Tomoaki Shirao
Yuko Sekino   *Editors*

Drebrin

From Structure and Function to Physiological and Pathological Roles

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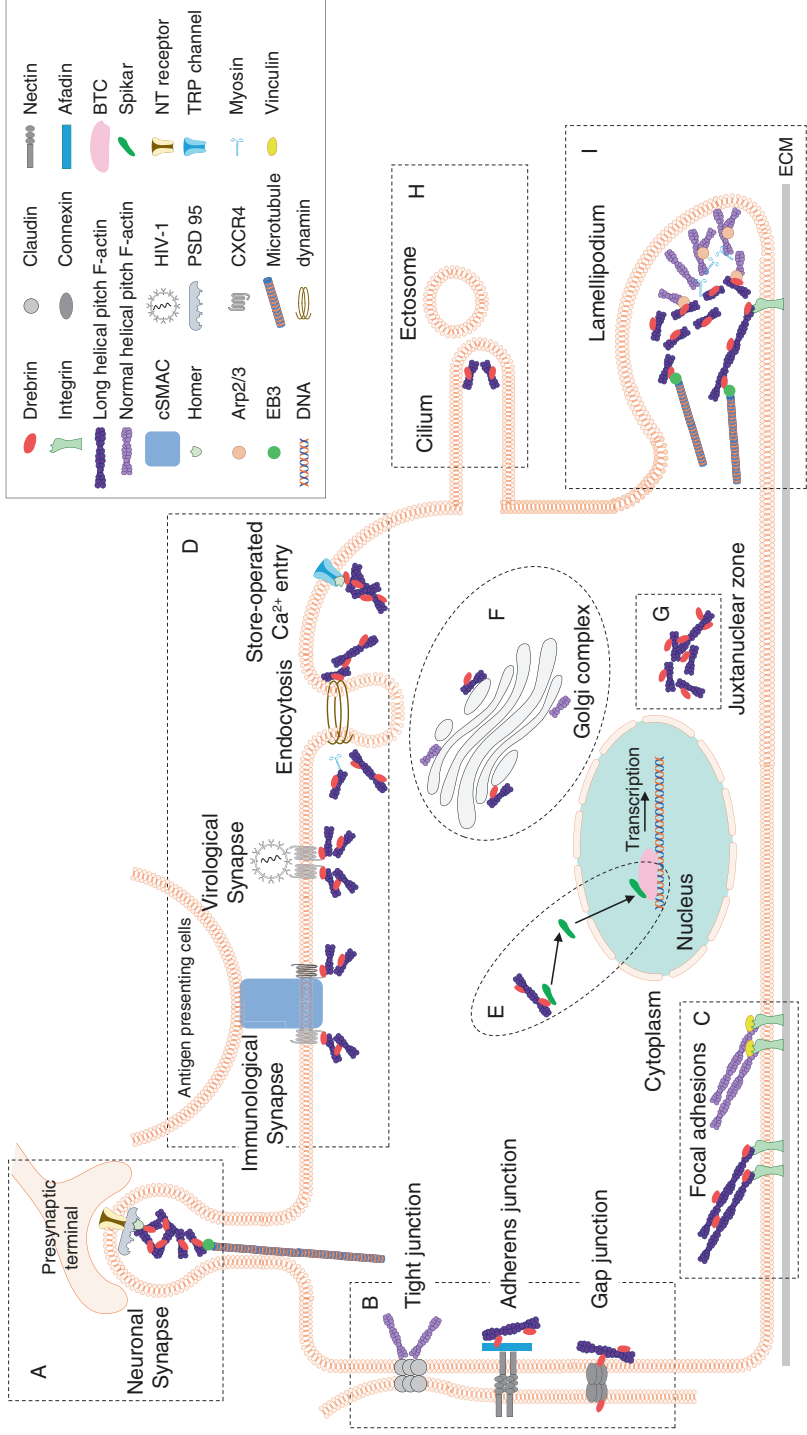
Drebrin forms unique stable F-actin with a long helical crossover and plays a pivotal role in intercellular communication at neuronal synapses, adherens and gap junctions, and immunological and virological synapses. Additionally, drebrin is involved in the cellular mechanisms of cell migration, cell process formation, cancer metastasis, and gene transcription through a transcription co-activator spikar. Drebrin is also found on the Golgi complex, at the juxtanuclear zone, and at a tip of cilia

Tomoaki Shirao • Yuko Sekino
Editors

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and Pathological Roles

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*This book is dedicated to our mentor
Dr. Kunihiko Obata whose wise counsel has
led us to the discovery and a deeper
understanding of drebrin.*

Preface

This book consolidates drebrin studies that have accumulated over three decades, since the first identification of drebrin by our group in 1985. Although in the 1980s we could not envision exactly how the study of drebrin would develop, the progress reflected in the chapters presented here was beyond our wildest expectations. This book begins with a general introduction of drebrin from a historical perspective, and then the chapters in the second part provide the molecular characterization of drebrin and drebrin-decorated F-actin. The third and fourth parts discuss its function in the nervous and non-nervous system, respectively.

This review will appeal to researchers who are interested in synapse formation and synaptic plasticity, as well as subcellular local morphogenesis, such as cell protrusion formation, cell migration, intercellular junction formation, and endocytosis. The book will also appeal to researchers who use drebrin as a tool, such as a marker of synaptic function or a disease marker. This book was kept as concise as possible, to be understood by readers from diverse scientific disciplines. Because of the clarity of its presentations, it can also serve as a textbook in graduate courses.

We wish to express our gratitude to the authors who so willingly contributed to this book. We would also like to thank the staff of Springer Japan, in particular Ms. Momoko Asawa and Dr. Yasutaka Okazaki.

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Contents

Chapter 8
Making of a Synapse: Recurrent Roles of Drebrin A at Excitatory Synapses Throughout Life

Chiye Aoki and Ang D. Sherpa

Abstract Mature excitatory synapses are composed of more than 1500 proteins postsynaptically and hundreds more that operate presynaptically. Among them, drebrin is an F-actin-binding protein that increases noticeably during juvenile synaptogenesis. Electron microscopic analysis reveals that drebrin is highly enriched specifically on the postsynaptic side of excitatory synapses. Since dendritic spines are structures specialized for excitatory synaptic transmission, the function of drebrin was probed by analyzing the ultrastructural characteristics of dendritic spines of animals with genetic deletion of drebrin A (DAKO), the adult isoform of drebrin. Electron microscopic analyses revealed that these brains are surprisingly intact, in that axo-spinous synaptic junctions are well-formed and not significantly altered in number. This normal ultrastructure may be because drebrin E, the alternate embryonic isoform, compensates for the genetic deletion of drebrin A. However, DAKO results in the loss of homeostatic plasticity of N-methyl-D-aspartate receptors (NMDARs). The NMDAR activation-dependent trafficking of the NR2A subunit-containing NMDARs from dendritic shafts into spine head cytoplasm is greatly diminished within brains of DAKO. Conversely, within brains of wild-type rodents, spines respond to NMDAR blockade with influx of F-actin, drebrin A, and NR2A subunits of NMDARs. These observations indicate that drebrin A facilitates the trafficking of NMDAR cargos in an F-actin-dependent manner to mediate homeostatic plasticity. Analysis of the brains of transgenic mice used as models of Alzheimer's disease (AD) reveals that the loss of drebrin from dendritic spines predates the emergence of synaptic dysfunction and cognitive impairment, suggesting that this form of homeostatic plasticity contributes toward cognition. Two studies suggest that the nature of drebrin's interaction with NMDARs is dependent on the receptor's subunit composition. Drebrin A can be found co-clustering with NR2B-containing NMDARs at the plasma membrane,

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while NR2A-containing NMDARs co-traffic into the spine cytoplasm but do not co-cluster at the plasma membrane. Most recently, we encountered a physiological condition that supports this idea. When adolescent female rats are reared under a condition of restricted food access and ad libitum wheel access, they paradoxically become excessive runners, choosing to run, even during the limited hours of food availability. This behavioral pattern is termed activity-based anorexia (ABA) and has served as an animal model for anorexia nervosa. Those animals that exhibit the greatest ABA vulnerability, in that they lose the most amount of body weight and run with greatest exuberance to the point of risking their lives, exhibit the highest levels of NR2B-NMDARs and drebrin at the postsynaptic membrane of hippocampal pyramidal neurons. Those animals that exhibit the greatest resilience to ABA, in that they run minimally under such condition, thereby losing minimal amount of weight, exhibit the highest level of NR2A-NMDARs in the spine cytoplasm and lowest levels of drebrin at the postsynaptic membrane. This pattern suggests that drebrin has dual roles: retention of NR2A-NMDARs in the reserve pool and trafficking of NR2B-NMDARs to the postsynaptic membrane, ultimately contributing to an individual's reactivity to stress. Altogether, these observations indicate that drebrin is a protein that is important for synaptic plasticity and deserves the attention of neuroscientists studying the neurobiological basis of cognition and stress reactivity.

