Thesis 3

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# Introduction

## Importance of Voltage-gated Sodium Channels in the Cortex

The action potential is an electrical signal that facilitates interneuronal communication. Action potentials occur in response to excitatory input which depolarizes the neuronal membrane and activates voltage-gated sodium channels (VGSCs). These channels drive neuronal depolarization, set the threshold for action potential generation, conduct the action potential, and propagate the signal to sites of neurotransmitter release (Hodgkin and Huxley 1952). In doing so, VGSCs prove vital to neuronal excitability. This process takes place over milliseconds and is shaped by a multitude of factors including both the intrinsic properties of these channels and extrinsic interactions with additional signaling molecules. While intrinsic factors are coupled to neuronal identity, extrinsic factors provide more dynamic systems to modulate and refine the electrical signal (for review (Catterall 2012; Ahern et al. 2015)).

## Properties of Voltage-gated Sodium Channels

In all excitable cells, VGSCs are closed at negative potentials, activate in response to a depolarization, and then rapidly inactivate at positive potentials. Within hundreds of microseconds of activation a large macroscopic current is produced and the proportion that inactivates rapidly within 1-2 milliseconds is described as the transient, fast-inactivating VGSC current  (Hodgkin and Huxley 1952; Hodgkin and Huxley 1952). The proportionately small residual VGSC current following is defined as the persistent VGSC current (Hotson, Prince, and Schwartzkroin 1979; Llinás and Sugimori 1980; Stafstrom et al. 1985). Relief of inactivation occurs only at strongly negative potentials and primes the channels for further activation (Hodgkin and Huxley 1952; Kuo and Bean 1994). Certain cell types, such as cerebellar Purkinje neurons, exhibit a resurgent VGSC current produced when depolarization-inactivated VGSCs reopen in response to repolarization after strong depolarizing pulses (Raman et al. 1997). Resurgent current exhibits its own voltage-dependence and kinetics (Raman and Bean 1997).  Voltage-dependent activation, voltage-dependent inactivation, persistent current, and resurgent current of VGSCs all influence the action potential waveform. The innate kinetics of any of these properties or modulation of them will influence signal conduction.

## Intrinsic VGSC Properties Influencing the Action Potential

Once the threshold for action potential firing is breached, an action potential will reliably fire with a set amplitude independent of the stimulus size (Noble and Stein 1966). While action potentials are a digital all-or-none signal in this regard, the VGSC conductance underlying this phenomenon is influenced by a host of variables, giving rise to wide-ranging action potential shapes among neuronal types (Bean 2007). The kinetics of sodium currents differ immensely between different types of neurons and, remarkably, even between different regions of the same neuron (Engel and Jonas 2005). While the explicit mechanisms producing variability in both the VGSC signal and action potential waveform are unknown, some variation can be attributed to VGSC subunit composition and distribution (Bean 1997).

At the structural level, VGSC properties are isoform-specific and modulated by auxiliary β subunits. Of the nine VGSC α subunits (NaV1.1-1.9), the mammalian central nervous system is primarily populated by isoforms NaV1.2 and NaV 1.6 (Catterall 2012). NaV1.2 is expressed almost exclusively in unmyelinated axons (Miyazaki et al. 2014),  NaV1.6 in myelinated axons of mature neurons at the initial segment and nodes of Ranvier (Boiko et al. 2003), NaV1.1 and NaV1.3 on neuronal soma (Westenbroek, Merrick, and Catterall 1989), and NaV1.1, NaV1.3, and NaV1.6 have been identified in dendrites (Westenbroek, Merrick, and Catterall 1989; Westenbroek, Noebels, and Catterall 1992; Caldwell et al. 2000). The differential distribution of these neuronal isoforms affect action potential waveform, initiation, propagation, frequency, and even action potential back propagation toward the postsynaptic terminal (Araya et al. 2007), changing how signals are integrated in the cortex. The differences in VGSC properties between isoforms are diverse and not yet fully quantified. Each isoform exhibits unique gating properties, voltage-dependence, and current profiles, with even more nuanced differences shown in use-dependent potentiation or use-dependent reduction of channel current (Zhou and Goldin 2004).

Adding another level of complexity, VGSCs in the mammalian brain are heterotrimeric complexes of an α subunit and flanking β subunits (Messner and Catterall 1985)(Hartshorne and Catterall 1981; Isom et al. 1995). While the α subunit can form a functional unit on its own with voltage sensor, sodium selectivity filter, and pore-forming domains, adjacent β subunits modulate voltage-sensitivity, gating kinetics, and membrane trafficking of the α subunit  (Catterall 2000; Kazen-Gillespie et al. 2000; Qin et al. 2003; Yu et al. 2003). The five mammalian β subunits (OMalley and Isom 2015) can increase peak current density (Brackenbury and Isom 2011), shift the voltage range over which activation and inactivation occur, enhance rates of inactivation and recovery from inactivation (Yu et al. 2005; Chen and Cannon 1995), and alter persistent and resurgent VGSC current in a tissue-specific pattern  (reviewed in (Calhoun and Isom 2014)). In addition, the β subunit can receive posttranslational modifications such as phosphorylation and glycosylation that alter channel gating (Johnson et al. 2004; Calhoun and Isom 2014). β  subunits therefore influence many of the key conformational changes that VGSCs undergo during the action potential (Brackenbury and Isom 2011; Patino and Isom 2010). Specific combinations of α and β subunits contribute to the particular physiological characteristics of divergent cell types (Winters and Isom 2016).

In addition, the location of VGSC expression can modify action potential shape to control the spatial and temporal features of the signal and, in many cases, define the role of that input in neuronal processing (for review (Lai and Jan 2006)). A classic example of this is VGSCs clustering at the axon initial segment and nodes of Ranvier to insure saltatory conduction along the axon (reviewed in (Freeman et al. 2015)). Further studies of VGSCs have revealed novel physiological roles of VGSCs at specific physical locations along excitatory cells. For example, VGSCs were once though to be absent in dendrites but now data show that sodium channels at these sites play a role in the back propagation of action potentials (Losonczy, Makara, and Magee 2008; Larkum et al. 2007; Gasparini and Magee 2006; Gasparini, Migliore, and Magee 2004).  Patch-clamp experiments have demonstrated that axonal and somato-dendritic VGSC currents differ in their voltage-dependent properties, indicating either different α and  β subunit compositions and/or distinct posttranslational modifications of identical subunits (Gasparini and Magee 2002; Colbert and Johnston 1998). These findings reveal how action potential waveform can vary widely along individual neurons and between divergent cell types.

VGSC subunit identity and composition are intrinsic factors that are coupled to neuronal identity and known to influence the firing properties of neurons.  These in addition to spatial distribution influence the electrical behavior of neurons at a base level (for review (Lai and Jan 2006; Cusdin, Clare, and Jackson 2008)). We now find that these foundational properties can be further shaped and refined through protein signaling pathways and posttranslational modifications to add additional complexity to the electrical contribution of VGSCs.

## Extrinsic Voltage-gated Sodium Channel Modulation

It was once though that the only point of regulation of the sodium channel signal was voltage-dependent gating on a millisecond time scale. Early voltage-clamp studies of VGSCs in neurons indicated that VGSCs are not subject to regulation via second messenger pathways (Hille 2001)(pgs 201-236). An explanation for this finding is that the methodology of these studies allowed for dialysis of the cytoplasm resulting in the loss or dilution of critical signaling components. These studies led to the widespread idea that VGSC channels are functionally similar in different excitable cells and that they are not modulated by cellular regulatory pathways. Now this picture has evolved with the identification of second messenger signaling cascades that modulate VGSC activity. A multitude of enzymes, including among others protein kinases A and C (Sigel and Baur 1988; Scheuer 2011; Murphy et al. 1993), calcium/calmodulin-dependent protein kinase (CAMK) II (Ashpole et al. 2012; Thompson et al. 2017), Fyn tyrosine kinases (Ahn et al. 2007)(Scheuer 2011), calcineurin (Murphy et al. 1993), induce posttranslational modifications of VGSCs culminating in short- and long-term alteration of sodium currents (reviewed in (Laedermann, Abriel, and Decosterd 2015)). Because the action potential is the final common pathway for neuronal output, VGSCs are ideally positioned to serve as an important point of regulation of neuronal signal transduction. Herein we identify a novel G-protein-dependent pathway for the dynamic regulation of VGSCs, investigate the components of this pathway and the kinetics of VGSC modulation, and discuss the significance of this regulation to neuronal function.

