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The latrophilins, "split-personality" receptors

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Abstract

Latrophilin, a neuronal "adhesion G-protein-coupled receptor", is the major brain receptor for α-latrotoxin, a black widow spider toxin which stimulates strong neuronal exocytosis in vertebrates. Latrophilin has an unusual structure consisting of two fragments that are produced by proteolytic cleavage of the parental molecule and behave independently in the plasma membrane. On binding an agonist, the fragments reassociate and send an intracellular signal. This signal, transduced by a heterotrimeric G-protein, causes release of calcium from intracellular stores and massive release of neurotransmitters. Latrophilin represents a phylogenetically conserved family of receptors, with orthologues found in all animals and up to three homologues present in most chordate species. From mammalian homologues, latrophilins 1 and 3 are expressed in neurons, while latrophilin 2 is ubiquitous. Latrophilin 1 controls synapse maturation and exocytosis, while latrophilin 2 is involved in breast cancer. Latrophilins may play different roles during development and in adult animals: thus, LAT-1 determines cell fate in early embryogenesis in *Caenorhabditis elegans* and controls neurotransmitter release in adult nematodes. This diversity suggests that the functions of latrophilins may be determined by their interactions with respective ligands. The finding of the ligand of latrophilin 1, the large postsynaptic protein lasso, is the first step in the quest for the physiological functions of latrophilins.

Introduction

Latrophilin was isolated in 1996[1,2]. This was a result of extensive efforts of a number of laboratories trying to identify the functional receptor(s) of α-latrotoxin, a neurotoxin from black widow spider venom whose study had begun more than thirty years ago.

Alpha-latrotoxin causes exhaustive release of neurotransmitters from nerve terminals of vertebrates even in the absence of extracellular Ca^{2+}.[3,4] Due to its stimulating effect on all types of synapses and endocrine cells, the toxin has been widely used to study the mechanisms of regulated exocytosis (for a review see Ref. [5,6]). When the toxin was found to require specific cell-surface receptors for its actions, several groups began looking for a high-affinity α-latrotoxin receptor[7-9] which was envisaged to play a key role in exocytosis.

This protracted search eventually resulted in the isolation of latrophilin which has not only become one of the first members of the adhesion G protein-coupled receptors (GPCRs) to be identified, but also the only member with a known exogenous agonist (α-latrotoxin). Latrophilin research has greatly contributed to the general understanding of this peculiar receptor family. Thus, the idea of posttranslational cleavage of adhesion GPCRs, although first described for CD97,[10] was developed into a conceptualised theory based on the studies of latrophilin.[11] Latrophilin has also served as a model to propose the "split personality" hypothesis stating that the fragments of adhesion GPCRs can behave as independent proteins capable of ligand-induced reassociation and concomitant signalling.[12,13] In addition, latrophilin is the first of the few adhesion GPCRs for which physical and functional association with G proteins has been directly demonstrated,[12,14-16] justifying the name of the whole family.

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The isolation of latrophilin

In 1990, by means of α-latrotoxin affinity chromatography in the presence of Ca^{2+}, Petrenko et al. isolated several proteins from solubilised bovine brain.[9] Two large components of this mixture were subsequently sequenced, cloned and termed neurexins[17]. Neurexins, a polymorphic family of neuronal cell-adhesion proteins, have later been shown to participate in synapse formation/stabilisation[18,19] and to contribute to predisposition to autism.[20] Although neurexin Iα was able to bind α-latrotoxin and mediate some of its toxic effects,[21,22] its binding was Ca^{2+}-dependent and its structural features did not suggest a signal transduction capability. This indicated the existence of another receptor that would bind α-latrotoxin in the absence of Ca^{2+} and have an ability to send intracellular signals.

In fact, another major protein was always present in the α-latrotoxin column eluate, but it was initially dismissed because, having the same molecular mass as α-latrotoxin (120-130 kDa), it was mistakenly considered to be the toxin leaching off the column. This protein actually was the N-terminal fragment (NTF) of latrophilin, a hitherto unknown brain protein which was simultaneously isolated by two groups in 1996, using α-latrotoxin affinity chromatography in the absence of calcium.[1,2] This protein's characteristics were consistent with the predicted receptor functions and, based on its high affinity for α-latrotoxin (~0.5 nM), one group termed it latrophilin,[1,14] while the other called it CIRL (calcium-independent receptor of α-latrotoxin).[2,11] This protein is also sometimes referred to as CL (CIRL/latrophilin) in the literature.[23]

The cDNA of latrophilin was cloned on the basis of peptide sequences from the 120 kDa protein (p120) isolated from bovine and rat brain.[11,14] One long open reading frame was detected that encoded a protein consisting of 1466[14] or 1471[11] amino acid residues. Since the predicted size of the cloned protein (~162 kDa) was significantly larger than 120 kDa, the presence of the entire latrophilin sequence in the α-latrotoxin column eluate was investigated using antibodies.[11] While an antibody against p120 recognised this band only, an antibody against the predicted C-terminal peptide failed to stain p120 but instead labelled some aggregated material at the top of the gel.[11] This aggregate was resolved by supplementing SDS-polyacrylamide gels with 8 M urea and by not boiling the electrophoretic samples. The resulting C-terminal fragment (CTF) appeared as a fuzzy band of ~85 kDa and was termed p85.[11] It has been subsequently shown, however, that CTF can be successfully analysed in standard SDS-gels if the samples are heated to 50 °C only;[12,13] under these conditions the protein consistently migrates as a concise group of bands with an average molecular mass of ~69 kDa, exactly as predicted for this fragment (see below).