8.1 The Critical Period for Developmental Plasticity Overlaps with the Period of Robust Synaptogenesis

So much of who we are is a result of the sensory, emotional, and cognitive experiences that we encounter during the first phase of life—the juvenile period. The unique environmental circumstances that we encounter during the juvenile period stimulate the afferents entering the neocortex, thereby driving the formation of new synapses—synaptogenesis—within the neocortex. For rodents and cats, almost all of the synaptogenesis in the neocortex begins postnatally. Synapse number increases by more than 15-fold, attaining adult-like levels toward the end of the juvenile stage of development (Blue and Parnavelas 1983; Cragg 1975; De Felipe et al. 1997; Kageyama and Robertson 1993; Micheva and Beaulieu 1997; Ramaswamy and Markram 2015). The critical period for developmental plasticity is defined as the time during which sensory experience sculpts receptive field properties of neocortical neurons to last a lifetime. This critical period coincides with the latter half of the juvenile phase for exuberant synaptogenesis (Gordon and Stryker 1996;

Hubel and Wiesel 1970; Wiesel 1982). Synaptogenesis in the primate neocortex follows a slightly different time course, since synaptogenesis begins prenatally: however, at least a twofold increase in synapse density occurs postnatally, again coinciding with the critical period for the formation of the receptive field properties (Bourgeois 1997).

The critical period for developmental plasticity is constant within any one species, but varies across species, indicating that most of the molecular events dictating the beginning and the end of the critical period are genetically defined. There are also slight differences in the timing of developmental plasticity across cortical regions within any one species. One cortical region with a significantly prolonged phase of plasticity and synaptogenesis is the hippocampus. Here, the dendritic growth phase is prolonged to the end of adolescence, rather than ending with the juvenile stage. The formation of dendritic spines keeps pace with dendritic branch growths, resulting in a roughly constant density of spines (Chowdhury et al. 2014b). However, a closer look at the spines of adolescent hippocampus indicates that as many as half of the spines are filopodia-like: immature, elongated, and thin (Chowdhury et al. 2014b). In accordance with this prolonged phase of synaptogenesis, mature place fields do not emerge until late adolescence (Martin and Berthoz 2002). The reason for this prolonged phase of synaptogenesis in the hippocampus and other neocortical regions that are primarily cortico-cortical (e.g., area V4) may be that they must wait for their afferents to attain maturity, before they initiate the activity-dependent synaptogenesis.

Mature excitatory synapses are composed of more than 1500 proteins postsynaptically and hundreds more that operate presynaptically (Ryan and Grant 2009). One among them that increases in level during synaptogenesis is drebrin, an F-actin-binding protein that occurs highly enriched, specifically on the postsynaptic side. In this chapter, I will review findings indicative of the function of the adult isoform, drebrin A, that could be gleaned from electron microscopic analyses of intact brain tissue. One idea that emerges from those studies is that drebrin A participates in the activity-dependent trafficking of NMDARs (*N*-methyl-D-aspartate receptors) via interaction with F-actin.

8.2   Drebrin A Is Involved in Excitatory Synaptic Function

Drebrin A is a soluble neuronal protein with a distinctive property of binding to F-actin (Hayashi and Shirao 1999; Shirao and Obata 1986). Although F-actin occurs abundantly in presynaptic axon terminals and dendritic spines, drebrin A occurs only on the postsynaptic side (Aoki et al. 2005), indicating that its preferential trafficking to dendrites and spines is somehow specified. Its putative involvement in synaptic function was first surmised, based on the dramatic increase of its level during the first postnatal week within chicken brains (Shirao et al. 1988). It was later shown that one of the two isoforms of drebrin, called drebrin A, emerges after around

Fig. 8.1 Drebrin A immunoreactivity within dendritic spines is revealed by using silver-intensified gold (SIG) as the immunolabel for electron microscopic immunocytochemistry. SP1 and SP2 are both dendritic spines. Five SIG particles are evident within SP2, while SP1 contains a greater number of SIG particles, indicating higher level of drebrin A within. The presynaptic axon terminal for SP1 and SP2 are labeled as T1 and T2, respectively. The synaptic junctions associated with T1 and T2 are asymmetric and presumed to be excitatory, based on the presence of thick PSDs (*white asterisks*). This figure was adapted from Fig. 2 of a published paper (Mahadomrongkul et al. 2005)
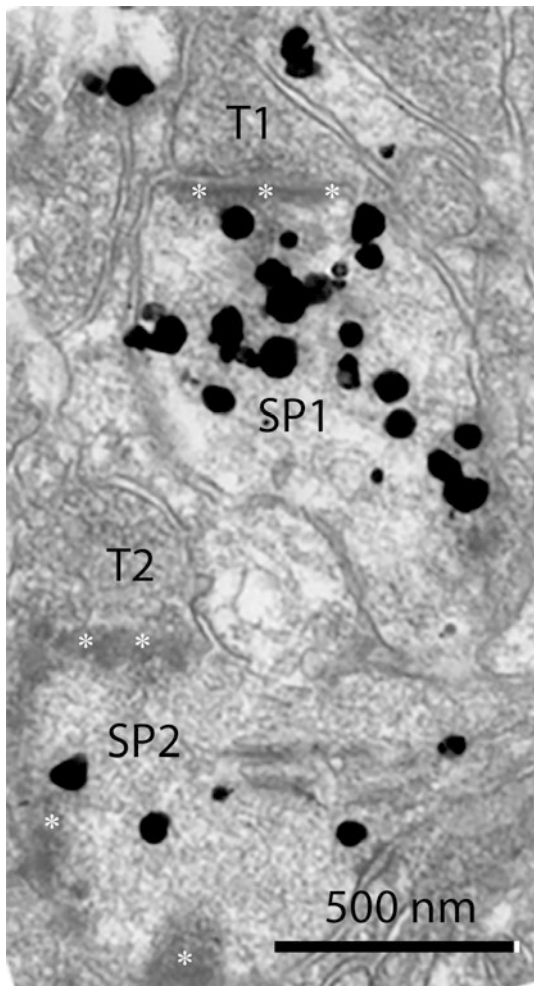
postnatal day (PNd) 10 in the cortex and the hippocampus of rat brains to replace an embryonic isoform, drebrin E (Aoki et al. 2005). Using an antibody that is specific to drebrin A, electron microscopic immunocytochemistry (EM ICC) was performed to determine the subcellular distribution pattern of drebrin A. In adulthood, it was shown that drebrin A is neuronal, detectable on the postsynaptic side of about 70% of the asymmetric (presumably excitatory) axo-synapses (Fig. 8.1). These asymmetric synapses belong to spines of pyramidal (i.e., glutamatergic) neurons and dendritic shafts of GABAergic interneurons. In contrast, none of the symmetric synapses are immunoreactive to drebrin A, whether on dendritic shafts of pyramidal neurons or on GABAergic interneurons. Thus, at least in the hippocampus and cortex, drebrin A is involved in excitatory synaptic function of all cell types and is not involved in inhibitory postsynaptic function, regardless of the cell types (Fig. 8.2).
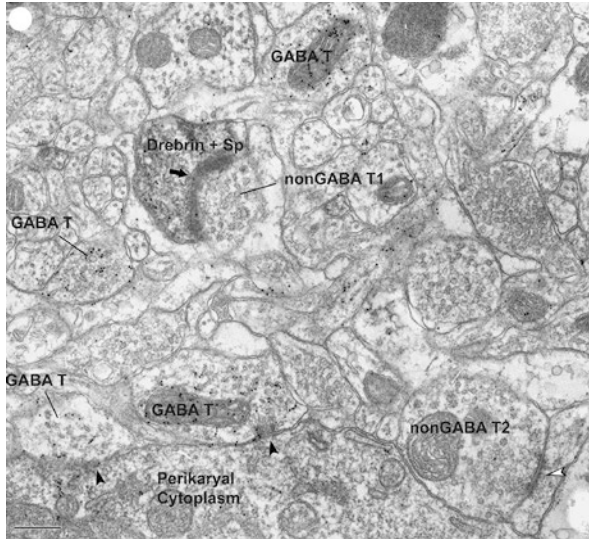
Fig. 8.2 Drebrin A immunoreactivity occurs postsynaptic to excitatory synapses but are absent at GABAergic inhibitory synapses. Adult hippocampal tissue was immunolabeled dually—by DAB—to immunolabel drebrin A and by post-embed gold (PEG) to immunolabel GABA. The spine immunoreactive to drebrin A (Drebrin + Sp) exhibits a thick PSD (*arrow*), indicating that it is forming an excitatory synapse. The presynaptic terminal lacks PEG particles, indicating that it is not GABAergic. There are three GABAergic terminals shown in this field, all of which contain numerous (an excess of ten) PEG particles that are 10 nm in diameter. Arrowheads point to the postsynaptic side of the inhibitory synaptic junctions, which lack the thick PSDs. This figure was modified from Fig. 5 of a published paper (Aoki et al. 2005)

