

G-protein regulation of neuronal calcium channels: back to the future

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Running title: Regulation of calcium channels by G-proteins

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Non-standard abbreviations

AA, arachidonic acid; AID, alpha interaction domain; AMPc, cyclic adenosine monophosphate; CSP, cysteine string protein; DAG, diacylglycerol; DRG, dorsal root ganglion; FHM-1, familial hemiplegic migraine type-1; FRET, fluorescence resonance energy transfer; GDP, guanosine-5'-diphosphate; GK, guanylate kinase; GPCR, G-protein coupled receptor; GTP, guanosine-5'-triphosphate; HEK293, human embryonic kidney 293; IP3, inositol trisphosphate; M2, muscarinic receptor 2; MAGUK, membrane-associated guanylate kinase; NOP, nociception receptor; ORL-1, opioid receptor like-1; PIP2, Phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; RGS, regulator of G-protein signaling; RIM, rab3-interacting molecule; SNAP-25, synaptosomal-associated protein 25; SNARE, soluble NSF (N-ethylmaleimide-sensitive fusion protein) attachment protein receptor; *Synprint*, synaptic protein interaction; VGCC, voltage-gated calcium channel.

Abstract

Neuronal voltage-gated calcium channels have evolved as one of the most important players for calcium entry into presynaptic endings responsible for the release of neurotransmitter. In turn, and in order to fine tune synaptic activity and neuronal communication, numerous neurotransmitters exert a potent negative feedback over the calcium signal provided by G-protein-coupled receptors. This regulation pathway of physiological importance is also extensively exploited for therapeutic purposes, for instance in the treatment of neuropathic pain by morphine and other mu-opioid receptor agonists. However, despite more than three decades of intensive research, important questions remain unsolved regarding the molecular and cellular mechanisms of direct G-protein inhibition of voltage-gated calcium channels. Here, we revisit this particular regulation and explore new considerations.

Introduction

Within neurons, calcium ion (Ca^{2+}) represents an essential important signaling molecule, responsible for regulation of a large number of diverse cellular functions (Berridge, 1998). Voltage-gated Ca^{2+} channels (VGCCs) have evolved as one of the most important players in the initiation of the Ca^{2+} signal by converting electrical impulse into intracellular Ca^{2+} elevation (Catterall, 2011). VGCCs are pore-forming multi-subunits plasma membrane complexes that are activated upon membrane depolarization (i.e. action potentials) to permit entry of Ca^{2+} along its electrochemical gradient. To date, ten genes encoding the pore-forming subunits of mammalian VGCCs have been identified (Figure 1). Seven genes encode the high-voltage activated (HVA) channel subfamily consisting of L-type ($\text{Ca}_v1.1$ to $\text{Ca}_v1.4$), P/Q-type ($\text{Ca}_v2.1$), N-type ($\text{Ca}_v2.2$), R-type ($\text{Ca}_v2.3$), and three genes encode the low-voltage-activated (LVA) channel subfamily composed exclusively of T-type channels ($\text{Ca}_v3.1$ to $\text{Ca}_v3.3$) (Ertel et al., 2000). The Ca_v pore-forming subunits of VGCCs share a similar transmembrane topology built of four homologous domains each of them containing six putative transmembrane helices (S1-S6), plus a re-entrant loop (P-loop) that forms the pore of the channel. The four domains are connected via large cytoplasmic linkers (loops I-II, II-III, III-IV), and cytoplasmic amino- and carboxy-terminal domains, which form interaction sites for various regulatory proteins. In addition to the Ca_v pore-forming subunit, HVA channels contain ancillary subunits (Arikkath and Campbell, 2003): β (β_1 to β_4 , a 55 kDa cytosolic protein of the membrane-associated guanylate kinase (MAGUK) family), $\alpha_2\delta$ ($\alpha_2\delta_1$ to $\alpha_2\delta_{\text{B}}$, a 170 kDa highly glycosylated extracellular protein with a single transmembrane domain), and in some cases γ (γ_1 to γ_8 , a 33 kDa transmembrane protein), that control channel trafficking, gating and function at the plasma membrane.

In order to make use and regulate the amplitude, duration and subcellular localization of the Ca^{2+} signal, VGCCs are under tight regulatory control. One of the most important regulatory mechanisms involves G-protein coupled receptors (GPCRs), also known as seven-transmembrane domain receptors. GPCRs are a large protein family of integral membrane receptors (Vassilatis et al., 2003) that sense extracellular molecules such as neurotransmitters and in turn activate intracellular signaling pathways by regulating the activity of heterotrimeric G-proteins. Heterotrimeric G-proteins consist of a $G\alpha$ -subunit that binds and hydrolyses GTP into GDP, and $G\beta$ - and $G\gamma$ -subunits that remain constitutively associated and form the $G\beta\gamma$ -dimer (Wettschureck and Offermanns, 2005). In the absence of stimulus, GDP-bound $G\alpha$ -subunit and $G\beta\gamma$ -dimer are associated with the receptor. Binding of an extracellular ligand onto the GPCR induces a conformational change that promotes the exchange of GDP for GTP from the $G\alpha$ -subunit, resulting in the dissociation of the GTP-bound $G\alpha$ and $G\beta\gamma$ -dimer from the receptor. Intrinsic hydrolysis of GTP by $G\alpha$ -subunit can be speeded up by GTPase activating proteins such as regulators of G-protein signaling (RGS) that allows reassociation of GDP-bound $G\alpha$ -subunit with the $G\beta\gamma$ -dimer, which terminates G-protein signaling (Figure 2). Although both GTP-bound $G\alpha$ -subunit and the $G\beta\gamma$ -dimer mediate intracellular signaling by modulating the activities of neuronal VGCCs, this review is focused on the so-called direct “voltage-dependent” regulation mediated by the $G\beta\gamma$ -dimer. Interested readers may also refer to the recent review of Zamponi and Currie for an interesting discussion on the regulation of VGCCs by $G\alpha$ - and protein kinase-dependent signaling (Zamponi and Currie, 2013).

Description of the phenomenon

Interestingly enough, while Ca^{2+} entry at synaptic endings can trigger transmitter release, numerous neurotransmitters released from synaptic contacts and hormones secreted at proximity of the synaptic cleft are in turn able to modulate presynaptic VGCCs via activation of G-protein-coupled receptors (GPCRs) to terminate the Ca^{2+} signal and neurotransmitter discharge. This regulation is not only of physiological importance but it is also extensively exploited as a therapeutic avenue. For example, one of the most remarkable usages of G-protein-mediated inhibition of VGCCs is the management of pain symptoms by specific opioid receptor agonists (e.g. natural opiates like morphine and its synthetic opioid derivatives).

