serine protease tissue plasminogen activator (tPA)

Extracellular proteolysis might be another important mechanism that links neuronal activity to structural changes within the nervous system. For example, several studies from recent years indicate that tissue plasminogen activator (tPA), an extracellular serine protease, is implicated in synaptic plasticity throughout the brain, in particular in ocular dominance plasticity [118–120]. tPA converts inactive plasminogen to the active protease plasmin by proteolytical processing, which in turn might degrade extracellular matrix and other proteins such as the precursor form of brain-derived neurotrophic factor (BDNF) [121]. tPA is released from extending growth cones [122], secreted from synaptic terminals [123,124] and tPA mRNA synthesis is increased by stimuli that produce long-lasting increases in synaptic strength (such as LTP, long-term potentiation) [125]. tPA-deficient mice reveal an impairment of the late phase of LTP, while tPA-overexpressing mice have enhanced LTP [126–128]. These combined observations suggest that tPA facilitates structural changes during different forms of synaptic plasticity.

To study how tPA is implicated in structural changes, Hensch and colleagues investigated shifts in ocular dominance in the absence of tPA and its inhibitors [119]. After monocular deprivation in critical periods, tPA was increased in the binocular zone. When GABAergic transmission, which is known to be an important parameter in visual plasticity, was reduced by deleting glutamic acid decarboxylase (GAD) 65 [113, 115], this upregulation of tPA activity was not observed, suggesting that GABA acts upstream of tPA in a molecular cascade of cortical visual plasticity. Consequently, in the absence of tPA, ocular dominance shift of the non-deprived eye was impaired but could be restored by tPA infusion, indicating that tPA itself plays a role in visual plasticity. Although the structural basis for these ocular dominance shifts is unknown, recent experiments indicate that spine dynamics and spine loss in apical segments of dendrites of layer II/III pyramidal neurons in the binocular zone of mouse visual cortex are regulated by monocular deprivation. Spine motility was elevated by application of tPA, and tPA gene disruption prevented monocular deprivation-induced spine loss [120, 129, 130]. These investigations on this system suggest that structural remodelling of synapses requires tPA activity (fig. 6).

Structural remodelling of dendritic trees by CPG15 in the tectum

cpg15 (candidate plasticity gene 15), also termed neuritin, was identified in a differential screen for activity-

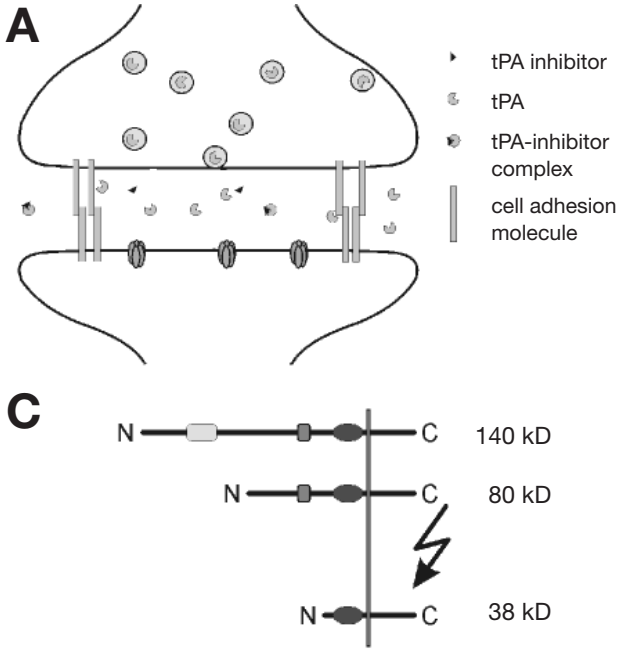

Figure 6. Proteins modulated by neuronal activity. (A) tPA is a serine protease and an immediate early gene. It is released from neurons and appears to be involved in structural remodelling of dendritic protrusions of the binocular zone of the primary visual system by degrading cell adhesion molecules and extracellular matrix components. (B) Scheme of tPA. (C) CALEB is generated as a transmembrane form which is converted to a truncated form that remains attached to the plasma membrane. This conversion is facilitated by neuronal activity and most likely ensures that the EGF domain becomes accessible. The absence of CALEB in knockout mice decreases the probability of neurotransmitter release.

regulated genes induced by kainate-stimulated seizures in the rat dendate gyrus and therefore considered to be implicated in synaptic plasticity [131, 132]. The gene of *cpg15* encodes a membrane-bound protein, attached by a glycosylphosphatidylinositol (GPI) anchor. Other stimuli such as light [133] or treatment of neural cultures with reagents that interfere with action potential propagation in neural cultures modulated mRNA levels of *cpg15*. Activity-dependent promotion of *cpg15* mRNA transcription required both calcium influx via NMDA receptors and voltage-gated calcium channels but did not require protein synthesis. Further investigations using pharmacological reagents suggested that activity-dependent expression of *cpg15* in cell culture requires activation of the Ca^{2+} calmodulin-dependent protein kinase (CaM kinase) and mitogen-activated protein kinase (MAP kinase) signal transduction pathways [134].