8.3 Is Drebrin A for Morphogenesis or Trafficking of Synaptic Molecules?

Soon after its discovery, drebrin (i.e., drebrin A or E) was recognized to have multiple properties conducive of orchestrating the interaction of cytoskeletal protein complexes within spines. For one, drebrin competes with tropomyosin for binding to F-actin (Shirao 1995). Drebrin also inhibits the actin-activated myosin ATPase and reduces the sliding velocity of actin filaments along immobilized myosin (Hayashi et al. 1996). These were clues that drebrin could be involved in one or both types of cellular processes: (1) neuronal morphogenesis and (2) actomyosin trafficking of cargos.

In support of the idea that drebrin A is involved in spine morphogenesis, it was shown that when drebrin is overexpressed in fibroblasts or CHO (Chinese hamster ovary) cells, F-actin filaments bind preferentially to drebrin rather than tropomyosin, the consequence of which is that thick, curving F-actin bundles form, leading to the appearance of neurite-like processes (Hayashi and Shirao 1999; Shirao 1995).

EM-ICC revealed that, unlike the adult tissue, which showed drebrin A to be predominantly cytoplasmic within dendritic spines, drebrin A in neonatal tissue (rat cortex at PNd7) occurs more frequently associated with the dendritic plasma membrane (Fig. 8.3). These dendritic plasma membranes are part of the flat dendritic shafts, since most dendrites at this stage are immature and have yet to form spines (Fig. 8.3, panel A) or are spinous membranes lacking well-defined PSDs (Fig. 8.3, panel B) and contain only low levels of NMDARs (Fig. 8.4) (Aoki et al. 2005). Through elegant molecular techniques to increase or decrease the expression of drebrin A, it has been shown that dendritic spines of cultured hippocampal neurons contain drebrin A-F-actin clusters before the arrival of PSD-95. When PSD-95 arrives to the spines, they co-cluster precisely where drebrin A-F-actin clusters are located. If drebrin A-F-actin clusters are experimentally depleted, then PSD-95 is no longer able to enter and be retained within spines (Takahashi et al. 2003). Conversely, if the F-actin-binding domain of drebrin A is deleted, drebrin A does not cluster in spines, even if overexpressed: instead, it remains diffusely distributed in both shafts and spines (Hayashi and Shirao 1999). These results suggest that the drebrin A-F-actin complex arrives to newly forming synaptic junctions and enables their maturation.