## The Intersection of G-Proteins and Ion Channels

GPCRs are a large protein family of integral membrane receptors (Vassilatis et al. 2003) that sense extracellular molecules such as peptides, lipids, or neurotransmitters and in turn activate intracellular signaling pathways by regulating the activity of bound heterotrimeric G-proteins. Heterotrimeric G-proteins consist of a G*α* subunit that binds and hydrolyzes GTP into GDP, and *β*  and *γ* subunits that form the G*βγ* dimer (Wettschureck and Offermanns 2005). In the absence of stimulus, the GDP-bound G*α*  subunit and G*βγ* dimer are associated with the receptor (Sondek et al. 1996). Binding of an extracellular ligand onto the GPCR induces a conformational change that is transduced to the G-protein and promotes the exchange of bound GDP for GTP from the G*α*  subunit. GTP-bound G*α*  dissociates from the GPCR and can freely diffuse away from the membrane. Upon activation, the G*βγ* dimer also dissociates from the heterotrimer and diffuses away from the GPCR in a membrane-delimited fashion. Intrinsic hydrolysis of GTP by the G*α*  subunit allows reassociation of GDP-bound G*α*  subunit with the G*βγ*dimer, terminating G-protein signaling. Both the GTP-bound G*α*  subunit and the G*βγ* dimer can mediate intracellular signaling. The activity of GPCRs has been implicated in a diverse repertoire of processes including metabolism, development, hormonal homeostasis, and synaptic plasticity (reviewed in (Rosenbaum, Rasmussen, and Kobilka 2009; Hoffmann et al. 2008)).

G-proteins have been shown to modulate ion channels for dynamic roles in synaptic transmission, exerting effects on both voltage-gated calcium (VGCCs) and voltage-gated potassium channels (VGKCs). Data show that G-protein modulation of these channels is widespread throughout the cortex. To date, up to 20 neurotransmitters and corresponding receptors have been described to modulate VACCs, including noradrenaline (Bean 1989; McFadzean and Docherty 1989; Lipscombe, Kongsamut, and Tsien 1989), GABA (Deisz and Lux 1985; Grassi and Lux 1989; Dolphin and Scott 1987), dopamine (Drolet et al. 1997) and acetylcholine (Bernheim, Beech, and Hille 1991; Shapiro et al. 1999).  Upwards of fifteen GPCRs have also been shown to modulate VAKCs, including M2-muscarinic (Bünemann et al. 1995), A1 adenosine (Soejima and Noma 1984), D2dopamine (Lacey, Mercuri, and North 1988; Saugstad, Segerson, and Westbrook 1996), μ-, δ-, and κ-opioid (Grudt and Williams 1993; North et al. 1987), 5-HT1A serotonin (Oh, Ho, and Kim 1995), mGluRs (Saugstad, Segerson, and Westbrook 1996), and GABAB receptors (Lacey, Mercuri, and North 1988) (reviewed in (Hille 1992; Yamada, Inanobe, and Kurachi 1998)). GPCR-induced modulation of VGCCs and VGKCs includes the indirect mechanisms of phosphorylation, lipid signaling pathways, and channel trafficking (for review (Zamponi and Currie 2013; Inanobe and Kurachi 2014)). Interestingly, both VGCCs and VGKCs are subject to direct modulation by the *βγ* dimer for reduced neuronal excitability. VGCC inhibition by G*βγ*provides negative feedback over VGCCs and terminates further neurotransmitter discharge  (Herlitze et al. 1996; Ikeda 1996) while G*βγ*activation of G-protein-coupled inwardly-rectifying potassium channels (GIRKs) (Lüscher et al. 1997) reduces the propagation of neuronal inputs from excitatory synapses as well as the back propagation of action potentials from the soma to the synapse (Ponce et al. 1996; Morishige et al. 1996; Liao, Jan, and Jan 1996).

Given the pervasiveness of G-protein and ion channel interactions, high structural homology between VGSCs and VGKCs, and the positioning of VGSCs as critical targets for the modulation of neuronal excitability, there exists a convincing probability and fortuitous opportunity for G-protein interaction with these channels. We already know that G-protein and ion channel interactions modulate the integrative properties of neuron; it is certain that further research will reveal additional functional relationships between these two key players in neuronal excitability and delineate more pathways for the control of synaptic transmission.

## G-Proteins and Voltage-gated Sodium Channels

The first studies of the G-protein modulation of VGSCs were undertaken following the identification of several putative phosphorylation sites on the pore-forming portion of the channel structure (Barchi 1988). This suggested that the channel may be subject to regulation by second messenger pathways that activate phosphorylating kinases. Sigel and Bauer, 1988, presented the first electrophysiological evidence of G-protein modulation of VGSCs by demonstrating that activators of PKC reduce the amplitude of VGSC currents in *Xenopus* oocytes injected with chick brain mRNA (Sigel and Baur 1988). It was then shown that GTP*γ*S, an activator of G-protein signaling, inhibited VGSC current in *Xenopus* oocytes injected with rat brain RNA (Cohen-Armon, Sokolovsky, and Dascal 1989). Since the initial observations that linked G-proteins to VGSCs, a complete picture has been painted between GPCRs at the cellular membrane and changes in VGSC output. Studies now show that VGSCs in the cortex can be modulated by dopamine, acetycholine, serotonin and other transmitters acting through GPCRs (ALL THE REFS). This regulation provides an important form of cellular plasticity, which controls the excitability of central neurons in response to synaptic inputs, the threshold for excitability, and the frequency and form of action potentials.

Evidence for G-protein modulation of VGSCs points to prolific regulation throughout the cortex as well as a common theme of G-proteins producing significant reduction in VGSC current. VGSCs have been shown to be inhibited by activation of dopamine D1-like receptors, metabotropic glutamate receptor 1 (mGluRs) , muscarinic acetylcholine receptor (mAChR) M1, and serotonin 5-HT1a/c receptor. While some studies report a decrease in channel conductance without a change to the voltage-dependent properties of VGSCs (Cantrell, Scheuer, and Catterall 1999; Cantrell et al. 1996), others indicate G-protein interactions can substantially shift voltage-dependent inactivation of the channels (Carlier et al. 2006; Carr et al. 2002).  The primary biophysical mechanism of VGSC modulation by GPCRs is phosphorylation downstream of PKA and PKC activation. This relationship have been shown to modulate VGSCs in preparations from the hippocampus (scheuer 1999)(Cantrell ma schemer catterall 1996), neocortex (Carlier et al. 2006), and prefrontal cortex (Maurice summerier 2001)(surmier 2002). These pathways have been proposed to function as negative feedback loops during periods of intense synaptic transmission. Preliminary studies has also suggested that VGSCs are also subject to direct modulation via the G*βγ* of heterotrimeric G-proteins, in much the same fashion as VGCCS and VGKCS, and that this increases persistent VGSC current of NaV1.1 and NaV1.2 (Mantegazza 2005), though additional research is necessary to fully understand the extent and nature of G*βγ* modulation of VGSCs. Overall, data is amassing for the G-protein modulation of VGSCs and the varied roles this relationship plays in synaptic transmission.

## The Calcium-Sensing Receptor

The calcium-sensing receptor (CaSR) is a GPCR activated by extracellular calcium expressed in many tissues including the nervous system (Leach et al. 2015; Butters et al. 1997; Rogers et al. 1997). The CaSR has been studied extensively for its roles in maintaining calcium homeostasis in the kidney and parathyroid gland. CaSRs expressed on the parathyroid gland sense serum calcium levels and modulate parathyroid hormone synthesis and secretion to maintain serum calcium within a physiological range. For this role, allosteric modulators of the CaSR provide therapeutic promise for disorders of calcium homeostasis (Brauner-Osborne, Wellendorph, and Jensen 2007).

In the cerebellum and cerebral cortex, the CaSR is expressed at nerve terminals (Chen et al. 2010; Ruat et al. 1995) where it modulates evoked and spontaneous synaptic transmission (Smith et al. 2012; Smith et al. 2004). Previous research in our lab has shown that presynaptic CaSRs decrease release probability at excitatory synapses in the neocortex through the regulation of a nonselective cation channel (NSCC) (Smith et al. 2004). This function forms a feedback loop wherein the CaSR represses a NSCC current as extracellular calcium increases and relieves inhibition on the channel as extracellular calcium falls (Smith et al. 2004). Decreases in extracellular calcium depress synaptic transmission due to the sensitivity of neurotransmitter release to extracellular calcium following its entry via VGCCs (Heidelberger et al. 1994; Schneggenburger and Neher 2000). When calcium falls at the cleft during periods of high synaptic activity CaSR activity is reduced, leading the relief of suppression of the NSCC conductance. The addition of the NSCC current depolarizes the terminal to allow for continued neurotransmitter release during bursts of synaptic activity.  In our current theoretical model, increased NSCC activity may increase action potential duration, prolong calcium entry at the terminal, and thus increase release probability. Consequently, the CaSR may be operating as part of a homeostatic pathway to prevent synaptic failure when extracellular calcium falls (for review (Jones and Smith 2016)).

