The Roles of Tubulin and CRMP2 in Neuronal Excitability

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Abstract

This paper shows that tubulin may modulate excitability at dendritic spines, axon terminals and the axon initial segment (AIS). Tubulin is a highly negatively charged molecule. Its close contact with the neuronal membrane would be able to inhibit the generation of action potentials. At dendritic spines, tubulin has been demonstrated to interact with NMDA receptors (NMDARs). The collapsin response mediator protein 2 (CRMP2) interacts with both tubulin and NMDARs. Therefore, CRMP2 may enhance the tubulin-NMDAR binding, thereby modulating synaptic strength. The antibodies against CRMP2 was shown to induce amnesia, which underscores the importance of CRMP2 in memory retrieval. At the axon terminals, tubulin may cooperate with parkin and leucine rich repeat kinase 2 (LRRK2) - two proteins implicated in Parkinson's disease. Parkin is an E3 ubiquitin ligase, capable of promoting tubulin degradation. LRRK2 is a large protein, whose binding with tubulin may prevent tubulin from entering the actin/spectrin layer beneath the neuronal membrane, thus facilitating synaptic transmission. At AIS, intensive neural activity would cause Ca^{2+} overload, which can induce microtubule depolymerization to produce free tubulin, consequently inhibiting neuronal firing. This could be the mechanism underlying seizure termination.

Introduction

This paper contrasts with Paper 2 which explores the roles of microtubules and Tau in neuronal excitability. Tubulin is the building block of microtubules. It has two isoforms, α and β, that usually form a heterodimer. In a tubulin heterodimer, the number of negatively charged amino acids exceeds that of positively charged amino acids by about 50 (Minoura and Muto, 2006). As a result, a microtubule is highly negatively charged along the entire length (Baker et al., 2001). This underappreciated physical property may play key roles in

neuronal excitability. At the axon initial segment (AIS), microtubules could work with the Tau protein to modulate neuronal excitability. Elevated Tau (especially the 4-repeat isoform) has been shown to cause hyperexcitability, and consequently neurodegenerative disorders, including Alzheimer's disease (Paper 4).

Beneath the plasma membrane of a neuron, there is a submembrane cytoskeleton composed of actin and spectrin. The actin/spectrin layer exists not only in axons (Xu et al., 2013), but also in the somatodendritic compartments of various neuronal types, across different animal species (He et al., 2016; Han et al., 2017). Due to the presence of the submembrane cytoskeleton, a microtubule cannot contact the intracellular membrane surface. However, the free tubulin dimer is a small molecular complex capable of penetrating the actin/spectrin layer to inhibit action potentials with its negative electric field. This explains how microtubule depolymerization can attenuate neuronal excitability (Paper 2). During a seizure, the intensive neural activity would cause Ca^{2+} overload, resulting in microtubule depolymerization (O'Brien et al., 1997). The free tubulin dimers produced by microtubule depolymerization may reach the membrane surface to inhibit neuronal firing, thereby terminating the seizure (Paper 14). The crucial role of tubulin in excitability is further supported by the finding that biallelic mutations in *TBCD* (encoding the tubulin folding cofactor D) reduces free tubulin level (Flex et al., 2016), and causes intractable seizures (Pode-Shakked et al., 2017).

The collapsin response mediator protein (CRMP), like Tau, is a microtubule-associated protein (Hensley and Kursula, 2016). To date, five members of the CRMP family have been identified, designated as CRMP1 - 5 (Schmidt and Strittmatter, 2007). CRMP2 was the most studied member. The binding of CRMP2 with tubulin dimers promotes their polymerization into a microtubule. The antiepileptic drug, lacosamide (LCM), has been shown to bind with CRMP2 and impair tubulin polymerization (Wilson and Khanna, 2015). Thus, LCM can increase the level of free tubulin dimers to inhibit neuronal firing. This may account for, at least in part, the antiepileptic action of LCM.

While the Tau protein binds preferentially with microtubules to free tubulin dimers, CRMP2 has higher affinity with free tubulin dimers than microtubules (Fukata et al., 2002), suggesting that the primary function of CRMP2 could be mediated by its interaction with free tubulin dimers, rather than microtubules. The antibodies against CRMP2 have been demonstrated to induce amnesia (Mileusnic and Rose, 2011), which underscores the importance of CRMP2 in memory. This paper will describe how tubulin can modulate excitability at dendritic spines, axon terminals and AIS. The mechanism of memory retrieval and consolidation will be discussed in next several papers, with focus on tubulin

and CRMP2 as well as their regulation by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), cyclin dependent kinase 5 (Cdk5), and others.