The first observation that synaptic activity is modulated by neurotransmitters goes back to the late 1970s with the pioneer work of Dunlap and Fischbach on sensory neurons (Dunlap and Fischbach, 1978), and later attributed the phenomena to the inhibition of VGCCs (Dunlap and Fischbach, 1981). To date, up to 20 neurotransmitters and corresponding receptors have been described to modulate VGCCs (Table 1), including noradrenaline (Bean, 1989) (Docherty and McFadzean, 1989) (McFadzean and Docherty, 1989) (Lipscombe et al., 1989), somatostatin (Bean, 1989) (Ikeda and Schofield, 1989a, Ikeda and Schofield, 1989b), gamma-aminobutyric acid (GABA) (Deisz and Lux, 1985) (Dolphin and Scott, 1987) (Grassi and Lux, 1989) and acetylcholine (Bernheim et al., 1991) (Shapiro et al., 1999).

Based on the observation that application of pertussis toxin on rat dorsal root ganglion neurons (DRG), or intracellular injection of non-hydrolysable GDP β S, prevents inhibition of voltage-activated Ca^{2+} currents by noradrenaline or GABA, it was proposed that heterotrimeric G-proteins certainly mediate GPCR-dependent modulation of VGCCs (Holz et al., 1986) (Scott and Dolphin, 1986). Using an original approach, Forscher and Collaborators

further demonstrated that this regulation is spatially delimited and does not involve diffusible second messengers, suggesting proximity between the Ca^{2+} channel and the GPCR (Forscher et al., 1986). The functional importance of channel / GPCR coupling in G-protein mediated inhibition of Ca^{2+} currents will be further discussed later in this review. A period of intensive work and controversy followed (1989-1996) to determine which of the G-protein subunits mediate inhibition of the Ca^{2+} channel. Using specific antibodies and antisense oligonucleotides to block or knockdown $\text{G}\alpha$ -subunits of heterotrimeric G-proteins, it was initially proposed that $\text{G}\alpha\text{o}$ is the mediator of the inhibition (McFadzean et al., 1989) (Baertschi et al., 1992) (Campbell et al., 1993) (Menon-Johansson et al., 1993). However, during the same period of time, various studies suggested as well an implication of $\text{G}\alpha\text{i}$ (Ewald et al., 1989) or $\text{G}\alpha\text{s}$ and $\text{G}\alpha\text{q}$ (Shapiro and Hille, 1993) (Golard et al., 1994) (Zhu and Ikeda, 1994). The divergent results led to the hypothesis that GPCR-mediated inhibition of VGCCs is not mediated by $\text{G}\alpha$ -subunits but rather by a common G-protein determinant, and Herlitze and Colleagues (Herlitze et al., 1996) and Ikeda (Ikeda, 1996) eventually established that inhibition of Ca_v2 channels is mediated by the $\text{G}\beta\gamma$ -dimer concomitantly produced with $\text{G}\alpha$ -GTP following GPCR activation. Indeed, the overexpression of $\text{G}\beta1\gamma2$ - or $\text{G}\beta2\gamma3$ -dimers in sympathetic neurons is sufficient to mimic noradrenaline-mediated inhibition of N-type currents, and prevents subsequent inhibition by α_2 -adrenergic agonists. The question is how can we incorporate those results in the observation of other groups suggesting a functional implication of $\text{G}\alpha$ -subunits? Interestingly enough, it appears that even though $\text{G}\beta\gamma$ -dimer is the mediator of the inhibition, $\text{G}\alpha$ -subunits might have an important role in the ability and specificity of GPCRs to functionally couple with VGCCs. Hence, inhibition of neuronal VGCCs is usually mediated by GPCRs coupled to $\text{G}\alpha\text{i}$ or $\text{G}\alpha\text{o}$ (Holz et al., 1986, Scott and Dolphin, 1986) such as α_2 -

adrenergic receptors, clarifying why depletion of the $G_{\alpha o}$ -subunit in NG108-15 cells prevents noradrenaline-mediated inhibition of Ca^{2+} currents (McFadzean et al., 1989).

Landmarks of GPCR-mediated inhibition of VGCCs

Inhibition of VGCCs by GPCRs involves the direct binding of G-protein $\beta\gamma$ -dimer onto various structural molecular determinants of the Ca_v2 -subunit (see next section). At the whole-cell level, this regulation is characterized by various phenotypical modifications of the Ca^{2+} current properties (Figure 3). The most obvious is a decrease of the inward current amplitude (Boland and Bean, 1993) (Wu and Saggau, 1997) that usually varies from 15 to 80% depending of the Ca_v channel / GPCR involved. Based on the observation that $G\beta\gamma$ -mediated inhibition of VGCCs is less pronounced at depolarized membrane potential, this regulation was named “voltage-dependent”. In some cases, this inhibition is also accompanied by a depolarizing shift of the voltage-dependence curve of current activation (Bean, 1989), and a slowing of activation (Marchetti et al., 1986) and inactivation kinetics (Zamponi, 2001). In addition, short highly depolarizing voltage step, usually applied around +100mV before the current eliciting pulse (double-pulse protocol), is sufficient to reverse, at least partially, most of the landmarks of G-protein inhibition and producing a so-called “prepulse facilitation” (Scott and Dolphin, 1990) (Ikeda, 1991) (Doupnik and Pun, 1994). Current inhibition has been attributed to the direct binding of $G\beta\gamma$ -dimer to the Ca_v2 -subunit (referred as “*ON*” landmark), whereas all the other landmarks including the slowing of current kinetics and prepulse facilitation can be described as variable time-dependent dissociation of $G\beta\gamma$ -dimer from the channel (referred as “*OFF*” landmarks) and consequent current recovery from inhibition (Elmslie and Jones, 1994, Stephens et al., 1998, Weiss et al., 2006). It is worth noting that “*ON*” and “*OFF*” landmarks do not represent two

independent regulations but rather the transition from G $\beta\gamma$ -bound channels to G $\beta\gamma$ -unbound channels, and *vice versa*. Furthermore, although the dissociation of G $\beta\gamma$ -dimer was previously defined as “voltage-dependent”, it was then proposed that channel opening after membrane depolarization and associated conformational changes of the Ca_v2-subunit was likely the trigger for G $\beta\gamma$ dissociation from the channel (Patil et al., 1996). More recently, this concept was further analyzed and it was shown that the voltage-dependence of the kinetic of G $\beta\gamma$ dissociation correlates to the voltage-dependence of the channel activation (Weiss et al., 2006). Hence, it is likely that the trigger of G $\beta\gamma$ dissociation is not the electrical membrane potential *per se*, but rather the conformational change that occurs within the Ca_v2-subunit during the opening of the channel, making the regulation intrinsically “channel-opening-dependent” rather than “voltage-dependent”.

What are the Ca_v channel molecular determinants of G-protein inhibition?

Various structural determinants of the Ca_v2-subunit have been characterized either as direct biochemical binding loci for G $\beta\gamma$ -dimer or of functional importance for GPCR-mediated regulation of Ca²⁺ currents. These determinants are located within the I-II loop of the Ca_v2-subunit, as well as in the amino- and carboxyl-terminal regions of the channel.