Within mature tissue, there appears to be a limited capacity for drebrin A to enter spines. This idea is based on an observation that overexpression of drebrin A by as much as 20% has no effect of increasing the proportion or level of drebrin A within spines, although the number of spines per unit volume is increased (Kobayashi et al. 2005). This indicates that drebrin A does not diffuse passively into spines. Indeed, there is evidence to indicate that the spine's capacity to house drebrin A is activity dependent. An in vivo study has shown that blockade of NMDARs within intact neocortex by superfusing the pial surface with the NMDAR antagonist, D-APV (D-2-aminopho-5-phosphovalerate), increases the proportion of spines immunoreactive to drebrin A as well as the levels of drebrin A within individual spines within 30 min (Fujisawa et al. 2006) (Fig. 8.5). The same tissue also increases the level of F-actin within spines (Fujisawa et al. 2006), together with the NR2A-containing NMDARs (Aoki et al. 2003), which are followed by a rise of PSD-95 within another 30 min (unpublished observations). The D-APV blockade-evoked upregulation of NMDARs can be considered to be a homeostatic plasticity mechanism that compensates for the pharmacologically induced reduction of NMDAR activity. A converse experiment, conducted using hippocampal neurons grown in vitro, confirms the link between NMDAR activity and drebrin-F-actin complexes in spines. Specifically, it has been shown that activation of NMDARs evokes the exodus of F-actin-bound drebrin A via activation of myosin II ATPase but not by the myosin light chain kinase or the Rho-associated kinase (Mizui et al. 2014). A scenario compatible with these observations is that drebrin A enters spine heads as a complex with F-actin from dendritic shafts. The net influx of drebrin A-F-actin complexes into spines could contribute toward trapping cargos containing NMDARs and PSD-95 within spine heads, once they have entered through the spine necks from dendritic shafts
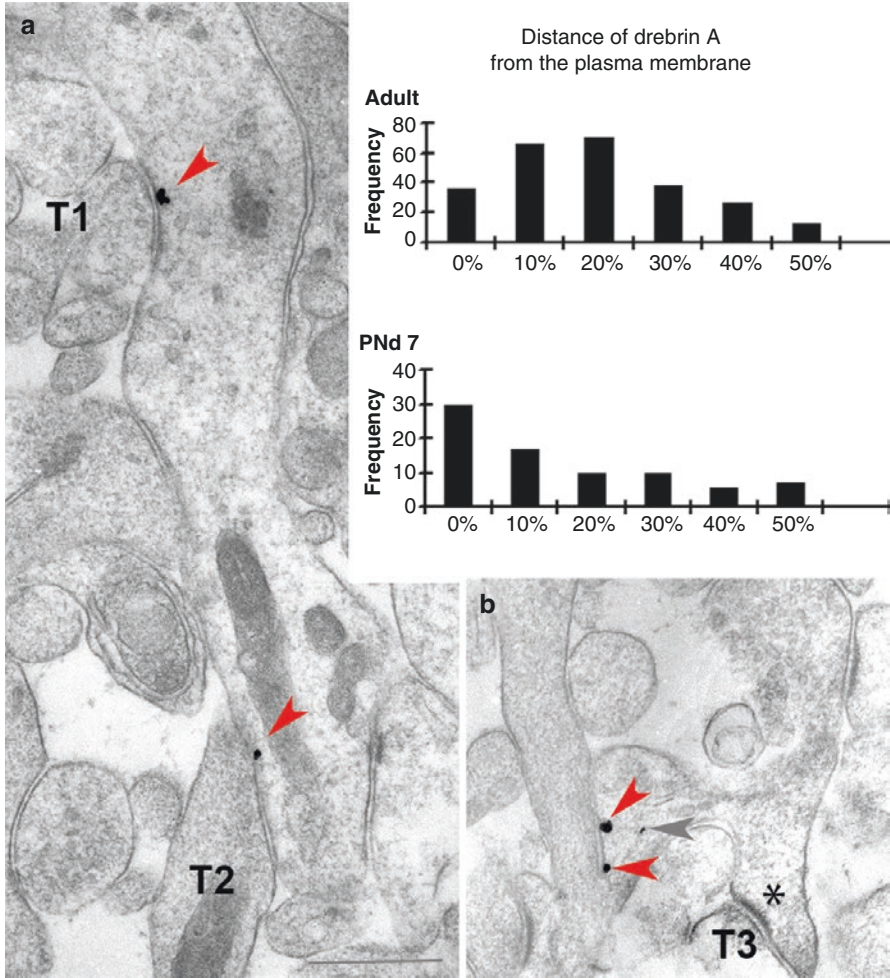
Fig. 8.3 Drebrin A immunoreactivity occurs more often over the plasma membrane of dendrites at PNd 7, when compared to adulthood. Silver-intensified gold particles (SIG) reflecting drebrin A immunoreactivity were quantified across multiple dendritic profiles in the hippocampus and tallied, based on their position as being exactly on the plasma membrane (0% distance from the plasma membrane in the bar graph), 50% from the plasma membrane, if equal distant from the two opposing plasma membranes sides of the dendrite, or at an intermediate distance between the midpoint and on the plasma membrane (10–40%). The frequency of SIG particles on the plasma membrane was higher at PNd7 than in adulthood (histogram on *top right*). Panels A and B show examples of such plasmalemmal labeling of drebrin A (*red arrowheads*) at PNd 7. Both immunolabels in panel A occur along smooth aspiny portions of the dendritic plasma membrane. T1 and T2 are presynaptic axon terminals, indicating that drebrin A is associated with postsynaptic membranes. In panel B, two drebrin A immunolabels occur along the plasma membrane of a spine head that has yet to acquire PSDs, such as the one that is postsynaptic to T3 (*asterisk*). The gray arrowhead is pointing to an example of an SIG that resides at a position removed from the plasma membrane. The calibration bar in panel A = 500 nm and also applies to panel B. This figure was modified from Figs. 10 and 11 of a published paper (Aoki et al. 2005)
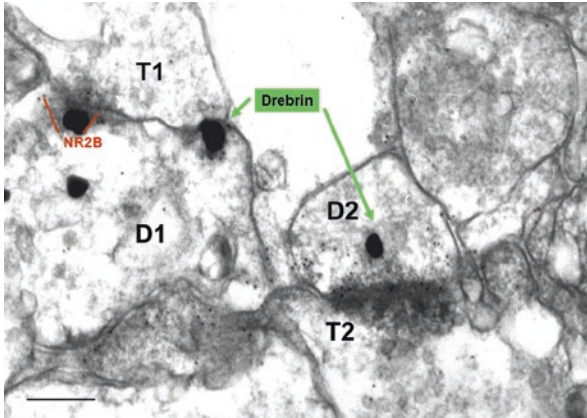
Fig. 8.4 Drebrin A immunoreactivity of PNd7 hippocampal tissue occurs at the plasma membrane patches of dendrites expressing low levels of immunoreactivity for the NR2B subunit of NMDA receptors and no PSD but occur removed from the plasma membrane of synapses with PSDs and higher levels of NR2B. Two dendrites, D1 and D2, with contrasting morphological features and immunoreactivity for the NR2B subunit of NMDA receptors are shown. Immunoreactivity of synapses to the NR2B subunit of NMDA receptors was visualized using 10 nm colloidal gold (PEG, post-embed gold). Red lines point to two examples of PEG particles reflecting NR2B immunoreactivity that are associated with the synaptic junction between dendrite D1 and axon terminal T1. PEG particles are more numerous for D2 than for D1, indicating higher levels of NR2B immunoreactivity in D2. T2 that is presynaptic to dendrite D2 contains more vesicles than in T1. Another difference between the two synapses is that D2 exhibits a PSD, while D1 does not. These three features together indicate that the synapse T2–D2 is relatively more mature than synapse T1–D1. Drebrin A immunoreactivity was detected using silver-intensified gold (SIG, *green arrows*) as immuno-labels. SIG particles reside over the plasma membrane of D1 but is removed from the plasma membrane of D2. These contrasting patterns, together with the quantification shown in Fig. 8.3, indicate that drebrin A tends to be associated with the plasma membrane of immature axo-dendritic synapses but becomes displaced from the plasma membrane once synapse stabilization is attained. Calibration bar = 500 nm. This figure was modified from Fig. 8 of a published paper (Aoki et al. 2005)

via inhibition of the actomyosin ATPase (Hayashi et al. 1996). Such a sequence of events explains the net rise of NMDARs and PSD-95 that we have observed at spines following NMDAR-blockade with D-APV. However, one important point to make here is that although the D-APV treatment increases the influx of NR2A immunoreactivity in the spine cytoplasm, we have not observed a rise of NR2A-NMDARs at the postsynaptic plasma membrane. This may be because the final step involving the exocytosis of cargos carrying NR2A-NMDARs requires NMDAR activity (Barria and Malinow 2002). The signal that promotes the entry of the drebrin A-F-actin complex into spines could be the lowered level of intracellular calcium: NMDARs permeate calcium, when activated, so blockade of NMDARs by D-APV would have the consequence of lowering intracellular levels of calcium.

A critical test of this working hypothesis was to determine the consequence of removing drebrin A from cortical spines. Indeed, when the D-APV treatment of
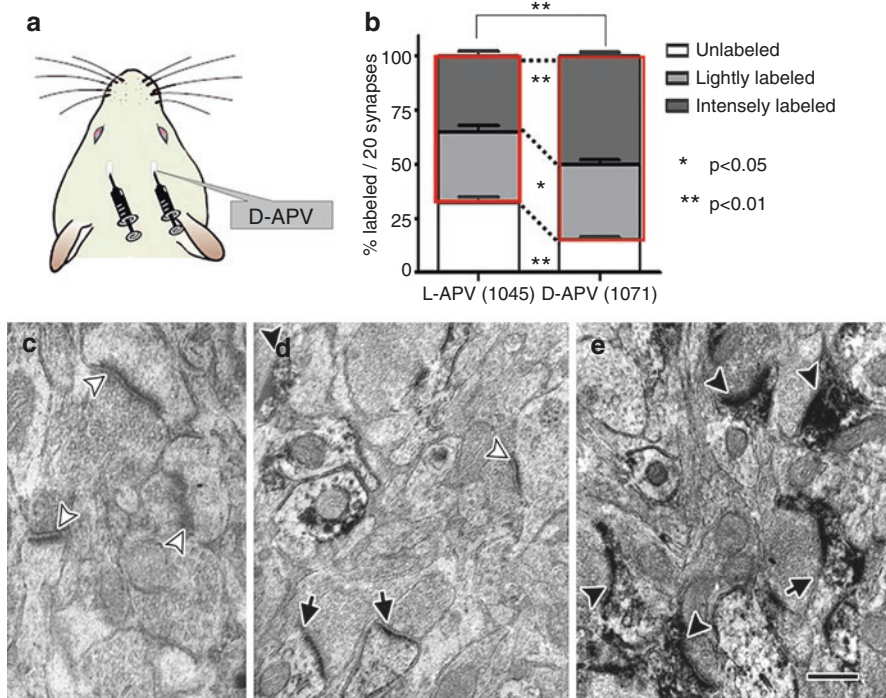
Fig. 8.5 D-APV blockade of NMDARs elicits trafficking of drebrin A into dendritic spines. Panel *A* illustrates the procedure followed for in vivo blockade of NMDARs. Small holes overlying the somatosensory cortex were drilled, and D-APV, an NMDAR blocker, was infused over the pia matter of one hemisphere while maintaining the animal anesthetized with isoflurane. The contralateral hemisphere received L-APV, an inactive enantiomer. 30 min to 2 h later, animals were transcardially perfused with a fixative to localize drebrin A and F-actin in place. Panel *C* shows an example of an electron micrograph of a tissue that was incubated with all immunoreagents minus the primary antibody directed against drebrin A. Panel *D* shows an example from the hemisphere that received L-APV, while panel *E* shows an example of the neuropil from the hemisphere that received D-APV. *Black arrowheads* point to spines labeled intensely for drebrin A, using DAB as the immunolabel. White arrowheads point to unlabeled spines. *Arrows* point to less completely immunolabeled spines. Panel *B* shows the results of the quantification, indicating that the proportion of spines intensely immunolabeled, lightly labeled, and unlabeled were all significantly different between the two hemispheres. D-APV significantly increased the proportion of spines that were immunolabeled for drebrin A. Each bar of the graph represents mean + SEM. Horizontal calibration bar in panel *E* = 500 nm and applies to all three micrographs. Adapted from Fig. 4 of a published article (Fujisawa et al. 2006)

