Lipid rafts and the control of neurotrophic factor signaling in the nervous system: variations on a theme

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Lipid rafts are specialized, liquid-ordered subdomains of the plasma membrane. Through their ability to promote specific compartmentalization of lipids and membrane proteins, lipid rafts have emerged as membrane platforms specialized for signal transduction. In recent years, signaling by neurotrophic factors and their receptors has been shown to depend upon the integrity and function of lipid rafts and associated components. It has also been shown that these microdomains play critical roles in selective axon–dendritic sorting and the proteolytic processing of several neurotrophic ligands and receptors in neuronal cells. The available evidence supports an important role for lipid rafts in the initiation, propagation and maintenance of signal transduction events triggered by different neurotrophic factors and their receptors in the nervous system.

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Abbreviations

EGFRs	epidermal growth factor receptors
Erk	extracellular signal-regulated kinase
FGFRs	fibroblast growth factor receptors
FRS2	FGFR substrate 2
GDNF	glial cell-line derived neurotrophic factor
GFRαs	GDNF family receptor αs
GPI	glycosyl-phosphatidylinositol
GRIP	glutamate–receptor interacting protein
GSLs	glycosphingolipids
MAPK	mitogen-associated protein kinase
nrg-1	*neuregulin-1*
NGF	nerve growth factor
PDZ	PSD95/Discs Large/ZO-1
PI3K	phosphatidyl inositol 3′ kinase
PLC	phospholipase C
SMDF	sensory and motor neuron-derived factor

Introduction

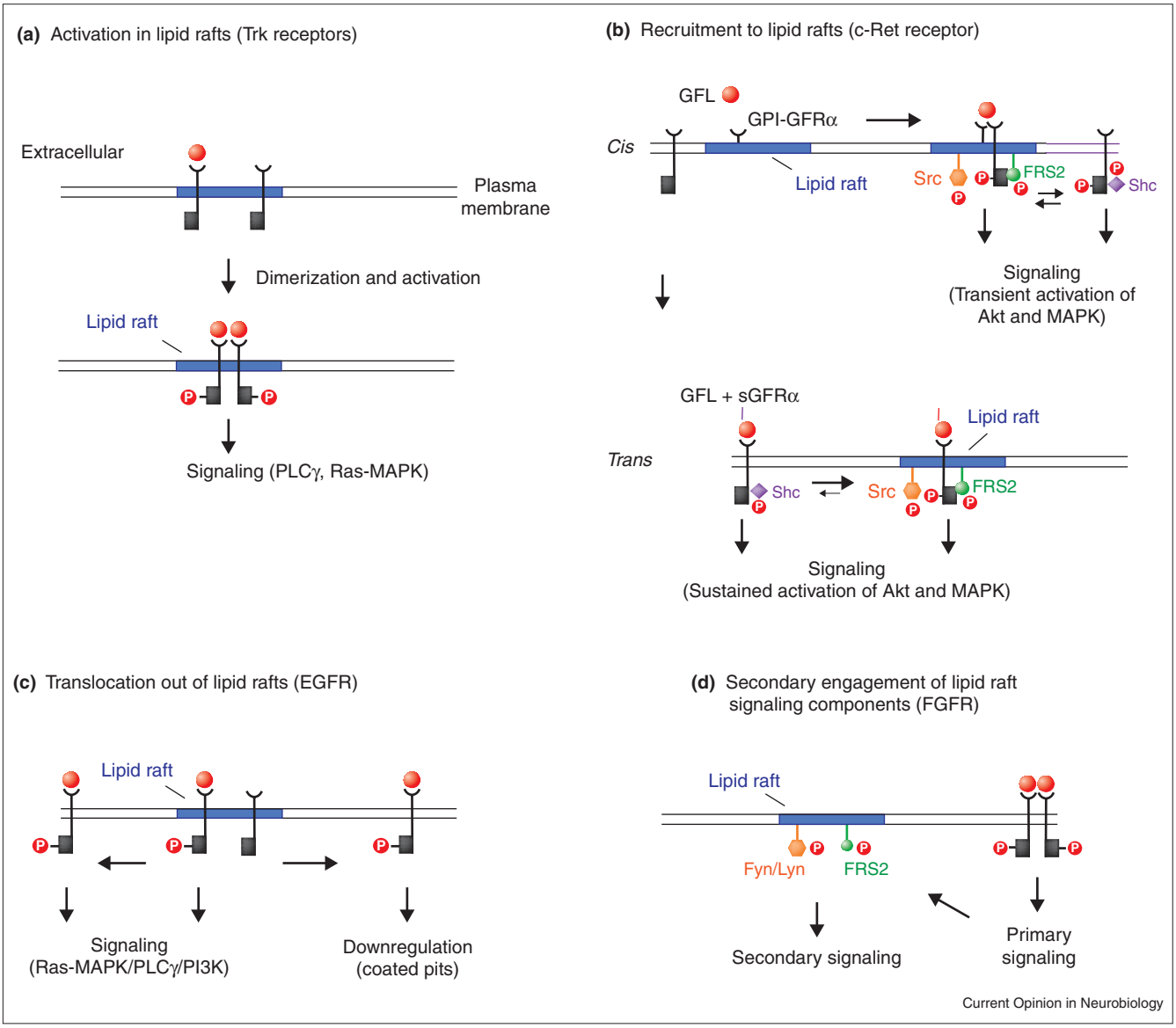
Electron microscopy studies performed during the 1950s revealed the presence of multiple, small flask-shaped invaginations in the plasma membrane of epithelial and endothelial cells [1]. These structures were named 'caveolae' by Yamada [2], on the basis of their characteristic morphology. The cytoplasmic surfaces of caveolae are enriched with members of a small family of integral membrane proteins known as the caveolins. Subsequent biochemical characterization of caveolae indicated that these substructures of the plasma membrane are rich in a variety of cell signaling molecules [3]. The shift in research from morphological to biochemical studies brought a new perspective to the study of caveolae and to the challenge of isolating and characterizing their components. Taking advantage of their light buoyancy, caveolae can be isolated by density gradient centrifugation of plasma membrane fractions solubilized in non-ionic detergents. This procedure was later found to result in the isolation of many of the same components found in caveolae, even in cells lacking caveolae and devoid of caveolin expression. However, similarly to caveolae, these detergent-resistant membranes were also rich in cholesterol and sphingolipids.