Role of the I-II loop of Ca_v2 channels

The observation that G $\beta\gamma$ -dimer is able to functionally interact with the Ca_v β -subunit (Campbell et al., 1995) led some groups to question the role of the I-II loop of Ca_v2 channels in G-protein regulation. It is well established that G $\beta\gamma$ -dimer is able to interact with the type II adenylate cyclase and the phospholipase C β ₂ via a consensus motif QXXER (Chen et al., 1995). Interestingly, this consensus site is also present within the I-II loop of Ca_v2 channels,

and is located in a proximal region of the so-called AID (Alpha Interaction Domain), a 18 amino-acids sequence (**QQUIERELNGY--WI--AE**) initially described as the main interaction locus of the $\text{Ca}_v\beta$ -subunit (Pragnell et al., 1994), suggesting a possible interaction between the I-II loop and the $\text{G}\beta\gamma$ -dimer. Consistent with this idea, injection in HEK293 cells of the synthetic peptide of the $\text{Ca}_v2.2$ -subunit FLKRR**QQUIERELNGYL** (peptide I-II_{s2}, Figure 4) prevents $\text{G}\beta\gamma$ -mediated inhibition of $\text{Ca}_v2.2/\alpha_2/\beta_{1b}$ channel, suggesting that $\text{G}\beta\gamma$ -dimer is able to interact with the peptide (Zamponi et al., 1997). This interaction was then demonstrated by in vitro binding of $\text{G}\beta 1\gamma 2$ -dimer onto the GST-AID_A fusion protein with an affinity of 63 nM (De Waard et al., 1997). For comparison, $\text{Ca}_v\beta_{1b}$ binds to the GST-AID_A fusion protein with an apparent K_d of 5 nM that is a dozen times more efficient than the binding of $\text{G}\beta\gamma$ -dimer (De Waard et al., 1995). In addition, the role of the QXXER domain in the binding of $\text{G}\beta\gamma$ -dimer was further analyzed by site-directed mutagenesis. Hence, substitution of the arginine (R) by a glutamic acid (E) is sufficient to prevent the binding of $\text{G}\beta 1\gamma 2$ -dimer to the GST-AID_A fusion protein, but also prevents GTP γ S-induced inhibition of $\text{Ca}_v2.1/\beta_4$ channels expressed in *Xenopus* oocyte (De Waard et al., 1997). This functional result is however in contradiction with another observation that the same amino acid substitution in the $\text{Ca}_v2.1$ channel expressed in tsA-201 cells together with the $\text{Ca}_v\beta_{1b}$ subunit promotes channel inhibition by GTP γ S (Herlitze et al., 1997). At this point, it is worth noting that substitution of the arginine located in the QXXER motif to a glutamic acid not only prevents the binding of the $\text{G}\beta\gamma$ -dimer, but also slows down the inactivation kinetics of the channel (Herlitze et al., 1997). Hence, the QXXER domain appears to be not only a binding locus of the $\text{G}\beta\gamma$ -dimer within the I-II loop of the Ca_v2 -subunit, but also an important molecular determinant of the fast inactivation of the channel. Considering that channel inactivation is by itself an important component for back modulation of G-protein

inhibition, as we will see later, it is delicate to conclude on the exact functional role of the QXXER domain. It was also shown that substitution of the isoleucine (I) to a leucine (L) within the QQIER domain of the $Ca_v2.1$ -subunit decreases GTP γ S-induced inhibition of $Ca_v2.1/\beta_{1b}$ channel (Herlitze et al., 1997). Altogether, these results provide a structural explanation for the weak sensibility of Ca_v1 channels, which instead contain a QQLEE motif, to GPCR inhibition (Bell et al., 2001). However, insertion of the consensus QXXER domain into the $Ca_v1.2$ -subunit is not sufficient to make the channel sensitive to G-protein inhibition (Herlitze et al., 1997), suggesting that despite an important role in the biochemical coupling of G $\beta\gamma$ -dimer with the channel, the QXXER domain is not the sole molecular determinant for potent channel inhibition by G-proteins. Hence, additional molecular loci of the I-II loop have been identified. For example, injection in HEK293 cells of the GVLGEFAKERERVENRRA peptide of the $Ca_v2.2$ -subunit (peptide I-II_{SL}, Figure 4), localized upstream the AID domain and straddling the IS6 transmembrane segment and the I-II loop, is able to prevent G $\beta\gamma$ -dependent inhibition of $Ca_v2.2/\alpha_2/\beta_{1b}$ channels, suggesting a second G $\beta\gamma$ -dimer binding locus (Zamponi et al., 1997). Finally, the GID domain (G-protein Interaction Domain) localized 13 amino acid residues upstream the AID domain has also been proposed as a molecular locus for G $\beta\gamma$ binding with an affinity of 20 nM (De Waard et al., 1997), and the corresponding synthetic peptide is likewise able to prevent G $\beta\gamma$ modulation of Ca^{2+} currents (Zamponi et al., 1997). Interestingly enough, PKC-dependent phosphorylation of the GID domain on a threonine residue prevents binding of G $\beta\gamma$, suggesting a functional crosstalk between direct (i.e. mediated by G $\beta\gamma$ -dimer) and indirect (i.e. involving second messengers) GPCR modulation of VGCCs (Zamponi et al., 1997).

Altogether, it is unambiguous that the cytoplasmic I-II loop of Ca_v2 channels contains various structural determinants for G $\beta\gamma$ -dimer binding. However, the functional importance

of those determinants in the modulation of the Ca^{2+} currents remains largely discussed and other groups rather suggested that the I-II loop was not critical for $\text{G}\beta\gamma$ -mediated inhibition of Ca^{2+} channels. Hence, it has been shown that the substitution of the I-II loop of the $\text{Ca}_v2.2$ -subunit by the I-II loop of the $\text{Ca}_v2.1$ channel (less sensitive to $\text{G}\beta\gamma$ inhibition) or by the I-II loop of the $\text{Ca}_v1.2$ channel (insensitive to direct G-protein modulation) does not alter somatostatin-mediated inhibition of the $\text{Ca}_v2.2/\beta_{1b}$ channel (Zhang et al., 1996). Conversely, the replacement of the I-II loop of the $\text{Ca}_v1.2$ -subunit by the I-II loop of the $\text{Ca}_v2.2$ -subunit is not sufficient to restore a direct G-protein modulation of the $\text{Ca}_v1.2/\beta_{2a}$ channel by D2 dopaminergic receptors (Canti et al., 1999). Finally, the substitution of the I-II loop of the $\text{Ca}_v2.3$ channel (rat rbE-II isoform (Soong et al., 1993)) - that is less sensitive to direct G-protein modulation - by the I-II loop of the $\text{Ca}_v2.2$ -subunit, induces a slowing of activation kinetics of $\text{Ca}_v2.3/\beta_{2a}$ channel upon activation of G-proteins by injection of $\text{GTP}\gamma\text{S}$, but no net inhibition of the maximal amplitude of the Ca^{2+} current (Page et al., 1997). Hence, it was proposed that the I-II loop could be the channel molecular determinant mediating the slowing of current kinetics under direct G-protein regulation, and that current inhibition *per se* could require other molecular determinants (Page et al., 1997). Considering that the apparent slowing of current activation kinetics is attributed to the dissociation of $\text{G}\beta\gamma$ -dimer from the channel (“OFF” landmarks) (Elmslie and Jones, 1994) (Stephens et al., 1998) (Weiss et al., 2006), the I-II loop might be the channel molecular determinant involved in the dissociation of $\text{G}\beta\gamma$ -dimer from the Ca_v2 -subunit.

