Chapter 13
Drebrins and Connexins: A Biomedical Perspective

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Abstract In this chapter we summarize knowledge on the role of drebrin in cell–cell communications. Specifically, we follow drebrin-connexin-43 interactions and drebrin behavior at the cell–cell interface described earlier. Drebrin is a part of the actin cytoskeleton which is a target of numerous bacteria and viruses invading mammalian cells. Drebrin phosphorylation, self-inhibition and transition between filaments, particles, and podosomes underlie cellular mechanisms involved in diseases and cognitive disorders. Cytoskeletal rearrangements influence the state of gap junction contacts which regulate cell signaling and metabolic flow of information across cells in tissues. Taking into account that connexin-43 (Cx43) (together with Cx30) is heavily expressed in astrocytes and that drebrin supports cell–cell contacts, the understanding of details of how brain cells live and die reveals molecular pathology involved in neurodegeneration, Alzheimer's disease (AD), other cognitive disorders, and aging.

Bidirectional connexin channels are permeable to Ca^{2+} ions, IP3, ATP, and cAMP. Connexin hemichannels are important for paracrine regulation and can release and exchange energy with other cells using ATP to transfer information and to support damaged cells. Connexin channels, hemichannels, and adhesion plaques are regulated by assembly and disassembly of the actin cytoskeleton. Drebrin degradation can alter gap junction communication, and drebrin level is decreased in the brain of AD patients. The diversity of drebrin functions in neurons, astrocytes, and non-neuronal cells still remains to be revealed. We believe that the knowledge on drebrin summarized here will contribute to key questions, "covering the gap" between cell–cell communications and the submembrane cytoskeleton.

Keywords Drebrin • Actin • Myosin • Connexins • CDRs • Calcium • cAMP • Small GTPases • Astrocytes • Neurodegeneration

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13.1 Introduction

Brain cells use two main ways to communicate with each other: one way is through the synaptic release of neuromediators (transmitters and modulators) and the other is through direct cell–cell interactions that are mediated by gap junction (GJ) channels (reviewed by Guthrie and Gilula 1989; Goodenough et al. 1996; Bennett and Zukin 2004). Among the 37 trillion cells existing in our body (Bianconi et al. 2013), there are approximately 100 billion neurons and ~1 trillion astroglial cells (Pakkenberg and Gundersen 1988 Herculano-Houzel 2009), (see also Fig. 13.1). Astrocytes form GJ contacts with up to ten partners in the three-dimensional space of the brain, thus providing thousands of highly permeable gap junction contacts that are constantly being built de novo, reorganized, and degraded in accord with physiological signals, moreover, astrocytes control the amount of synaptic contacts (Ullian et al. 2001).

In contrast to synaptic vesicles, which require time to fuse in order to release their signal mediators, gap junction channels and connexin hemichannels operate by the non-vesicular direct release of signal molecules and are thus much faster than synaptic vesicle-mediated signal transmission (Moore et al. 2014). Gap junction channels mediate transmission of signals between the cytoplasms of connected cells. This way, paracrine and regulatory signals propagate information encoded in the gradients of ATP, cAMP, IP3, and Ca^{2+} through gap junctions that may facilitate or terminate signals directed to the distant parts of the brain (Dell'Acqua et al. 2006; Berridge et al. 2012). Thus, a complex relationship between neurons and the surrounding astroglial networks supports brain physiology in health and disease (Alvarez-Maubecin et al. 2000; Orellana et al. 2012). Connexin hemichannels, on the other hand, can release ATP, Ca^{2+}, and other messenger molecules into the extracellular space, thus affording yet another mode of paracrine cell–cell signaling in the brain.

13.2 Connexin-43 Identified as a *Bona Fide* Interactor of the Actin-Binding Protein Drebrin

Which molecules can structurally modify cell–cell interfaces, regulate formation of spines and gap junction contacts, tune their function in accord with activity-dependent tasks, and operate in the extremely complex architectural network (as one shown in Fig. 1) composed of contacting lipid-based cell membranes?

In search of molecules used by brain cells to regulate highly permeable cell–cell contacts, we analyzed interacting partners of connexin-43 (Cx43), one of the more ubiquitous connexins expressed in astrocytes and in many other human tissues including heart muscle (Duffy et al. 2002). Toward this end, we cloned the cytosolic C-terminus of Cx43 into a GST-tagging plasmid, expressed and purified the chimeric protein, and used it as a bait to find binding partners. We fractionated homogenates of mouse brain and tried a series of different concentrations of ATP in our in vitro binding

reactions. This approach recovered a number of proteins that were resolved/identified by MALDI-TOF analyses. In different fractions we obtained, in addition to the already known interaction partners of Cx43 molecule ZO-1 (Toyofuku et al. 1998, 2001 Giepmans and Moolenaar 1998) and tubulin (Giepmans et al. 2001), a previously undescribed binding partner- which proved to be drebrin. Indeed, in primary astrocytic cell cultures, probed with antibodies by -immunofluorescence, drebrin was colocalized with Cx43 at cell–cell interfaces, and downregulation of drebrin using specific RNAi resulted in dramatic connexin internalization (Butkevich et al. 2004). We observed a close molecular proximity of these two proteins at cell–cell interfaces in transfected living cells by donor-acceptor FRET signals after co-expression of drebrin-CFP and Cx43-YFP-tagged proteins (Butkevich et al. 2004). The role of drebrin interactions with Cx43 channels was reviewed by Stout et al. (2004).

Drebrin was first described by Tomoaki Shirao (Shirao and Obata 1985) and originally identified in neuronal cells. Soon they recognized the enormous membrane modifying the morphogenic potential of this protein, when it was shown to be able to form neurite-like outgrowths upon overexpression in cells (Shirao et al. 1992). A decade later it was established that three isoforms of drebrin are present in mammalian cells: neuron-specific drebrin A, ubiquitous drebrin E (Shirao and Obata, 1986; Shirao et al. 1988), and finally the so-called s-drebrin A, which represents a form of a C-terminally truncated drebrin A (Jin et al. 2002). Drebrin A was later shown to be involved in the formation of dendritic spines (Takahashi et al. 2003; Mizui et al. 2005) and in synaptic plasticity (Sekino et al. 2006; Takahashi et al. 2006; Aoki et al. 2009; Mizui et al. 2014). During development all, not only neuronal drebrin A, contribute to a triangle of protein-protein interacting partners—actin, drebrin E, and myosin II and V that have been shown to be necessary to drive axon growth (Mizui et al. 2009).