These results suggested that the translated protein was cleaved. This hypothesis was directly confirmed by N-terminal sequencing of CTF (p85) which demonstrated that the N-terminus of p85 corresponded to Thr-838 of the full-size protein.[11] Thus, the cleavage at this position produced two fragments, or subunits. The predicted molecular mass of the α subunit was ~95 kDa (this corresponded to p120, which was known to be highly glycosylated).[1,11] The predicted size of the β subunit was ~69 kDa,[11] and its aberrant migration as p85 could be explained by the effect of urea.[12] To avoid confusion and due to the partial independence of the two fragments, in the rest of this review we will refer to p120 (95 kDa fragment, α subunit) as NTF and to p85 (69 kDa fragment, β subunit) as CTF.

The latrophilin family

When the cDNA encoding rat and bovine latrophilin/CIRL was isolated and sequenced, three types of sequences were identified and found to represent a family of homologous mRNAs.[23-25] One of these, encoding the protein purified by α-latrotoxin chromatography,[1,2,11,14] was termed latrophilin 1 (CIRL1), while the other two sequences

were assigned numbers (2 and 3) based on their homology to latrophilin 1. Consistent with its higher resemblance of latrophilin 1, latrophilin 2 also binds α-latrotoxin but with a much lower affinity,[26] which does not allow its purification on α-latrotoxin columns, while latrophilin 3 does not bind the toxin appreciably.[24] The genes encoding these mRNAs were termed *lphn1, lphn2*, and *lphn3*, the most widely used nomenclature. These genes are located, respectively, on chromosomes 19, 1 and 4 in humans and 8, 3 and 5 in mice. In a separate later study,[27] three homologous genes encoding lectomedins (proteins containing lectin and olfactomedin domains) were identified by genome sequencing and termed *lec1, lec2* and *lec3*. Sequence comparisons demonstrate that *lec1* is identical to *lphn2* and *lec2* has the same sequence as *lphn1*, while *lec3* corresponds to *lphn3*. Also, human "latrophilin 1" (*lphh1*)[28] is in fact the human orthologue of rat and bovine latrophilin 2.

The latrophilin mRNAs have several sites of alternative splicing (two have been directly identified by cDNA sequencing in *lphn1*, five in *lphn2* and four in *lphn3*).[23] The exon boundaries in the three *lphn* genes are essentially the same, with a few exceptions, and many alternative splice sites coincide. The most notable are splice sites 5 and 7. The former alters the sequence of the third cytoplasmic loop between transmembrane regions (TMRs) 5 and 6 and is thus likely to affect G-protein coupling (see 'Latrophilin as a GPCR'). The latter leads to the expression of different cytosolic domains in latrophilin 3. Alternative splicing at splice site 2 in latrophilin 1 truncates the protein immediately downstream of the N-terminal lectin-like domain (see 'NTF' below), producing a short protein which is probably secreted.

Variably spliced latrophilin gene orthologues are present in all animals, from coelenterates, nematodes and insects to tunicates and vertebrates,[29,30] and have apparently evolved from a primordial adhesion GPCR gene. The nematodes possess two homologous genes, while one gene is found in arthropods. Coelenterates have three genes only weakly homologous to latrophilin and lacking many domains. Three proper latrophilin homologues are present in most chordates: from sea squirt to fish, platypus and humans; however, only two genes have been identified in the chicken genome. The family of three genes probably resulted from two rounds of gene duplication in early *Chordata*, with latrophilin 1 being the latest evolutionary acquisition. Given the early divergence of the three vertebrate *lphn* genes and their location on different chromosomes, it is not surprising that their introns differ vastly in size and sequence. However, the intron positions are highly conserved and almost precisely coincide with the borderlines between the domains of respective proteins (Fig. 1).

Interestingly, the latrophilin orthologues from such distant vertebrates as fish and humans are much more homologous than the three latrophilins within one organism, indicating that the three latrophilins possess different functions which are strictly preserved in the evolution of chordate animals. This is further supported by the different expression patterns of the three latrophilin homologues in various tissues.

Expression patterns of latrophilins

Northern blot analyses of different tissues have shown that latrophilins 1 and 3 are strictly brain specific, while latrophilin 2 is expressed in many tissues.[14,23,24,26] Similar to neurexin Iα,[17,31] very small levels of latrophilin 1 mRNA can be detected outside brain, in particular in kidneys and pancreas. It is possible that in the samples of these latter tissues latrophilin 1 mRNA is actually present in neurons from autonomic ganglia or in endocrine cells. Indeed latrophilin 1 mRNA has been found in many endocrine cells, e.g. pancreatic β-cells[32] and chromaffin cells.[33]

Recently, a real-time PCR profiling of mRNA levels of all known adhesion GPCRs, including latrophilins, was conducted.[34] Consistent with Northern blot studies carried out previously,[14,23,24,26] it was shown in that study that latrophilin 1 (Lec2) and latrophilin 3

