THE $\alpha_2\delta$ SUBUNIT AND ABSENCE EPILEPSY: BEYOND CALCIUM CHANNELS?

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Abstract

The spike-wave discharges (SWDs) underlying absence seizures, are generated by hyperactivity of T-type voltage sensitive calcium channels (VSCCs) within the cortico-thalamo-cortical network, and the first-line drug used in the treatment of absence epilepsy inhibit T-type calcium channels. The $\alpha_2\delta$ subunit associates with all types of VSCCs, including T channels, and facilitates VSCC activation. Hence, one expects that drugs that bind to, and inhibit the $\alpha_2\delta$ subunit, e.g., gabapentin and pregabalin, are protective against absence epilepsy and that mice lacking the $\alpha_2\delta$ subunit are resistant to evoked absence seizures. In contrast, gabapentin and pregabalin are not clinically useful, and may even be detrimental, in the treatment of absence epilepsy, and *ducky* mice lacking the $\alpha_2\delta$ subunit *develop* absence seizures. This suggests that the $\alpha_2\delta$ subunit displays functions that go beyond the regulation of VSCCs, and that these functions are involved in the regulation of the cortico-thalamo-cortical network. This viewpoint critically examines the role of the $\alpha_2\delta$ subunit in the pathophysiology of absence seizures focusing on the potential role of the $\alpha_2\delta$ ligands, thrombospondins.

1. Role for the $\alpha_2\delta$ subunit in the modulation of VSCCs

VSCCs are formed by a multimolecular complex that includes the α_1, β_1, γ, and $\alpha_2\delta$ subunits. The α_1 subunit forms the ion channel and accounts for the basic properties of the channel. Based on the specific type of the α_1 subunit, VSCCs are classified into T-type (containing the α_1G, α_1H and α_1I subunits), L-type (containing the α_1C, α_1D, α_1S, and α_1F subunits), P/Q-type (containing the α_1A subunit), N-type (containing the α_1B subunit), and R-type (containing the α_1E subunit). There are four isoforms of the intracellular β subunit, and eight isoforms of the transmembrane γ subunits, which do not confer a particular specificity to the VSCCs, although the γ subunit is preferentially found in L-type channels of the skeletal muscle. The γ_2 subunit belongs to the group of transmembrane AMPA-receptor regulating proteins (TARPs) and is also named stargazine (reviewed by Tomita et al., 2007; Kato et al., 2010). Interestingly, *stargazer* mice lacking the γ_2 subunit show a severe neurological phenotype characterized by ataxia and absence epilepsy (Chen et al., 2000; Coombs and Cull-Candy, 2009; Menuz and Nicoll, 2008; Payne, 2008). Convincing evidence for the role of this protein in absence epilepsy comes from a study in adult GAERS (Genetic Absence Epilepsy Rats of Strasbourg), a well validated genetic absence model (Depaulis et al., 2016) , in which it was found that elevated levels of stargazin in the cortical focal region (the somatosensory cortex) are associated with an increase in AMPA receptor proteins, GluA1 and GluA2. Elevated stargazin expression is not seen in the epileptic WAG/Rij rat (Wistar Albino Glaxo rats from Rijswijk), another well described genetic absence model endowed with face, construct, and pharmacological validity (Coenen and van Luijtelaar, 2003; Ngomba et al., 2011), suggesting that these changes are not likely a consequence of the hundreds daily SWDs, but it might be one of the many causal factors that play a role in the occurrence of absence seizures and or in epileptogenesis.

The transmembrane $\alpha_2\delta$ subunit is formed by the α_2 and δ subunits covalently linked by a disulfide bridge. Interestingly, the two subunits are generated by a single gene product, which is post-translationally cleaved. The α_2 subunit is glycosylated and interacts extracellularly with the α_1

subunit. The δ subunit has a single transmembrane domain and interacts with anchoring intracellular proteins *via* the C-terminal region (De Jongh et al., 1990). There are four isoforms of the $\alpha_2\delta$ subunit, produced by different genes. $\alpha_2\delta$-1, originally described in the skeletal muscle, is ubiquitous; $\alpha_2\delta$-2 and $\alpha_2\delta$-3 are specifically found in neurons, whereas $\alpha_2\delta$-4 is non-neuronal.