Prevalence of Silent Neurons

In the hippocampus, nearly two-thirds of all pyramidal neurons are behaviorally silent (Thompson and Best, 1989) and over 90% of granule cells opt for early "retirement" (Alme et al., 2010). The prevalence of silent neurons has also been observed in the neocortex where a minority of neurons are responsible for the majority of spikes (Barth and Poulet, 2012). Regarding sparse firing, the dentate gyrus (DG) of the hippocampus is probably the most unique. A singe dentate granule cell receives inputs from 3600 to 5600 neurons in the entorhinal cortex. Yet the majority of DG neurons are silent. On the other hand, DG is located in a pathway that connects hippocampal regions with high propensities for generating seizures. This has led to the hypothesis that DG may serve as a control point for seizures in the hippocampus and that a breakdown of the dentate gate causes seizures (Lothman et al., 1992). Recent studies support the dentate gate hypothesis (Krook-Magnuson et al., 2015).

What makes neurons silent? A plausible mechanism is the inhibition by GABAergic interneurons. In DG, the axons of granule cells are present at the hilus where 50% of their target cells are GABAergic interneurons, providing feedback inhibition onto dentate granule cells (Dengler and Coulter, 2016). This feedback inhibition was hypothesized to contribute to the sparse firing in DG. "However, in both kindling and post-status-epilepticus models of temporal lobe epilepsy, numerous studies have shown a surprising **upregulation** of $GABA_A$ receptor expression, both synaptically and in whole-cell recordings from chronically epileptic rats" (Dengler and Coulter, 2016). Furthermore, THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol) is a $GABA_A$ receptor agonist with strong potency at δ-subunit-containing receptors. Despite functional δ-subunit-containing $GABA_A$ receptors in dentate granule cells, THIP showed no anticonvulsive effect in the pentylenetetrazole kindling model (Simonsen et al., 2017). Therefore, GABA inhibition does not seem to be the major mechanism for sparse firing.

Several studies have revealed a number of intrinsic properties about silent neurons, such as more negative resting membrane potential (Staley et al., 1992) and lack of Ca^{2+}-mediated burst firing mode (Fricke and Prince, 1984; Epsztein et al., 2011). Recently, Diamantaki et al. (2016) uncovered an additional feature of silent dentate granule cells: silent cells have less dendritic branches than active cells (Figure 1). CRMPs play a pivotal role in dendritic branching (Niisato et al., 2012; Niisato et al., 2013; Zhang et al., 2016). Tubulin is a canonical binding partner of CRMPs. As mentioned above, biallelic mutations

in the tubulin folding cofactor D (TBCD) has been demonstrated to reduce free tubulin level and causes intractable seizures. Therefore, tubulin could interact with CRMPs to silence neurons.