Role of the amino-terminal domain of Ca_v2 channels

Interestingly enough, while Ca^{2+} currents generated by the BII-2 brain rabbit isoform of the $\text{Ca}_v2.3$ -subunit are subject to direct G-protein modulation (either upon somatostatin

application (Yassin et al., 1996) or by direct injection of GTP γ S (Meza and Adams, 1998)), currents generated by the rat rbE-II isoform that presents a truncated amino-terminal domain remain insensitive (Page et al., 1997), suggesting an implication of the amino-terminal domain of the Ca_v2 channel in direct G-protein modulation. Consistent with this idea, extension by PCR of the amino-terminal region of the rbE-II isoform, leading to a Ca_v2.3-subunit homologue to the rabbit one, is sufficient to restore quinpirole (D2 dopaminergic agonist)-mediated inhibition of Ca²⁺ currents (Page et al., 1998). Moreover, the substitution of the amino-terminal region of the Ca_v1.2-subunit by the amino-terminal domain of the Ca_v2.2-subunit makes the Ca_v1.2 channel sensitive to quinpirole-mediated inhibition (Canti et al., 1999). Further work identified a highly conserved 11 amino-acids sequence (YKQSIAQRART) in the amino-terminal region of Ca_v2 channels critical for direct G-protein modulation (Canti et al., 1999) (Figure 4A), and alanine scan of the YKQ and RAR motifs abolished quinpirole-induced inhibition of Ca_v2.3/ β_{2a} / $\alpha_2\delta$ channels (Canti et al., 1999). In addition, it was proposed that the amino-terminal region of the Ca_v2.2-subunit might constitute a G-protein-gated inhibitory module acting via interaction with the cytoplasmic I-II linker of the channel (Agler et al., 2005). More recently, Ca_v2.2 amino-terminal-derived peptides have been shown to prevent noradrenaline-induced G-protein inhibition of Ca²⁺ currents in superior cervical ganglion neurons (SCGNs) strengthening the implication of the Ca_v2 amino-terminal region in G-protein modulation in native environment (Bucci et al., 2011).

Role of the carboxy-terminal domain of Ca_v2 channels

The carboxy-terminal region of the Ca_v2-subunits has also been proposed as an important determinant for direct-G-protein regulation and a binding domain of G $\beta\gamma$ -dimer was

identified in the distal carboxy-terminal region of the $\text{Ca}_v2.3$ -subunit (Qin et al., 1997) that presents some homologies with the corresponding region of the $\text{Ca}_v2.1$ and $\text{Ca}_v2.2$ -subunits (Simen et al., 2001) (Figure 4A). Hence, the substitution of the carboxy-terminal region of the $\text{Ca}_v2.3$ -subunit by the corresponding one of the $\text{Ca}_v1.2$ -subunit is sufficient to abolish M2 muscarinic receptor-mediated inhibition of the $\text{Ca}_v2.3/\beta_{2a}$ channel (Qin et al., 1997). Association of G-protein $\beta\gamma$ -subunit with the carboxy-terminal region of the $\text{Ca}_v2.1$ channel was also observed using a fluorescence resonance energy transfer approach (FRET) (Hummer et al., 2003). In addition, based on the observation that FRET signal between $\text{Ca}_v2.1/\text{Ca}_v\beta_{1b}$ FRET pairs is increased in the presence of $\text{G}\beta_2$ -subunit and requires the presence of the carboxy-terminal region of the channel, it was proposed that binding of G-proteins to the carboxy-terminal region may produce a conformational change of the channel that might contribute to channel inhibition (Hummer et al., 2003). However, deletion of the carboxy-terminal region of the $\text{Ca}_v2.2$ -subunit containing the binding determinant of $\text{G}\beta\gamma$ -dimer does not alter inhibition of Ca^{2+} currents mediated by injection of $\text{GTP}\gamma\text{S}$ (i.e. agonist/receptor-independent) (Meza and Adams, 1998), but only alters somatostatin-mediated inhibition (i.e. agonist/receptor-dependent) of $\text{Ca}_v2.2$ channels (Hamid et al., 1999) suggesting that the carboxy-terminus might rather be involved in the functional coupling of the channel with agonist-dependent activation of G-proteins. Interestingly, the binding determinant of $\text{G}\beta\gamma$ -dimer in the carboxy-terminal region of Ca_v2 -channels is localized close to the binding domain of $\text{G}\alpha_o$ on $\text{Ca}_v2.1$ - and $\text{Ca}_v2.2$ -subunits (Furukawa et al., 1998b) and $\text{G}\alpha_q$ on the $\text{Ca}_v2.2$ -subunit (Simen et al., 2001) (Figure 4A), suggesting that the carboxy-terminal region could be involved in the functional and biochemical coupling of Ca_v2 channels with the GPCR via the $\text{G}\alpha/\beta\gamma$ trimmer (Kitano et al., 2003)(Beedle et al., 2004, Kisilevsky et al., 2008) (Weiss, 2009). Paradoxically, the

importance of the carboxy-terminal region of Ca_v2 channels in the direct regulation by G-proteins was poorly investigated compared to the intensive work that was done for the I-II loop and the amino-terminal region. One possible reason is that injection of $\text{GTP}\gamma\text{S}$, or overexpression of $\text{G}\beta\gamma$ -dimer to trigger direct G-protein modulation of Ca_v2 channels was not suitable to highlight the functional importance of the carboxy-terminal region.

Toward the notion of $\text{G}\beta\gamma$ -Protein-Binding Pocket

As previously seen, the Ca_v2 -subunit contains various molecular determinants for $\text{G}\beta\gamma$ -dimer binding, and it was initially proposed that several $\text{G}\beta\gamma$ -dimers could interact simultaneously with the Ca_v2 -subunit in a cooperative manner (Boland and Bean, 1993). However, further analysis revealed that the kinetics of $\text{G}\beta\gamma$ -dimer interaction with the $\text{Ca}_v2.2$ channel (evidenced by the functional modulation of the Ca^{2+} current) can be described by a mono-exponential function with a time constant directly correlated to the free $\text{G}\beta\gamma$ -dimer concentration, suggesting that only a single $\text{G}\beta\gamma$ -dimer interacts with the Ca_v2 -subunit (Zamponi and Snutch, 1998). Based on this observation, it was proposed that the various $\text{G}\beta\gamma$ -binding sites located within the Ca_v2 -subunit could be spatially structured to form a unique interaction domain called the $\text{G}\beta\gamma$ -Protein-Binding Pocket (GPBP) (De Waard et al., 2005) (Figure 4B), where various binding loci of the GPBP could be responsible of a particular feature of the G-protein regulation. Hence, the carboxy-terminal region of the Ca_v2 -subunit would play a critical role in the inhibition of Ca^{2+} currents by GPCRs (Qin et al., 1997) (Furukawa et al., 1998b) (Furukawa et al., 1998a) through favoring the biochemical and functional coupling of the receptor with the channel via the $\text{G}\alpha/\beta\gamma$ -trimmer (Kitano et al., 2003) (Beedle et al., 2004). In that context, the carboxy-terminal domain of the Ca_v2 -subunit would not be directly involved in the inhibition of the Ca^{2+}