(Lec3) mRNAs are strongly enriched in the mouse brain and are essentially absent from, for example, mouse lung and liver. Again in line with the previous publications, latrophilin 2 (Lec1) mRNA was found in most mouse tissues. On the other hand, in the rat, the levels of latrophilin 1 and 3 mRNA in the liver and lung appeared as high as in the brain.[34] This result is rather surprising because (1) it contradicts the multiple direct mRNA hybridisation experiments; (2) it is inconsistent with the close evolutionary relationship between mouse and rat and (3) latrophilin 1 cannot be detected in rat liver using α-latrotoxin chromatography or anti-latrophilin antibodies.[1,24] It is possible that either the primers used in this work were able to amplify the latrophilin 2 message or that the samples of rat tissues used were fortuitously enriched with neuronal (e.g. autonomic ganglia) or endocrine cells.

It is also conceivable that even if some latrophilin 1 mRNA is present in non-neuronal tissues, it is not translated efficiently. From this point of view, tissue distribution of latrophilin protein should be studied directly. Indeed, our analysis of different rat tissues by Western blotting[1] and especially immunohistochemistry (which permits unequivocal typifying of positive cells; paper in preparation) show that latrophilin 1 is absent from any non-neuronal cells and is present in very small amounts in adrenals, but not in liver, lung or kidneys. This result has been confirmed by immunoelectron microscopy demonstrating latrophilin 1 presence in synaptic junctions only (data not shown).

The structure of latrophilin

Latrophilin 1, due to its strong affinity for α-latrotoxin, is by far the most studied of the three latrophilins. However, the primary structures of these proteins are 48-63% identical, and there is no reason to believe that the processing and behaviour of latrophilins 2 and 3 should be grossly different. Therefore, the general protein architecture and behaviour as described below apply to all latrophilins and may be relevant for all adhesion GPCRs.

The primary structure of latrophilin comprises the following domains: an 851 residues-long extracellular domain; seven hydrophobic TMRs which, together with the intra- and extracellular loops, encompass 243 residues; and a cytoplasmic tail of 372 amino acids. Constitutive proteolysis within the extracellular domain (19 amino acids upstream of the first TMR) produces NTF (832 residues) and CTF (634 residues).

NTF

The extracellular domain, which gives rise to NTF, begins with a hydrophobic signal peptide. Immediately downstream lies a 108 residues-long cysteine-rich region homologous to galactose-binding lectin (GBL).[11,14] GBL is present in most latrophilin orthologues found in animals from to nematodes (*Caenorhabditis elegans*) to vertebrates (but not in coelenterates).[29] GBL is indeed able to bind D-galactose but shows much stronger preference for L-rhamnose[35,36] and ouabain.[35] The solution structure of this region, alternatively termed rhamnose-binding lectin, has been solved recently.[35] This study argues, however, that carbohydrates are unlikely to be the endogenous ligands of this domain[35] because (1) rhamnose is not normally found in animals; (2) the affinity of the monomeric lectin domain for rhamnose and especially other monosaccharides is insufficient for specific binding and (3) the residues critical for carbohydrate binding are not conserved in GBLs of latrophilin orthologues from different organisms. However, many GPCRs are dimeric, and it would be interesting to determine the affinity of GBL for D-galactose-containing glycans when latrophilin dimerises in response to agonist (α-latrotoxin) binding.[12] In addition, the strong evolutionary conservation of GBL (amino acid sequence identity between latrophilins from nematodes, insects and mammals is 36-40%)[29] suggests that GBL plays a very important role in the function of this receptor.

The GBL domain is followed by the region (260 amino acids) of homology to olfactomedin, a glycoprotein of the extracellular matrix of the olfactory neuroepithelium (for a review see Ref. 37). Olfactomedin domain is found in many different proteins all of which, apart from latrophilin, are secreted and most are expressed in the nervous system. Olfactomedin domain-containing proteins have been implicated in cell-cell interactions important for neurogenesis, neural crest formation, dorsoventral patterning and cell cycle regulation, while mutations in these proteins may be involved in various neurological diseases from glaucoma to psychiatric disorders. Interestingly, this domain is absent in all invertebrate orthologues of latrophilin and could be acquired during the early evolution of vertebrates.[29]

The 79 amino acid sequence downstream of the olfactomedin domain contains multiple serines, threonines and prolines (STP)[25] but shows no significant homology to any known proteins, except some proline-rich bacterial proteins with low sequence complexity and unknown functions. The STP domain is found in insect and vertebrate orthologues of latrophilin, but not in *C. elegans*.