Drebrin was of interest to researchers from both cytoskeletal and gap junction fields. The group of Werner Franke working on desmosomes and other cytoskeletal proteins described drebrin E as an important protein necessary to anchor actin filaments (Peitsch et al. 1999), similar to drebrin A (Ikeda et al. 1995, 1996). Moreover, an excess of drebrin in cells was shown to be deposited in the form of drebrin particles (Asada et al. 1994; Peitsch et al. 2001). At the same time, the group of Daniel Goodenough working on connexins reported that drebrin E was specifically bound to F-actin in the stomach and in kidney cells (Keon et al. 2000). After we discovered that drebrin is a Cx43-interacting protein (Butkevich et al. 2004), it was then shown that apart from the Cx43 permeability function, unopposed membranes with Cx43 hemichannels are necessary to mediate cell–cell adhesion function important for correct neuronal precursor migration and layer formation in early development (Schaar and McConnell 2005; Elias et al. 2007; Marin et al. 2010) (see Fig. 13.1).

The ability of drebrin to support cell–cell adhesion and cell–cell interfaces may be vitally important for plasticity-dependent tasks. The localization of drebrin at cell–cell interfaces or outgrowths often seen in living cells suggests that this molecule may function to integrate intracellular and extracellular signals to accordingly align cell–cell contacts (cartoon Fig. 13.2) and reorganize actin networks in response to cell activity (see Figs. 13.3 and 13.4).

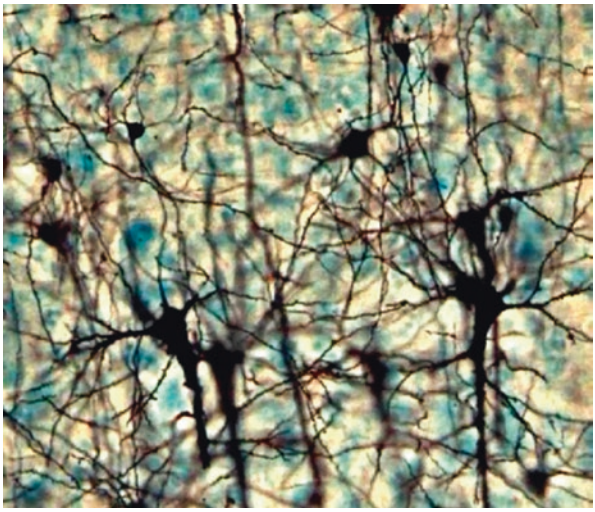

Fig. 13.1 Cortex slice impregnated with silver Golgi reagent reveals neurons (seen in *black*), while astroglial nuclei (seen in *blue*) were stained with Nissl cresyl violet (*left*), and intra-filopodial distribution of expressed drebrin-A (*right*)

The dual nature of connexin-drebrin interactions resembles the opposing force of yin-yang black-white complementary. Drebrin's task together with Cx43 is not only to align opposite membranes to form highly permeable GJ channel domains (i.e. GJ plaques) but also to constantly reposition these domains in 3-D space dictated by cellular activity.

13.3 Drebrin and Connexin at Cell–Cell Interfaces

Astrocytes in the brain express both drebrin E and Cx43. Submembrane drebrin is often seen accumulated in cell–cell contact zones and is colocalized there with connexin channels in GJ plaques (Figs. 13.2 and 13.3). During formation of cell–cell interfaces, the delivery of connexin hemichannels from the Golgi is regulated by complex trafficking steps (Majoul et al. 2009) and involves a microtubular transport machinery (for review see Akhmanova et al. 2009). Interestingly, on the way to the PM, connexins transiently accumulate in the Golgi; drebrin that is seen on the bottom of the cell with actin fibers in the upper 3-D Z-sections appeared again around the Golgi (Fig. 13.4), where its function is less known. Both molecules are often colocalized at cell–cell interfaces repeating similar complex geometry that can be visualized after fixation. We used the mouse monoclonal anti-drebrin antibody M2F6 (created by T. Shirao's group and recognizing both the drebrin A and E isoforms) and our anti-Cx43 polyclonal epitope-specific C-terminal antibody to reveal their colocalization (Fig. 13.3). Live cell analyses confirmed that within less than 6 h after

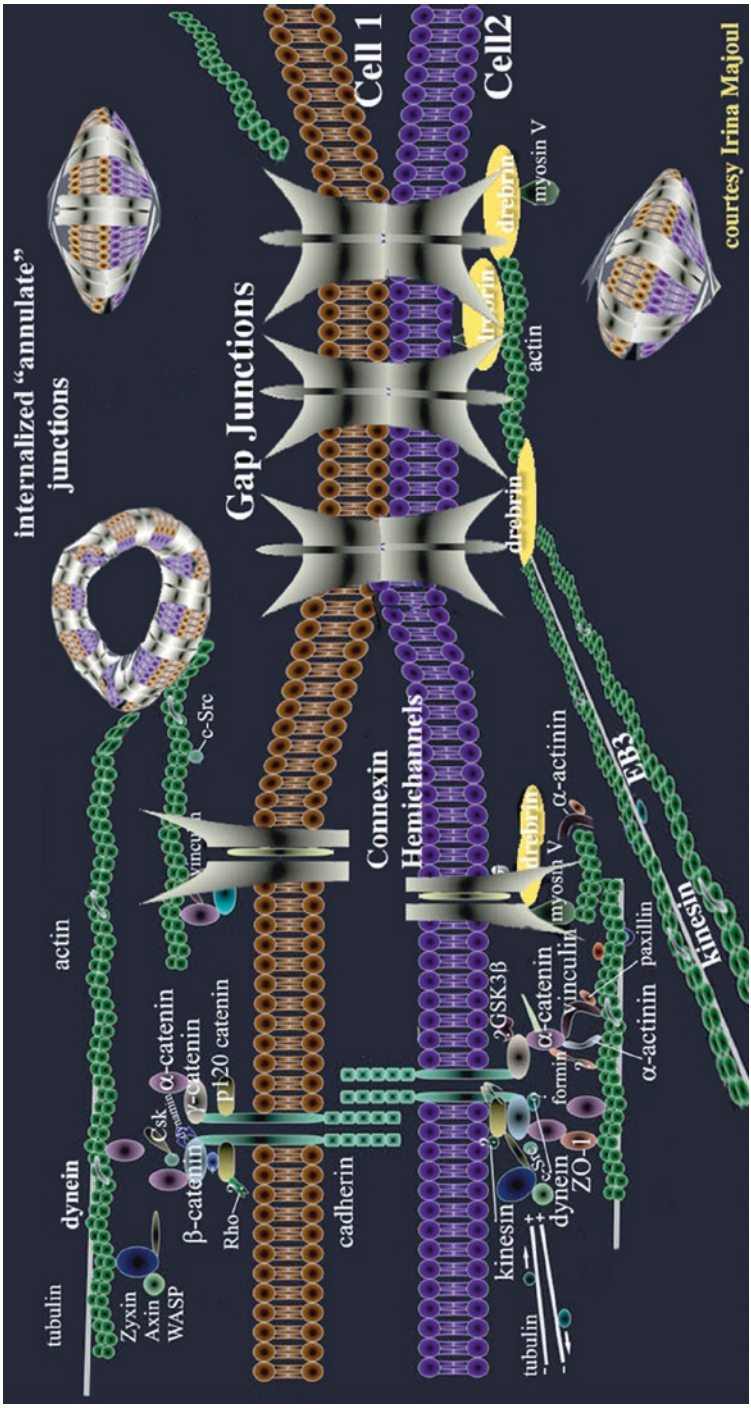

Fig. 13.2 Schematic drawing of a cell–cell interface. Initial proximity of cell–cell contacts is probably mediated by cadherins operating in a calcium-dependent manner. Next, drebrin may assist the connexin hemichannels from two opposing plasma membranes to establish highly-permeable GJ contacts. The accumulation of Cx43 hemichannels will require formation of multi-subunit complexes composed of molecules interacting with the cytosolic part of connexin tails. The drebrin that participated in the lateral alignment of connexin hemichannels is not present after Cx43 is internalized in the form of annular junctions (see Fig. 13.4)