currents but would rather allow the rapprochement of the GPCR with the channel required for the direct G-protein modulation (Forscher et al., 1986). The I-II loop of the Ca_v2 -subunit represents an important domain of interaction with the $G\beta\gamma$ -dimer via three motifs clearly identified. However, the binding of $G\beta\gamma$ -dimer onto the I-II loop does not appear critical for the inhibition of the Ca^{2+} current (“*ON*” landmark) (Zhang et al., 1996) (Qin et al., 1997), but rather seems to be involved in the relaxation of the inhibition (“*OFF*” landmarks, i.e. unbinding of $G\beta\gamma$ -dimer from the Ca_v2 -subunit) in response for example to a depolarizing prepulse (Herlitz et al., 1997) (Simen and Miller, 2000). Finally, the amino-terminal region of the Ca_v2 -subunit appears as the main determinant of direct G-protein inhibition of Ca^{2+} currents by $G\beta\gamma$ -dimer (Page et al., 1998) (Canti et al., 1999). In that context, the GPBP emerges as a dynamic structure, composed of various loci that on one hand bring the Ca^{2+} channel and the GPCR together, and on the other hand differentially mediates the “*ON*” and “*OFF*” G-protein landmarks.

How $G\beta\gamma$ -dimer inhibits Ca_v2 channels?

Mutagenesis studies have identified a number of amino acid residues on the surface of the $G\beta$ -subunit important for inhibition of the Ca^{2+} channel (Ford et al., 1998) (Tedford et al., 2006) (Mirshahi et al., 2002). Interestingly, most of these residues are located on the G_b surface that interacts with $G\alpha$ and are essentially masked when $G\alpha$ is present. However, the molecular mechanism by which binding of $G\beta\gamma$ -dimer to the channel inhibits the Ca^{2+} current remains largely unknown. It has to be mentioned that beside the extensive work that was done to understand the molecular mechanisms of G-protein regulation of VGCCs, it still remains unclear how the binding of $G\beta\gamma$ -dimer to the Ca_v2 -subunit inhibits the Ca^{2+} current. Based on the observation that the voltage-dependence curve of the Ca^{2+} current

under G-protein modulation is significantly shifted to more depolarized potentials, it was initially proposed that the Ca^{2+} channel undergoes a switch from a “*willing*” mode (i.e. easily activated) to a “*reluctant*” mode (i.e. hardly activated) upon GPCR activation (Bean, 1989). The channel returns to the “*willing*” state after dissociation of the $\text{G}\beta\gamma$ -dimer from the Ca_v2 -subunit (Bean, 1989) (Elmslie et al., 1990). Consistent with this idea, single channel recordings revealed latency in the first opening of the $\text{Ca}_v2.2$ channel upon M2 muscarinic receptor activation, after what the channel presents a similar behavior as the non regulated control channel (Patil et al., 1996). Hence, it was proposed that the first opening latency could correspond to the time for $\text{G}\beta\gamma$ -dimer to dissociate from the Ca_v2 -subunit in response to the membrane depolarization prior to channel opening (Patil et al., 1996). It is worth noting that low-probability openings of $\text{Ca}_v2.2$ channels under G-protein modulation have been observed, corresponding most likely to transient dissociations of the $\text{G}\beta\gamma$ -dimer from the channel upon moderate membrane depolarizations (30 mV) (Lee and Elmslie, 2000). This simple model of regulation appears to be sufficient to support both the inhibition of the Ca^{2+} current and the depolarizing shift of the current/voltage activation curve. However, the observation that the amplitude of gating currents of $\text{Ca}_v2.2/\alpha_2/\beta_{1b}$ channels is reduced upon injection of $\text{GTP}\gamma\text{S}$ led the authors to propose that G-proteins mediate inhibition of Ca^{2+} current by altering intrinsic gating properties of the channel (Jones et al., 1997). Gating currents are produced by the movement of the charged voltage-sensor domains, including positively charged S4 segments, that move into the lipid bilayer in response to electrical membrane depolarizations and leading to the opening of the channel. It is conceivable that $\text{G}\beta\gamma$ -dimer could bind either to the intracellular end of the S4 segment of the Ca_v2 -subunit, or to another structural determinant of the voltage-sensor exposed on the intracellular surface of the channel, preventing the proper movement of the voltage-

sensor and thus inhibiting the channel. Consistent with this idea, it was shown that the point mutation G177E localized in the third transmembrane segment of the first domain (IS3) of the Ca_v2.2a-subunit (rat rbB-I isoform) and induces a tonic inhibition of the channel that can be reversed by a depolarizing prepulse as the classical G-protein inhibition (Zhong et al., 2001). Interestingly, while this inhibition shares some of the features of the G-protein modulation, activation of G-proteins does not produce additional inhibition. Similarly, introduction of the G177E mutation into the Ca_v2.2b-subunit (rat rbB-II isoform) produces a tonic inhibition of the channel and prevents G-protein-mediated modulation of the Ca²⁺ current (Zhong et al., 2001). In contrast, the E177G mutation introduced into the Ca_v2.2a-subunit reverses tonic inhibition of the channel and restores a normal G-protein regulation. Hence, it was proposed that the negative charge introduced by the mutation G177E could interact with a positively charged residue of the S4 segment of the first domain of the Ca_v2-subunit, pushing the channel to a “reluctant” mode via a mechanism of “voltage-sensor-trapping”, suggesting that Gβγ-dimer could possibly regulate the channel in a similar way (Zhong et al., 2001) (Flynn and Zamponi, 2010). Intriguingly, Gem2 that belongs to the RGK (Rad, Gem and Kir) family of small G-proteins is also able to inhibit Ca_v2.2 channels (Chen et al., 2005). However, in contrast to the direct G-protein inhibition by Gβγ-dimer, depolarizing prepulses do not reverse Gem2-mediated inhibition (Chen et al., 2005). Remarkably, structure analyses of Rad, an homologue of Gem, indicates the presence of a molecular motif similar to the seven repeated motifs of the Gβ-subunit, suggesting a common mechanism by which Gβγ-dimer and RGK proteins modulate VGCCs. Another possible model of channel regulation by G-proteins that does not require any alteration of gating parameters was also proposed in which “willing” and “reluctant” modes are intrinsic to the channel, whereas G-proteins and other effectors simply shift the fraction of channels