The STP region is linked to a domain (~60 residues) characteristically found in Class II (or secretin family) peptide hormone GPCRs.[38] It is variably called “hormone-binding domain”,[38] “signature domain”[24] or “hormone receptor motif” (HRM, adopted here).[29] HRM contains two conserved tryptophan residues and three to four conserved cysteines, which may form internal disulphide bridges. This region appears in many but not all adhesion GPCRs; it is absent from the latrophilin orthologues from insects and in latrophilin 3 from the chicken, but is present in the *C. elegans* latrophilins.[29]

A unique 68 amino acid region downstream of HRM connects it to a glycosylated domain of 180 residues which is analogous (although only 20 % identical) to the “Stalk” region of another adhesion GPCR, EMR3[39] and its homologues. In EMR proteins, this region is essential for the cleavage of the ectodomain[39,40] and is thought to be an autoproteolytic enzyme.[41,42] Truncations of this domain in latrophilin render this receptor unable to bind its ligand, α-latrotoxin.[25]

The Stalk domain is attached to a short, highly conserved[29] sequence containing four cysteine residues and termed GPCR proteolysis site (or GPS).[11] To avoid confusion with the actual site of cleavage, we will be referring to this region as the “GPS motif”. It is difficult to define the borderlines of this motif because of the variable conservation of the sequences surrounding this region in different adhesion GPCRs. Therefore, we suggest to set the limits of the GPS motif according to the ends of the exon encoding this entire domain (see ‘The latrophilin family’ above). This would mean that the GPS motif in latrophilin is 57 amino acids-long (starting at Ala-788 and ending at Ile-844). Most importantly, this motif contains the site of posttranslational cleavage that divides latrophilin into the non-covalently bound NTF and CTF. The cleavage occurs between Leu-832 and Thr-833,[11] which is 8 amino acids upstream of the C-terminal end of the GPS motif or 19 amino acids upstream of the first TMR. As a result of this cleavage, the GPS motif itself becomes unequally split between NTF and CTF.

Both the Stalk domain and the GPS motif are present in latrophilin orthologues from all animal taxa,[29] as well as in all other adhesion GPCRs. Sequence identity between latrophilins from vertebrates, insects and worms is 16-33 % within the Stalk domain and 45-49 % within the GPS motif.[29] These domains are even more conserved among the three latrophilin homologues found in any vertebrate animal, where sequence identity is 50-60 % between the Stalks and 72-82 % between the GPS motifs. It is possible that the Stalk and GPS motif form a single functional unit that is involved in the posttranslational cleavage of latrophilin.

CTF

CTF begins at the site of cleavage within the GPS motif (Thr-833).[11,12] The most prominent feature of this fragment is the presence of seven TMRs that are highly homologous to those of the secretin family GPCRs (50-60 % sequence similarity and 30 % identity). Similar to peptide hormone GPCRs, extracellular loops 1 and 2 contain two cysteine residues which are believed to form an intramolecular disulphide bridge. In fact, CTF possesses many other features thought to be important for GPCRs, e.g. a negatively charged amino acid within the third TMR, proline residues in the fourth and fifth TMRs and potential sites of palmitoylation in the N-terminal part of the cytoplasmic tail.

The cytoplasmic tail is the least conserved domain among latrophilins. Thus, sequence identity in this region is 13-28% among latrophilin orthologues from worms, insects and vertebrates, and 35-49 % among the three latrophilin homologues found in each mammal. For comparison, the average sequence identity of the whole protein molecules among the three homologues is 49-63%, while within the TMRs (including loop regions) it is 69-80 %.

There are numerous potential phosphorylation sites for several types of protein kinases on the cytoplasmic C-terminal portion of CTF. In fact, CTF of latrophilin isolated from rat brain is phosphorylated on multiple positions, and this explains the behaviour of this fragment in SDS-electrophoresis (see Section ‘The isolation of latrophilin’). Phosphorylation does not normally occur in cultured cells expressing latrophilin, which suggests that this modification in the brain is a result of normal latrophilin function. The phosphorylation plays an important role in the interaction of latrophilin fragments: the phosphorylated forms of CTF bind NTF much stronger than the non-phosphorylated CTF (paper in preparation). This may have important implications for the behaviour of the two fragments after their reassociation and signalling.

Cleavage and unusual behaviour of latrophilin fragments

Latrophilin was the first adhesion GPCR for which the site of intramolecular posttranslational cleavage was identified by direct sequencing of CTF.[11] Since then all adhesion GPCRs studied in respect of cleavage have been proven to undergo proteolysis at a strictly conserved position within the GPS motif. This autoproteolytic cleavage and the two-subunit structure probably represent a common feature of all the members of the adhesion GPCR family.[11,43,44] Moreover, the cleavage site between NTF and CTF coincides with the tentative borderline between the “adhesion” and the “GPCR” halves of these chimerical receptors.[43,44]

The cleavage occurs constitutively in the endoplasmic reticulum and is required for latrophilin trafficking to the cell surface[12,43] Full-size, non-cleaved adhesion-GPCRs are apparently short-lived and not normally detectable in live tissues. Although full size GRP56 (another member of the adhesion GPCR family) was reported to appear in large quantities in some mouse tissues,[45] this was later disproved.[46]

As described above, the cleavage site is localised in the GPS motif, 19 amino acids upstream of TMR1. Thus NTF contains no TMRs, but it is not released into the medium and remains non-covalently associated with the membrane. Given that NTF and CTF have an ability to interact strongly with each other when isolated by α-latrotoxin chromatography and immunoprecipitation,[11,12,43] NTF has been thought[11,43] to attach to the cell surface through its interaction with CTF, a transmembrane protein. This is also supported by the fact that proteolysis at a second site (located between the GPS and TMR1) releases some NTF into the medium.[47]