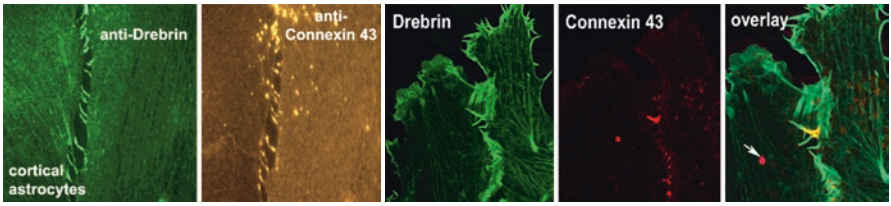

Fig. 13.3 (*Left*) Native distribution of drebrin and Cx43 seen in a primary culture of rat cortical astrocytes co-stained with anti-drebrin (monoclonal) and anti-Cx43 (polyclonal) antibodies. Both molecules are present at specific points of cell–cell interface. (*Right*) Living Vero cells express GFP-tagged drebrin E that colocalized with Cx43-RFP at cell–cell interfaces. No drebrin fluorescence is seen around internalized annular junctions of Cx43 (overlay, *white arrow*)

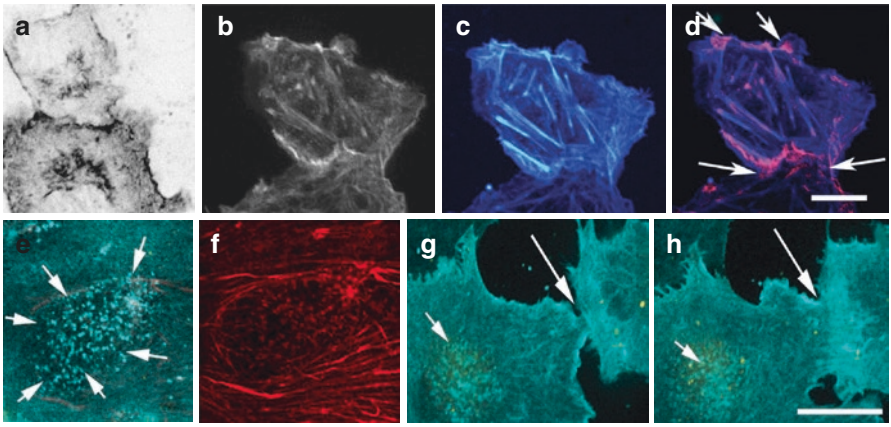

Fig. 13.4 (**a**) Drebrin E reactivity is often present in the Golgi region. (**b**, **c**) Distribution of drebrin E-CFP (seen in **b**) at the cell–cell interface in cells co-transfected with actin-red (seen in **c**). (**d**) Overlay: actin (*blue*), drebrin (*red*). In contrast to actin, drebrin is preferentially present at the cell-cell interface and at the leading edge. (**e**) Highly resolved image of "Drebrin particles" accumulated in the cytoplasm in the perinuclear region indicated by *arrows.* (**f**) actin-red present in some of "drebrin particles" but most of it fluorescence seen in the surrounding fibers. (**g**) Drebrin particles are less prominent in cells during formation cell–cell contacts, big arrows. (**g–h**) Time-dependent images of drebrin show the development of the cell–cell contact, images are taken with intervals of 20 min (connexin is not shown). Scale bar, 20 μm

co-expression drebrin can be colocalized with Cx43. Their dynamic proximity remained even during dramatic cytoskeletal rearrangements of GJ plaques at cell–cell interfaces (Fig. 13.3, right panel). In contrast to cell–cell interfaces where drebrin is colocalized with Cx43, internalized Cx43 in the form of annular junctions is usually depleted from drebrin immunoreactivity (Fig. 13.2, overlay). Inside the cell, drebrin remains organized along the actin stress fibers (seen in Fig. 13.4).

Live cell imaging revealed how drebrin participates in the reorganization of new GJ Cx43 interfaces, and drebrin-directed RNAi oligo treatment resulted in the disorganization and partial removal of GJ contacts (Butkevich et al. 2004). Drebrin is

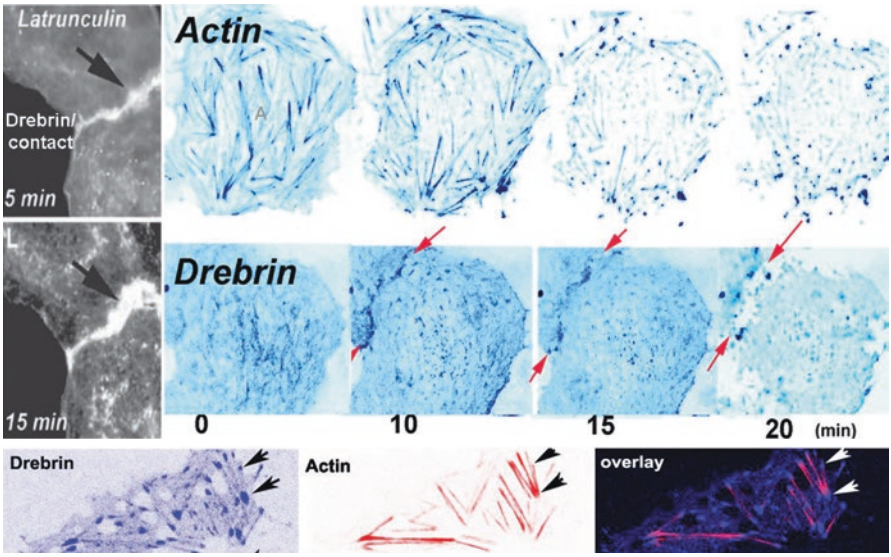

Fig. 13.5 (*Upper left panel*) Accumulation of drebrin-GFP at cell–cell interface seen after the disassembly of actin by latrunculin A (original data stack used in Butkevich et al. 2004). (*Upper right panel*) Actin and drebrin were co-transfected into the same cell to follow the disassembly of actin by latrunculin A. Upon the disassembly of actin, drebrin is accumulated at cell–cell interfaces (*red arrows*) and apparently prevents the rupture of cell–cell contacts (seen in the last image around 20 min). (*Lower panel*) Cells co-expressing tagged drebrin-CFP and actin-red were treated with cholera toxin (wt) to increase cAMP/PKA-dependent phosphorylation that accumulated drebrin in dense clusters. Actin filaments were disassembled by a short application of latrunculin A that was then reversibly washed away. After washout and addition of fresh media, actin filaments (*red*) are growing to reconnect the remaining clusters of drebrin (*blue*). Drebrin nodes remained densely packed throughout the experiment (overlay)

participating in other protein-protein interactions, thus fulfilling multiple other cellular functions (Fig. 13.5).