in these two states without modifying their intrinsic gating properties (Herlitze et al., 2001). This “willing” to “reluctant” model could also potentially explain the decrease in gating currents initially observed during G-protein regulation (Jones et al., 1997). Finally, based on the observation that sodium currents recorded through $Ca_v2.2$ channels are less affected than calcium currents by G-protein inhibition, it was proposed that binding of $G\beta\gamma$ -dimer onto the Ca_v2 -subunit alters ion permeation of the channel (Kuo and Bean, 1993). Hence, consistent with the observation that a 35 amino-acids peptide (residues 271-305) of the $G\beta_2$ -subunit is sufficient to inhibit Ca^{2+} currents (Li et al., 2005), it is also conceivable that $G\beta\gamma$ -dimer simply inhibits the channel via a “pore-modifier” mechanism. However, such a mechanism could only contribute partially to the overall current inhibition given the effect of $G\beta\gamma$ -dimer on single channel gating properties (essentially latency of the first opening). Either way, the exact molecular mechanisms by which $G\beta\gamma$ -dimer inhibits VGCCs remains essentially unknown and certainly deserves further investigation. Recent structural information obtained from voltage-gated sodium channels (Payandeh et al., 2011, Zhang et al., 2012) will certainly provide key information to further analyze the binding of G-proteins to the channel subunit and find out the molecular determinant of the inhibition.

What is the functional role of the $Ca_v\beta$ -subunit in direct G-protein inhibition?

The involvement of the $Ca_v\beta$ -subunit in the direct regulation of VGCCs by GPCRs was extensively examined. Hence, the $Ca_v\beta$ -subunit and $G\beta\gamma$ -dimer share a structural binding site (QXXER located in the AID domain of the I-II loop) on the Ca_v2 -subunit suggesting a possible cross talk. The initial observations that expression of the $Ca_v\beta_3$ -subunit in *Xenopus* oocytes decreases G-protein inhibition of $Ca_v2.1$ and $Ca_v2.2$ channels (Roche et al., 1995),

and conversely that inhibition of Ca_v2.1 channels by μ -opioid and M2 muscarinic receptors is increased in the absence of Ca_v β -subunit (Bourinet et al., 1996, Roche and Treistman, 1998a) led the authors to propose that the Ca_v β -subunit is antagonistic to the direct G-protein modulation of Ca_v2 channels. In addition, it was shown that binding of G $\beta\gamma$ -dimer to the carboxy-terminal region of the Ca_v2-subunit is abolished by the GK domain of the Ca_v β_{2a} -subunit, which also prevents M2 muscarinic receptor-dependent inhibition of the Ca²⁺ current, providing a possible explanation for the functional antagonism existing between the Ca_v β -subunit and direct G-protein inhibition of VGCCs (Qin et al., 1997). However, considering that the carboxy-terminal domain of the Ca_v2-subunit plays an important role in the functional coupling with the GPCR, it is likely that the Ca_v β -dependent antagonism observed relies on an altered coupling of the channel with the GPCR rather than on the direct alteration of G $\beta\gamma$ -dimer binding to the channel. In contrast to the antagonistic hypothesis, it was also proposed that the Ca_v β -subunit could rather be essential for potent G-protein modulation of the Ca_v2 channels (Meir et al, 2000). Consistent with this idea, it was proposed using a FRET approach that G $\beta\gamma$ -dimer induces Ca_v2.1 channel reluctance by displacement of the Ca_v β -subunit from the channel (Sandoz et al., 2004). However, besides the fact that direct G-protein modulation of Ca_v2 channels has been observed numerous times in the absence of the Ca_v β -subunit (Bourinet et al., 1996, Roche et al., 1995)(Canti et al., 2000), this observation is balanced by a study using a similar FRET approach showing that binding of Ca_v β_{1b} and G β to the Ca_v2.1-subunit is not exclusive but rather synergetic (Hummer et al., 2003). In addition, it was shown that recovery of the Ca_v2.2 channel from G-protein inhibition is not only influenced by the presence of a Ca_v β -subunit (Roche and Treistman, 1998b) but also depends on the Ca_v β isoform ($\beta_3 > \beta_4 > \beta_{1b} > \beta_{2a}$) (Canti et al.,

2000). Considering that channel activity is an important factor of the “OFF” G-protein landmark, these results suggest that the $\text{Ca}_v\beta$ -subunit could modulate G-protein inhibition indirectly by biophysical changes induced on the channel. Consistent with this idea, the respective kinetics of channel inactivation induced by various $\text{Ca}_v\beta$ -subunits nicely correlates with the kinetic of recovery from G-protein inhibition (Weiss et al., 2007a). It was thus proposed that the $\text{Ca}_v\beta$ -subunit, by controlling channel inactivation, indirectly influence the speed of G-protein dissociation from the channel. Hence, fast inactivating $\text{Ca}_v\beta$ -subunits (example $\text{Ca}_v\beta_3$) act as a cofactor to speed the recovery from the G-protein inhibition. However, the situation becomes more complicated considering the fact that fast inactivating $\text{Ca}_v\beta$ -subunits, by accelerating the inactivation kinetics of the channel, also reduce the temporal window for G-protein dissociation (Figure 5). Hence, fast inactivating $\text{Ca}_v\beta$ -subunits, while speeding up the rate of G-protein dissociation, also reduce the maximal extent of current recovery from inhibition that leads to an apparent decrease of the prepulse facilitation. In contrast, a slow inactivating $\text{Ca}_v\beta$ -subunit (for instance $\text{Ca}_v\beta_{2a}$) that slows down channel inactivation, has minor effect on the kinetic of G-protein dissociation, but prolong the temporal window of opportunity for G-protein dissociation and leads to a far more complete current recovery from inhibition, evidenced by an apparent increased prepulse facilitation (Weiss et al., 2007a). It is worth noting that the amino-terminal domain of the Ca_v2 channels that has been implicated in the direct G-protein inhibition of VGCCs mediates $\text{Ca}_v\beta$ -dependent fast inactivation of $\text{Ca}_v2.2$ channel (Stephens et al., 2000). In addition, the R387E mutation in the QXXER motif of the I-II loop that alters G-protein regulation (De Waard et al., 1997) (Herlitze et al., 1997), also affects channel inactivation. Hence, it is possible that these molecular channel determinants contribute to

G-protein regulation not only by providing an anchor for the G $\beta\gamma$ -dimer but also via their intrinsic effect on channel gating.

How synaptic proteins modulate direct G-protein inhibition?