However, this does not seem to be true for a large proportion of NTF and CTF. Mutagenesis of CTF showed that only eight[43] or even seven[12] residues in the C-terminal part of the GPS motif (after cleavage forming the N-terminus of CTF) are both necessary and sufficient for the cleavage. These seven amino acids are also sufficient for the interaction between NTF and CTF. Despite such a short sequence holding NTF and CTF together, NTF cannot be removed from the membranes by most chaotropic conditions: pH 2.5, pH 12, 4 M Mg^{2+} or 8 M urea (unpublished observations). Most detergents, while solubilising the membrane, do not affect the NTF-CTF complexes, and the latter can be isolated from solution. However, upon solubilisation of cells expressing latrophilin, a large percentage of each fragment remains free from the other. Furthermore, a weak detergent, perfluorooctanoic acid, which does not break up the membrane bilayer, removes a large amount of NTF from the plasma membrane, while leaving all CTF behind.[13] Together, these data suggest that at least some proportion of NTF is anchored in the membrane independently of CTF, perhaps via hydrophobic amino acids or modification/s.[12,13]

This “split personality” hypothesis has been tested in a comprehensive series of experiments,[12,13] which demonstrate that the two fragments do not always colocalise with each other on the cell surface and can even migrate in the membrane and internalise independently. When patched on live cells using antibodies, the two fragments behave as non-interacting free proteins.[12] This corroborates the idea that NTF could have a hydrophobic anchor of its own.

Under certain conditions, e.g. the binding of agonists (see ‘Extracellular ligands’ below) and also upon membrane solubilisation with detergents, the free latrophilin fragments can reassociate.[12] Treatment of latrophilin-expressing cells with α-latrotoxin or its mutant LTX^{N4C} results in the formation of large ternary complexes (α-latrotoxin-NTF-CTF) on the plasma membrane. This leads to intracellular signal transduction to intracellular Ca^{2+} stores (described in ‘Latrophilin as a GPCR’). The mechanism of this reassociation apparently involves dimerisation of NTF domains, which increases the affinity between NTF and CTF.[12,13]

Most intriguingly, the ligand-induced reassociation of latrophilin fragments does not always occur within the same cleaved receptor molecule, as the ligand-bound NTF can interact with CTF from another latrophilin molecule.[13] Moreover, due to the high conservation of GPS motifs in all adhesion GPCRs, NTF of latrophilin can even bind to CTF from another member of this receptor family. Such criss-cross association of NTFs and CTFs produced by the cleavage of different receptors creates functional complexes capable of intracellular signalling and has the potential of greatly diversifying the transduced signal.[13]

This “split personality” architecture of receptors, consisting of two independent modules that associate interchangeably on binding their ligands, is rather enigmatic but not entirely unprecedented. Several other signalling systems require co-receptors. For example, there are two receptors for the Wnt signalling proteins: Frizzled (a GPCR) and low-density-lipoprotein receptor-related protein.[48] Normally such co-receptors are phylogenetically unrelated proteins, both of which bind the same ligand molecule. However, in the case of latrophilin and possibly other adhesion GPCRs, both “co-receptors” are the complementary fragments of the same (or structurally related) parental proteins. In addition, at least when α-latrotoxin is used, the ligand apparently only interacts with NTF, which then serves as an activated ligand of CTF.[12,13] Pleiotropy of downstream effects in this case is achieved not by one ligand activating two different receptors (as in the Wnt pathway), but by the ligand-bound NTF activating CTF from one or another adhesion GPCR. This mechanism may have important implications for the biology of all adhesion GPCRs.

Latrophilin as a GPCR

Latrophilin was classified as a GPCR[14] on the basis of its high sequence homology with the TMRs of the secretin family GPCRs and the features within CTF that are important for G protein coupling (see Section 'CTF' above). However, this cannot be considered a proof that this protein signals through G proteins. This aspect of receptor function was studied in some detail and, as a result, latrophilin has become the first receptor of the adhesion GPCR group for which specific binding to G proteins, namely $G\alpha_0$ and $G\alpha_{q/11}$, was demonstrated.[14-16] This interaction is strong because it persists through two consecutive affinity chromatographies on different adsorbents that bind NTF only.[14,15] (It needs to be pointed out that G proteins can only interact with CTF, and their isolation on NTF-binding columns is only possible due to NTF and CTF forming strong complexes). Moreover, the interaction between CTF and G proteins is dynamic and depends on the ability of G proteins to cleave GTP.[15] Thus, G proteins copurify abundantly with latrophilin only when GDP and EGTA are added to the solubilisation buffer, i.e. under the conditions when the GTPase activity of G proteins is inhibited and they normally interact with respective GPCRs. The addition of GTP and Mg^{2+} to purification buffer supports the GTPase activity and causes the dissociation of G proteins, resulting, as expected, in their loss from the column eluate.[15] Thus, excess of GTP is able to reverse the interaction of latrophilin with its requisite G protein(s), suggesting that this association is not only physical but also functional.