## Calcium-Sensing Receptor Modulators

The drugs cinacalcet and calindol serve as positive allosteric modulators of the CaSR, enhancing its sensitivity to extracellular calcium. In an antagonistic action, negative allosteric modulators NPS 2143 and calhex decrease the sensitivity of the CaSR to extracellular calcium. All four drugs exhibit high selectivity for the CaSR and are known to possess overlapping though not identical binding sites to the seventh transmembrane domain (TMD) of the receptor (for review (Ward and Riccardi 2012; Conigrave and Ward 2013)).

Interestingly, cinacalcet, marketed under the name Sensipar, was the first drug of its class to become available for clinical use and been shown to treat severe secondary hyperparathyroidism in patients receiving hemodialysis treatment for chronic kidney disease (Block et al. 2017). In this role, cinacalcet acts on targets on the parathyroid to stimulate the CaSR, thereby decreasing circulating parathyroid hormone levels and producing a concurrent decrease in serum calcium levels. Cardiovascular disease is the leading cause of death in patients with chronic kidney disease and a risk factor for secondary hyperparathyroidism. Cardiovascular disease results from premature cardiovascular aging characterized by vascular calcification on a background of hypercalcemia secondary to high levels of parathyroid hormone. Cinacalcet prevents vascular calcification by reducing parathyroid hormone and consequentially reduce the risk of cardiovascular events in patients with chronic kidney disease.

A trial called EVOLVE was designed to evaluate if cinacalcet reduced the risk of death and nonfatal cardiovascular events among patients with secondary hyperparathyroidism who were undergoing dialysis. The trial, published in *The New England Journal of Medicine* in 2012, concluded that while cinacalcet reduced the levels of parathyroid hormone effectively, treatment with cinacalcet did not lead to a statistically significant reduction in the risk of death or cardiovascular events (Chertow et al. 2012). The results indicated that adverse or unknown effects of cinacalcet might be undermining its effectiveness in the treatment of hyperparathyroidism. A criticism of the EVOLVE trial was the high nonadherence rate for cinacalcet-medicated patients due to intolerable side effects (Moe and Thadhani 2013; Parfrey et al. 2016). Cinacalcet is lipid-soluble, meaning that it readily crosses the blood brain barrier. Commonly-reported neurological side effects of cinacalcet include paresthesia, vertigo, severe hearing loss, and altered mental status (Borchhardt et al. 2008). Despite its ability to readily diffuse into the nervous system and the results of the EVOLVE trial, the effects of cinacalcet in the brain have yet to be studied.

In investigating the role of the CaSR in synaptic transmission, we found that allosteric CaSR modulators reduced GABAergic transmission between neocortical neurons. The nature of this inhibition indicated CaSR-mediated conduction block of the presynaptic action potential and this hypothesis was validated by the discovery that CaSR allosteric modulators strongly inhibit VGSCs. This finding was immediately relevant to the clinical usage of cinacalcet as well as our understanding of the role of the CaSR in the cortex. Further examination showed that both allosteric agonists and allosteric antagonists of the CaSR completely inhibited VGSC current. This inhibition, in all cases, was shown to be dependent on a G-protein pathway. In Chapter 1, we look at the mechanism underlying CaSR modulator-regulation of VGSCs, investigating the kinetics of this effect as well as potential effector proteins within the pathway.

## Anandamide and the Calcium-Sensing Receptor

Endocannabinoids (eCBs) are a family of lipid molecules that serve as key players in synaptic plasticity. The two major cannabinoid-signaling molecules in the central nervous system are anandamide (AEA) and 2-Arachidonoylglycerol (2-AG). Cannabinoid type 1 (CB1) receptors have been shown to possess the highest sensitivity to AEA, however, data support the existence of additional receptors that respond to eCBs (reviewed in (Castillo et al. 2012; Kano et al. 2009; Katona and Freund 2012; Alger 2012)) . We have identified AEA as a positive allosteric modulator of the CaSR, acting to increase the sensitivity of the receptor to extracellular calcium in the same fashion as has been observed for calindol and cinacalcet.

## Significance of Anandamide in Physiology and Disease-States

In the canonical pathway for cannabinoid function, eCBs are produced on-demand in postsynaptic neurons in response to stimulation then travel in a retrograde fashion to targets receptors on the presynaptic component. eCBs function primarily through presynaptically expressed CB1 receptors throughout the cortex on both excitatory and inhibitory terminals (Herkenham et al. 1990). Activation of presynaptic cannabinoid receptors can initiate both short- and long- term synaptic depression (reviewed in (Castillo et al. 2012; Kano et al. 2009; Katona and Freund 2012; Alger 2012)). Recent work shows that cannabinoid function is more diverse than previously believed. In addition to their role as instigators of synaptic depression, cannabinoids have also been shown to participate in non-retrograde signaling (Grueter, Brasnjo, and Malenka 2010; Chávez, Chiu, and Castillo 2010) and signaling via astrocytes (Stella 2010; Navarrete and Araque 2008). Furthermore, studies show that eCBs act on as-yet unidentified receptors to contribute to these processes of synaptic plasticity (reviewed in (Begg et al. 2005)).

Importantly, eCBs contribute to a range of physiological processes, including synaptic plasticity and learning (Heifets and Castillo 2009), pain (Guindon and Hohmann 2009), metabolism and energy homeostasis (Viveros et al. 2008), and neural development (Fride 2008). Dysregulation of the eCB system has been implicated in neuropsychiatric conditions, such as depression, autism, schizophrenia, addiction, stress and anxiety (Ruehle et al. 2012; Hillard 2014; Mechoulam and Parker 2013; Parsons and Hurd 2015; Volkow, Wise, and Baler 2017). eCBs have also shown therapeutic promise for Tourette’s syndrome (Müller-Vahl 2013), Huntington’s disease (Pazos, Sagredo, and Fernández-Ruiz 2008), epilepsy (Alger 2004), Alzheimer’s disease (Maroof, Pardon, and Kendall 2013), depression (Huang, Chen, and Zhang 2016), and stroke (Hillard 2008).  Advancing our understanding of the many roles of eCBs in the nervous system will enhance our knowledge of disease states and synaptic transmission.

## Allosteric Modulators of the Calcium-Sensing Receptor Modulate Voltage-gated Sodium Channels via a G-protein pathway

In Chapter 1 of this dissertation, we show that synthetic allosteric modulators of the CaSR substantially reduce VGSC current.  In a detailed look at the kinetic properties of VGSCs, it is shown that CaSR modulators produce a hyperpolarizing shift in steady-state inactivation of the channels, pushing them into a deeply inactivated state relieved only by strong, prolonged hyperpolarization. We establish that this pathway is independent of the CaSR but dependent on G-protein signaling.   Cumulatively, these data indicate an important new pathway for modulating neuronal excitability in the cortex. Following this study, we sought to identify the VGSC modulatory properties of the endogenous signaling ligand AEA. In Chapter 2 , we show that AEA completely inhibited VGSC current also via a change in the inactivation properties of VGSCs. This block of VGSC current was also independent of the CaSR but required G-protein activation. The VGSC inhibition also appeared independent of CB1 receptors, indicating a novel site of action of AEA as well as a new G-protein-dependent pathway for VGSCs regulation in the cortex.

The findings presented here are exciting in a number of ways. First, they lend further support to the idea that VGSCs are subject to dynamic modulation by second messenger pathways. Second, they show AEA has actions independent of CB1 receptors in the cortex. And third, they point to the existence of an as-yet unexplored eCB-activated pathway for synaptic plasticity in the cortex.

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# Discussion

Voltage-gated sodium channels are integral to a number of physiological processes in excitable cells: setting the action potential threshold, action potential conduction, signal integration, firing properties, and synaptic transmissions. The regulation of these channels can alter the transmission of cellular signals in response to local stimuli. Neurotransmitters such as acetylcholine and dopamine have been shown to inhibit VGSCs via G-protein pathways to change the firing properties and input-output relationships of cortical neurons. The data presented in this dissertation examines new pathways for the G-protein modulation of VGSCs by allosteric modulators of the CaSR.

The five allosteric modulators of the CaSR tested all exhibited similar mechanisms and kinetics of VGSC inhibition. Cinacalcet, calhex, NPS 2143, calindol, and AEA reliably produced near-complete VGSC inhibited with slow kinetics (full inhibition occurring in 100s of seconds), dependence on G-proteins, and a shift in the steady-state inactivation of affected VGSCs (shown with cinacalcet, AEA). Inhibition in all cases was shown to be independent of the CaSR. Furthermore, AEA-induced inhibition was independent of CB1 receptors, all indicating off target effects of these ligands. Of the CaSR allosteric modulators tested, AEA is the only endogenous ligand. The participation of this major cortical signaling molecule in the G-protein-dependent mechanism for VGSC inhibition points to a significant physiological role for this pathway in the process of synaptic transmission.