Two different drebrin isoforms, E and A, in the brain have different distributions and functions (Shirao and Obata 1986; Shirao et al. 1989). Both the ubiquitously expressed drebrin E and the neuron-specific drebrin A have the same N-terminal ADF (actin-depolymerizing factor) domain that is followed by a coiled-coil domain, and both domains are believed to be involved in the interaction with actin (Hayashi et al. 1999; Grintsevich et al. 2010) (see Fig. 1.3 in Chap. 1). Drebrin interaction with actin filaments is likely to provide a dynamic cellular plasticity different from actin-tropomyosin interactions. In different parts of the cell, actin seems to have a variety of interacting partners, and drebrin may act by disrupting actin interaction with other proteins such as tropomyosin, cofilin, and/or α-actinin (Ishikawa et al. 1994; Grinstevich and Reisler 2014). It remains unclear how at different cellular locations tropomyosin and troponin from one side and drebrin from the other form different and physiologically distinct complexes that are controlled in a domain-specific and phosphorylation-dependent manner by other interacting partners. Cytoskeletal molecules in drebrin proximity include actin, tropomyosin, α-actinin,

actin motors, myosin II, myosin V, myosin VI, and postsynaptic scaffolds (Shirao and Sekino 2001). The N-terminal domain of drebrin provides interaction with submembrane partners, e.g., Cx43 and other proteins of the cellular machinery (Ambrosi et al. 2016). The morphogenic property of drebrin can allow this molecule to participate in fast vital cellular decisions that provide plasticity to the submembrane cytoskeleton and regulatory systems important for the early development in neuronal precursor migration, layer formation, neurite outgrowth, cell–cell contact formation, and control of growth cones. At later stages of cellular expansion, drebrin seems to be involved in synaptic connectivity, and many other activities of the developing brain (Song et al. 2008; Mizui et al. 2009; Sonego et al. 2015).

13.4 Drebrin, Small GTPases, and Bacterial Toxins

It is not surprising that the bacterial toxins that co-evolved with mammalian cells developed direct approaches to deplete cellular energy and to disrupt cell–cell communications. Thus, internalization and intracellular transport of cholera toxin (a member of the AB5 toxin family) into mammalian cells leads to conversion of cellular ATP energy into cAMP the concentration of which can be increased up to 20-fold (Majoul et al. 1998). Both Cx43 and drebrin can be phosphorylated by protein kinase A during toxin transport that will modify cell–cell contacts.

Rho GTPases are well-known regulators of the submembrane cytoskeleton (Hall 1998), but interestingly they may also provide a supplementary link between the cytoskeleton and Ca^{2+} signaling (Uehata et al. 1997). A better understanding of Rho GTPase action on the submembrane actin cytoskeleton was obtained from the study of bacterial toxins that invade cells and act on Rho GTPases (Aktories and Just 1995). These data revealed multiple layers of complexity in the action of bacterial toxins that influence both the regulation of the actin cytoskeleton and cellular junctions.

Both live cell analyses of drebrin behavior, and drebrin depletion analyses, suggested that drebrin at cell–cell contacts is rather dependent on the GTPase activity of Cdc42 and from RhoA at noncontacting membranes (our unpublished observations). These data are in agreement with the known functions for Cdc42 and Rho-family GTPases as critical regulators of cell polarity, actin cytoskeleton, and cell–cell communication (Ridley 2000; Etienne-Manneville and Hall 2002; Derangeon et al. 2008).

Drebrin expression in living cells is strongly influenced by signaling mechanisms and the GTPase activities of Rho, Rac, and Cdc42 that are believed to act primarily on actin. Without properly assembled actin, cells cannot rearrange cell–cell contacts or migrate and remain attached to the substrate. Specific dependence of drebrin on Cdc42 and RhoA may explain correlation between the cellular distribution of drebrin at cell–cell interfaces and at noncontacting membranes, where drebrin protrusion-repulsion activity is also dependent on a RhoA gradient. Other drebrin interacting partners at cell–cell interfaces may be critical for GJ-mediated cell–cell communication.

RhoA activity also affects Cx43 permeability without a significant change in the geometry of the cell–cell interface or the distribution of Cx43 phosphorylation

bands on blots. Nevertheless, a high level of RhoA expression can disrupt submembrane Cx43 interactions with drebrin (our unpublished observations). Because cell–cell communications rely on the integrity of the drebrin-actin cytoskeleton, the remodeling of cortical actin by small GTPases and/or bacterial toxins can directly influence the state of GJ communications. Dye transfer and gating of Cx43 gap junctions are usually disrupted by bacterial toxin internalization, and both actin and RhoA GTPases are targets of many toxins.

Disruption of cell–cell communications was shown by Suh et al. (2012) when they grew cells on glass coated with laminin-111. It turned out that decreased permeability of gap junctions between cells growing on laminin-111 was due to an increased activity of the small GTPase RhoA. The application of C3 (a RhoA inhibitor) reversed the effect of laminin-111, increased Cx43/drebrin/ZO-1 integrity and Cx43 phosphorylation, and recovered the control levels of dye transfer. These data reveal a perspective picture where the stability of a drebrin-Cx43/ZO-1 complex favors the existence of GJ coupled cell populations with defined levels of dye transfer. The reduction of Cx43/ZO-1/drebrin complex resulted either in an increase of cell proliferation (in some cells) or in apoptosis with a specific pattern of Cx43 phosphorylation in other clusters (Suh et al. 2012).

Small GTPases change spine morphologies similar to those like the phenotypes that result from the application of GEFs (GTP-for-GDP exchange factors) or specific bacterial toxins. We began to understand how bacterial toxins act on small GTPases specifically toward the Rho family, with the discovery that bacterial toxins are able to deamidate Gln 63 of RhoA (Schmidt et al. 1997). A modification of the cytoskeleton by bacterial toxins seems to include the modification of gap junctions and dendritic spines that are particularly vulnerable during development (Matsuzaki et al. 2004).