The $\alpha_2\delta$-1 protein is mainly present in axon terminals of excitatory neurons (Cole et al., 2005), whereas the $\alpha_2\delta$-2 protein is expressed by cerebellar Purkinje cells (Barclay et al., 2001). The $\alpha_2\delta$-3 protein is ubiquitously expressed in the forebrain (Cole et al., 2005).

The $\alpha_2\delta$ subunit regulates the activity of VSCCs by increasing current amplitude and causing a shift of voltage-dependent activation towards more hyperpolarized membrane potentials (Arikkath and Campbell, 2003; Dolphin, 2003; Cantí et al., 2005; Hendrich et al., 2008; Davies et al., 2010). In addition, the $\alpha_2\delta$ subunit regulates cellular trafficking and membrane expression of VSCCs. Accordingly, transient expression of the $\alpha_2\delta$-1, -2, and -3 subunits in cultured hippocampal neurons targets P/Q channels in presynaptic terminals, thereby enhancing depolarization-evoked neurotransmitter release (Hoppa et al., 2012). This mechanism may be particularly relevant under pathological conditions, as suggested by the evidence that the $\alpha_2\delta$-1 subunit translocates from neuronal cell bodies of dorsal root ganglia to axon terminals in the superficial layers of the dorsal horns of the spinal cord in a mouse model of neuropathic pain (Bauer et al., 2009).

2. The ambiguous role for the $\alpha_2\delta$ subunit in absence epilepsy

a) T-types VSCCs: the key molecular players in absence epilepsy

Absence seizures, the hallmark of absence epilepsy, are characterized by transient lapses of consciousness associated with bilateral symmetrical SWDs in the electroencephalogram (EEG). Absence seizures are generated by pathological oscillations in a cortico-thalamo-cortical network with a cortical origin; sleep spindles, another type of thalamo-cortical oscillations, are generated in the reticular thalamic nucleus, which is part of this network. This SWDs generating network includes interconnected pyramidal cells and GABA-ergic interneurons in the somatosensory cortex

(SSCtx), thalamo-cortical relay cells in the ventrobasal thalamic (VBT) nuclei, and the reticular thalamic nucleus (RTN) (reviewed by Blumenfeld, 2005; Avoli and Gloor, 1982; Meeren et al., 2005; van Luijtelaar et al., 2011). Highly excitable cells in deep layers of the somatosensory cortex send excitatory fibers to both the VBT and RTN, VBT neurons in their turn send excitatory fibers to the cortical pyramidal neurons and to the GABA-ergic RTN. RTN neurons project to the VBT, and are interconnected by gap junction (reviewed by Blumenfeld, 2005; Ngomba et al., 2011; Kohmann et al., 2016).

T-type VSCCs play a critical role in the generation of pathological oscillations underlying the electrophysiological correlate of absence seizures, the SWDs. These channels are activated at negative membrane potentials by the hyperpolarization-activated cyclic nucleotide-gated cationic I h pacemaker channels (HCN), and show a fast voltage-dependent inactivation with respect to other VSCCs. Owing to these peculiar functional properties, T channels are involved in the generation of repetitive firing in the thalamo-cortical network (Perez-Reyes, 2006; Talavera and Nilius, 2006). In the generation of SWDs, an increased excitatory drive from the somatosensory cortex to the RTN generates pathological bursts of RTN GABAergic projection neurons leading to $GABA_B$ receptor-mediated inhibitory postsynaptic potentials (IPSPs) in VBT neurons. The resulting hyperpolarization enhances the activity of T-type VSCCs in VBT neurons producing the pathological 3-4 Hz oscillations that are typical of absence seizures in humans and 7-11 Hz in the genetic rat models (reviewed by Blumenfeld, 2005). Based on pioneering in vitro studies it is thought that the therapeutic activity of ethosuximide in absence epilepsy occurs via reducing the low-threshold Ca^{2+} current (I_T) in thalamocortical neurons (Coulter et al., 1989; Crunelli and Leresche, 2002). Although modern views emphasize a leading role for the cortex in the initiation of SWDs, the above described thalamic cellular properties, the anatomical projection and an intact cortico-thalamo-cortical network remain imperative for SWDs to occur.