Following the nomenclature suggested by Simons and Toomre [4••], we use here the generic term of lipid rafts to denote dynamic assemblies of cholesterol and sphingolipids scattered within a fluid, disordered phase of the lipid bilayer [5]. According to this terminology, caveolae are considered a specialized kind of lipid raft containing caveolin and are characterized by morphologically defined cell surface invaginations. The lipid raft concept emerged from the early observations of differential lipid composition in the apical and basolateral surfaces of epithelial cells [6]. The external leaflet of the apical surface is enriched in glycosphingolipids (GSLs) whereas on the basolateral side of the external leaflet, phosphatidylcholine predominates. The enrichment of GSLs is postulated to occur in the lumenal face of the trans-Golgi network membrane, where GSLs, sphingomyelin and a distinct complement of proteins cluster together forming rafts. Lipid rafts were in this way proposed as vehicles for the specific targeting of certain classes of proteins to the apical side of epithelial cells. More recently, a similar mechanism has been proposed to mediate the sorting of protein–lipid complexes in detergent-insoluble glycolipid-enriched domains to the axonal compartment of polarized hippocampal neurons [7]. The participation of lipid rafts in the initiation, propagation and maintenance of trophic factor signaling is currently under intensive study. This review focuses on recent advances made in our understanding of the role of lipid rafts in the signaling mechanisms used by different trophic factors in the nervous system.

Lipid rafts and caveolae in signal transduction

Lipid rafts concentrate lipids and proteins that function in transmembrane signaling events [8], allowing them to interact with each other and preventing them from interacting with molecules excluded from rafts. Thus, for example, membrane proteins anchored by a glycosyl-phosphatidylinositol (GPI) link accumulate on the extracellular face of these membrane microdomains, whereas certain G-proteins and members of the Src family of