## G-Protein Modulation of Voltage-gated Sodium Channels

While it was once believed that VGCs were not subject to regulation by second messenger systems, recent studies have upended prior claims and accrued evidence for G-protein modulation of VGSCs in the cortex. G-protein-dependent inhibition of VGSCs has been measured downstream of dopamine D1-like, mAChR M1, mGluR1, and serotonin 5HT2a/c receptors in the cortex (reviewed in Cantrell & Catterall, 2001).

## Other Pathways and how ours is different

The four known pathways for VGSC modulation thus far (mAChR, mGluR1, D1-like, 5HT2a/c) all rely on the activity of a protein kinases; either PKA or PKC (Cantrell, Ma, Scheuer and Catterall 1996, Cantrell, Smith, Goldin, Scheuer and Catterall 1997, Carr, Cooper, Ulrich, Spruston and Surmeier 2002, Carlier, Sourdet, Boudkkazi, D\selectlanguagengermanéglise, Ankri, Fronzaroli-Molinieres and Debanne 2006). These kinases phosphorylate a serine residues in the intracellular loop connecting domains I and II of the VGSC alpha subunit (Cantrell & Catterall, 2001). This common theme is one major divergence from the pathway of CaSR allosteric modulators. Experiments with cinacalcet showed that the G-protein inhibition induced by this CaSR allosteric modulator was independent of either PKA or PKC. Interestingly, the phosphorylation of VGSCs by PK\_ downstream of \_\_\_ and \_\_ was shown to shift VGSC inactivation in the hyperpolarizing direction, much the same as observed with Cinacalcet and AEA. While this study targeted PKA and PKC with specific inhibitors chelerythryine chloride, PKI, PKI\_\_\_\_\_, we cannot rule out the involvement of staurosporine resistant kinases. Phosphorylation of VGSCs downstream of AEA may still influence this pathway and produce the shift in VGSC inactivation.

## Protein Kinases Involvement

Here we attempt to identify the full pathway from ligand to GPCR to effector proteins and, finally, to changes in VGSC modulation. We approached this with inhibitors of G-protein signaling and protein kinases. Several pitfalls prevented a more complete exploration; namely, the absence of selective, reliable tools by which to inhibit proposed components of this pathway. One mechanism that has been proposed is direct Gbetagamma modulation of VGSCs. The first evidence of Gbetagamma modulation of ion channels shown activate cardiac potassium channels downstream of acetylcholine on muscarinic receptors (Logothetis et all 1987). Since that discovery, it has been well-established that the Gbetagamma particle serves a role in inhibiting \_\_\_ and activating \_\_\_.  Betagamma has been shown to interact with a wide range of effectors and proteomic methods and yeast two-hybrid screening have revealed that no readily apparent consensus sequence or structure mediates binding of these proteins to G\selectlanguagegreekβγ. \selectlanguageenglishThe binding of Gbetagamma to VGSCs has been briefly addressed by Ma et al, 1997. The group showed that Gby increased persistent VGSC current from NAV1.2 and NaV1.1 but not NaV1.4 or NaV 1.5 when coexpressed in \_\_\_\_ system. This study also garnered evidence that G-protein \selectlanguagegreekβγ \selectlanguageenglishsubunits interact with that C-terminal site of NaV1.2 (Ma et al., 1997). Consistent with this idea, G-protein \selectlanguagegreekβ \selectlanguageenglishsubunits immunoprecipsitate with sodium channel \selectlanguagegreekα \selectlanguageenglishsubunits from cortical neuron preparations (Marin et al., 2001). Persistent VGSC current through Nav1.1 and Nav1.2 conduct is modulated by G\selectlanguagegreekβγ, \selectlanguageenglishbut current of Nav1.4 and Nav1.5 is not. CHECK THESE STUDIES FOR TRANSIENT It has been proposed that because Gbetagamma signaling is membrane-delimited, modulation by betagamma occurs in membrane microdomains (Galbiati et al., 2001) that are strategically positioned to affect action potential generation in the axon hillock and back-propagation at dendritic branches. Such tightly delimited modulation might be critically important for integrative properties of the neuron. Determining if Gbetagamma interaction with VGSCs is responsible for modulation of VGSCs by CaSR allosteric modulators requires reliable, specific Gbetagamma signaling blockers for which controls can be designed within our system. The tools currently available to test this hypothesis are insufficient and we therefore cannot verify whether or not Gbetagamma participates in this pathway.

## Inactivation Kinetics: Slow inactivation

Upon depolarization, VGSCs move from several closed (C) resting states though an open (O) state to one or several inactivated states (I). Recovery from inactivation does not pass through the O state, except in the case of resurgent VGSC current (Afshari et al. 2004)(Raman and Bean 2001). While there is variation between models, it has been suggested that VGSCs in the cortex can exhibit up to five closed stated (Kuo and Bean 1994) and multiple forms of inactivation. These inactivation states vary in time scale, biophysical mechanism, and pharmacological sensitivity. Fast inactivation takes place over the timescale of a single action potential. The forms of non-fast inactivation have been termed “intermediate,” “slow,” and “ultraslow.” Since the molecular basis clearly defining these forms of inactivation are not yet known, they are often clumped under the general term “slow inactivation.” Slow inactivation is produced by prolonged depolarizations over tens of seconds or a series of brief, consequential depolarizations.

Sodium channels are capable of fast inactivation (complete within a few milliseconds), and different forms of slow inactivation (time constants ranging from  100 ms to several minutes) [18]. The mechanism of fast inactivation is well-studied and occurs by a ‘hinged lid’ mechanism wherein an intracellular linker between domains III and IV occludes the channel pore after the open state is reached. In contrast, the structural basis for slow inactivation has not been fully resolved. Slow inactivation is thought to occur by a conformational rearrangement or ‘collapse’ of the outer pore region [38]. Mutational studies also indicate long distance interactions with other structural regions including involvement of residues in domains IV S4 (19), II S5-S6(30), and II S6 (21). It was demonstrated that channels could enter the slow-inactivated state from the closed, open, and fast-inactivated states (10). Slow inactivation decrease VGSC availability and serves to decrease repetitive action potential firing and back propagation  (Ruff 1988)  in a way that is dependent on previous history of action potential firing (Mickus 1999, Toib 1998).  Slow-inactivated state-perferring drugs have been identified including lacosimide, an antiepileptic   [19], [23].  However, their specific action as state-preferring is contentious. Electrophysiological protocols used to study the slow inactivated state utilize a prolonged depolarization (to induce slow inactivation), followed by a hyperpolarizing gap (to allow recovery from fast, but not slow inactivation). Because availability in such protocols is solely determined by the extent of slow inactivation, a drug that decreases availability is considered to be slow-inactivated state-preferring. However, gating rates (the rate of inactivation and rate of recovery from inactivation) are altered by drug binding. A fast-inactivated state-preferring drug stabilizes this state by delaying recovery. A delayed recovery does not necessarily indicate actual modification of the gating rate. For example if the bound drug prevents recovery from inactivation, then recovery will appear to be slowed because the drug needs first to dissociate [24], [25]. Because of the altered gating, the rate of recovery from fast inactivation in the presence of the drug can easily overlap with the rate of recovery from slow-inactivated state.

The rate of state-dependent association and dissociation of the drug should also be taken into account. As a result, interpretation of data obtained with these protocols is not straightforward (e.g. [9], [30]).

shifts inactivation voltage dependence

and slows recovery from inactivatiData show that both cinacalcet and AEA shift the steady-state inactivation of VGSCs toward more hyper polarized potentials. This produces reduced VGSC current during depolarizations and severely diminishes neuronal excitabilty. However, the application of prolonged, hyperpolarizing pulses provided for complete recovery of VGSC in the presence of AEA and cinacalcet (Figure \_\_\_). This slowing of recovery from inactivation can result from two processes: 1) slow rate of dissociation of the ligand or 2) increased stability of a slow-inactivated state, on the basis of an increased rate of entry into the slow-inactivated state in the presence of ligand. These are two fundamentally different mechanisms that have qualitatively similar effects on excitability.

Slowed rate of dissociation of a ligand can occur when drugs bind with high affinity to a specific state-dependent conformation of the channel (Hille, 1977; Hondeghem and Katzung, 1977) or drugs may be trapped by the channel gates during specific gating states (Starmer, 1986). If the blocking effect requires activation it will demonstrate use-dependence. With regard to the dissociation hypothesis, drugs are thought to bind with high affinity to a specific state-dependent conformation of the binding site (“modulated receptor theory”) (Hille, 1977; Hondeghem and Katzung, 1977) or drugs may be trapped by the channel gates during specific gating states (“guarded receptor theory”) (Starmer, 1986). if drug binds preferentially to a slow inactivated state, then it would accelerate the development of this state during high frequency stimulation (or series of APs). Cinacalcet was shown to demonstrate use-dependence (Figure \_\_\_) with inhibition slowed \_\_\_ BLAH BLAH BLAH EXPERIMENT.