The two types of Ca²⁺ channels (Ca_v2.1 and Ca_v2.2) that are most responsible for voltage-dependent release of neurotransmitter at nerve terminal endings biochemically associate with presynaptic vesicles of transmitters (Bennett et al., 1992) (Yoshida et al., 1992) (Leveque et al., 1994) by interacting with some of the proteins of the vesicular machinery release complex (SNAREs) including syntaxin-1A and SNAP-25. Molecular characterization of channel / SNAREs interaction has identified a *synprint* (*synaptic protein interaction*) locus in Ca_v2.1 and Ca_v2.2 located within the intracellular loop between domains II and III of the channels (Sheng et al., 1994) (Rettig et al., 1996). It is believed that the functional relevance of this interaction is to bring presynaptic vesicles close to the Ca²⁺ source for efficient and fast neurotransmitter release and disruption of Ca_v2 / SNARE complex alters Ca²⁺-dependent release of neurotransmitter (Mochida et al., 1996) (Rettig et al., 1997) (Harkins et al., 2004) (Keith et al., 2007). Furthermore, direct binding of syntaxin-1A and SNAP-25 to the Ca_v2.1 and Ca_v2.2 subunits also potently modulates channel gating by shifting the voltage-dependence of channel inactivation toward more negative membrane potentials (Bezprozvanny et al., 1995) (Wiser et al., 1996) (Zhong et al., 1999) (Degtiar et al., 2000) (Weiss and Zamponi, 2012) (Zamponi and Currie, 2013), although the physiological relevance of this regulation is not fully understood. Interestingly, while the *synprint* site of Ca_v2.1 and Ca_v2.2 subunits, and in general the II-III intracellular linker, are not essential molecular determinants of G $\beta\gamma$ -mediated modulation of the channel activity, proteolytic cleavage of syntaxin-1A with botulinium neurotoxin C1 in primary neurons was shown to

completely prevent GPCR-dependent inhibition of presynaptic Ca^{2+} channels (Stanley and Mirotznik, 1997). However, because G-protein modulation of transiently expressed $\text{Ca}_v2.2$ channels in *Xenopus* oocytes (Bourinet et al., 1996) or HEK cells (Zamponi et al., 1997) does not require coexpression with syntaxin-1A, it is unclear why the cleavage of syntaxin-1A in native neuronal environment would lead to a total loss of G-protein modulation. In contrast, Jarvis et al., reported that syntaxin-1A is not essential but facilitates G-protein modulation of the $\text{Ca}_v2.2$ channel. Indeed, while $\text{Ca}_v2.2$ transiently expressed in tsA201 cells in combination with $\text{G}\beta 1\gamma 2$ dimer is susceptible to a tonic inhibition that can be assessed (relieved) by a preceding strong membrane depolarization, coexpression of syntaxin-1A produced an even larger tonic G-protein inhibition, suggesting that syntaxin-1A facilitates $\text{G}\beta\gamma$ -dependent inhibition of the channel (Jarvis et al., 2000). However, it remains unclear whether syntaxin-1A facilitates G-protein inhibition or promotes $\text{G}\beta\gamma$ -dimer dissociation from the channel upon prepulse depolarization. Indeed, as previously discussed in the case of the $\text{Ca}_v\beta$ -subunit, it is possible that syntaxin-1A, by affecting channel gating, indirectly influence the kinetic of G-protein dissociation from the channel, producing a larger prepulse facilitation that could be misinterpreted as the result of an increased initial G-protein inhibition. In support of this idea, coexpression of SNAP-25 that reverses syntaxin-1A-dependent channel gating also reduces the magnitude of the prepulse-induced current recovery upon tonic G-protein inhibition (Jarvis and Zamponi, 2001). However, this concept should also be toned-down as coexpression of a mutant syntaxin-1A that is locked permanently in an “open” conformation (the conformation adopted by syntaxin-1A when in interaction with SNAP-25 or synaptobrevin-2) (Dulubova et al., 1999) (Brunger, 2001) has no effect on $\text{Ca}_v2.2$ gating but still modulates $\text{G}\beta\gamma$ -dependent inhibition of the channel (Jarvis et al., 2002). Conversely, syntaxin-1B affects $\text{Ca}_v2.2$ channel gating but does not

support G-protein modulation (Jarvis and Zamponi, 2001). Altogether, these results suggest that syntaxin-1A may present specific features responsible for G-protein modulation independently of its effect on the channel gating, possibly by facilitating the co-localization of G-proteins with the Ca^{2+} channel. Consistent with this idea, a syntaxin 1 / $\text{G}\alpha\text{o}$ / $\text{Ca}_v2.2$ complex has been described at presynaptic nerve terminals of chick ciliary ganglion cells (Li et al., 2004).

Several other types of presynaptic proteins have been documented as important in the modulation of G-protein inhibition of VGCCs. For example, co-expression of Rim1 significantly decrease direct DAMGO-induced direct G-protein inhibition of $\text{Ca}_v2.2$ channels expressed in HEK293 cells (Weiss et al., 2011). Interestingly, careful kinetic analyses revealed that Rim1 does not prevent inhibition of the channel (“ON” landmark, i.e. binding of $\text{G}\beta\gamma$ -dimer to the channel) but rather favor channel recovery from inhibition (“OFF” landmarks, i.e. unbinding of $\text{G}\beta\gamma$ -dimer from the channel), likely by slowing-down channel inactivation (Kiyonaka et al., 2007) increasing the time window for functional recovery from G-protein inhibition similarly to $\text{Ca}_v\beta$ -subunits (Weiss et al., 2011)(Weiss et al., 2007a). Modulation of G-protein inhibition of VGCCs has been also documented with cysteine string protein (CSP). Although CSP has been shown to stimulate $\text{G}\alpha$ -subunit activity by promoting the exchange of GTP for GDP (Natochin et al., 2005) that in turn is expected to reduce free $\text{G}\beta\gamma$ -dimers, coexpression of CSP increases apparent $\text{G}\beta\gamma$ -dependent inhibition of $\text{Ca}_v2.2$ channel similarly to what was observed with syntaxin-1A (Magga et al., 2000). Although the molecular mechanism underlying CSP-dependent modulation of G-protein inhibition remains to be further explored, it is possible that CSP brings G-proteins together with the Ca^{2+} channel since binding of CSP has been documented with $\text{Ca}_v2.1$ and

Ca_v2.2 subunits (Leveque et al., 1998)(Magga et al., 2000), as well as with G-proteins G α -subunit and G $\beta\gamma$ -dimer (Magga et al., 2000).

How the termination of the signal occurs?

In order to be efficient, cellular events have to be localized and timely regulated. Hence, we have seen that direct G-protein modulation of VGCCs requires 1) the activation of the GPCR by specific extracellular ligands, 2) the release and binding of G $\beta\gamma$ -dimer to the channel leading to a complex regulatory phenotype, and finally 3) that this regulation can be temporally relieved, beside the continuous presence of the extracellular agonist, in response for example to a train of action potentials. However, how does this regulation definitively end? While the recapture and/or degradation of the agonist terminate the activation of the GPCR, how does the channel bound G $\beta\gamma$ -dimer reassociates with the G α -subunit? Because channel recovery from G-protein inhibition can occur at membrane potentials above the reversal potential of Ca²⁺, ion influx is not the driving force for G $\beta\gamma$ -dimer dissociation from the channel. From a biochemical and thermodynamic point of view, it is conceivable that intrinsic hydrolysis of GTP by the G α -subunit and formation of GDP-bound G α -subunit - which presents a high affinity for G $\beta\gamma$ -dimer - could chelate free G $\beta\gamma$ when dissociating from the channel according to a binding/unbinding thermodynamics equilibrium. It is also possible that the GDP-bound G α -subunit is able to bind G $\beta\gamma$ -dimer while still on the Ca_v-subunit. This concept implies the molecular determinants of G α and G $\beta\gamma$ binding being accessible when the G $\beta\gamma$ -dimer is bound to the channel. Binding of GDP-G α to G $\beta\gamma$ could produce a conformational change of the channel Ca_v-subunit and/or G $\beta\gamma$ -dimer, unfavorable to the formation of Ca_v / G $\beta\gamma$ complex, possibly by altering an essential channel molecular determinant important for G $\beta\gamma$ binding. Although this molecular

determinant has not been identified yet, it is likely to contribute to $G\beta\gamma$ dissociation upon channel activation, and possibly involves channel structures sensitive to electrical membrane potential, like S4 segments for example. However, this concept remains to be investigated to be able to better understand the molecular dynamic of GPCR-induced inhibition of VGCCs.