Figure 1



Different ways in which lipid rafts may participate in neurotrophic factor signaling. **(a)** Receptors permanently associated with lipid rafts. Activation of these receptors results in signal propagation via components that are intrinsic to lipid rafts, including members of the Src kinase family, adaptor molecules and small lipid second messengers such as ceramide. Examples of this type of receptor include GPI-linked molecules such as class A ephrins and GFRαs, and some transmembrane molecules such as class B ephrins and the neurotrophin receptors $p75^{NTR}$ and TrkA, B and C. **(b)** Recruitment of receptors to lipid rafts. Receptors with weak or no affinity for rafts may get recruited to this compartment upon ligand binding by a number of different mechanisms, all of which involve an increase in the affinity of the receptor for one or more resident component of lipid rafts. This recruiting component may be extracellular, such as a GPI-anchored coreceptor (recruitment in *cis*), or intracellular, such as lipid-anchored adaptor molecules (recruitment in *trans*). Signaling may be different from inside and outside lipid rafts. An example of such a receptor is the receptor tyrosine kinase c-Ret. **(c)** Release of receptors from lipid rafts. Activation of receptors that, under resting conditions, reside in lipid rafts may decrease their affinity for raft components and trigger their translocation outside this compartment. Signaling may be different from inside and outside lipid rafts. This event may be linked to the eventual inactivation and endocytosis of ligand-bound receptors, thereby contributing to the termination of the signal. The example here is the EGFR. **(d)** Secondary engagement of lipid raft signaling components. Receptors that are not normally associated with lipid rafts may nevertheless utilize lipid raft components for signal propagation or amplification. Ligand binding may lead to activation of downstream targets that either translocate themselves to lipid rafts, or facilitate the activation of a component that normally resides in lipid rafts. An example of such a receptor is the FGFR. GFL, GDNF family ligand.

protein tyrosine kinases are found associated with the inner leaflet of the rafts. In addition, several membrane-associated proteins are found only transiently associated with lipid rafts, by either entering or leaving these compartments in response to extracellular or intracellular events, such as ligand binding or phosphorylation. Thus,

the specific and dynamic localization of various signaling molecules in these compartments has helped to bolster the idea of lipid rafts as platforms for signal transduction integration.

Lipid rafts participate at several different stages of signaling cascades, from the initiation, to the propagation, to the maintenance of signals originating in the plasma membrane. In Figure 1, we illustrate this concept with the example of signaling through receptors. Some receptors are more or less permanently associated with lipid rafts, some either leave or enter this compartment upon ligand binding and some receptors, although not normally present in lipid rafts, still utilize components in this compartment for the propagation and maintenance of downstream signaling.

In the first case, activation of receptors permanently associated with lipid rafts results in signal propagation via components that are intrinsic to these compartments, including members of the Src kinase family, and small lipid second messengers such as ceramide (Figure 1a). In the second case, receptors with weak or no affinity for rafts may get recruited to this compartment upon ligand binding by several different mechanisms, all of which involve an increase in the affinity of the receptor for one or more resident components of lipid rafts (Figure 1b). Increasing the residence time spent by an activated receptor within lipid rafts allows its interaction with a different complement of adaptor proteins and other downstream targets, thereby contributing to the diversification of intracellular signaling. In the third case, activation of receptors that, under resting conditions, reside in lipid rafts may decrease their affinity for raft components and trigger their translocation outside this compartment (Figure 1c). In the example considered below, this event appears to be linked to the inactivation and endocytosis of ligand-bound receptors, thereby contributing to the termination of the signal. The fourth and somewhat less committed case that we consider includes receptors that do not normally become associated with lipid rafts, but that nevertheless utilize lipid raft components for signal propagation or amplification (Figure 1d). In this case, binding of ligand to the receptor leads to the activation of downstream targets that either translocate themselves to lipid rafts, or facilitate the activation of a component that normally resides in lipid rafts.

Finally, it should also be noted that ligand-induced multimerization of components associated with lipid rafts may lead to the formation of lipid raft clusters, containing novel combinations of adaptor, scaffolding and anchoring proteins and enzymes [4••]. The coalescence of individual rafts to form raft clusters may contribute both to signal amplification (e.g. by the synergistic engagement of protein kinases with cognate substrates) and to signal attenuation (e.g. by bringing activated protein kinases and phosphorylated substrates in contact with protein phosphatases). Lipid raft clustering has been observed during the assembly of the immunological synapse — a specialized cell–cell contact interphase involved in the activation of lymphocytes by antigen-presenting cells [9,10] — and in more artificial settings, such as in the crosslinking of lipid raft components with specific antibodies. Although the physiological relevance of this model of signal initiation/propagation for neurotrophic factor signaling remains to be demonstrated, it is possible, by analogy to the immune system, that it may play a role in synaptogenesis and synaptic remodeling.