cortical tissue was repeated upon mice with genetic deletion (knockout) of drebrin A (DAKO), these cortical spines no longer responded with increased levels of NR2A-NMDARs in the cytoplasm (Aoki et al. 2009) (Fig. 8.6). While these results indicate that trafficking of NR2A-NMDARs into spines is dependent on the expression of drebrin A, we have not tested the interdependence of drebrin A expression
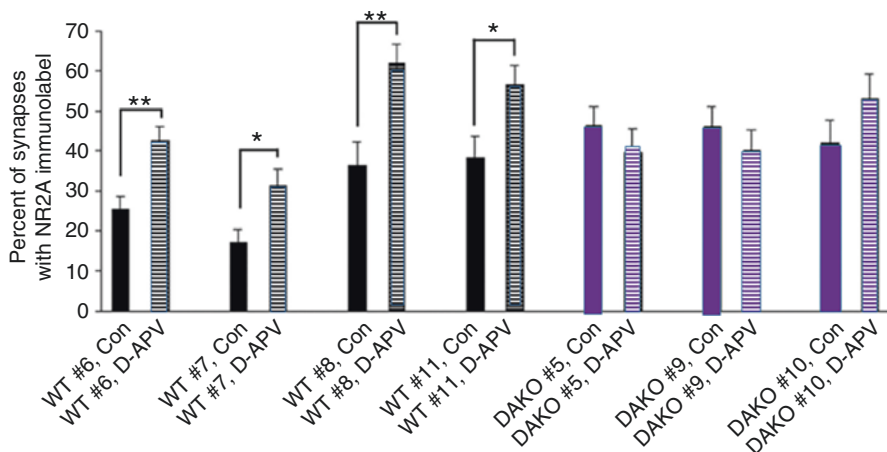
Fig. 8.6 Drebrin A knockout (DAKO) eliminates the D-APV blockade-evoked increase of NR2A immunoreactivity within dendritic spines. Each pair of bars depicts the proportion of dendritic spines in layer 1 of somatosensory cortex with immunoreactivity to the NR2A subunit of NMDARs within the two hemispheres of a single animal (mean + SEM). One hemisphere received D-APV, the NMDAR blocker (*striped bars*), while the contralateral hemisphere received the inactive enantiomer, L-APV ("Con," *solid bars*). Black pair of bars depict the results obtained from wild-type adult mice, while the purple pair of bars depict results from DAKO mice. In four out of four wild-type cases, the interhemispheric differences in immunolabeling for the NR2A subunit of NMDARs were significant. In three out of the three DAKO cases, the interhemispheric differences in immunolabeling were not significant. Modified from Fig. 5 of a published article (Aoki et al. 2009)

with the activity-dependent trafficking of NR2B-NMDARs using DAKO tissue. This point is relevant, because the trafficking of NR2A-NMDAR and NR2B-NMDAR is in opposite directions (NR2B-NMDAR declines, while the NR2A-NMDAR levels rise) following D-APV blockade of wild-type neocortex (Fujisawa and Aoki 2003).

8.4 Additional Phenotypes of Drebrin A KO: Beyond the Loss of Homeostatic Plasticity?

As was summarized above, a number of works involving cultured hippocampal neurons have indicated that drebrin A facilitates spine morphogenesis. Moreover, since our previous EM analysis had revealed that drebrin A level within spines correlates positively with spine size within adult cortex (Kobayashi et al. 2007), we expected DAKO to cause a reduction in the number and size of dendritic spines. Contrary to

Fig. 8.7 Perforated and non-perforated PSDs. Some of the PSDs of excitatory synapses occur perforated. Typically, perforated PSDs are associated with the larger mushroom head spines. This micrograph was taken from layer 1 of a young adult mouse cortex
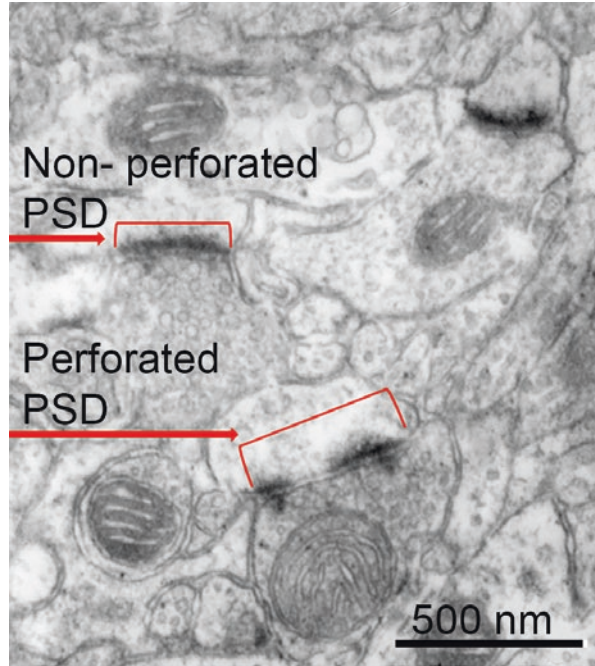
this expectation, DAKO does not induce any obvious alteration in spine density or spine morphology. The parameters we measured were the number of synapses per unit volume, the width of spines, and the width of PSDs. We also could not detect any difference in the synaptic or cytoplasmic localization of NR2A-containing NMDARs within spines at basal levels. Such lack of phenotypic difference suggests that when drebrin A is genetically deleted, drebrin E persists and may provide the compensatory biochemical pathway necessary for spine morphogenesis, even though they cannot compensate for the biochemical pathway necessary for the activity-dependent trafficking of NMDARs.