The stabilization hypothesis suggests that drugs bind to native “slow” inactivated states that under drug-free condition require a long time to develop (Khodorov et al., 1976; Zilberter Yu et al., 1991; Balser et al., 1996b; Kambouris et al., 1998; Chen et al., 2000). However, drug-binding to a slow-inactivated state accelerates the time course of development of this state during a depolarization (“stabilization”), thereby increasing the fraction of channels recovering from that state during a subsequent hyperpolarization. Slow drug dissociation from fast-inactivated states and stabilization of slow-inactivated states need not be mutually exclusive, but both mechanisms may operate in concert, as noted by Nuss et al. (2000).

Slow inactivation of sodium channels may be pivotal in regulating firing properties of a range of neurons by increasing spike threshold, curtailing prolonged action potential bursts, and limiting active back propagation of action potentials into dendritic regions (Jung et al., 1997; Maurice et al., 2004). Furthermore, slow-inactivated state preference has been proposed as a therapeutic advantage [19]–[21],  in such diseases as epilepsy, neuropathic pain and certain arrhythmias [19]–[22]. Defective slow inactivation is associated with several nervous system diseases, including myotonia, periodic paralysis, and long-QT syndrome.

## How this fits into what we know of AEA in synaptic transmission

While the investigation of synthetic allosteric modulators of the CaSR’s role in this pathway gives us mores detailed insight into the change in kinetic properties of VGSCs downstream of G-proteins and explores potential effector proteins, the addition of AEA as an activator of this pathway to VGSC inhibition presents the greatest evidence for the physiological relevance of this pathway. AEA are manufactured on demand from membrane phospholipid through an enzymatic pathway triggered by neuronal activity, large depolarization-induced calcium increases, the activation of D2-like dopamine receptors (Giuffrida et al., 1999), and MAYBE mGLURS???. AEA is canonically thought of a retrograde signaling molecule that decreases the release of neurotransmitters, both inhibitory and excitatory, via the activation of presynaptic receptors (Ohno-Shosaku et al., 2001; Wilson & Nicoll, 2001; Alger, 2002). AEA is removed by uptake from its site of action and the enzymes that catalyze the conversion of AEA to its degradation compound FAAH (Cravatt 1996, Goparaju 1998) so that it’s action is tightly spatiotemporally regulated. This indicates that the action of AEA is diffusible but local to its point of production and short-lived. Recent studies show that eCBs can have diverse functions are mediators of both long and short term synaptic depression.

The concentration of AEA within synaptic clefts of the cortex during periods of high activity is unknown. Measurements of basal AEA levels vary widely between studies due to quantification technique, parameters for lipid extraction and purification as well as post-mortem handling of tissue prior to extraction. However the basal AEA level is quite high and may reach 0.8 umoles/kg brain tissue (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC55427/). Cannabinoid receptors CB1 and CB2 and the CaSR are high-affinity targets for AEA. CB1 is primarily expressed in CNS (reviewed by Howlett, 2002) and CB2 receptor expression is restricted to the periphery (Galiegue et al., 1995). The CaSR is known to be ubiquitously expressed throughout the cortex. Pharmacological experiments in this manuscript ruled out CB1 and the CaSR as AEA target for the initiation of this pathway. AEA is also known to act on GPCRs Muscarinic M1 & M4, Adenosine A3, 5-HT1 & 5-HT2, S1P1, GPR18, GPR55, GPR119, plus a large number of ion channels and nuclear receptors (https://link.springer.com/chapter/10.1007%2F978-3-319-20825-1\_1). Several cannabinoid-like orphan receptors have been identified as AEA targets, including GPR18, GPR55, and GPR119, with affinities for AEA in the nM-uM range nM (https://link.springer.com/chapter/10.1007%2F978-3-319-20825-1\_1) (https://onlinelibrary.wiley.com/doi/full/10.1002/mnfr.201500449). A discussion of those receptors can be found in the Future experiments section of this discussion. The role of many of these receptors as part of an endocannabinoid pathway is largely unknown. In our current understanding of AEA action in the cortex through the activation of CB1 receptors, the inhibition of VGSCs would prove to be synergistic. Cannabinoid agonists have been shown to reduce GABA-mediated IPSCs and Glutamate-mediated EPSCs in postsynaptic terminals and this is blocked by selective cannabinoid antagonists and mimicked by cannabinoid receptor agonists, suggesting roles limited to CB1 (Levenes et al., 1998) (Maejima et al., 2001; Kreitzer and Regehr, 2001b) (https://www.sciencedirect.com/science/article/pii/S0896627301002471). The inhibition of VGSCs by AEA acting acting independently of CB1 receptors would serve to further reduce presynaptic excitability. VGSC inhibition in this instance would reduce or fully block conduction of presynaptic action potentials to response to and postsynaptic retrograde signal. Therefore, VGSC inhibition by AEA may contribute to eCB-mediated STD and LTD. How neurotransmitter release is actually inhibited by cannabinoids is still under investigation. There are several possibilities, as cannabinoids are known to have actions on ionic conductances that could, if exerted on ion channels at the nerve terminal, inhibit release. The classic work of Ikeda (1996) and Herlitze et al. (1996) clearly established that the \selectlanguagegreekβγ-\selectlanguageenglishsubunits of heterotrimeric G-proteins directly interact with and inhibit the high voltage-activated Ca2+ channels the mediate transmitter release at most synapses. CB1Rs are pertussis-sensitive G-protein-coupled receptors, and their activation reduces N-type Ca2+ current (Mackie and Hille, 1992; Caulfield and Brown, 1992; Caulfield et al., 1992; Pan et al., 1996; Twitchell et al., 1997). CB1R activation also increases the conductances of various voltage-gated K channels in neurons (Deadwyler et al., 1993, 1995; Schweitzer, 2000), and activates GIRKs in heterologous expression systems (Henry and Chavkin, 1995; Mackie et al., 1995). Effects on any of these ion channels could reduce transmitter release. Direct inhibition of transmitter release machinery independent of Ca2+ is also possible.

Wilson and Nicoll (2001) reported that DSI is expressed on TTX-insensitive mIPSCs in the presence of a high-K+/-Ca2+ solution. Evidently by directly depolarizing the inhibitory nerve terminals, this solution promotes spontaneous, Ca2+-dependent vesicular release from terminals possessing CB1Rs, and permits DSI to occur. Clearly DSI can be exerted at the level of the nerve terminal, and does not depend entirely on axonal conduction block for its expression, because it can be observed in the presence of TTX. It is not yet clear whether DSI of the Ca2+-dependent, TTX-insensitive mIPSCs represents a Ca2+-dependent, or a downstream consequence of CB1R activation. Conceivably, DSI-susceptible nerve terminals produce a very low level of basal, i.e. Ca2+-insensitive, mIPSCs. Depolarization by high [K+]o in the presence of high Ca2+, by initiating mIPSC occurrence, may simply make cannabinoid-sensitive release evident, i.e. the fact that these mIPSCs are Ca2+-dependent does not mean that their inhibition is also Ca2+-dependent. DSI of the mIPSCs also seems to be a more variable and less robust phenomenon than DSI of eIPSCs or of sIPSCs in the absence of TTX. Moreover, the decrease in mIPSCs has not been shown to account quantitatively for the decrease in eIPSCs. Hence, the data do not rule out other mechanisms for DSI expression (see Section 2.7.4).

AEA are manufactured on demand from membrane phospholipid through an enzymatic pathway triggered by neuronal activity, large depolarization-induced calcium increases. It is also reported that anandamide is released in the striatum by activation of D2-like dopamine receptors (Giuffrida et al., 1999). In the canonical AEA pathway, AEA travels postsynaptically to nerve terminals where it decreases the release of neurotransmitters, such as GABA or glutamate, via the activation of presynaptic receptors (Ohno-Shosaku et al., 2001; Wilson & Nicoll, 2001; Alger, 2002).AEA is removed by uptake from its site of action and the enzymes that catalyze the conversion of AEA to its degradation compound FAAH (Cravatt 1996, Goparaju 1998). This indicates that the action of AEA is diffusible but local to its point of production and short-lived. Recent studies show that eCBs can have diverse functions are mediators of both long and short term synaptic depression. eCBs are implicated in a range of physiological processes, on a cellular level: only synaptic plasticity and learning (Heifets and Castillo, 2009), pain (Guindon and Hohmann, 2009), metabolism and energy homeostasis (Viveros et al., 2008), and neural development (Fride, 2008). They are also implicated in behavior experiments in motivation and reward (Solinas et al., 2008), emotional state (Lutz, 2009), the aetiology of drug addiction (Lopez-Moreno et al., 2008; Parolaro and Rubino, 2008), as well as numerous models of neurodegenerative disease. In many instances, the evidence for an eCB mechanism is indirect and pathways not fully delineated. Accordingly, there has been substantial interest in the quantification of eCB content in a variety of biological matrices.

https://www.sciencedirect.com/science/article/pii/S0301008202000801?via%3Dihub#BIB160

synaptic cleft concentrations of the endocannabinoids at times of activity are unknown. However the basal

Regional brain tissue eCb levels in rats 20 pmol/g in cortex (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2931546/)

Measurements vary with brain tissues and between studies due to the parameters for lipid extraction and purification can have a large impact of eCB recovery, as can the post-mortem handling of tissue prior to extraction

ECs are released from neurons in a nonvesicular manner in response to large depolarization-induced Ca2+ elevation (Cadas *et al* ., 1996; Stella *et al* ., 1997).