There are three subtypes of T-type channels: $Ca_V3.1$, $Ca_V3.2$ and $Ca_V3.3$, containing the pore-forming α_1G, α_1H and α_1I subunits, respectively (Catterall et al., 2005). $Ca_V3.2$ and $Ca_V3.3$ channels

are expressed in RTN neurons and at least $Ca_V3.3$ has been implicated in the generation of RTN bursts. $Ca_V3.1$ channels are highly expressed in thalamocortical neurons, and all types of T channels are found in cortical neurons (Ernst et al., 2009; Cain and Snutch, 2010; Cheong and Shin, 2013).

b) The $\alpha_2\delta$ subunit associates with T channels and enhances T channel activity in heterologous expression systems

A few studies suggest that the $\alpha_2\delta$ subunit positively modulates the activity of T channels, although the physiological significance of the association between the $\alpha_2\delta$ subunit and T channels is still debated. Overexpression of $\alpha_2\delta$ influences the voltage-dependence of activation of native T-type channels in neuroblastoma/glioma cell lines (Wyatt et al., 1998). In *Xenopus* oocytes and mammalian cells the $\alpha_2\delta$ subunit enhances membrane expression and current amplitude of T channels (Dolphin et al., 1999; Dubel et al., 2004; Thompson et al., 2011). The $\alpha_2\delta$ subunit can also alter the activation and inactivation kinetics and gating properties of T channels (Hobom et al., 2000; Lacinová and Klugbauer, 2004).

c) The *ducky* mouse: a counterintuitive association between the lack of $\alpha_2\delta$ subunit and absence epilepsy

The association between the *Cacna2d2* gene and childhood absence epilepsy suggests a role for the $\alpha_2\delta$ subunit in the pathophysiology of absence seizures (Chioza et al., 2009). Because the $\alpha_2\delta$ subunit facilitates the activity of T channels (see above) it is reasonable to predict that the lack of the $\alpha_2\delta$ subunit confers protection against absence seizures. In contrast, *ducky* mice, characterized by a spontaneous loss-of-function mutation of the *Cacna2d2* gene, show generalized bilateral SWDs with a frequency of 5-7 Hz, and are considered as a genetic model for absence epilepsy (Barclay et al., 2001). *Ducky* mice also show severe ataxia, dyskinesias, dysgenesis of the cerebellum and other central nervous system (CNS) regions, axonal dystrophy and demyelination

(Meier, 1968). Another spontaneous mutation of the *Cacna2d2* gene (*entla*) encodes a mutant form of the $\alpha_2\delta$-2 protein with an intact C-terminus. This mutation also causes generalized epilepsy (Brill et al., 2004), as does a targeted knockout of *Cacna2d2* (Ivanov et al., 2004). The epileptic phenotype of mice lacking $\alpha_2\delta$-2 is counterintuitive if one postulates that the $\alpha_2\delta$ subunit positively modulates T-type channels in thalamic neurons.

d) Lack of therapeutic activity of the two $\alpha_2\delta$ ligands, gabapentin and pregabalin, in absence epilepsy