The one phenotypic feature that was different within brains of DAKO mice was the frequency of synapses with perforated PSDs, which were increased by 40% within brains of DAKO cortex (Fig. 8.7). The perforated PSDs are about 60% larger than the non-perforated PSDs and are usually associated with mushroom spines. Such well-developed larger spines are hypothesized to be sites for storage of memory, while the smaller spines are hypothesized to be the sites for acquiring new memory (Matsuzaki et al. 2004). If this association between perforated PSDs and memory storage holds for the DAKO brains, then DAKO animals might be predicted to exhibit deficits with extinction of memory and LTD, due to the excess number of mushroom spines.
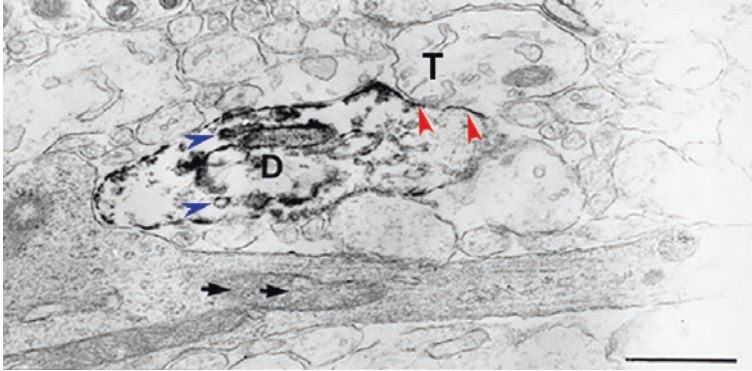
Fig. 8.8 NMDAR immunoreactivity appears along dendrites prior to the formation of spines. This micrograph was taken from the visual cortex of a PNd 4 rat. Immunoreactivity for the NR1 subunit of NMDAR was visualized using DAB as the immunolabel within a dendrite, D. A terminal with few vesicles (T) is directly contacting the dendrite at the portion highlighted (*small red arrowheads*). Immunoreactivity is also associated with membranous organelles in the cytoplasm, two of which are highlighted (*blue arrowheads*). The two black arrows highlight a glial process coursing below the dendrite. Calibration bar = 500 nm. This figure was taken from a published paper (Aoki et al. 1994)

8.5　The Putative Role of Drebrin in Transporting NMDARs to the Plasma Membrane of Newly Forming Spines

As was described earlier, drebrin A is found at the plasma membrane of dendritic shafts at early developmental stages, when dendritic spines are still scarce and PSDs are not yet evident (Aoki et al. 2005). By EM-ICC, NR1 subunits of NMDARs within neonatal cortices have also been visualized along smooth portions of the plasma membrane of dendritic shafts, prior to the formation of dendritic spines (Aoki et al. 1994, 1997) (Fig. 8.8). Synaptogenesis proceeds through fusion of membranous packets containing newly formed NR2B-NMDARs (Washbourne et al. 2002). An intriguing possibility is that these NR2B-NMDAR cargos are transported along actomyosin lattices toward the plasma membrane and become trapped at the membrane surface, where drebrin presides, remaining stabilized at the plasma membrane while "waiting" for the eventual arrival of other anchoring proteins, such as PSD-95 and the NR2A-NMDARs (Fig. 8.9). In support of this idea, EM-ICC has revealed that those plasma membrane patches expressing drebrin A overlap with patches expressing NR2B-NMDARs (Fig. 8.4).
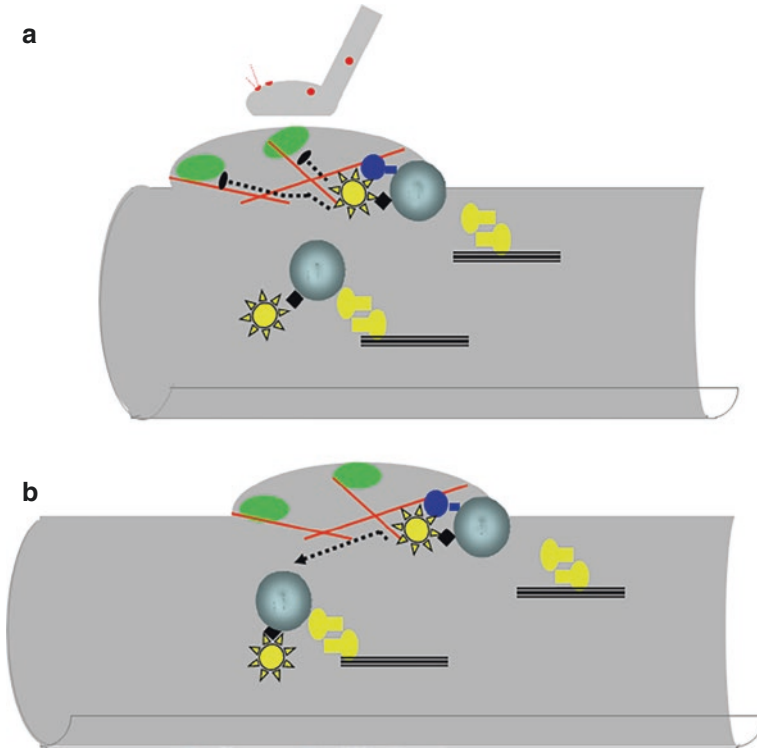
Fig. 8.9 A working model of drebrin A's involvement in the trafficking of NMDAR cargos to the plasma membrane of a newly forming spine. *Green ball* = drebrin A; *yellow sun* = actomyosin ATPase motor; *red lines* = F-actin bundles; *cobalt blue spheres* = membranous cargo carrying PSD-95 and NMDARs; *small blue clubs* = α-actinin; *yellow clubs* = motor proteins associated with microtubules; *double lines* = microtubules in the dendritic shaft microtubules; *red dots* = vesicles within a nascent axon terminal. Panel *A*: a cargo containing NMDAR and PSD-95 moves along F-actin bundles via the actomyosin ATPase motor. The same cargo can move into and toward the plasma membrane until it encounters drebrin A at the plasma membrane, as indicated by the black dotted lines. Once the cargo encounters drebrin A, the cargo becomes stationary, due to drebrin A's inhibitory influence upon actomyosin ATPase. In this way, NMDAR cargos accumulate wherever drebrin A is located. If drebrin A is located at the plasma membrane, NMDAR cargo will accumulate by the plasma membrane. These events lead to a buildup of receptors into membranous clusters at newly forming spines, becoming increasingly sensitive to the neurotransmitter that is released from the nearby axon terminal (located above the spinous protrusion). Panel *B*: the same cargo can be transported elsewhere within spines and out of spines by following the portion of F-actin lattice that is not associated with drebrin A