Recent studies have revealed the essential role of ECs in retrograde modulation of synaptic transmission in the central nervous system: strong depolarization of postsynaptic neurons releases ECs, which act backwards onto presynaptic CB1 receptors and decrease the release of other neurotransmitters, such as \selectlanguagegreekγ\selectlanguageenglish-\selectlanguageenglishaminobutyric acid (GABA) or glutamate, from presynaptic terminals (Ohno-Shosaku *et al* ., 2001; Wilson & Nicoll, 2001; Alger, 2002).

;Wilson &a\selectlanguagepolishĖ\selectlanguageenglishrdJ

DSI shows a substantial delay, several hundred milliseconds to a second or more, suggesting the involvement of second messengers (https://ac.els-cdn.com/0896627394904308/1-s2.0-0896627394904308-main.pdf?\_tid=85e10993-1659-4d30-876b-04afbe6625d9&acdnat=1521588031\_11fb139ed11b6c05be0e412c226a6499)

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normal’r\_J

EXAMPLE WHERE OMISSION OF GTP DOES NOT STOP G PROTEIN PROCESS https://ac.els-cdn.com/0896627394904308/1-s2.0-0896627394904308-main.pdf?\_tid=85e10993-1659-4d30-876b-04afbe6625d9&acdnat=1521588031\_11fb139ed11b6c05be0e412c226a6499

GPR55, GPR119, and GPR18 are three cannabinoid-like orphan GPCRS (Mackie and Stella 2006; Pertwee, 2010). Little is yet known of the physiological functions of these receptors. All three receptors are expressed in brain tissue. AEA is identified as a partial agonist of GPR18, a GPCR expressed in the central nervous system, including the cortex (Vassilatis et al., 2003). GPR18 was only recently deorphanized and its role in physiological processes consequentially unexplored. GPR18 serves roles in microglial migration in the central nervous system. GPR18 is suggested to have a sympathoinhibitory role in neurons of the rostral ventrolateral medulla (https://www.ncbi.nlm.nih.gov/pubmed/24431468). However, further physiological roles for this receptor are as yet unexplored. GPR119 is also expressed in the cortex (Bonini 2002 Methods of identifying compounds that bind to SNORF25 receptors.). Research on GPR 119 has focused on it’s role in the modulation of insulin release by pancreatic \selectlanguagegreekβ-\selectlanguageenglishcells and of GLP-1 secretion by gut enteroendocrine cells (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2268073/). It’s action in the cortex is not yet known. GPR55 was identified as a cannabinoid receptor. Activation stimulates RhoA, cdc42, rac1.Expressed in the brain (M. Sawzdargo, et al.Identification and cloning of three novel human G protein-coupled receptor genes GPR52, PsiGPR53 and GPR55: GPR55 is extensively expressed in human brain). These three GPCRs serve as putative targets for AEA to stimulate the pathway identified in this research. Exploring the role of these receptors in the pathway will require the development of knockout mice and/or highly specific receptor inhibitors. Without these tools, testing for VGSC inhibition via GPR18, GPR55, or GPR119 will be difficult to conclude.

## What we already know about AEA on VGSCs

## Potential GPRCs

### Binding sites of CaSR Modulators

R 568  cinacalcet is a derivative of

NPS 2143, Calindol, Calhex 231 and cinacalcet are structurally related phenylalkylamines, containing two aromatic rings connected by a flexible chain with a positively charged amino group. Studies have revealed calcimimetics and calcilytics target a similar interaction sites in a crevice formed by transmembrane domains (TMs) 3, 5, 6, and 7. The binding pocket for both calcimimetics and calcyliytics of the CaSR has been suggested to involve Trp-818, Phe-821, Glu-837, and Ile-841 located in transmembranes (TM) 6 and TM7, with differences observed between each family of compounds (petrel and ruat evernote). All four compounds are exhibit ionic interactions with a negatively charged amino acid (Glu-837) located in TM7 (https://academic.oup.com/edrv/article/32/1/3/2354777). The binding sites for all four compounds are largely overlapping but not identical and located within a bundle formed by the seven transmembrane domains. The shared binding pocket for all four CaSR allosteric modulators points to, though does not prove, the existence of a structurally similar binding pocket on a separate GPCR mediating this effect. It is not yet known how if AEA interacts with the same allosteric modulator site on the CaSR, though the presence of a positively charged amino group may indicate similarities in the mechanism of binding.

### GPR55, GPR18

GPR55, GPR119, and GPR18 are three cannabinoid-like orphan GPCRS (Mackie and Stella 2006; Pertwee, 2010). Little is yet known of the physiological functions of these receptors. All three receptors are expressed in brain tissue. AEA is identified as a partial agonist of GPR18, a GPCR expressed in the central nervous system, including the cortex (Vassilatis et al., 2003). GPR18 was only recently deorphanized and its role in physiological processes consequentially unexplored. GPR18 serves roles in microglial migration in the central nervous system. GPR18 is suggested to have a sympathoinhibitory role in neurons of the rostral ventrolateral medulla (https://www.ncbi.nlm.nih.gov/pubmed/24431468). However, further physiological roles for this receptor are as yet unexplored. GPR119 is also expressed in the cortex (Bonini 2002 Methods of identifying compounds that bind to SNORF25 receptors.). Research on GPR 119 has focused on it’s role in the modulation of insulin release by pancreatic \selectlanguagegreekβ-\selectlanguageenglishcells and of GLP-1 secretion by gut enteroendocrine cells (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2268073/). It’s action in the cortex is not yet known. GPR55 was identified as a cannabinoid receptor. Activation stimulates RhoA, cdc42, rac1.Expressed in the brain (M. Sawzdargo, et al.Identification and cloning of three novel human G protein-coupled receptor genes GPR52, PsiGPR53 and GPR55: GPR55 is extensively expressed in human brain). These three GPCRs serve as putative targets for AEA to stimulate the pathway identified in this research. Exploring the role of these receptors in the pathway will require the development of knockout mice and/or highly specific receptor inhibitors. Without these tools, testing for VGSC inhibition via GPR18, GPR55, or GPR119 will be difficult to conclude.

GPR18 is Galpha i/o coupled and Gq

Studies of  G\selectlanguagegreek*βγ* \selectlanguageenglish  binding to VGKCs and VGCCs indicate that there is no consensus sequence for protein-protein interactions (Lin and Smrcka 2011), rather a structurally flexible protein interaction hot spot that accommodates different structures on the beta gamma particle(DeLano 2002). This is unsurprising given the diversity in protein sequence and structure that are known to be accommodated by this single binding site.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4237895/

GPR18 deorphanized when it was found to be activated by AEA, NAGly, abn-CBD

expressed in the CNS including cortex (Vassilatis et al., 2003).