Lipid rafts and neurotrophic factor signaling

Neurotrophin receptors: Trks and p75^{NTR}

The neurotrophins nerve growth factor (NGF), brain-derived neurotrophic factor, neurotrophin 3 and neurotrophin 4 represent the best known group of neurotrophic factors. In addition to being the first growth factor discovered, NGF has remained the best-studied neurotrophic factor during the past fifty years. The neurotrophins regulate neurite outgrowth and neuronal survival during development by interacting with two types of cell surface molecules: the receptor tyrosine kinases TrkA, B and C, and the so-called low-affinity receptor p75^{NTR}, a member of the tumour necrosis factor receptor superfamily.

p75^{NTR} lacks intrinsic catalytic activity, and signals through a series of protein–protein interactions mediated by its intracellular juxtamembrane and death domains [11]. In cells expressing Trk receptors, neurotrophins promote cell survival by stimulating sustained activation of the phosphatidyl inositol 3′ kinase (PI3K)/Akt and Ras/Erk (extracellular signal-regulated kinase) pathways, which in turn intercept nuclear and mitochondrial cell death programs [12]. When coexpressed with appropriate Trk receptors, p75^{NTR} increases neurotrophin binding affinity and assists in ligand discrimination by different Trk family members. p75^{NTR} can also contribute to cell survival directly by activation of the nuclear factor κB (NFκB) pathway, to neurite outgrowth by regulation of Rho activity, and to cell migration [11,12]. When Trk activation is reduced or absent, high levels of p75 expression can make cells susceptible to apoptotic cell death, through increased ceramide production, activation of c-Jun kinase and p53 [11,12].

TrkA and B and p75^{NTR} are highly enriched in membranes of low buoyant density prepared from synaptic plasma membranes of rat forebrain and PC12 cells [13,14]. In addition, many of the intermediates in the signaling cascade activated by Trk receptors are also present in lipid rafts, including PI3K, phospholipase Cγ (PLCγ), Shc, Grb2, Ras, Raf-1 and mitogen-associated protein kinase (MAPK) [4••]. Moreover, some of the effects of p75^{NTR} on cell death and growth may in part be mediated by generation of the bioactive lipid metabolite ceramide, a product of the hydrolysis of sphingomyelin, a lipid enriched in lipid rafts [15–18]. It has been shown that NGF binding to p75^{NTR} and TrkA occurs mainly in lipid rafts [19]. The majority of the high-affinity TrkA binding sites are present in these membrane microdomains. NGF can be chemically crosslinked to p75^{NTR} in lipid rafts, under conditions that favor binding to high-affinity receptors, suggesting the

participation of lipid rafts in the formation of high-affinity binding sites for NGF.

Although the molecular basis for this increased high-affinity binding within lipid rafts still needs to be defined, a distinct possibility is the facilitation of higher order complexes between the two NGF receptors, as demonstrated by Ross *et al.* [20]. Moreover, the localization of these receptors is in agreement with the increased tyrosine phosphorylation of TrkA detected in lipid rafts upon ligand binding. Virtually all the activated TrkA is found in these microdomains, where it can be coimmunoprecipitated with phosphorylated crucial downstream components such as Shc and PLCγ [19]. NGF binding does not alter the partition of $p75^{NTR}$ or TrkA in lipid rafts, suggesting that these receptors are either internalized in membranes derived from caveolae-like rafts, or are sorted into membranes with similar composition and physicochemical properties [21•]. Additional experiments will be required to address these possibilities.