8.6   Drebrin's Role During Adolescence

Recently, we have begun to explore the role of drebrin A in the hippocampus during adolescence, after realizing that synaptogenesis is still robust during this phase of development for this brain region (summarized above). Our recently collected data indicate that during adolescence, not only is synaptogenesis robust but also that the level of expression of NMDARs at spines in stratum radiatum is strongly influenced by the animal's experience. For example, just 4 days of food restriction or 8 days of wheel running activity or the combination of these two environmental factors can each evoke changes to the dendritic branching pattern in stratum radiatum of the CA1 (Chowdhury et al. 2014a, b). Paradoxically, when adolescent rodents are subjected to the combination of food restriction and running wheel access, these animals become excessive runners, choosing to run even during the limited period of food availability. Those animals that become obsessive wheel runners must be removed from the environment of wheel+food restriction within 4 days, or else they will die from self-starvation. This phenomenon, called activity-based anorexia (ABA), can be induced in about half of the adolescent female rodents and has been used as an animal model of anorexia nervosa. ABA captures four core features of this mental illness: (1) voluntary food restriction, (2) hyperactivity, (3) elevated anxiety-like behavior, and (4) severe weight loss (Aoki et al. 2012; Gutierrez 2013). There are a number of hypotheses regarding the reason that food-restricted animals become hyperactive, with one being that starvation induces an innate foraging-like behavior, which is converted to wheel running for animals held in captivity (Guisinger 2003; Gutierrez 2013). Individuals respond to the wheel+food restriction environment with different degrees of excessive running, which, in turn, is strongly correlated to the extent of weight loss and anxiety-like behavior (the more that the animals run, the stronger is their anxiety-like behavior, as tested on the elevated plus maze (Wable et al. 2015). We examined whether spine density or dendritic branching in the hippocampus might be correlated with these behavioral measurements and found none. However, it is possible that what is altered is not simply the number of excitatory synaptic junctions formed upon dendrites of the hippocampus but, instead, the strength of these synapses and modifiability of their strengths. Indeed, we have found that dendritic spines of the animals that underwent this wheel+food restriction treatment exhibit elevated levels of both the NR2B- and NR2A-NMDARs at the plasma membrane (Klingensmith et al. 2015; Chen et al. 2016). Closer examination of these changes reveals that NR2B-NMDARs are increased precisely at the postsynaptic plasma membrane and especially among individuals that respond to food restriction with greatly excessive wheel running— i.e., the ABA-vulnerable individuals (ABA vulnerable, Fig. 8.10). Conversely, although the NR2A-NMDARs also rise, the pattern is the opposite from that observed for the NR2B-NMDARs. NR2A-NMDARs increase at the cytoplasm, and this rise in the cytoplasm correlates negatively with activity: the more that NR2A-NMDARs are at the cytoplasm, the less that they are hyperactive (ABA resilient, Fig. 8.10), because they are better able to suppress the innate response of foraging. Membranous NR2B-NMDAR levels are inversely correlated with cytoplasmic
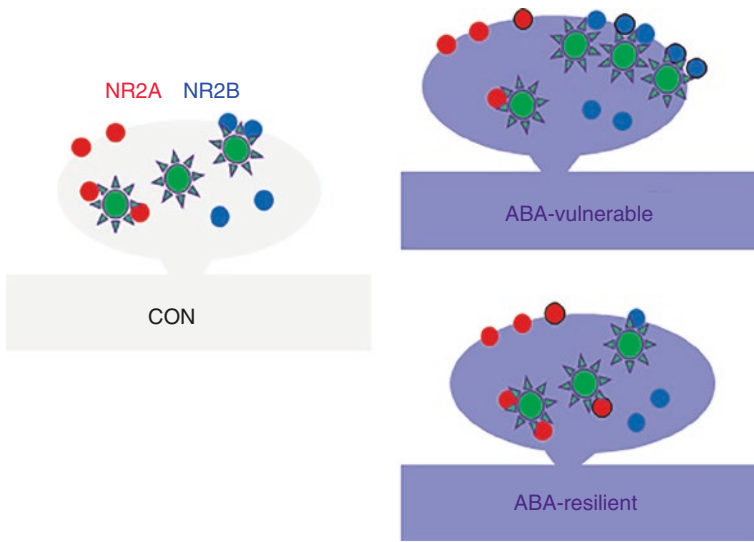
Fig. 8.10 The proposed role of drebrin A in the trafficking of the NR2A- and the NR2B-NMDAR content within spines of the hippocampus after activity-based anorexia (ABA) induction. ABA vulnerability is defined by the degree of hyperactivity evoked by food restriction, leading to exacerbated body weight loss. This behavior correlates with anxiety-like behavior. We propose that hyperexcitability of hippocampal pyramidal neurons may contribute to this. *Red dots* = NR2A-NMDAR immunoreactivity; *blue dots* = NR2B-NMDAR immunoreactivity; *green stars* = drebrin A interacting with the actomyosin ATPase. *Dots with black outlines* reflect those NMDARs that are hypothesized to have been added as a consequence of ABA induction. Within spines of ABA-vulnerable adolescent rats, both NR2A and NR2B-NMDAR immunoreactivity have increased at the postsynaptic plasma membrane, as has the proportion of spines with drebrin A immunoreactivity. Those animals with heightened expression of drebrin A also exhibit heightened levels of NR2B-NMDARs at the postsynaptic plasma membrane. Within the spines of animals exhibiting ABA resilience, defined as animals that have suppressed the food restriction-evoked hyperactivity, levels of drebrin A and postsynaptic membranous NR2B-NMDAR are lower, while the level of the non-synaptic (including cytoplasmic) NR2A-NMDAR is high. Correlations of the level of NMDARs at specific locations within spines with drebrin A are shown by the close association of the symbols. The correlation analysis suggests that drebrin A interacts directly with both the membranous NR2B-NMDARs but only with the cytoplasmic NR2A-NMDARs. It is assumed that the interaction between drebrin A and NMDARs remains the same before and after ABA induction. Modified from Chen et al. 2016

NR2A-NMDARs. This tight and inverse relationship suggests the existence of an exchange mechanism between membranous NR2B-NMDARs and cytoplasmic NR2A-NMDARs. In light of the topic of this chapter, drebrin A, we speculate that drebrin A may be involved in this exchange mechanism. First, it is interesting to note that ABA induction during adolescence also increases drebrin A levels within spines and that this, too, is related to the animal's behavior. The proportion of spines of the ABA hippocampus immunolabeled for drebrin A ranged from 30% to 67%, and this proportion strongly correlated positively with NR2B-NMDAR labelings at the membrane and negatively with NR2A-NMDAR labelings within spine

cytoplasm (Fig. 8.10) (Chen et al. 2016). The rise of drebrin A within spines of ABA-induced animals may enable the delivery of ABA-evoked newly synthesized NR2B-NMDARs (blue circles with black outlines in Fig. 8.10) to the spine plasma membrane, as we have already hypothesized for newly forming synapses of neonatal tissue (Fig. 8.4). Conversely, the role of drebrin A may also be to favor the location of NR2A-NMDARs to the cytoplasm of spines of resilient animals, such that some of the newly synthesized NR2A-NMDARs entering the spine head of ABA-induced animals (red circles with black outlines in the "ABA-resilient" spine of Fig. 8.10) are prevented from reaching the postsynaptic plasma membrane. In support of this idea, we have observed that a pharmacological treatment that enhances the rise of cytoplasmic NR2A-NMDAR immunoreactivity also increases cytoplasmic drebrin A and F-actin (Fig. 8.5 and Fujisawa et al. 2006). Although highly speculative, this working model makes specific predictions that are testable: for this model to work, drebrin A's affinity to cytoplasmic NR2A-NMDARs would have to be higher than drebrin A's affinity to cytoplasmic NR2B-NMDARs or plasmalemmal NR2A-NMDARs. Moreover, drebrin A's affinity for the membranous NR2B-NMDARs would have to be higher than drebrin A's affinity for the membranous NR2A-NMDARs or cytoplasmic NR2B-NMDARs.

The contrasting pattern of regulation for the NR2A and NR2B-NMDARs is a recurrent theme. During development, the expression of NR2B-NMDAR is earlier than that of the NR2A-NMDAR [rev. in Haberny et al. (2002)], with the NR2A-NMDAR expression becoming relatively more dominant sometime between P10 and P35 in the rat hippocampal CA1 (Petralia et al. 2005). In this review article, we described results demonstrating that D-APV treatment to intact adult cortical neuropil evoked a rise of NR2A-NMDARs and a decline of NR2B-NMDARs in the spine cytoplasm (Aoki et al. 2003; Fujisawa and Aoki 2003).

What might be the consequence of the rise of membranous NR2B-NMDARs at spines of the adolescent hippocampus? One simplest scenario is that the rise of membranous NR2B-NMDARs increases excitability of hippocampal pyramidal neurons. Since increased excitability of the hippocampus is linked to anxiogenesis (Shen et al. 2007) and elevated anxiety is a strong contributor to hyperactivity that leads to self-starvation (Wable et al. 2015), the rise of NR2B-NMDARs could be the culprit contributing toward self-starvation. However, an opposite scenario is also possible, involving NR2B-NMDAR-mediated LTD (long-term depression). Due to the difference in the NMDAR channel kinetics, the frequency used to induce long-term depression (LTD; 1 Hz) generates a larger charge transfer of calcium influx via the NR2B-NMDARs than the NR2A-NMDARs (Erreger et al. 2005). Therefore, LTD will be enhanced within a network receiving low-frequency stimulation and heightened NR2B-NMDAR expression. Conversely, the frequency used to induce long-term potentiation (LTP; 100 Hz) is more effective in evoking calcium charge transfer via the NR2A-NMDARs than of the NR2B-NMDARs (Erreger et al. 2005). In the perirhinal cortex at ages 7–12 weeks (adults) and in the juvenile hippocampus (3–4 weeks), NR2B-NMDAR activation has been shown to be required for the long-term depression (LTD), while NR2A-NMDAR activation leads to long-term potentiation (LTP) or depotentiation (Liu et al. 2004; Massey et al. 2004). If the

same rule applies to the adolescent hippocampus, then both the rise of NR2B-NMDARs at the synaptic membrane (for the ABA vulnerable) and the trafficking of NR2A-NMDARs into the cytoplasm (for the ABA resilient), which we speculated to involve drebrin A, may dampen excitability of the hippocampus. If so, then drebrin A could ultimately be serving to protect individuals from self-starvation by dampening the anxiety-driven hyperactivity through NR2B-NMDAR-mediated LTD and reduction of the NR2A-NMDAR-mediated LTP.