Gabapentin and pregabalin have been developed for the treatment of focal epilepsy and are currently used in the treatment of neuropathic pain, migraine, anxiety disorders, and bipolar disorders (Bialer, 2012; Johannessen Landmark, 2008). Gabapentin and pregabalin bind selectively to $\alpha_2\delta$-1 and $\alpha_2\delta$-2 with nanomolar affinity (reviewed by Cantí et al., 2003; Klugbauer et al., 2003; Davies et al., 2007; Lana et al., 2014). An arginine residue in position 217 of $\alpha_2\delta$-1 is part of the binding pocket, and binding of both [^{3}H]gabapentin and [^{3}H]pregabalin is largely reduced in the brain regions of transgenic mice with the $\alpha_2\delta$-1 R217A mutation (Gee et al., 1996; Gong et al., 2001; Hendrich et al., 2008; reviewed by Dooley et al., 2007; Dolphin, 2012). Interaction of gabapentin and pregabalin with $\alpha_2\delta$ has two major effect on VSCCs: (i) a reduced membrane localization of $\alpha_2\delta$ and α_1 subunits; and (ii) a reduced calcium channel current. It is generally believed that both effects contribute to the therapeutic action of gabapentin and pregabalin in focal epilepsy, neuropatic pain, and generalyzed anxiety disorder (Dooley et al., 2007, Stahl et al., 2013).
By interacting with the $\alpha_2\delta$ subunit, gabapentin and pregabalin are expected to have therapeutic potential in absence epilepsy as a result of T channel inhibition. In contrast, clinical studies have consistently shown that the two drugs have no activity in absence epilepsy (Trudeau et al., 1996; Chadwick et al., 1996), and gabapentin can precipitate absence and myoclonic status epilepticus (Perrucca et al., 1998; Thomas et al., 2006). In animal studies, pregabalin did not affect the

incidence of SWDs in GAERS (Vartanian et al., 2006) and gabapentin had no effect on absence-like seizures in the lethargic mouse model of human absence epilepsy (Hosford and Wang, 1997).

3. Potential explanations for the ambiguous role of the $\alpha_2\delta$ subunit in absence epilepsy

a) Modulation of non-T type VSCCs

One possible explanation for the epileptic phenotype of *ducky* and *entla* mice is that the loss of $\alpha_2\delta$ subunit causes absence seizures by restraining the activity of high voltage-activated (HVA) Ca^{2+} channels (P/Q, N, L and R channels). Although T channels are one of the major players in the generation of pathological oscillations in the cortico-thalamo-cortical network, a large body of evidence suggests that HVA channels are also involved in the pathophysiology of absence seizures. Pharmacological activation of L-type HVA Ca^{2+} channels alters the firing rate of thalamocortical neurons (Kanyshkova et al., 2014), while the systemic administration of L-type Ca^{2+} channel blocker nimodipine increased SWDs, while a decrease was found after i.c.v. (intracerebroventricular) BAY K8644, a L-type Ca^{2+} channel opener. BAY K8644 was also able to antagonise the effects of nimodipine in rats with spontaneous occurring SWDs (van Luijtelaar et al., 1995; van Luijtelaar et al., 2000). R-type channels are also involved in the generation of oscillatory burst discharges in RTN neurons and absence epilepsy (Zaman et al., 2011). Preliminary evidence for a role in the R-type Ca^{2+} blocker was obtained after the administration of the conotoxin GVIA, it reduced SWDs (van Luijtelaar et al., 2000). Mutations of the *Cacna1a* gene encoding for the α_1 subunit of P-type channels are associated with absence seizures in rodents and humans (Burgess and Noebels, 1999; Zamponi et al., 2010; Serikawa et al., 2015). It is possible that in *ducky* mice a reduced expression/activity of HVA VSCCs causes a componsatory increase in the activity of T-type channels resulting into absence seizures.

b) Beyond VSCCs: the $\alpha_2\delta$ subunit mediates the action of thrombospondins in the CNS

The $\alpha_2\delta$ subunit is the receptor for a class of proteins, called thrombospondins (TSPs) , which were first isolated from platelets stimulated with thrombin and show a widespread distribution in various organs, including the CNS. TSPs are a large oligomeric extracellular matrix (ECM) proteins, found in vertebrates and lower metazoa (Adams, 2001; Bentley and Adams, 2010; Mosher and Adams, 2012). The human TSP protein family consists of five members (TSP-1, TSP-2, TSP-3, TSP-4, and TSP-5, or COMP, cartilage oligomeric matrix protein) which are subdivided into two groups (Adams, 2001; Adams and Lawler, 2004; Adams and Lawler, 2011) on the basis of their oligomerization state and domain structure. Subgroup A includes TSP-1 and TSP-2, which form homotrimers; subgroup B includes TSP-3, TSP-4, and TSP-5, which form homopentamers (Lawler, 2002). The various isoforms of TSPs are the products of different genes, and display differential non-overlapping functions (Stenina-Adognravi, 2013).