Intracellular signalling mechanisms of latrophilin have so far been studied using its exogenous agonist, α-latrotoxin. The signalling induced by α-latrotoxin is also consistent with the activation of G protein pathways. In particular, non-neuronal cells expressing latrophilin respond by stimulation of adenylate cyclise and phospholipase C (PLC) and release of intracellular calcium.[14] Similarly, the toxin triggers activation of PLC and increase in cytosolic Ca^{2+} in PC12 cells[49] and synaptosomes.[50] However, studies of signal transduction from α-latrotoxin have been complicated by the fact that the toxin binds to at least two disparate receptors (neurexin and latrophilin) and also forms Ca^{2+}-permeable transmembrane pores.[5,6] The effect of Ca^{2+} influx through the toxin pore may obscure any physiological signalling. A breakthrough has been achieved with the creation in Tom Südhof's laboratory of a mutant α-latrotoxin, LTX^{N4C}.[51] This mutant lacks the ability to form pores[33] but still stimulates neurotransmitter secretion in hippocampal slices, neuronal cultures, neuromuscular junctions and synaptosomes,[6,52-55] indicating that its effect is based on stimulation of a receptor. The subsequent receptor transduction pathway involves a G protein coupled to activation of PLC, production of inositol-trisphosphate and release of Ca^{2+} from intracellular stores.[53] To determine which receptor is involved in this signalling, neuroblastoma cells expressing latrophilin or neurexin were stimulated with LTX^{N4C}. It was demonstrated that only the cells expressing latrophilin (but not neurexin or latrophilin mutant with a single TMR) reacted by activating PLC and producing cytosolic Ca^{2+} waves.[12] These results unequivocally indicate that latrophilin – via its CTF – can send intracellular signals to PLC and intracellular Ca^{2+} stores.

Until recently, however, it has been difficult to prove that the same signalling cascade (LTX^{N4C} – latrophilin – G protein – PLC – Ca^{2+} stores)[12,13] also underlies the LTX^{N4C}-induced neurotransmitter release in nerve terminals.[33,53] This is because LTX^{N4C} also binds two other neuronal receptors (neurexin Iα and PTPσ) which might contribute to or mediate its effect in neurons. In addition, LTX^{N4C} action in nerve terminals requires extracellular Ca^{2+},[33,53] possibly suggesting that the toxin might induce influx of Ca^{2+}_e rather than release of Ca^{2+}_i. Indeed, when large LTX^{N4C} binds to any receptor, it might interact with ion channels and thus cause exocytosis irrespective of latrophilin. These arguments have been remarkably answered by our recent finding that a recombinant single-chain antibody A1 against latrophilin 1, isolated from a phage display library, can cause burst-like

neurotransmitter exocytosis similar to that induced by LTX^{N4C} (paper in preparation). This ultimately proves that stimulation of neuronal latrophilin by agonists sends an exocytotic signal via a G protein pathway.

Neuronal studies also demonstrate that the main signals sent by latrophilin are relatively fast and reach a maximum within several seconds or minutes. Furthermore, this signalling retains all its characteristics even in synaptosomes or neuromuscular junctions, subcellular systems consisting of severed nerve terminals and lacking the neuronal somata. This leads to an important conclusion that, consistent with Ca^{2+} signalling, latrophilin acts locally, within presynaptic nerve terminals and does not necessarily send signals to the cell body and the nucleus.[52,55]

Of course, it is also possible that latrophilin can link to other signalling pathways, especially considering the promiscuous reassociation of its NTF with CTF's from other adhesion GPCRs. Therefore, in-depth studies of both G protein-coupled and any alternative mechanisms are required.

Ligands and interacting partners of latrophilins

Extracellular ligands

α-Latrotoxin—The main exogenous ligand of latrophilin 1 is α-latrotoxin. The toxin stimulates its receptor and thus can be classed as an agonist. The interaction of α-latrotoxin with latrophilin was tested using various truncated constructs of latrophilin.[25] This study demonstrated that a very large fragment of NTF containing HRM, Stalk and GPS (390 amino acids) may be necessary to bind α–latrotoxin strongly, suggesting a multi-point interaction between toxin and latrophilin, with low-affinity binding at each point. In particular, HRM, a putative ligand-binding region of hormone receptors, alone was unable to mediate toxin binding. It may be possible that some peptide within the toxin molecule mimics a natural ligand of HRM but interacts with this domain only weakly. The association of toxin with NTF at additional sites may provide sufficient retention of the hormone-mimetic toxin peptide in contact with HRM, leading to receptor activation.

Black widow spider venom also contains another component (possibly ε-latroinsectotoxin) that kills *C. elegans* worms on injection.[56] Knockout and RNAi studies have shown that the toxic effects of the venom is mediated by the LAT-1 orthologue of latrophilins in *C. elegans*, but not by LAT-2.[56]

Cyclooctadepsipeptides—Latrophilin orthologues from the parasitic nematode *Haemonchus contortus* (HC110-R) and *C. elegans* (LAT-1) were thought to bind the anthelmintic cyclical depsipeptides, PF1022A (a natural secondary metabolite of the fungus *Mycelia sterilia*) and its semisynthetic derivative emodepside.[57] Electrophysiological studies revealed that emodepside inhibited pharyngeal pumping of the nematodes in a concentration-dependent manner.[58] In *C. elegans* LAT-1 knockout mutants emodepside had a decreased paralysing effect on the pharyngeal muscle.[59] These studies suggested that cyclodepsipeptides were exogenous agonists of the latrophilin-like proteins in nematodes, leading to the release of an unidentified inhibitory transmitter which acted postsynaptically to relax both pharyngeal and somatic body wall muscle, causing flaccid paralysis of the nematode.