(https://onlinelibrary.wiley.com/doi/full/10.1002/mnfr.201500449)

GLP-1 se\selectlanguageenglishrqb

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EC50 for AEA on GPR55 is 18 nM (https://link.springer.com/chapter/10.1007%2F978-3-319-20825-1\_1) but also activated by AM 251

EC50 AEA on GPR18 3.83 uM (https://onlinelibrary.wiley.com/doi/full/10.1002/mnfr.201500449) (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4806671/)

an style=’fon3rb

### GPRC6A

## Future Experiments

As illustrated by our lack of complete understanding about the identity and composition of the inward current following mGlu1 and mGlu5 receptor activation, there is still much to be answered regarding Group I mGluR activation in dopamine neurons. The data presented in this dissertation add new insights for understanding how these receptors modulate dopamine neuron activity. But, these data also present new questions that hadn’t been appreciated before. The most relevant and approachable questions will now be addressed, along with ideas for experiments

## Summary and Conclusions

In this work, I identify five ligands that inhibit VGSC current via a G-protein pathway. All of the ligands are known to act on the CaSR with either negative or positive allosteric effects. The action of synthetic modulators of the CaSR, cinacalcet, calhex, NPS 2143, and Calindol, were characterized in Chapter 1. All produce complete inhibition of VGSC current with time in a dose-dependent fashion. The inhibition by Cinacalcet, NPS 2143, and calindol was alleviated in the presence of G-protein signaling inhibitor GDPbetaS, indicating a G-protein dependent mechanism. This finding became more intriguing as I determined that cinacalcet, Calindol, NPS 2143, and calhex exert their effects independently of the CaSR, their known target GPCR.  I investigated the identity of the target GPCR for these ligands by first looking at candidates within the Class C GPCR family, second, eliminating a class of G-protein with PTx, and third, determining the involvement of downstream effector proteins with PKA and PKC inhibitors. Using this methodology, data showed that cinacalcet does not act through mGluR1, mGlurR5, or GABAB receptors. Cinacalcet inhibition of VGSCs also does not require Gi/o G-proteins, eliminating a class of G-protein and narrowing the range of GPCR candidates. Lastly, cinacalcet inhibition of VGSCs does not require the activation of PKA or PKC. These findings leave a number of possibilities for the composition of this pathway: Gq, G12, and Gs G-proteins, staurosporine-resisitance kinases, phospholipases, Gby signaling, intracellular calcium-signaling, and a multitude of other factors, some of which I will explore in this discussion. The kinetics of cinacalcet-induced inhibition were quantified in-depth. It was shown that cinacalcet produces inhibition of VGSC current by shifting the steady-state inhibition of the channels toward more polarized potentials. This inhibition could therefore be completely reversed by long hyperpolarizing steps. This finding first suggests that cinacalcet pushes VGSCs into a deeply inactivated state. In Chapter 2, I show that this type of inhibition is also enacted by endogenous cannabinoid anandamide. This is important as AEA is natively present in the cortex and this establishes that the pathway may play a role in synaptic communication. AEA, as the other CaSR modulators, produced complete inhibition of VGSC current over time. This inhibition was relieved with GDPbetaS and independent of the CaSR. Surprisingly, inhibition was also independent of AEA target CB1. Like cinacalcet, AEA shifted inactivation of VGSCs and this inactivation-produced inhibition was completely reversed with strong hyperpolarizing pulses. While it cannot be conclusively stated that AEA, Cincalcet, Calindol, Calhex, and NPS 2143 act via the same pathway and same GPCR, it is highly likely given their commonality as CaSR allosteric modulators and the highly similar way in which they affect VGSC inactivation. While NPS 2143, cinacalcet, calhex, and Calindol aided in the characterization of this pathway, AEA’s participation in this pathway is the finding that emphasizes the importance of this pathway to synaptic physiology. Here, we establish a pathway from ligand to G-protein to changes in VGSC output. This pathway may represent a novel homeostatic mechanism to reduce neuronal excitability.

## Extra Information without a home

Gallein is a cell-permeable, small molecule inhibitor of betagamma signaling. It binds to betagamma particle with high affinity Kd ~400 nM. Gallein acts by blocking the interactions between Gbetagamma and its effectors. Gallein does not promote dissociation of Galpha from Gbetagamma.

Since then, beta gamma has been shown to act on Adenylyl cyclase (AC) I” (Tang and Gilman, 1991; Sunahara and Taussig, 2002), G protein-coupled receptor kinase 2 (GRK2) (Pitcher et al., 1992), phospholipase C (PLC) \selectlanguagegreekβ\selectlanguageenglish2 and \selectlanguagegreekβ\selectlanguageenglish3 isoforms (Camps et al., 1992; Park et al., 1993; Smrcka and Sternweis, 1993), inwardly rectifying potassium channels (GIRK) (Logothetis et al., 1987; Nakajima et al., 1996), phosphoinositide 3-kinase \selectlanguagegreekγ \selectlanguageenglish(PI3K\selectlanguagegreekγ\selectlanguageenglish) (Stephens et al., 1994, 1997), and N-type calcium channels (Ikeda, 1996)”

Proteomic methods and yeast two-hybrid screening have revealed multiple novel G\selectlanguagegreekβγ \selectlanguageenglishbinding proteins. These include PDZ domain containing proteins (Li et al., 2006); guanine exchange factors (GEFs) for small G proteins such as P-Rex1 (Mayeenuddin et al., 2006), FLJ00018, also known as pleckstrin homology domain containing family G member 2 (a G\selectlanguagegreekβγ-\selectlanguageenglishactivated Rac and Cdc42 guanine nucleotide exchange factor) (Ueda et al., 2008), and p114-RhoGEF (Niu et al., 2003); protein kinase D (PKD) (Jamora et al., 1999); receptor for activated C kinase 1 (RACK1) (Dell et al., 2002); soluble NSF attachment protein (SNAP) receptor (SNARE) complex (Yoon et al., 2007); and a Radil-Rap1A complex (Ahmed et al., 2010). \selectlanguagegreek**A striking observation for all of these Gβγ binders is that no readily apparent consensus sequence or structure mediates binding of these proteins to Gβγ.**

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english Gby has been shown to modulation 1.2 and 1.2 NaV. InaP persistent is increased when NaV1.2 coexpressed with Gby (Ma et al 1997).

This increase was prevented by a putative G\selectlanguagegreekβγ-\selectlanguageenglishbinding peptide from the C terminus of the Nav1.2 channel, suggesting that G-protein \selectlanguagegreekβγ \selectlanguageenglishsubunits interact with that C-terminal site during modulation (Ma et al., 1997). Consistent with this idea, G-protein \selectlanguagegreekβ \selectlanguageenglishsubunits immunoprecipitate with sodium channel \selectlanguagegreekα \selectlanguageenglishsubunits from cortical neuron preparations (Marin et al., 2001).

We show that both Nav1.1 and Nav1.2 conduct *I* NaP (Nav1.1 > Nav1.2) that can be modulated by G\selectlanguagegreekβγ, \selectlanguageenglishbut Nav1.4 and Nav1.5 do not.

Analysis of channel chimeras reveals that the transmembrane bodies of Nav1.4 and Nav1.5 channels are responsible for their lack of G-protein modulation rather than their C-terminal domains (Scheuer, MAntegazza, Powell, Yu.

Modulation by free G\selectlanguagegreekβγ \selectlanguageenglishin neurons might occur locally in membrane microdomains (Galbiati et al., 2001) strategically positioned to affect action potential generation in the axon hillock, action potential back-propagation at dendritic branches, or other localized electrical events. Such tightly delimited modulation might be critically important for integrative properties of the neuron but would

The cocrystal structures reveal that effector/binding proteins share a critical interaction interface on the top of the torus of G\selectlanguagegreekβ \selectlanguageenglishcreated by the \selectlanguagegreekβ \selectlanguageenglishpropeller fold that binds to switch II helix of the G\selectlanguagegreekα \selectlanguageenglishsubunits (Fig. 1).

As discussed, small-molecule G\selectlanguagegreekβγ \selectlanguageenglishinhibitors (M119/gallein) have been used extensively to investigate G\selectlanguagegreekβγ \selectlanguageenglishfunctions in cell biological and animal models of diseases. In the course of these studies, many questions concerning off-target effects have been addressed. For example, in the presence of M119/gallein, the following were observed:

1. Unimpaired isoproterenol- and G\selectlanguagegreekα\selectlanguageenglishs-dependent cAMP production (Casey et al., 2010).

2. Unimpaired [d-Ala2,N-MePhe4,Gly-ol]-enkephalin (a \selectlanguagegreekμ-\selectlanguageenglishopioid receptor-specific agonist), G\selectlanguagegreekα\selectlanguageenglishi-dependent decrease in cAMP and unimpaired \selectlanguagegreekμ-\selectlanguageenglishopioid receptor, G\selectlanguagegreekβγ-\selectlanguageenglishdependent analgesia, as discussed under Biased Agonist Signaling (Mathews et al., 2008).

3. No effect of compounds on \selectlanguagegreekδ \selectlanguageenglishand \selectlanguagegreekκ \selectlanguageenglishopioid receptor signaling (Mathews et al., 2008).

4. Unimpaired fMLP- and G\selectlanguagegreekβγ-\selectlanguageenglishdependent extracellular signal-regulated kinase activation in HL60 neutrophil-like cells (Bonacci et al., 2006).

5. Unimpaired M3 muscarinic acetylcholine receptor, G\selectlanguagegreekα\selectlanguageenglishq- dependent Ca2+ regulation (Bonacci et al., 2006).

6. Unimpaired stromal cell-derived factor-1- and G\selectlanguagegreekα\selectlanguageenglishi-dependent inhibition of cAMP levels (Kirui et al., 2010).

All these data indicate that GPCRs function normally in the presence of G\selectlanguagegreekβγ \selectlanguageenglishinhibitors and that there is selectivity for different G\selectlanguagegreekβγ \selectlanguageenglishtargets.