Interestingly, the spatial and temporal pattern of expression of *cpg15* in the developing nervous system correlates with active phases of dendritic elaboration and synaptogenesis, in particular when axon arbor structure is refined through retraction and growth of branches [135-137]. To test a possible involvement of CPG15 protein in axon arbor formation, CPG15 was overexpressed in vivo in *Xenopus* optic tectum neurons by using a recombinant vaccinia virus. This treatment resulted in an elaboration of dendritic arbors in projection neurons in that the total dendritic branch length increased [138, 139]. These time-lapse imaging studies are consistent with observations on the human homologue of CPG15 (neuritin) which was found to promote neurite formation in vitro [132]. Interestingly, tectal interneurons were unaffected by CPG15 overexpression suggesting that regulation of dendritic development is different in these types of neurons. To study whether CPG15 could mediate intercellular signaling, the branching of retinal ganglion cell axons was imaged when invading the tectum and when tectal neurons simultaneously overexpress CPG15 [139]. A selective decrease in branch retractions and stabilization of retinal axon arbors was observed, indicating that CPG15 can function as a ligand on neighbouring neurons. The stabilization of arbors was accompanied by an increase in the strength of synaptic transmission by recruiting AMPA receptors to the synapse. The observations of axon branch formation on neurons of the visual system were recently extended by time-lapse imaging on motor neuron axon terminal arbors in intact *Xenopus* tadpoles. In contrast to GFP-expressing and similar to tectal cells, motor neurons overexpressing CPG15 revealed a different dynamic in the branching behaviour which also resulted in a higher synapse density [140]. Most interestingly, many initiation sites of new branches occur at putative presynaptic sites.

In addition to CPG15 expression at phases of synapse maturation, CPG15 is also found in several but not all proliferative zones of the brain very early in development before circuit formation occurs, suggesting that it may have a different function at this stage. Indeed, a soluble, secreted form of CPG15 was identified in embryonic rat brain which protects cultured cortical neurons from apoptosis [141]. Knockdown of CPG15 expression by RNA interference increased apoptosis of cortical progen-

itor cells in vitro, suggesting that CPG15 is required for their survival. Similarly, knockdown of CPG15 in vivo resulted in an increase of apoptosis in the neocortex in contrast to the diencephalon, where normal expression of CPG15 is low. Conversely, overexpression of CPG15 resulted in an enlarged cortical plate and fewer apoptotic cells.

The biphasic expression pattern, activity-independent in early stages of brain development and activity-dependent at later stages, is consistent with a dual role of *cpg15*. Early in development it acts as survival factor during brain morphogenesis and may help to regulate the number of neurons by preventing apoptosis in specific subpopulations and therefore affects the final size and shape of the brain. At later stages it plays a role as ligand in structural remodelling and synaptic maturation. This would be reminiscent of the functions of neurotrophins, although a specific receptor protein for CPG15 has not yet been identified.

The conversion of CALEB, an EGF-like transmembrane protein, is facilitated by neuronal activity

Several screens were conducted to identify genes or directly proteins that are modulated by neuronal activity [131, 142–147]. Proteins that are regulated by activity are considered to be candidate components involved in synaptic refinement and plasticity. One such screen led to the identification of the transmembrane protein CALEB (chicken acidic leucine-rich epidermal growth factor (EGF)-like domain-containing brain protein) [148], also termed neuroglycan C [149]. A characteristic feature of CALEB is an EGF-like domain close to its plasma membrane-spanning region that is related to transforming growth factor α (TGFα) or neuregulin-1 [150]. CALEB contains an acidic box that binds to tenascin-C and -R and previously published in vitro antibody perturbation experiments suggested a participation of CALEB in neurite formation [150, 151]. CALEB becomes glycosylated by chondroitinsulfate chains at the N-terminus [150, 152] and is generated in at least two isoforms that differ in their cytoplasmic region [153]. The EGF domain and the cytoplasmic stretch are highly conserved in vertebrates, but related proteins are not found in invertebrates.