However, the expression of depsiphilin, a LAT-1 orthologue from the canine hookworm *Ancylostoma caninum*, did not correlate with emodepside sensitivity.[60] Also, in *C. elegans* LAT-1 knockout worms only pharyngeal pumping was resistant to the inhibitory effect of emodepside, while locomotion was blocked by the drug even in the double mutant *lat-1,*

lat-2 worms.[61] Ultimately, emodepside has been found to target directly a Ca^{2+}-activated potassium channel, SLO-1, which is expressed in both neurons and muscles. One pathway involving neuronal SLO-1 and controlled by LAT-1, is responsible for pharynx pumping. The second pathway, based on both neuronal and muscle SLO-1 that is independent of LAT-1 or LAT-2, is responsible for locomotion.[61]

Putative small endogenous ligands—The 54 kDa N-terminal fragment of latrophilin-like receptor HC110-R from *H.* contortus has been tested for its ability to bind different FMRFamide-like neuropeptides.[62] Three of these peptides (AF1, AF10 and PF2) exhibited low-affinity interaction with the receptor with dissociation constants of 11 μM, 52 μM and 583 μM, respectively. These data suggest that AF1, AF10, and PF2 might represent natural ligands of HC110-R and might be involved in controlling pharyngeal pumping in nematodes.

Endogenous adhesion ligand—The structure of latrophilin, with its large adhesion-like N-terminal domain, and the finding of large protein ligands for other adhesion GPCRs[63,64] suggest that latrophilin may be capable of interacting with ligands on adjacent cells or in the extracellular matrix. Therefore, in our attempts to isolate an endogenous ligand of latrophilin, we used NTF. Several variants of recombinant NTF were expressed, purified and used to synthesise an affinity adsorbent. Out of these constructs, only one resulted in an active column, which allowed us to isolate a protein from solubilised rat brain that specifically binds latrophilin (paper in preparation). This protein, termed lasso (latrophilin-associated synaptic surface organiser), is a large glycoprotein expressed on the postsynaptic membrane. The binding of lasso to NTF of latrophilin involves multiple sites in each molecule and causes specific association of cells expressing these proteins. Moreover, the interaction between latrophilin and lasso is required for synapse formation and maturation.

Intracellular partners

Most importantly for its signalling function, latrophilin has been found to bind two types of α-subunits of heterotrimeric G proteins[14-16] (described in detail in ‘Latrophilin as a GPCR’).

Close to the C-terminus of CTF, there is a proline-rich region,[14] which could bind SH3 domains of proteins involved in signalling.

In addition, CTF may stably or transiently interact with structurally important proteins. In a yeast two-hybrid system, the C-terminal cytoplasmic tail of latrophilin was able to bind Shank, a proline-rich postsynaptic scaffolding protein.[65,66] The significance of this interaction is unclear: Shank contains a PDZ domain that binds proteins with a consensus sequence Ser/Thr-X-Φ, where Φ is a bulky hydrophobic amino acid. However, this sequence is present in all three latrophilin homologues, and consistently both latrophilins 1 and 2 were isolated in this artificial system.[66] It is possible that the ubiquitous latrophilin 2 is the physiological target of Shank in the postsynaptic density, while latrophilin 1 is normally found in the presynaptic terminals (discussed in Section ‘Expression patterns of latrophilins’).

Finally, CTF of latrophilin has been shown to interact with TRIP8b, tetratricopeptide repeat-containing Rab8b-interacting protein,[67,68] a cytosolic protein that binds clathrin and the adaptor protein AP2. This indicates that latrophilin may play a role in receptor-mediated endocytosis or trafficking of neuronal channels.[69]

Latrophilin gene knockouts

Mouse

To study the physiological role of latrophilin 1, its gene has been knocked out in mice by deleting exon 2[70] or the distal part of exon 1 plus the proximal part of exon 2 (unpublished data). In our laboratory the first *lphn1*$^{-/-}$ mouse only appeared after about 40 rounds of mating heterozygous animals, suggesting that the *lphn1* deletion is actually embryonically lethal. This is supported by our consistent finding of dead embryos in pregnant heterozygous female mice. However, both knockout approaches eventually resulted in live and fertile *lphn1*$^{-/-}$ mice, indicating that the lack of latrophilin 1 can be effectively compensated for by a mutation or upregulation of another gene that either occurs spontaneously or is introduced by C57BL/6 backcrossing. Both compensated mutant strains display lack of maternal behaviour (pup nursing, nest building, etc.). In our colony, these maternal nurturing defects reciprocally correlate with the dose of *lphn1* gene. In addition, our knockout mice show increased aggression. It should be noted that a very similar phenotype has been reported for mice lacking Gαq[71] or PLC-β1,[72] the proteins known to be involved in the downstream signalling cascade of latrophilin. Despite the mild phenotypic manifestation of latrophilin 1 deletion, which is apparently due to compensatory changes in the genetic background, further behavioural studies are needed to throw more light on the functions of latrophilin 1 in those cells and brain regions where the genetic compensation is less pronounced. In addition, it would be especially revealing to determine the nature of the compensatory mutation/s.

At the biochemical level, knockout mice demonstrated a decreased binding of α-latrotoxin and a great decrease in toxin-evoked glutamate release from nerve terminals, both in the presence and absence of Ca^{2+},[70] indicating that latrophilin is the major receptor for α-latrotoxin. However, this study employed the wild-type toxin, whose ability to form Ca^{2+}-permeable pores complicated the results and made it impossible to detect an inhibition of latrophilin signalling in knockout mice. An in-depth exploration of the role of latrophilin in nerve terminals must be conducted using the non-pore-forming mutant LTX^{N4C} or other tools.