Ephrins and Eph receptors

Ephrins are cell surface-bound ligands for members of the Eph receptor family, the most extensive group of receptor tyrosine kinases found in vertebrates. The ephrin/Eph system mediates cell–cell contact signaling and plays critical roles during neuronal development, by regulating axonal guidance and fasciculation, cell migration and the formation of boundaries [22,23]. Ephrins can either have a transmembrane domain followed by a short cytoplasmic domain (class B) or be anchored to the plasma membrane via a GPI link (class A). Soluble ephrins are unable to stimulate Eph receptor signaling unless they are either membrane anchored or artificially clustered. Intriguingly, ephrin/Eph signaling is bidirectional, as membrane-bound ephrins can also function as signaling receptors, when engaged by cognate Eph receptors expressed on the surface of nearby cells [24]. Both GPI-anchored and transmembrane ephrins, as well as the Eph receptors, have been localized to lipid raft microdomains [13,25], and several signaling events triggered by this system appear to critically depend on this localization. For example, activation of ephrin-B1 has been shown to promote the recruitment of glutamate–receptor interacting protein (GRIP)1 and GRIP2 to lipid rafts, through their direct association with the carboxy (C)-terminal PDZ (PSD95/Discs Large/ZO-1) domain of the ephrin [25]. This event results in the formation of large raft patches containing GRIPs and in the recruitment of a GRIP-associated serine/threonine kinase activity [25].

Similarly to many other GPI-anchored proteins, class A ephrins are clustered on the cell surface in lipid raft microdomains [26••]. It has been shown that the GPI-anchored ephrin-A5 is able to generate a signaling cascade from these domains upon binding to the extracellular region of its cognate Eph receptor. Ligation of ephrin-A5 results in the recruitment and activation of the Src family kinase Fyn within the lipid raft compartment and subsequent tyrosine phosphorylation of a Fyn substrate of 80 kDa [26••]. Ephrin-A signaling has been shown to modulate cell adhesion and morphology via an integrin-dependent mechanism [27,28]. In these studies, it was found that sustained activation of the MAPKs Erk1 and Erk2 in response to ephrin-A5 ligation correlated with the extension of cellular processes, and that MAPK activation involved both Src family kinase-dependent and Src Family kinase-independent pathways [27]. Although the exact mechanism by which the signal is transmitted across the membrane remains unknown, the ability of other GPI-anchored proteins to mediate a signal has typically been linked to their interaction with a transmembrane signaling component [29,30].

GDNF family receptors: c-Ret and GFRα

The glial cell line-derived neurotrophic factor (GDNF) family of ligands includes GDNF, neurturin, persephin and artemin, and promotes the survival and differentiation a broad spectrum of neuronal subpopulations in the central and peripheral nervous systems [31]. Outside the nervous system, GDNF also has important roles as a morphogenetic factor in the developing kidney and in the differentiation of spermatogonia [32,33]. GDNF family ligands promote their biological effects through a receptor system, in which the tasks of ligand binding and transmembrane signaling are subdivided between a GPI-anchored component — the GDNF family receptor αs (GFRαs) — and a receptor tyrosine kinase, the product of the c-Ret proto-oncogene [29,34]. Different GFRαs (GFRα1–4) mediate the specific actions of cognate members of the GDNF ligand family [31]. Binding of the complex formed by a GFRα and its cognate GDNF family ligand is followed by recruitment of c-Ret to the complex, receptor dimerization, autophosphorylation and intracellular signaling.

By virtue of their GPI anchor, GFRαs are localized into lipid rafts [35••,36••]. In cells coexpressing c-Ret and GFRαs, ligand binding triggers the recruitment of c-Ret molecules to a detergent-insoluble membrane compartment that colocalizes with the ganglioside GM1, a characteristic lipid raft marker [35••,36••]. Because low levels of c-Ret are also observed in this compartment in the absence of ligand [36••], it is possible that formation of the GDNF/GFRα/c-Ret complex merely increases the affinity of c-Ret for rafts, thereby stabilizing it within this compartment and making it resistant to detergent extraction. The stabilization of c-Ret in the lipid raft compartment does not depend upon an active c-Ret kinase, and is therefore likely to be mediated by the interaction of its extracellular domain with the GDNF/GFRα1 complex [36••]. GDNF signaling has been shown to dependent on the integrity of lipid rafts, because cholesterol depletion with methyl-β-cyclodextrin, a treatment known to disorganize lipid rafts, reduces GDNF-dependent activation of MAPK and Akt kinases [35••].