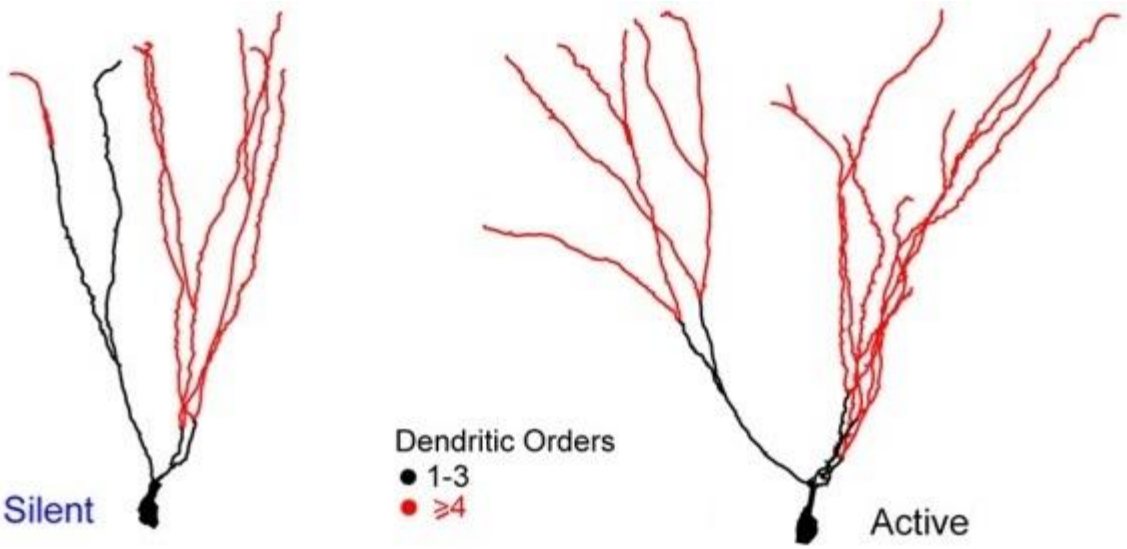

Figure 1. Morphological difference between active and silent granule cells in the dentate gyrus. Active cells have more dendritic branches than silent cells. [Adapted from: Diamantaki et al., 2016]

Inhibition by Tubulin at Dendritic Spines

Tubulin is present in the postsynaptic density (PSD), a structure under the postsynaptic membrane (Caceres et al., 1984; Sahyoun et al., 1986). The close contact with the membrane allows the negatively charged tubulin to exert a strong hyperpolarizing field at the membrane. By definition, the voltage between two points is given by the integration of electric fields from one point to another. Thus, the tubulin in PSD may contribute to more negative resting membrane voltage observed in silent neurons (Staley et al., 1992). This notion is supported by further evidence that tubulin interacts with N-methyl-D-aspartate receptors (NMDARs), specifically the C-terminal domains of NR1 (GluN1) and NR2B (GluN2B) subunits (van Rossum et al., 1999). NMDARs are crucial for learning and memory. Their opening requires both glutamate binding and the relief of Mg^{2+} block. The latter is voltage dependent. Hence, tubulin would be able to repress the opening of NMDARs by inhibiting the relief of Mg^{2+} block (Figure 2). Recalling that an intrinsic property of silent neurons is the lack of Ca^{2+}-mediated burst firing (Epsztein et al., 2011, Figure 5). NMDARs are permeable to Ca^{2+} ions. Remarkably, they are also responsible for burst firing (Zhu et al., 2004). Therefore, the

presence of tubulin in PSD can account for two intrinsic properties of silent neurons: more negative resting membrane potential and lack of burst firing.