13.5 Drebrin Self-Inhibition

We and other groups observed that many cell types natively regulate an excess of drebrin by forming drebrin particles, sometimes seen in the cytoplasm or by forming ring-like podosomes (Asada et al. 1994; Peitsch et al. 1999). Formation of drebrin complexes may reflect a native ability of cells to control drebrin levels to control its action on actin which would otherwise induce the formation of outgrowths and other uncontrolled changes of cell shape. Analyzing the action of drebrin in both cells and solutions, we hypothesize that conformation-dependent self-inhibition of drebrin or the formation of cytoplasmic multimeric drebrin-partner complexes is a way for cells to deal with the high morphogenic potential of this molecule (see Fig. 13.6).

The hypothetical mechanism of the conformational self-inhibition of drebrin still remains controversial, and complexity in the crystallization of this molecule has prevented further detailed structural analyses. Molecular machinery of reversible phosphorylation (or other modifications) of drebrin, its inactivation of actin-modifying ADF, middle 233-366 and coil-coil domains, and the role of the C-terminal remain to be analyzed in detail (Fig. 13.7).

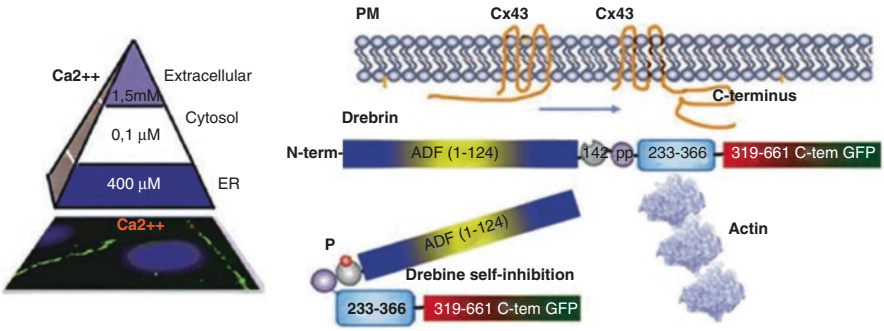

Fig. 13.6 Healthy cellular membranes maintain high gradients of Ca^{2+}concentration between the PM and cytoplasmic space (15,000:1) and between the endoplasmic reticulum (ER) and the cytosol (4,000:1). Cx43 channels and hemichannels (permeable for Ca^{2+}) keeping these gradients can regulate its in-coming concentration by channel closure. Drebrin domains, its self-inhibition and interaction with Cx43 and actin are depicted (*right*)

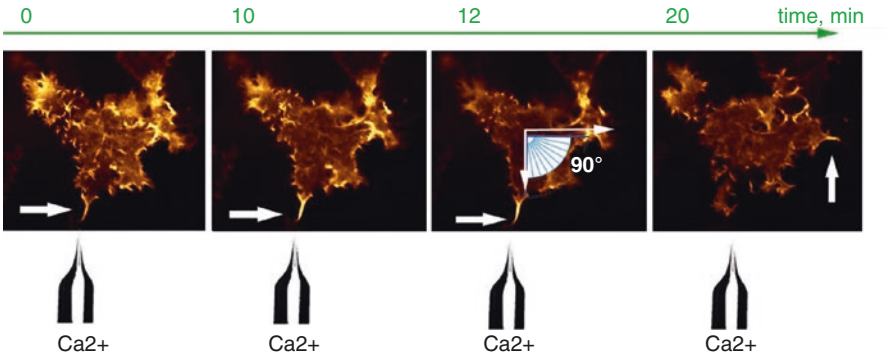

Fig. 13.7 Drebrin E-YFP is expressed in cells growing on glass coverslips. Time-dependent dynamic movement of drebrin was imaged in live cells. Some cells form rather immobile outgrowths with connexin hemichannels acting as an attachment tail. In this experiment Ca^{2+} concentration of the media was set to 2 mM, while the microelectrode positioned close to the immobile tail has a [Ca^{2+}] concentration 3 times higher. Within the next 20 min, the "immobile" tail of drebrin (indicated by *arrow*) was retracted and re-appeared away from the direction of Ca^{2+} flux in the 90° position corner (as indicated by the last *arrow*)

13.6 Similarity Between Two Molecular Complexes: Cx43-Drebrin and the Glycine Receptor-Gephyrin

Cx43-drebrin interactions have some degree of functional similarity with another well-known protein complex formed by the protein gephyrin and the glycine receptor. Gephyrin is expressed in both excitatory neurons (which release glutamate) and in inhibitory neurons which release GABA or glycine or both neuromediators. However, gephyrin localizes specifically to inhibitory synapses (that should contain stronger binding partners) on the postsynaptic side and is similar to drebrin in that it forms a

complex with a transmembrane channel molecule, the glycine receptor. Gephyrin is critical for glycine receptor clustering at inhibitory synapses and was also found in a complex with another inhibitory receptor—$GABA_A$, nevertheless, $GABA_A$ is functionally less dependent on gephyrin than the glycine receptor (Kneussel et al. 2001).

Next, similar to the dependence of drebrin on small GTPases described above, gephyrin in the PSD interacts with collybistin, which is a GEF (guanine nucleotide exchange factor) for the small GTPase Cdc42 (Kins et al. 2000).

13.7 Drebrin, Cx43, and Second Messengers

Gap junctions represent cellular platforms for the effective exchange of second messengers such as Ca^{2+}, IP3, cAMP, and cGMP between cells. Under normal conditions, these permeable "hot spots" of cellular connectivity are necessary to keep control over cell–cell connectivity and organ physiology. Gap junctions regulate cell–cell communications and can support the metabolic state of impaired cells in organs by releasing ATP. Live cell FRET analyses revealed that direct interaction between drebrin and the Cx43-C-terminus is ATP (i.e. phosphorylation) dependent. It can be that only healthy cells provide regulatory impact on channel stability and permeability because drebrin is involved in the rearrangements of Cx43 gap junction channels at different stages of contact formation (Butkevich et al. 2004; Majoul et al. 2009). Drebrin-dependent rearrangement of Cx43 channels on the plasma membrane is regulated by membrane phosphatidylinositol 4,5-bisphosphate (PIP2) and IP3 signaling to the ER. Residing in the inner leaflet of the plasma membrane, PIP2 is involved in signaling and is also a substrate for phospholipase C that generates diacylglycerol (DAG) and inositol triphosphate (IP3) (Rebecchi and Pentyala 2000; Berridge 2010). When surrounding GJ plaques, PIP2 may also dock clusters of drebrin, helping this molecule to interact with the C-termini of connexins in a signal- and activity-dependent manner. As we observed earlier, Cx43 permeability and dye transfer are dependent on the external ATP level, which upon physiological stimuli can modulate GJ permeability by affecting the open-closed state of GJ channels. Drebrin can filter cAMP, IP3, and Ca^{2+} information between coupled cells by supporting contacts, redirecting membrane GJ domain, or just removing them.