TSPs primarily regulate cell-cell and cell-extracellular matrix interactions (Bornstein, 2000) acting through a number of ECM proteins and cell surface receptors and controlling cytoskeletal dynamics, cell migration, and cell attachment (Bornstein et al., 2004). All these activities regulate many aspects of cell phenotype and contribute to angiogenesis and wound healing, vessel wall biology and synaptogenesis (Adams and Lawler, 2011).

Astrocyte-secreted TSP-1, TSP-2 promote synaptogenesis in cultured hippocampal neurons (Xu et al., 2010) and retinal ganglion cells (Christopherson et al., 2015), and TSP-1 can also promote neuronal migration (Iruela-Arispe et al., 1993) and axonal growth (Yu et al., 2008). TPS-1 and TSP-2 are highly expressed during development (Christopherson et al., 2005; Xu et al., 2010; Cahoy et al., 2008) and acts in specific time windows to promote synaptogenesis in the developing brain. TSP-4 is exclusively expressed by mature astrocytes, and represents the mature TSP isoform in the CNS (Bornstein et al., 2004).

Eroglu et al. (2009) have shown for the first time that the synaptogenic epidermal growth factor (EGF)-like repeat domains of TSPs interact with the VWF-A (von Willebrand factor) domain of $\alpha_2\delta$-1. This interaction mediates the synaptogenic activity of TSPs. Accordingly, overexpression of

$\alpha_2\delta$-1 enhanced synaptogenesis in mice, and the $\alpha_2\delta$ ligand, gabapentin, restrained the synaptogenic activity of TSPs (Eroglu et al., 2009).

A potential link between alterations in synaptogenesis and absence epilepsy is suggested by the evidence that stargazin mutation in mice causes an impairment in synaptic formation and maturation in the cerebellum (Meng et al., 2006). In addition, double mutant zi/zi, tm/tm rats, which spontaneously develop absence seizures after 6 weeks of age, show a low expression of the synaptic vesicle proteins, SV2A and synaptotagmin-1 (Hanaya et al., 2012). We have found a selective reduction of the transcript encoding for TSP-1 in the ventrobasal thalamus of WAG/Rij (Santolini et al., 2015), which represent a validated genetic rat model of human absence epilepsy (van Luijtelaar and Coenen, 1986; 1997).

An attractive hypothesis is that the TSP-$\alpha_2\delta$ axis plays a key role in synaptic formation within the cortico-thalamo-cortical network that lies at the core of the pathophysiology of absence epilepsy (Blumenfeld, 2005) and that defects in either TSPs or $\alpha_2\delta$ disrupt synaptic organization in the circuit resulting into absence seizures. TSPs may have a broader role in epileptogenesis that involves other types of epilepsy. Mendus et al. (2015) have recently found that mice with genetic deletion of TSP-1 show an increased sensitivity to chemical kindling induced by repeated administrations of a subconvulsive dose of pentylenetetrazole (PTZ). TSP-1 knockout mice developed generalyzed tonic-clonic seizures in response to the second administration of PTZ, whereas at least eight administrations were required to produce the same phenotype in wild-type mice (Mendus et al., 2015). Interestingly, TSP-1 knockout mice also showed a reduced expression of both $\alpha_2\delta$-1 and $\alpha_2\delta$-2 subunits in the frontal cotex (Mendus et al., 2015).

In conclusion, the $\alpha_2\delta$ subunit subserves pleiotropic functions that may be relevant for the pathophysiology of epilepsy and may be targeted by therapeutic intervention. The role of $\alpha_2\delta$ subunit in absence epilepsy cannot be reconducted to the modulation of T-type channels, and may involve other types of VSCCs. Increasing evidence suggests that the TSP-$\alpha_2\delta$ axis can be dysfunctional in absence epilepsy and other forms of epilepsy. Abnormalities in either TSPs and/or

$\alpha_2\delta$ subunit may contribute to the process of epileptogenesis, which is ultimately responsible for the recurrence of epileptic seizures. If proven to be correct, this may lay the groundwork for the design of novel disease modifying drugs that are directed towards mechanisms that lie at the core of epileptogenesis.