For many brain regions, adolescence is the final developmental stage for brain development. This stage coincides with the rampant rise of the first incidences of mental illness, risky behaviors, and environmental stresses that are associated with "leaving the nest" [rev. in Aoki et al. (2016)]. Given that dendritic branches in the hippocampus are still undergoing growth and retraction during adolescence, it is important that we strive to determine whether a number of cell biological principles that have been identified for adult and juvenile brains also apply toward adolescent brains. There is a possibility that at least some of those rules are turned upside-down during adolescence, as the dendrites replay the cellular events of synaptogenesis that have been characterized for neonatal tissue. I believe we have made one contribution toward this endeavor by identifying a role for drebrin A in the activity-dependent trafficking of NMDARs to spines of adolescent hippocampus.

8.7 Drebrin A's Role in the Aging Brain

One of the greatest challenges for neuroscientists of this century is to understand the etiology of Alzheimer's disease (AD). Brain samples from individuals diagnosed with Alzheimer's disease are reported to contain lowered levels of drebrin A than those of age-matched healthy adults (Counts et al. 2006; Harigaya et al. 1996; Hatanpaa et al. 1999; Shim and Lubec 2002). By the time patients are diagnosed with the pre-AD stage of mild cognitive impairment (MCI), the temporal cortex begins to exhibit the loss of drebrin A. In contrast, the frontal cortex is reported to exhibit an increase of drebrin A among those diagnosed with MCI, but this converts to drebrin A becoming lower than normal by the time the disease has progressed to AD (Counts et al. 2006). The loss of this protein cannot be accounted for simply by cell loss or neuronal loss, because these assays are normalized to F-actin and neuron-specific enolase levels (Shim and Lubec 2002). Is this a phenomenon associated with the loss of excitatory synapses and spines or the loss more specifically of drebrin A? We have addressed this question through the use of an animal model of familial AD (FAD) that was generated by targeted knock-in of two genes (2xKI)—one encoding the mutated amyloid precursor protein (APP: K670N/M671L, Swedish mutation with humanized amyloid beta sequence) and another encoding the mutated presenilin-1 (PS1, P264L). In this animal model, the expression of the wild-type gene is completely eliminated, and the two humanized genes are controlled by endogenous promotors, so as to avoid their overexpression (Flood et al. 2002). Histological studies had established that brains of these animals are not

distinguishable from those of the wild-type littermates at 6 months of age. However, by the ninth postnatal month, plaque load and amyloid beta42 emerge and rise monotonically thereafter. Hippocampal LTP deficiency emerges around the 7–9 months of age, and deficiency in AMPAR currents is apparent by the 9–12 months of age (Chang et al. 2006). In order to determine whether synaptic changes that are detectable by EM-ICC might precede even the biophysical and the histological changes, we analyzed drebrin A levels within brains of these animals at a time point preceding the emergence of the biophysical or histological changes—i.e., at 6 months of age. We analyzed drebrin A immunoreactivity in spines across three regions of the 2xKI mouse brains—the hippocampal CA1, the entorhinal cortex, and the somatosensory cortex (Aoki et al. 2007; Mahadomrongkul et al. 2005)—to determine whether the limbic areas (hippocampus and entorhinal cortex) exhibit greater and earlier signs of neurodegeneration than the neocortex, as has been reported to be for the brains of individuals with AD (Braak and Braak 1991). In the hippocampal CA1, we detected a 56% decrease in the density of drebrin A-containing spines, which contributed toward a 25% decrease in overall synapse density (i.e., counting both the drebrin A-containing and drebrin A-lacking spines). In the entorhinal cortex of the same age, we did not detect a change in spine density or in the proportion of spines containing drebrin A. However, those spines that were drebrin A positive contained only 30% of the normal levels. This pattern in the entorhinal cortex suggest to us that, with time, these would convert to becoming drebrin A negative, i.e., containing levels of drebrin A that are below the level of detectability. Finally, in the somatosensory cortex of the same 2xKI animals, we also detected a more modest (15%) decrease in the proportion of drebrin A-containing spines, but this was due mostly to the increase in the number of spines lacking drebrin A, rather than to a decrease in the number of drebrin A-containing spines (Mahadomrongkul et al. 2005). This pattern in the somatosensory cortex indicates to us that the sparing of the neocortex from degeneration (Braak and Braak 1991) may be related to their retention of synaptogenic properties. Conversely, the enhanced vulnerability of the hippocampus may be related to its high level of expression of drebrin A (Aoki et al. 2005). In support of this idea, we observed that the distribution of drebrin A within spines was more frequent at the synaptic plasma membrane than at non-synaptic portions of spines for the 2xKI aged brain (greater than 18 months old) and that this enhancement of synaptic localization was already evident by 6 months postnatal (Fig. 8.11). If, as was proposed for the plasmalemmal drebrin A during neonatal synaptogenesis (Fig. 8.9), drebrin A within brains of the 2xKI is triggered to become localized more to the plasma membrane, then drebrin A's actomyosin ATPase inhibiting property could cause NMDARs to become trapped more at the plasma membrane. If, as was proposed for the adolescent hippocampus, the synaptic drebrin A is enabling the localization of NR2B-NMDARs to the plasma membrane and retention of the NR2A-NMDARs in the cytoplasm, this could favor LTD over LTP in the somatosensory cortex, where modest levels of drebrin A was found, thereby protecting the somatosensory cortex from excitotoxicity and eventual death of neurons. It has been proposed that Alzheimer's disease is a manifestation of synaptic failure (Selkoe 2002). The loss of drebrin A, especially in brain regions such as the
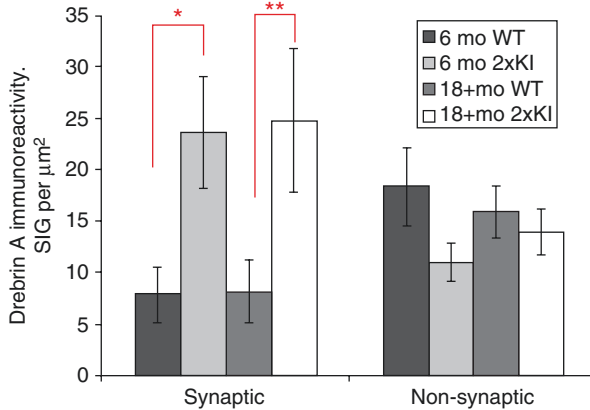
Fig. 8.11 Drebrin A immunoreactivity within spines of the somatosensory cortex of FAD (familial-type Alzheimer's disease) model mice (2xKI) occurs more often at the synaptic membrane, compared to spines from age-matched wild-type littermates. Drebrin A immunoreactivity was detected using silver-intensified gold (SIG) as the immunolabel and determined to be clustered over the postsynaptic region or removed from the synaptic membrane by analyzing electron micrographs of images acquired from layer 1. Adapted from Fig. 4 of a published paper (Mahadomrongkul et al. 2005)

hippocampus, where the loss is profound, may be one of the contributors to synaptic dysfunction, leading to excessive excitation of those spines via NR2A-NMDAR-mediated LTP, excitotoxicity of those neurons, and eventual cell death within brains of patients with FAD.

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