The IP3-dependent Ca^{2+} signaling was recently studied in astrocytes after a constitutive genetic receptor deletion in IP3R2 KO mice, where no significant changes were observed (Jego et al. 2014). Nevertheless, the effects of abnormal Ca^{2+} homeostasis, compensatory mechanisms, and inositol 1,4,5-trisphosphate receptor 1 (IP3 R1) which represents the main caspase-3-sensitive channel receptor for Ca^{2+} have not been evaluated. Indeed, in living cells we observed that the behavior of gap junctions at cell–cell interfaces is extremely sensitive to the level of Ca^{2+} and ATP in the surrounding media (our unpublished observations) and in in vitro reconstruction experiments of the binding of the Cx43-C-terminus to drebrin required at least 1.5 mM ATP in the binding solution. Cell–cell permeability is also dependent on the ATP concentration and when we lowered extracellular ATP to 1 mM binding

decayed. This energy-dependent decline of cell–cell communications and the instability of drebrin may also include other submembrane factors such as the cortical actin cytoskeleton, myosin motors, and microtubular transport activity. Noticeably, in young cells with undepleted energy level, drebrin modifications regulate numerous processes of migration and layer formation (Sonego et al. 2015).

13.8 Drebrin and Ca^{2+}

Ca^{2+} is involved in the regulation of numerous processes in astrocytes, neuronal and nonneuronal cells (Berridge et al. 2000; Berridge 2010), and the release of Ca^{2+} from the ER is important for many signaling events (Berridge 2012). Small dendritic spines operate with Ca^{2+} signaling (Sabatini et al. 2001). Very low background levels of Ca^{2+} in the cytoplasm of healthy cells (70–100 nano-molar range) allow signals to propagate via Ca^{2+} entry through the plasma membrane or via the release of Ca^{2+} from the endoplasmic reticulum, an important intracellular calcium store. Most of the Ca^{2+} ions propagating signals in non-excitable cells come from the ER, which is true as well for excitable muscle and secretory cells. In contrast, neurons, rely mainly on Ca^{2+} entry domains on the PM, and neuronal ER stores calcium for their local modular needs. This interesting fact may explain neuronal dependence on well-developed propagation of Ca^{2+} waves in astrocytes. Moreover, during complex cellular events under both physiological and pathophysiological conditions, Ca^{2+} is necessary to regulate mitochondrial activity and the production of cellular ATP. Cellular loss of Ca^{2+} is associated with the induction of the unfolded protein response (UPR), which results in cell death because the energy supply for the ER requires both Ca^{2+} homeostasis and mitochondrial ATP production (Berridge et al. 2000; Walter and Ron 2011).

Drebrin is sensitive to the Ca^{2+} concentration, and the release of Ca^{2+} from the ER is directly coupled to the plasma membrane channels known as store-operated Ca^{2+} entry (SOCE). SOCEs are composed of stromal interacting molecule 1 (STIM1) and the Ca^{2+} channel pore-forming unit ORAI1 positioned in the plasma membrane (reviewed by Hogan and Rao 2007). The ER Ca^{2+} sensor STIM1 binds directly to ORAI1 proteins in the plasma membrane, which comprise the pore-forming subunit of the Ca^{2+} release-activated Ca^{2+} channel. Ca^{2+} influx through the channels activates the key functional cellular responses, metabolism, cell movement, and gene expression. Gap junctions can spatially restrict Ca^{2+} signals by forming PM microdomains and can sense intracellular Ca^{2+} being activated by the emptying of intracellular Ca^{2+} stores. The same proteins can act to restore Ca^{2+} in the ER after global and local Ca^{2+} dependent cellular events. During the resting state, cells can accumulate up to 95% of the intracellular Ca^{2+} in the ER lumen. Surprisingly, it was discovered that 3,5-bis(trifluoromethyl)pyrazole (BTP) compound regulates store-operated calcium channels via the actin-binding protein drebrin (Mercer et al. 2010).

Connexin channels and hemichannels control gradients of signaling molecules, and specifically of Ca^{2+} (Benninger et al. 2008). Finally, astrocytes can use connexins to buffer Ca^{2+} ions as was shown recently by Mark Yeager group (Zhang et al. 2016)

who solved the crystal structure of the first extracellular loop of Cx26 bound to Ca^{2+}. This discovery demonstrated that extracellular loops of connexins can bind Ca^{2+} ions. Ca^{2+} oscillations in primary cultures of astrocytes and neurons can be monitored in living cells with the dye Fluo-4. We recorded Ca^{2+} oscillations with a Nikon Ti imaging system using an Andor Ixon EM-CCD camera and time-lapse imaging of cells excited with a 488 nm laser and reading the Ca^{2+} fluorescence with dichroic emission filters 505 nm beamsplitter and 530/20 nm barrier filter.

How can drebrin sense extracellular and intracellular (ER) Ca^{2+} and how may Ca^{2+} influence the behavior of drebrin? To understand whether drebrin is responding to Ca^{2+} changes, we tested live cell behavior of the drebrin A isoform expressed as a fluorescently tagged molecule. This experiment reveals how drebrins may respond to an elevation of Ca^{2+} level (Fig. 13.7). This way, drebrin rearrangements may help GJ channels and connexin hemichannels to respond to gradients of extracellular Ca^{2+} and to provide bidirectional control of cell–cell communications. Drebrin may also help astrocytes to outline connexin protrusions in proximity to neurons keeping control over the cellular territories. The recently resolved crystal structure of the extracellular loop of connexins revealed that the bound Ca^{2+}ion acted to close the channel electrostatically (Zhang et al. 2016). Connexins expressed in astrocytes may buffer extracellular calcium released by neurons via a similar mechanism.

Although gap junctions provide direct pathways for the exchange of Ca^{2+} between coupled cells, it would be important to know mechanisms of how extracellular information is transferred to the intracellular molecules, which regulate migration, cell–cell communication, and connectivity.

13.9 Degradation of Drebrin by Calpain

The direct relationship between drebrin and Ca^{2+} remained unexplored until recently when it was shown that drebrin can be proteolytically cleaved by calpain, a calcium-dependent, non-lysosomal cysteine protease although it was reported that drebrin degradation is calcium-dependent (Arai et al. 1991). It happens during excitotoxic neuronal death caused by activation of NMDA-type glutamate receptors (NMDARs), but interestingly, pharmacological inhibition of F-actin only facilitated the degradation of drebrin (Chimura et al. 2015). The authors concluded that degradation of drebrin during neurodegeneration is triggered by similar mechanisms and may modulate NMDAR-mediated signal transductions at different stages.

13.10 Cx43 and Drebrin in Astrocytes

In astrocytes Cx43 and Cx30 are the two main GJ channel-forming proteins. Cx43 is stabilized by ZO-1 (Giepmans and Moolenaar 1998; Giepmans et al. 2001) and modified by drebrin (Butkevich et al. 2004). The mobility of astrocytic outgrowth

and the rearrangement of GJ plaques in astrocytes are mediated by drebrin (as seen in Fig. 13.3), because not ZO-1 but drebrin can disassemble actin or bundle F-actin thus providing a tool to direct submembrane activity. Drebrin is one of the most strong morphogenic molecules known (Shirao et al. 1992, 1994; Majoul et al. 2007). In in vitro reconstruction experiments, we showed that drebrin-like protein can bind actin and fold it into ring structures (Butkevich et al. 2015).