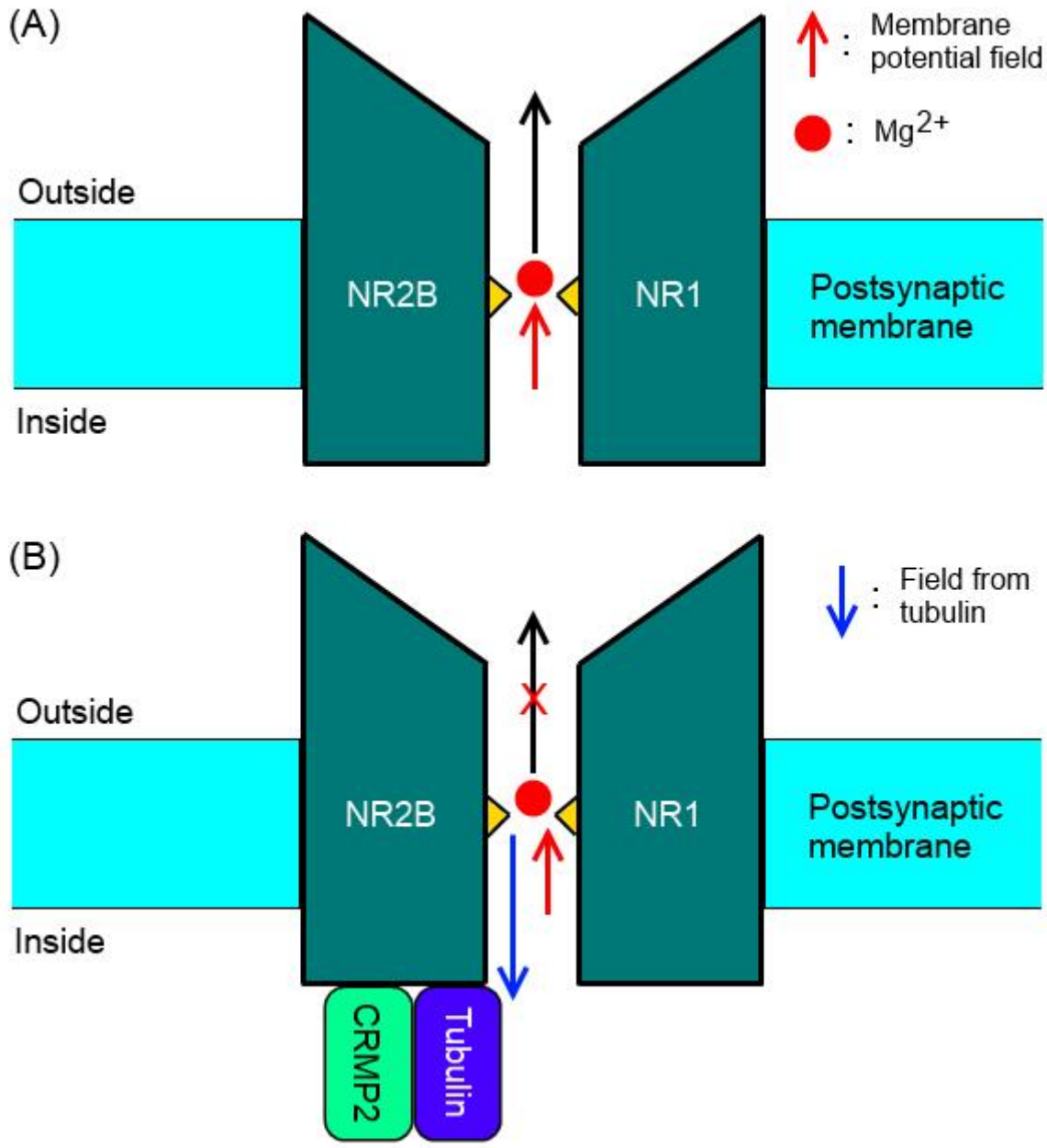

Figure 2. Schematic drawing of the NMDAR regulated by the tubulin/CRMP2 complex. (A) In the resting state, an NMDAR is blocked by Mg^{2+} ion. When an action potential is generated, the depolarizing field may expel the Mg^{2+} ion out of the pore, allowing Ca^{2+} and Na^{+} ions to pass through. (B) The tubulin tightly bound with CRMP2 and the NR2B subunit of NMDAR may exert a negative field, counteracting the depolarizing field, thus inhibiting the relief of Mg^{2+} block.

Like tubulin, CRMP2 also interacts with NR2B (Brustovetsky et al., 2014). CRMP2 knockout mice exhibited hyperactivity (Nakamura et al., 2016; Zhang et al., 2016). It seems that CRMP2 may interact with tubulin to enhance its binding with NR2B. In the absence of CRMP2, the binding between tubulin and NR2B is weakened, facilitating tubulin to exit PSD and consequently leading to higher neural activity. Therefore, in silent neurons the triad (tubulin, CRMP2 and NR2B) may form a tightly bound complex, preventing the opening of NMDARs. These silent neurons can be excited when the complex is interfered such that the tubulin leaves PSD. This could be the fundamental mechanism of memory retrieval.

It has been well documented that NR2A- and NR2B-containing NMDARs have distinct impact on long-term potentiation (LTP) and long-term depression (LTD) (Yashiro and Philpot, 2008; Shipton and Paulsen, 2013). NR2B is expressed at birth with high level, but decreases into adulthood, while NR2A expression increases with age (Shipton and Paulsen, 2013). The two subunits also play different roles in memory consolidation and retrieval (Holehonnur et al., 2016; Travaglia et al., 2016). Intriguingly, both tubulin and CRMP2 interact with NR2B, but not NR2A. Could this cause the difference between the two subunits?