Depolarization with elevated KCl or by treatment with GluR agonists facilitates the conversion of CALEB at the plasma membrane, resulting in a truncated transmembrane form with an exposed EGF domain. Different intermediate processing products are detectable in different cell types. The reason for this conversion is currently not known; however, it is conceivable that the EGF-like domain, which is likely to play a prominent role in the function of CALEB, becomes accessible upon processing for interaction with a yet unknown receptor. The converted form might function as an activity-dependent juxtacrine signalling system, and its regulation might provide a molecular basis for activity-dependent synaptic plasticity. CALEB is found throughout the nervous system and displays a developmentally regulated expression profile in many brain regions. For example, in the retina CALEB is predominantly localized in the optic fiber and inner plexiform layer, while in the cerebellum it is primarily associated with the Purkinje cells as well as the inner granular layer [148, 150, 154, 155].

In the absence of CALEB, the number of synapses, their morphological characteristics, such as the number of docked vesicles at the active zone, as well as their postsynaptic properties, such as decay time constants of IPSCs, remained unchanged. However, CALEB gene inactivation alters the release features of synapses, indicating that CALEB influences the function or the development of the presynapse [148]. In acute slices of the superior colliculus of a CALEB-deficient mouse, GABAergic synapses displayed higher paired-pulse ratios, less depression during prolonged repetitive stimulation, a lower rate of spontaneous postsynaptic currents and a lower neurotransmitter release probability. The molecular nature accounting for the CALEB dependency of the neurotransmitter release probability is not known. Interestingly, all measured effects of CALEB gene inactivation are confined to early stages of brain development. It is therefore conceivable that CALEB functions in the process of the assembly of synapses. Absence of CALEB could then lead to a change in synaptic transmission.

The influence of neuronal activity on synapse development is most obvious under competitive situations [109]. It would therefore be interesting to study the activity-dependent regulation of CALEB under conditions where CALEB-positive and -negative neurons are simultaneously available in a network and to ask whether CALEB-deficient neurons are less successful in generating and maintaining synapses compared with wild-type synapses.

The serum-inducible kinase is implicated in protein degradation

At initial phases of synaptogenesis, excess synapses are generated in many brain regions which are subsequently eliminated by competitive processes. Such a mechanism ensures that neurons and other target cells receive only an appropriate number of inputs. Experimental evidence revealed that synaptic efficacy stabilizes connections, while inactive connections are less favoured [156]. The molecular interactions responsible for this synaptic remodelling are at a very early stage of understanding but might involve the dismantling of cytoskeletal and scaffolding protein complexes. A recent report now presents data that serum-inducible kinase (SNK) may contribute

to these processes [157]. This serine/threonine kinase belongs to the polo family of kinases, and its mRNA becomes rapidly upregulated in the rat hippocampus after drug-induced seizures or LTP generation [158, 159]. Yeast two-hybrid screens and other biochemical data revealed that SNK interacts with SPAR [spine-associated Rap guanosine triphosphate (GTPase)-activating protein (GAP)], a PSD95 binding protein concentrated in spines. Previous studies indicated that this postsynaptic protein regulated spine morphology when overexpressed in neurons [160]. Surprisingly, overexpression of SNK caused phosphorylation and downregulation of SPAR in heterologous cells, while the 'kinase-dead' form of SNK had no effect. Further investigations on these observations revealed that co-expression resulted in a higher ubiquitination of SPAR. This finding indicated that downregulation is dependent on the ubiquitin-proteasome pathway. Consequently, inhibition of the proteasome pathway reduced SNK-induced SPAR degradation, while lysosomal degradation blockers did not prevent elimination of SPAR. In cultivated hippocampal neurons SNK was found to be enriched in dendritic spines, and similar to the results described above, overexpression of SNK in neurons caused elimination of endogenous SPAR and a clear reduction of PSD95. This reduction in staining intensities was accompanied by morphological changes in dendritic spines: a decrease in the densities of mature spines as well an increase in filopodia were observed. SNK is also inducible in cultivated neurons by application of glutamate or depolarization using elevated concentrations of KCl. This induction requires calcium influx through voltage-gated calcium channels, which activates the phosphatase calcineurin. Induction of SNK by neuronal activity also caused a loss of SPAR and PSD95. These findings therefore led to the hypothesis that increased expression of SNK by activity followed by its localization in dendritic spines through binding to SPAR allows phosphorylation of SPAR. This phosphorylation then leads to degradation of SPAR via the proteasome pathway which is associated with a loss of PSD95. As a result of these degradations, synapses are eliminated [157, 161]. However, the function of these proteins in vivo remains to be determined.

Conclusions

A fascinating question is how neuronal activity interacts with genetic instructions to form and modify synapses or circuits within the nervous system. It is known that synaptic activity can induce a number of molecular changes, including posttranslational modifications of synaptic proteins, regulation of gene activity and secretion of proteases [162]. Although several genes that are modulated by neuronal activity have been recently identified, as described above, our understanding of the function of these factors is at a very early stage. It is uncertain how these components restructure or direct the development of central synapses in vivo. Future research will show how some of the known activity-dependent factors act in concert with components involved in synaptogenesis and circuit formation.

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