In the brain, GJ contacts as well as synapses are constantly modified by specific electrical and metabolic patterns of neuronal activity (Rao et al. 2005; Robel and Sontheimer 2016). Drebrins are crucial for the support of both cell–cell communications and neuronal activity and drebrin-dependent morphological and functional changes contribute to both electrical and chemical signal propagation and to the astrocytic connectivity. Neuronal drebrin A-dependent changes are shown to be involved in learning, memory formation, and other cognitive functions that require spine assembly (Kojima et al. 2016). The dynamic redistribution of drebrin toward the plasma membranes is responsible for dendritic spine maturation (Aoki et al. 2005; Takahashi et al. 2003, 2006).

Drebrin-formed outgrowths help astrocytes to come in a close proximity to active synapses; similar to podocytes in the kidney, astrocytes in the brain form highly branched outgrowths that can sense distant gradients of passing ions (as, e.g., Ca^{2+}), electrolytes, and fluid. Structure-function relationships seen with electron microscopy reveal the distribution of neurons and astrocytes in brain slices and suggest that connexin hemichannels may support neurons by absorbing small metabolites in close proximity to neuronal terminals, dendritic spines, and clefts (Fig.13.8). Drebrin property to move connexins in a close proximity to the terminals (as shown here for Cx43) is likely to master Cx30 adhesive insertion of astrocytic processes into the synaptic cleft for a better control of the glutamate level (Pannasch et al. 2014). During synaptic stimulation, ions and toxic metabolite molecules are released by neurons in the surrounding space (Swanson et al. 2004). Close proximity of astrocytes can facilitate the absorbance of Na^+, K^+, and other molecules; moreover, astrocytes can release ATP in a close proximity to neurons (Farahani et al. 2005). Remarkably, the astrocytic Ca^{2+} is elevated only when the acetyl cholinergic synapses are activated, not when the glutamatergic one; moreover, only mGluR activation can induce Ca^{2+} signaling in astrocytes (Perea et al. 2014).

13.11 Cx43 and Brain Pathology

During the transition into pathological states, astrocytes undergo molecular and morphological changes. Although cases of reactive astrogliosis are clinically ubiquitous, the cellular and molecular mechanisms of astrocyte behavior remain poorly understood. Remarkably, human pathologies of CNS (central nervous system) involve neurons only rarely (neuroblastoma) and even this is mediated via astrocytes, with the most common type of reactive astrogliosis known as glioblastomas. Interestingly, neuroblastoma cells in culture constitutively express drebrin, and drebrin reactivity was attenuated with antisense to drebrin (Toda et al. 1999).

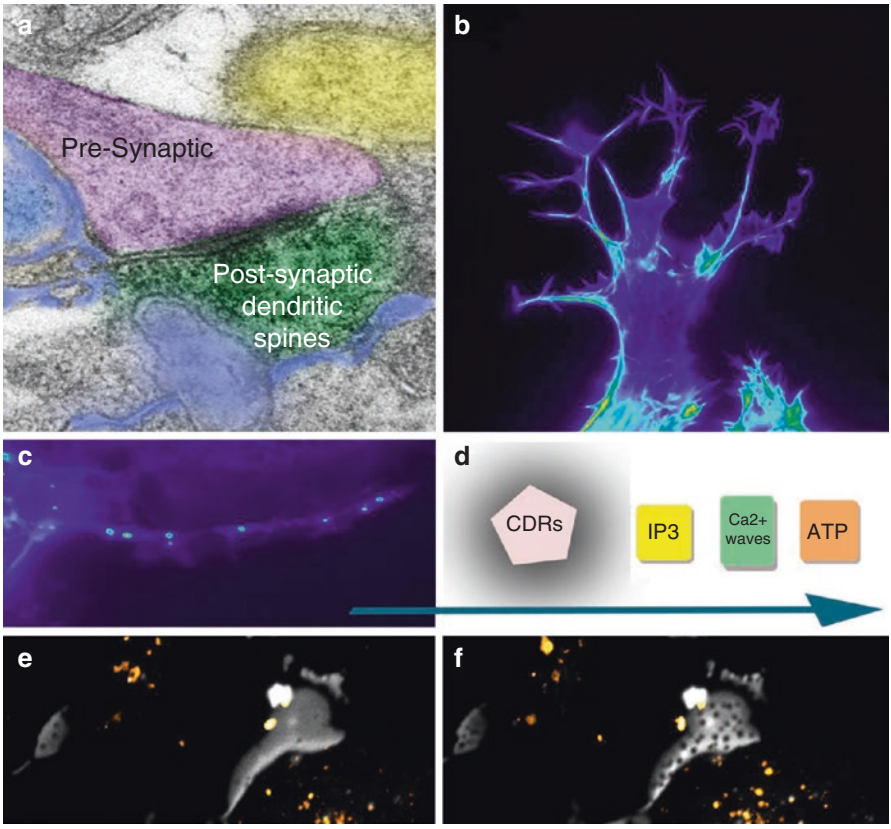

Fig. 13.8 (**a**) Close proximity of glial cells (in *blue*) to the synaptic terminals can be seen by electron microscopy (EM image courtesy of Dr. Vadim Rogachevsky, RAN, Synapse.ru). (**b**) Primary cortical astrocytes were co-transfected with drebrin-CFP and Cx43. (**c**) Cx43 is seen in dotted structures propagating along the drebrin-formed outgrowth. (**e**, **f**) Cx43 gap junction plaque before and during CDR responses (**d**). CDR-induced signaling from the GJ domain may include changes in the second messenger (IP3) release from the PM, Ca^{2+} homeostasis, and ATP level by which astrocytes can sense and respond to the changes in the molecular gradients in neuronal proximity

Reactive astrogliosis and scar formation should complete the neural repair by protecting neurons and regulating the inflammatory CNS responses. Instead, in many cases glial scar formation rather inhibits axonal regeneration. In an attempt to decrease reactive gliosis, conditional Cx43 KO animals have been studied. Reactive astrogliosis should regulate the uptake of glutamate that is toxic for neurons by producing glutathione and reducing oxidative stress and by releasing ATP and adenosine (Chen et al. 2001), and Cx43 expressed in astrocytes is functioning in form of channels and hemichannels upon hypoxic preconditioning (Bosch et al. 2014). In contrast to expected results, astrocytes from Cx43 KO mice show a decreased neuroprotective effect of hypoxic preconditioning. Instead, the KO of another channel molecule, the water-transporting aquaporin AQP4, in astrocytes reduced cytotoxic

swelling and improved the outcome after stroke (Zador et al. 2009). A possible role of drebrin was not addressed in these studies.