Inhibition by Tubulin at AIS

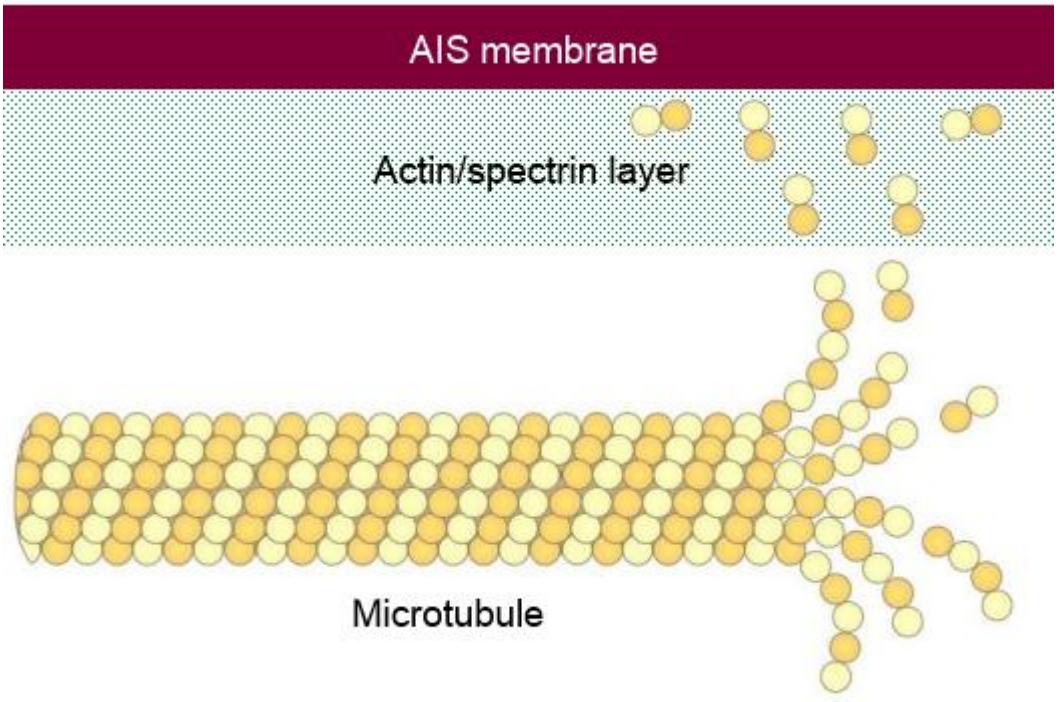

Figure 3. Schematic illustration of the inhibitory effects by free tubulin dimers at AIS. The Ca^{2+} overload induces microtubule

depolymerization, producing free tubulin dimers to penetrate the actin/spectrin layer and inhibit neuronal firing.

AIS is the initiation site of action potentials. It contains high density of voltage gated sodium channels (Buffington and Rasband, 2011), and several types of voltage-gated calcium channels (Bender et al., 2012; Yu et al., 2010). Intensive neural activities may cause Ca^{2+} overload, resulting in microtubule depolymerization (O'Brien et al., 1997). This process would produce free tubulin dimers to inhibit neuronal firing (Figure 3). Thus, the tubulin at AIS plays a key role in protecting neurons from over-excitation.

In contrast to dendritic spines, AIS does not seem to have specific proteins to hold free tubulin dimers in close contact with the plasma membrane. Rather, it may use Ca^{2+} ions to retain tubulin within the actin/spectrin layer. Both actin and spectrin are also highly negatively charged (Elzinga et al., 1973; Speicher et al., 1983), which would repel tubulin out of the submembrane cytoskeleton. It has been well established that like-charged molecules can attract each other in the presence of multivalent counterions (Ha and Liu, 1999). Paper 2 suggests that Ca^{2+} ions are likely to be the counterions mediating the attraction between tubulin and the actin/spectrin layer. Therefore, the inhibition by tubulin at AIS should depend on Ca^{2+} concentration. As the Ca^{2+} overload fades away, the tubulin will be repelled out of the actin/spectrin layer. In line with this notion, the studies of traumatic brain injury (Paper 13) have revealed that the excitability decreases between 1 and 4 hr post-trauma, and then gradually recovers in a couple of days (Ping and Jin, 2016). After seizure termination, there is also a refractory period that lasts about 4 hours (Minabe et al., 1989).

Inhibition by Tubulin at Axon Terminals

The close contact between tubulin and axon terminal membrane may inhibit local generation of action potentials, thus suppressing the release of neurotransmitters. This process could be regulated by parkin and leucine rich repeat kinase 2 (LRRK2) - two proteins implicated in Parkinson's disease (PD). CRMP2 also plays an important role in transmitter release, but via a different mechanism.

The Role of CRMP2

CRMP2 interacts with tubulin dimers to promote microtubule polymerization, thus stimulating axonal outgrowth. Phosphorylation of CRMP2 by Cdk5 at Ser522

disrupts its binding with tubulin, resulting in growth cone collapse (Schmidt and Strittmatter, 2007). At axon terminals, CRMP2 may regulate transmitter release by interacting with N-type calcium channels, CaV2.2 (Brittain et al., 2009). Cdk5-mediated phosphorylation of CRMP2 enhances its interaction with CaV2.2, thereby increasing Ca^{2+} influx (Brittain et al., 2012). This result suggests that tubulin may reduce transmitter release by interfering with the binding between CRMP2 and CaV2.2 .