Unexpectedly, in cells lacking GFRα receptors, c-Ret can be stabilized within the lipid raft compartment by cotreatment

with ligand and its cognate GFRα partner in soluble form. This event, however, is delayed and depends upon an intact c-Ret kinase domain [36••], suggesting the participation of an intracellular component of lipid rafts in c-Ret recruitment. The critical role of Tyr1062 located in the tail of the c-Ret receptor has implicated the adaptor protein FGFR substrate 2 (FRS2), itself a resident protein of lipid rafts due to its myristylation [37]. Phosphorylation of Tyr1062 has been found necessary for the activation and association with c-Ret of FRS2 [36••]. The activated c-Ret receptor may thus be recruited to lipid rafts as a result of the interaction between FRS2 and phosphorylated Tyr1062 in c-Ret. In addition to FRS2, $p60^{Src}$ — a member of the non-receptor Src kinase family — interacts with c-Ret in raft compartments [35••]. Activated c-Ret can also be detected outside lipid rafts, suggesting the existence of a dynamic equilibrium between raft and non-raft compartments. Notably, GDNF signaling through c-Ret located in the rafts is different from the signaling occurring outside rafts [36••]. Although such compartmentalization of signaling appears to be a unique aspect of this receptor system, it may represent a more general strategy to diversify downstream signaling, by allowing a single class of receptors to transmit different signals from different locations in the membrane.

Epidermal growth factor receptors

Several studies have reported that under resting conditions (i.e. in unstimulated cells) epidermal growth factor receptors (EGFRs) are concentrated in plasma membrane caveolae, and that they move out of caveolae following EGF stimulation [38,39]. Several rapid signaling events induced by EGF binding, including tyrosine kinase activation, recruitment of adaptor proteins, PLC hydrolysis of phosphatidylinositol 4,5-biphosphate, and activation of Ras/MAPK, appear to occur within caveolae, suggesting that EGFR signaling is initiated and organized in these microdomains.

In addition to forming EGFR homodimers, EGF binding initiates the formation of heterodimers with other members of the ErbB receptor family (ErbB2 in particular), which are also constitutively present in lipid rafts [39] (see also below). Association of the activated EGFR with ErbB2, which has a reduced internalization capacity compared to EGFR, decreases EGFR downregulation and enhances the mitogenic and transforming activity of this receptor [40,41]. Presumably, dimerization events occur within caveolae or rafts; however, direct demonstration of the presence of EGFR homodimers or heterodimers with ErbB2 in either compartment is yet to come. Termination of EGF-dependent signals appears to correlate with the disappearance of EGFRs from caveolae, suggesting that migration of receptors out of caveolae precedes their subsequent internalization and trafficking by clathrin-dependent endocytosis. Interestingly, manipulations that block the exit of activated EGFRs from caveolae, such as the inhibition of EGFR or Src family kinase activities, impair endocytosis and further receptor trafficking [39], indicating the requirement of proximal signaling events for the trafficking decision.

Highlighting some of the discrepancies generated by the use of different methodologies, a recent immunoelectron microscopy study found that the majority of EGFRs colocalized with a generic lipid raft marker — GPI-anchored placental alkaline phosphatase — but were outside caveolae in unstimulated human carcinoma cells [42•]. Moreover, the authors of this study [42•] found no detectable relocalization of EGFRs upon ligand binding [42•]. In another study [43], manipulation of cholesterol levels affected the fraction of EGFRs available for ligand binding, suggesting that non-caveolar lipid rafts may function as negative regulators of EGF receptor signaling, by sequestering a fraction of the EGFRs in a state inaccessible for ligand binding. Thus, the EGFR has been reported to generate signals from coated pits, endosomes, lipid rafts and caveolae [39,44,45], indicating a complex regulation of EGFR activation, signaling, and desensitization, and suggesting that several different, and perhaps cell-type-dependent, mechanisms may be at play in EGF-mediated signaling.

Neuregulin receptors: ErbB2–4

The products derived from the *neuregulin-1* gene (*nrg-1*) are cell–cell signaling proteins that interact with the receptor tyrosine kinases ErbB2–4 in the EGFR subfamily [46]. Members of the neuregulin family are expressed in the nervous system, where they are implicated in cell proliferation, differentiation and survival, and in the development of neuromuscular synapses, Schwann cells, motor and sensory neurons [47,48]. The most common NRG-1 isoforms in the nervous system are synthesized as transmembrane pro-proteins, which can be further processed by proteolytic cleavage in the stalk region to generate soluble or membrane-associated signaling molecules.