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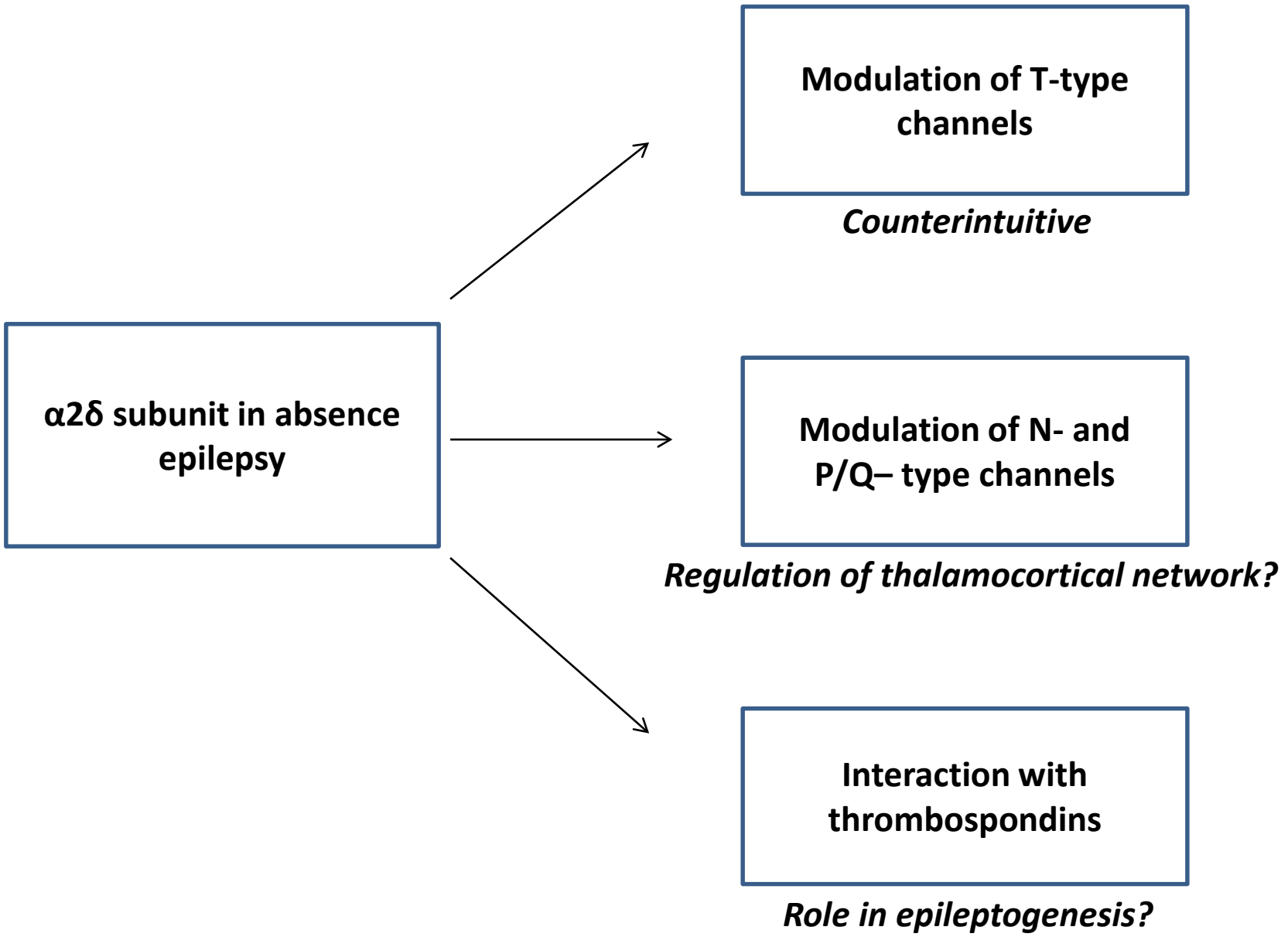
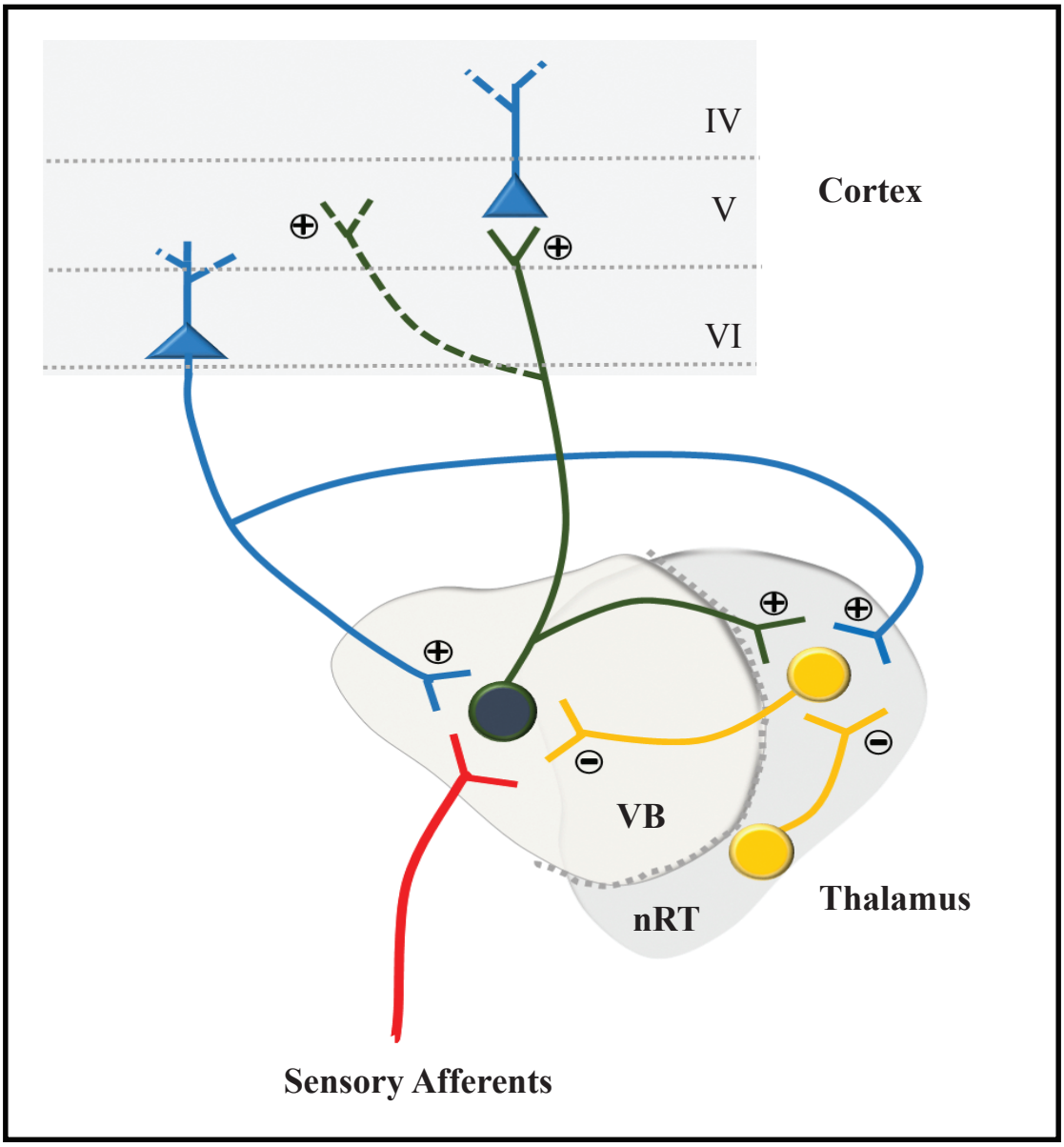

α2δ subunit in absence epilepsy
Modulation of T-type channels
Counterintuitive
Modulation of N- and P/Q– type channels
Regulation of thalamocortical network?
Interaction with thrombospondins
Role in epileptogenesis?


IV
V
VI
Cortex
VB
nRT
Thalamus
Sensory Afferents