The Role of Parkin

Parkin is an E3 ubiquitin ligase which has been shown to promote α/β-tubulin ubiquitination and degradation via direct interaction. The parkin mutants found in PD patients do not ubiquitinate or degrade tubulin (Ren et al., 2003). Hence, degradation of tubulin by parkin at axon terminals is expected to promote synaptic transmission. This prediction agrees with the experimental findings that synaptic excitability decreases in the absence of parkin (Goldberg et al., 2003) and parkin gene inactivation inhibits glutamate neurotransmission (Itier et al., 2003).

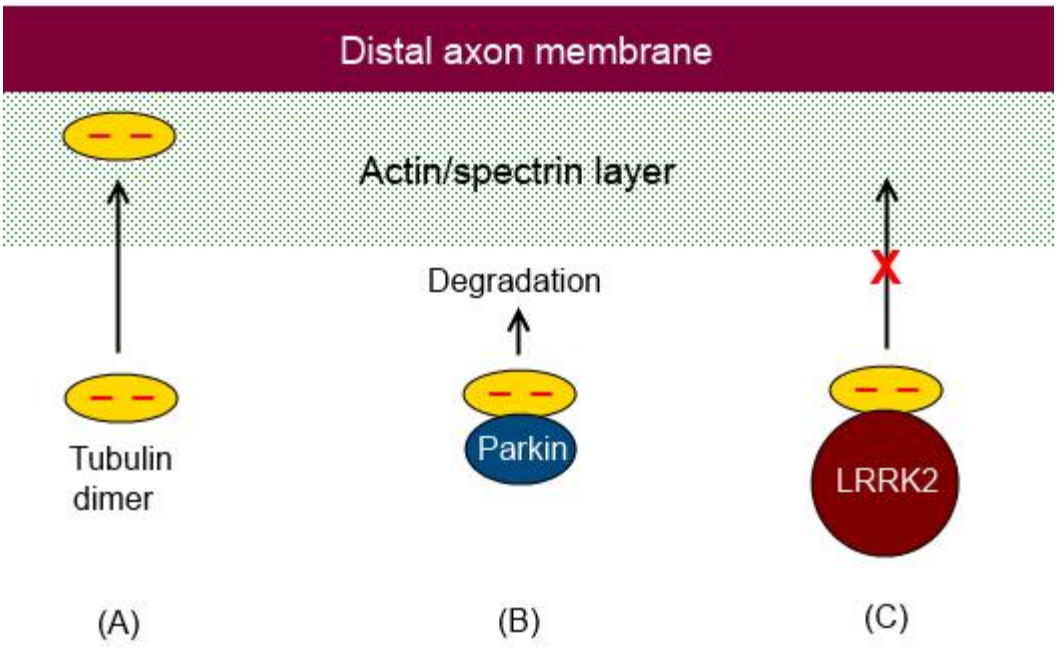

Figure 4. Possible roles of parkin and LRRK2 in the generation of action potentials at axon terminals. (A) Free tubulin dimers may enter the actin/spectrin layer to inhibit action potentials via negative electric field. (B) Parkin can enhance tubulin degradation, thereby promoting transmitter release from the axon terminal. (C) LRRK2, a large

tubulin-interacting protein, could maintain proper neurotransmission by preventing tubulin dimers from entering the actin/spectrin layer.

The Role of LRRK2

LRRK2 interacts with β-tubulin (Gillardon, 2009; Law et al., 2014). It is a large protein with over 2,500 amino acids, and exists as a dimer under native conditions (Greggio et al., 2008). The LRRK2/tubulin complex would not be able to penetrate the actin/spectrin layer. Therefore, LRRK2 overexpression may enhance synaptic transmission by preventing tubulin dimers from entering the actin/spectrin layer at axon terminals (Figure 4), in accord with observation (Li et al., 2010).

LRRK2 plays an important role in axon and dendrite growth (Sepulveda et al., 2013; Häbig et al., 2013). During neurite growth, massive tubulins are produced for the polymerization of microtubules. However, large amount of free tubulins inside the actin/spectrin layer would inhibit synaptic transmission. Therefore, LRRK2 may have a normal function to retain tubulin outside the actin/spectrin layer for microtubule polymerization and proper neurotransmission.