GABAB activates GiRKS baclofen Betterl + Tiao 2006 Pharmacol Ther

Kir3 type channel

Not blocked by galleon (casey, blaxall Circ res 2010) shows selectivity of Gbetagamma

**Lehmann**  *et al*  (2008) Small molecule disruption of G protein \selectlanguagegreekβγ \selectlanguageenglishsubunit signaling inhibits neutrophil chemotaxis and inflammation. Mol.Pharmacol. 73  410 PMID: 18006643

**Ukhanov**  *et al*  (2011) Phosphoinositide 3-kinase-dependent antagonism in mammalian olfactory receptor neurons. J.Neurosci. 31  273 PMID: 21209212

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Regulation of VGSCs by second messenger systems may have wide-ranging effects on cellular activity

5HT regulation is not enough to affect somatic action potentials of pyramidal neurons in PFC slice but may recue action potential amplitude back-propogating into apical dendrite (Carr 2002) This reduced dendritic excitability to negatively modulate acivity-dependent dendritic synaptic plasticity

Na channels regulate not only somatic spike generation but also dendritic integration of synaptic input (Schwindt and Crill, 1995; Stuart and Sakmann, 1995; Lipowsky et al., 1996;Gonzalez-Burgos and Barrionuevo, 2001),

ty as CaSR allor\*b

The experiments in this dissertation demonstrate…. . However, it is less clear …..Future experiments blocking the activity of the endogenous Isl1, Lhx3 or LMO4 enhancers and then examining the expression of each of these genes and motor neuron specification will allow us to better assess the role of each enhancer in motor neuron specification. Likewise, blocking these enhancers could prove to be an effective mechanism for knocking out, or knocking down Isl1, Lhx3 or LMO4 expression relatively late in motor neuron development. This late alteration in gene expression could be a useful model for learning more about the roles of 105 each of these genes in motor neuron development and motor neuron subtype specification.

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Since then, beta gamma has been shown to act on Adenylyl cyclase (AC) I” (Tang and Gilman, 1991; Sunahara and Taussig, 2002), G protein-coupled receptor kinase 2 (GRK2) (Pitcher et al., 1992), phospholipase C (PLC) \selectlanguagegreekβ\selectlanguageenglish2 and \selectlanguagegreekβ\selectlanguageenglish3 isoforms (Camps et al., 1992; Park et al., 1993; Smrcka and Sternweis, 1993), inwardly rectifying potassium channels (GIRK) (Logothetis et al., 1987; Nakajima et al., 1996), phosphoinositide 3-kinase \selectlanguagegreekγ \selectlanguageenglish(PI3K\selectlanguagegreekγ\selectlanguageenglish) (Stephens et al., 1994, 1997), and N-type calcium channels (Ikeda, 1996)”

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Modulation by free G\selectlanguagegreekβγ \selectlanguageenglishin neurons might occur locally in membrane microdomains (Galbiati et al., 2001) strategically positioned to affect action potential generation in the axon hillock, action potential back-propagation at dendritic branches, or other localized electrical events. Such tightly delimited modulation might be critically important for integrative properties of the neuron but would

Proteomic methods and yeast two-hybrid screening have revealed multiple novel G\selectlanguagegreekβγ \selectlanguageenglishbinding proteins. These include PDZ domain containing proteins (Li et al., 2006); guanine exchange factors (GEFs) for small G proteins such as P-Rex1 (Mayeenuddin et al., 2006), FLJ00018, also known as pleckstrin homology domain containing family G member 2 (a G\selectlanguagegreekβγ-\selectlanguageenglishactivated Rac and Cdc42 guanine nucleotide exchange factor) (Ueda et al., 2008), and p114-RhoGEF (Niu et al., 2003); protein kinase D (PKD) (Jamora et al., 1999); receptor for activated C kinase 1 (RACK1) (Dell et al., 2002); soluble NSF attachment protein (SNAP) receptor (SNARE) complex (Yoon et al., 2007); and a Radil-Rap1A complex (Ahmed et al., 2010). A striking observation for all of these G\selectlanguagegreekβγ \selectlanguageenglishbinders is that no readily apparent consensus sequence or structure mediates binding of these proteins to G\selectlanguagegreekβγ.

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Gby has been shown to modulation 1.2 and 1.2 NaV. InaP persistent is increased when NaV1.2 coexpressed with Gby (Ma et al 1997).

This increase was prevented by a putative G\selectlanguagegreekβγ-\selectlanguageenglishbinding peptide from the C terminus of the Nav1.2 channel, suggesting that G-protein \selectlanguagegreekβγ \selectlanguageenglishsubunits interact with that C-terminal site during modulation (Ma et al., 1997). Consistent with this idea, G-protein \selectlanguagegreekβ \selectlanguageenglishsubunits immunoprecipitate with sodium channel \selectlanguagegreekα \selectlanguageenglishsubunits from cortical neuron preparations (Marin et al., 2001).

We show that both Nav1.1 and Nav1.2 conduct *I* NaP (Nav1.1 > Nav1.2) that can be modulated by G\selectlanguagegreekβγ, \selectlanguageenglishbut Nav1.4 and Nav1.5 do not.

Analysis of channel chimeras reveals that the transmembrane bodies of Nav1.4 and Nav1.5 channels are responsible for their lack of G-protein modulation rather than their C-terminal domains (Scheuer, MAntegazza, Powell, Yu.

Modulation by free G\selectlanguagegreekβγ \selectlanguageenglishin neurons might occur locally in membrane microdomains (Galbiati et al., 2001) strategically positioned to affect action potential generation in the axon hillock, action potential back-propagation at dendritic branches, or other localized electrical events. Such tightly delimited modulation might be critically important for integrative properties of the neuron but would

(392 receptors in mice, 367 in humans)https://www.ncbi.nlm.nih.gov/pmc/articles/PMC153653/

The cocrystal structures reveal that effector/binding proteins share a critical interaction interface on the top of the torus of G\selectlanguagegreekβ \selectlanguageenglishcreated by the \selectlanguagegreekβ \selectlanguageenglishpropeller fold that binds to switch II helix of the G\selectlanguagegreekα \selectlanguageenglishsubunits (Fig. 1).

We found cinacalcet-induced inhibition occurs independently of not only the CaSR, but also class C GPRCS mGluR1, mGluR5, and the GABAB receptor. Eliminating mGluR1 as the target of cinacalcet allows us to establish this as a separate pathway for G-protein-dependent modulation of VGSC current. Furthermore, mAchR M1, D1 receptor, 5-HT2a/c receptor, and mGluR1 activation were all shown to depend on either PKA or PKC activity for VGSC inhibition (Cantrell, Ma, Scheuer and Catterall 1996, Cantrell, Smith, Goldin, Scheuer and Catterall 1997, Carr, Cooper, Ulrich, Spruston and Surmeier 2002, Carlier, Sourdet, Boudkkazi, D\selectlanguagengermanéglise, Ankri, Fronzaroli-Molinieres and Debanne 2006) whereas VGSC inhibition by cinacalcet occurred in the absence of PKA and PKC activity.. In addition, stimulation of mAchR receptors and dopamine D1/D5 receptors did not reduce VGSC in the same neurons that responded to cinacalcet (Fig. 9a,c,d). This further distinguishes this pathway from all other G-protein processes for VGSC modulation for which data currently exists (Cantrell and Catterall 2001).

VGSC current inhibition that relies on slow inactivation has been shown to reduce a neuron’s ability to sustain trains of spikes (Carr, Day, Cantrell, Held, Scheuer, Catterall and Surmeier 2003). We predict that the strong, slow inhibition of VGSC by CaSR modulators should have similar effects.

Indeed actions in the brain are of such concern that new CaSR modulators, that are not lipophilic and thus less likely to cross the blood brain barrier, are being synthesized and tested clinically (Martin, Bell, Pickthorn, Huang, Vick, Hodsman and Peacock 2014). Our findings may describe mechanisms of off-target effects of cinacalcet.

Reduction in peak VGSC current could serve as a neuroprotective safeguard, preventing excitotoxicity during periods of intense synaptic transmission. Consequently, the G-protein pathway investigated here may represent an avenue for new therapies characterized by neuronal hyperexcitability such as epilepsy, migraine, chronic pain, and periodic paralysis.

https://www.sciencedirect.com/science/article/pii/S0006899305013107?via%3Dihub

Kim et al. show that AEA blocks TTX-S and TTX-R VGSCs in rat dorsal root ganglion neurons of the periphery. Conc-dep manner with Kd 5.4 uM on TTX-S neurons. Produced hyperpolarization of steady-state inactivation. AEA affected inactivated Na channels rather than resting- indication for use-dependence. Inhibition not reversed by CB1 antagonist AM 251, CB2 antagonist AM 630, or Capsazepine (VR1 antagonist). They conclude this then means direct action but do not explore other G-protein dependent pathways.

family C GPCRs also includes a amino acid- and divalent cation-sensing receptor called GPRC6A

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