13.12 Drebrin and Neurologic Disorders

Cellular analyses revealed that small GTPases and their regulators are involved in disruption of cell–cell contacts and in dendritic spine pathology (Lan et al. 2005). Mutations in guanine nucleotide exchange factors (GEFs), whose crucial functions are to exchange GTP for GDP on these GTPases, are known to cause disease states. Taking into account the described above action of bacterial toxins on Rho GTPases and the dependence of drebrin on RhoA, described above, suggests that the disbalance between these proteins may result in human pathologies. Indeed, kalirin, a protein directly interacting with drebrin, is an exchange factor for Rho (i.e. a Rho-GEF) that is modifying dendritic spines in control and disease states (Penzes et al. 2003).

Even more severe diseases are related to the GTPase-activating proteins (GAPs) that accelerate GTP hydrolysis on small GTPases that regulate the actin cytoskeleton. The X-linked mental retardation protein oligophrenin-1 is a Rho-GAP protein and possesses not only GAP activity for small GTPase Rho but also affects Rac and Cdc42 as well. Oligophrenin-1 is found in both neuronal and astroglial cells, and it colocalizes with actin at the leading edges (Fauchereau et al. 2003). Interestingly, even more than actin, such leading edges are found to carry drebrin (as, e.g., seen in Figs. 13.4b, d and 13.5). GTPase activity is important for the spine formation and if blocked would increase unbalanced activity of Rho GTPases resulting in disruption of spines. However, the brain pathology and cellular function of oligophrenin-1 still remain to be elucidated in details.

Decreased levels of drebrin have been shown in the brains of Alzheimer patients (AD) (Harigaya et al. 1996; Shim and Lubec 2002). Analyzed at postmortem pathology levels, drebrin decay was restricted to specific brain regions. The decay of drebrin alters spine morphology and changes numerous functions of axons and dendrites. Drebrin decay disrupts connexin-mediated astrocytic connectivity thus decreasing metabolic support of synaptic connectivity. Drebrin was shown to be involved in both astrocytic action and neuronal experience-dependent plasticity of synaptic transmission, the process that represents the cellular basis of learning. Neurodegeneration and aging reveal morphologic tissue disruption, decreased spine density, loss of pyramidal neurons, and metabolic changes that are believed to be dependent on astroglial connectivity supported by drebrin.

13.13 Drebrin-Mediated Plasticity of Gap Junction Communications in Brain

High-resolution electron microscopy images revealed that astrocytes are often surrounding neuronal terminals. The functional significance of astrocytic gap junctions in a close proximity to synaptic terminals is of great interest (Jeanson et al. 2016;

Genoud et al. 2015). 3-D reconstructions of the hippocampal neurons revealed that astrocytes are covering newly formed spines rather than the larger established ones (Medvedev et al. 2014). It was also shown that astrocytes direct microglial cells to phagocytose weak synapses in the retino-geniculate system, thereby participating in the elimination of "wrong" and "weak" signaling (Stevens et al. 2007). Gap junctions not only connect cell interiors but can also unite cells in syncytium-like clusters that can provide additional help in establishment of common properties such as collective cell polarization and ion-dependent signal propagation. Indeed connexin-mediated astrocytic Ca^{2+} wave propagations spread much more efficiently than that produced by the firing of individual neurons. Astrocytic connectivity is based mainly on connexins Cx43 and Cx30 which provide fast bidirectional communications important for transmitting neurons Ca^{2+}, IP3, cAMP, cGMP, and metabolites. How drebrin activity influences Cx43 channels in different tissues remains unclear, but the downregulation of drebrin in Alzheimer's disease may contribute not only to deregulation of spines but also to a reduction in astrocytic connectivity of both channels and hemichannels. Massive opening of astrocytic hemichannels induced in hippocampal slices of AD mice correlates with the loss of ATP and resulted in cell death (Orellana et al. 2013; Bosch and Kielian 2014). Modulation of connexin hemichannels seems to impair memory in Alzheimer's mice (reviewed by Koulakoff et al. 2012). Except Cx43, drebrin activity may influence other connexins as, e.g., Cx36 that is upregulated in early development and acts before neuronal receptors are expressed (Arumugam et al. 2005).

Live cell reconstruction experiments allow us to hypothesize that highly dynamic drebrin-formed outgrowths of astrocytes may bring Cx43 GJ channels and hemichannels into close proximity with dendrites and synaptic terminals. We hypothesize that this proximity has functional implications that are based on signaling and on metabolic cooperation between the two main types of brain cells. Moreover, we discovered millisecond-fast rearrangements of connexin channel clusters—so-called CDRs—formed inside the GJ plaques in response to stress agents (Majoul et al. 2013). Similar fast mechanisms can be used by the astrocytic gap junctions to sense "hot spots" caused by neuronal terminals. This new aspect allows to suggest that close proximity of connexin channels can dramatically influence neuronal behavior (Alvarez-Maubecin et al. 2000; Malchiodi-Albedi et al. 2012). Based on these discoveries and other hypothetical considerations, we suggest a role of drebrin-connexin interactions important for brain function (Fig. 13.8).

Different types of neurons release specific neurotransmitters such as glutamate, GABA, ACh, and 5-HT to activate common Ca^{2+} and ATP-dependent responses in glial cells. Astrocytic connexin channels and hemichannels can induce and propagate IP3 and Ca^{2+} gradients across the 3-D space of astroglial circuits thereby providing a supplement layer of brain connectivity that is, as yet, less well understood (Bani-Yaghoub et al. 1999; Belliveau et al. 1997, 2006). In this way, astroglial connexins can contribute to dynamic neuro-glial interactions. Although still poorly understood, it seems likely that the drebrin-Cx43 interactions will assume an ever-larger role in our understanding of the conceptually exciting, structure - function relationships between astrocytes and neurons on brain function.

13.14 Perspectives and Future Directions

Drebrin's role in supporting cell–cell contacts in astrocytes is as important as its role affecting neuronal spines or post-synaptic density. The cytoskeletal rearrangements leading to cell–cell contacts are necessary to regulate cell signaling and the metabolic flow of information. Cx43 together with Cx30 are the two main isoforms expressed in astrocytes, and drebrin seems to modify the plasticity of cell–cell contacts to either, increase or limit the flow of information through the astrocytic GJs. The drebrin-dependent mobility of connexin hemichannels in the PM is vital to support neuronal activity by releasing the key cellular energy molecule ATP, where and when it is most needed (Belliveau et al. 2006). Although many questions covering neuronal receptors, gap junctions, membrane receptors, ion channels, effector molecules, and the submembrane cytoskeleton still remain open (Vargas et al. 2008), we believe that future studies of drebrin's role in brain connectivity will uncover new molecular targets for drugs designed to limit the loss of brain function associated with aging, neurodegeneration and Alzheimer's disease, and other cognitive disorders. New discoveries should bring light to these molecules and reveal drugs for treatment of cognitive disorders.

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