Recently, it has been reported that some members of the NRG-1 family (type I β1 and type III β1a) are segregated into lipid rafts [49•]. Subcellular localization studies demonstrated that the 40 kDa form, but not the 83 kDa form of the sensory and motor neuron-derived factor (SMDF; a type III NRG-1 isoform) is segregated into lipid rafts. Studies of the molecular determinants of SMDF topology revealed that its C-terminal domain is critical for compartmentalization into lipid rafts [50]. Lipid rafts are known to participate in the regulation of proteolytic processing of transmembrane proteins [51,52], and it has been shown that the C-terminal fragment that is generated by cleavage from the NRG precursor is concentrated in lipid rafts in brain and in transfected cells [49•]. The differential membrane compartmentalization of the two SMDF forms may represent a regulatory strategy of protein biosynthesis, to facilitate the correct posttranslational modifications that distinguish these two neuregulin isoforms. It has also been speculated to contribute to the differential sorting or selective delivery of SMDFs to specialized locations in neurons [50].

The physiological relevance of the distinct subcellular compartmentalization of SMDF isoforms may be related to

their differential ability to interact with ErbB receptors. Thus, whereas both ErbB2 and ErbB3 are able to bind the 83 kDa form of SMDF, only ErbB3 is capable of interacting with the shorter, lipid-raft-associated isoform. Differential compartmentalization between raft and non-raft membranes of different NRG isoforms could therefore represent a strategy to diversify signal transduction in this growth factor family.

Fibroblast growth factor receptors

Fibroblast growth factors trigger their effects by binding to a family of transmembrane tyrosine kinase receptors, the fibroblast growth factor receptors FGFR1–4. Signaling through FGFRs stimulates differentiation and survival of neuronal cells [53]. Ligand binding leads to FGFR dimerization and autophosphorylation on tyrosine residues, which then act as recruitment sites for cellular targets that couple FGFR signaling to other downstream effectors [54]. Although FGFRs do not appear to be present in lipid rafts, many proteins involved in FGF signaling reside within these domains. Thus, for example, the adaptor protein FRS2 is localized to rafts and is crucially involved in the effects of FGF on neuronal differentiation [37]. Stimulation of human neuroblastoma cells with FGF2 was shown to result in tyrosine phosphorylation of several proteins, including the two Src family kinases Fyn and Lyn, within lipid rafts [55•]. The authors of this study [55•] have shown that FGF treatment also induces the recruitment of the Src family substrate anexin II to lipid rafts. Because the FGFR itself has never been detected in lipid rafts, its activation outside these microdomains may lead to the translocation of intermediary molecules to this compartment by, for example, increasing their affinity for a lipid raft component through phosphorylation.

Conclusions: many unanswered questions

Many important aspects regarding the role of lipid raft microdomains in neurotrophic factor signaling still remain unanswered. One general but highly significant issue requires a more precise determination of the biochemical composition of rafts. Another issue consists of whether neurotrophic factor receptor signaling utilizes a specialized subset of microdomains or the entire lipid raft population in a cell. It will also be important in each case to verify precisely which functions of a given neurotrophic factor system require lipid raft compartmentalization. Furthermore, as illustrated in the case of the EGFR, new methodological advances are likely to have an impact on our understanding of the structure and function of lipid rafts. As with all operationally defined concepts, many of the arguments and confusions in this field have arisen from disagreements between datasets generated with different techniques. In this regard, it is interesting to note the recent development of a method to biochemically isolate lipid rafts at 37°C [56••], allowing their characterization under physiological conditions.

Finally, the possible role of lipid raft clustering in synaptogenesis and synaptic plasticity deserves greater attention. Many components of presynaptic and postsynaptic regions are known to be present in lipid rafts. The recent realization of the role of ephrin-B/EphB signaling in neuronal synapse formation [57] suggests that, as in the immunological synapse, lipid raft signaling and clustering may be an important step for the assembly of the many components that form neuronal synapses in the central nervous system.

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