C. elegans

The orthologues of mammalian latrophilins in the nematode *C. elegans* are encoded by two genes: *lat-1* and *lat-2*. The LAT proteins are 25-28 % identical to all latrophilins, but not particularly related to any one latrophilin.

The results obtained from *lat-1* knockout in *C. elegans* strongly support the hypothesis that LAT-1 is presynaptic in adult nematodes and that its stimulation, similar to the mammalian latrophilin pathway,[12] signals via activation of $G\alpha_q$ protein and phospholipase C-β1, leading to the mobilisation of diacylglycerol (DAG). DAG then activates UNC-13, an important protein that regulates the tethering of presynaptic vesicles to the plasma membrane, and synaptobrevin, a vesicular protein that binds vesicles to the plasma membrane. This is thought to result in transmitter release.[59,73]

In addition, loss-of-function mutations in *lat-1* (but not in *lat-2*) have indicated a different role for LAT-1 in *C. elegans* development.[74] The lack of this protein results in defects in anterior-posterior polarity, leading to arrest of larval development and suggesting that LAT-1, in parallel with the *wnt* pathway, controls the polarity of cell division and cell migration during nematode embryogenesis. Both the extracellular N-terminal region (including the GBL/RBL domain) and the C-terminal domain are required for this mechanism. This indicates that in the process of early worm development LAT-1 acts by transforming the interaction of NTF with adjacent cells into intracellular signals. These

signals are probably different from those sent by the protein in terminally differentiated cells of the adult worm.

Latrophilins in disease

To our knowledge, genetic links between the *lphn1* gene and an inheritable disease have not been established yet. This may suggest – in line with our knockout results above – that most mutations in latrophilin 1, as well as its ablation, are embryonically lethal. Indirect evidence suggests that latrophilin 1 may be associated with such mental disorders as schizophrenia and bipolar disorder. Thus, chronic administration of risperidone, an antipsychotic drug often used to treat schizophrenia, led to an upregulation of *lphn1* in rats.[75] Also, the lack of latrophilin in mice, despite the compensatory changes, led to behaviours consistent with schizophrenia phenotypes.[76] Schizophrenia is a complex neuropsychiatric disease, and multiple genes and environmental factors can contribute to its manifestation, making further research into latrophilin gene/s even more important.

On the other hand, mutations in the human gene *lphh1* encoding the ubiquitous latrophilin 2 have been associated with breast cancer.[28] Analyses of tumour cell lines showed that *lphh1* expression was variable. Also, gene product variability was higher in the tumour than in normal breast tissue.

Conclusions

Taken together these data suggest that the ancient physiological role of latrophilins in animals is to convert cell contacts into intracellular signals. However, the members of this family have distinct distributions and functions, from early patterning during embryogenesis to controlling release of neurotransmitters in neurons. The identification of specific ligands that bind different latrophilin homologues, or each latrophilin during different stages of animal development, will bring about a new level of understanding of these unusual receptors.

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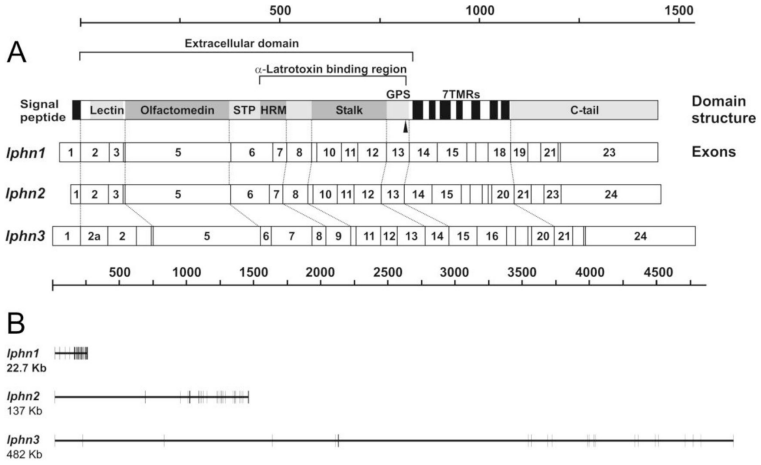

Figure 1.

The structure of latrophilin proteins and genes. A. The distribution of protein domains in latrophilin 1 (top diagram) is shown in comparison with the distribution of exons in the mRNAs of latrophilins 1-3. Only the translated exons are shown (the numbering starts at the first protein-coding exon). Arrowhead, the site of proteolytic cleavage. The scales show the size of the mature protein (top) and the mRNA (bottom). Exon 2a (alternative) in *lphn3* is alternatively spliced. Note that many exons (or groups of exons) encode specific protein domains. B. Exon-intron structures and relative sizes of the three mouse latrophilin genes. Exons are depicted as vertical bars; introns, as horizontal lines. The size of each gene (including the translated exons and introns) is shown below each gene's name. The gene structures shown are from the 129/SvJ mouse (some intron sizes differ between the 129/SvJ and CL57BL/6 mouse strains).

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