Are Ca_v2 channels the only calcium channels susceptible to direct G-protein inhibition?

It is thought that direct G-protein modulation of VGCCs is mostly confined to neuronal Ca_v2 channels. Indeed, structure/function studies indicate that Ca_v1 channels that lack most of the believed important structural determinants involved in the binding of the $G\beta\gamma$ -dimer to the channel, such as the QXER motif of the I-II loop (Herlitz et al., 1997), are not subject to direct G-protein regulation (Roche et al., 1995) (Bourinet et al., 1996) (Zhang et al., 1996) (Meza and Adams, 1998). However, additional studies on native channels clearly demonstrated an inhibition of L-type Ca^{2+} currents mediated by heterotrimeric G-proteins. For example, injection of $GTP\gamma S$ in cerebellar granule cells induces inhibition of L-type Ca^{2+} currents that is partly reversed upon channel activation by the dihydropyridine agonist $+(S)-202-791$ (Haws et al., 1993). Moreover, inhibition of L-type Ca^{2+} currents has been documented upon agonist stimulation of GPCRs. Indeed, baclofen activation of GABA-B receptors in retinal bipolar neurons induces inhibition of L-type Ca^{2+} currents, an inhibition that is potentiated by $GTP\gamma S$ injection (Maguire et al., 1989). In addition, activation of mGluR2/3 receptors by application of $(2S,1'S,2'S)-2-(\text{carboxycyclopropyl})$ glycine (L-CCG-1) on cerebellar granule cells also inhibits L-type Ca^{2+} currents (Chavis et al., 1994). Although these results do not provide evidence for a direct G-protein inhibition, it is worth noting

that GPCR-mediated inhibition of L-type Ca^{2+} currents in nerve cells is fully abolished by pertussis toxin treatment indicating the implication of $\text{G}\alpha\text{i/o}$ proteins. However, evidence for a direct G-protein modulation of Ca_v1 channels comes from the observation that inhibition of L-type Ca^{2+} currents in pancreatic β -cells can be reversed by application of a depolarizing prepulse (Ammala et al., 1992). Though work performed on nerve and pancreatic cells mostly involved $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ channels, G-protein modulation of native $\text{Ca}_v1.1$ channel isoform has also been described in skeletal muscle cells where L-type Ca^{2+} current inhibition was observed upon β -adrenergic stimulation by isoproterenol or $\text{GTP}\gamma\text{S}$ injection (Somasundaram and Tregear, 1993). In addition, it was shown that expression of $\text{G}\beta_1\gamma_2$ -dimer *in vivo* in adult skeletal muscle fibers specifically inhibits L-type Ca^{2+} currents and voltage-induced Ca^{2+} release (Weiss et al., 2010). Although this inhibition is not reversed by a classical depolarizing prepulse, the observation that expression of some other $\text{G}\beta\gamma$ -dimer combinations (for instance $\text{G}\beta_2\gamma_2$ -, $\text{G}\beta_3\gamma_2$ - or $\text{G}\beta_4\gamma_3$) has no effect on the Ca^{2+} current strongly suggest that $\text{G}\beta_1\gamma_2$ -dimer specifically mediates modulation of $\text{Ca}_v1.1$ channels. The situation is similar regarding the low-voltage-activated Ca_v3 channels. Hence, application of baclofen on dorsal root ganglion neurons (DRG) inhibits T-type Ca^{2+} currents, this inhibition being often counterbalance by the concomitant presence of a current potentiation (likely mediated by diffusible second messengers) (Scott and Dolphin, 1990). Similarly, it was shown that inhibition of T-type Ca^{2+} current by dopamine D1 receptor in rat adrenal glomerulosa cells requires the combined action of $\text{G}\beta\gamma$ -dimer and cAMP (Drolet et al., 1997). More recently, the molecular mechanism of T-type Ca^{2+} current inhibition by G-proteins was specified. It specifically affects $\text{Ca}_v3.2$ channel over the $\text{Ca}_v3.1$ isoform, and in contrast to $\text{Ca}_v1.1$ channels specifically requires the $\text{G}\beta_2\gamma_2$ combination ($\text{G}\beta_1\gamma_2$ -dimer having no significant effect on the Ca^{2+} current) (Wolfe et al., 2003). The inhibition relies on

the direct binding of $G\beta_2\gamma_2$ -dimer to the II-III loop of the $Ca_v3.2$ -subunit, replacement of this loop by the corresponding $Ca_v3.1$ region preventing G-protein modulation. This inhibition has been attributed to a diminution of the opening probability of $Ca_v3.2$ channels, without other alteration of channel gating or expression (DePuy et al., 2006). It is worth noting that similarly to what was observed for Ca_v1 channels, G-protein-mediated inhibition of the $Ca_v3.2$ channel is not reversed by depolarizing prepulse, although mediated by the direct binding of $G\beta\gamma$ -dimer to the channel.

Taken together, these results suggest that likely most of the VGCCs are affected by G-protein inhibition. However, in contrast to Ca_v2 channels, it remains unclear if inhibition of Ca_v1 and Ca_v3 channels is mediated by a direct binding of $G\beta\gamma$ -dimer to the channel or required activation of secondary signaling pathways. Inhibition of Ca_v1 and Ca_v3 channels that is usually not reversed by a depolarizing prepulse, is also not characterized by a depolarizing shift of the current / voltage activation curve (DePuy et al., 2006), typical feature of the direct $G\beta\gamma$ -dependent inhibition. However, considering the presence of numerous molecular channel determinants that contribute to the phenotype of the direct $G\beta\gamma$ -regulation of Ca_v2 channels, it is possible the Ca_v1 and Ca_v3 channels lack important molecular determinants required to reveal “OFF” landmarks of the regulation. Either way, future studies will certainly uncover the molecular mechanisms by which G-proteins modulate Ca_v1 and Ca_v3 channels.

Voltage-independent inhibition of Ca_v2 channels

While this review is essentially focused on the so-called fast voltage-dependent regulation mediated by direct binding of G-proteins onto the channel, various studies on native neurons and heterologous expression systems have identified other types of inhibition that

usually take tens of second to develop and involve diffusible second messengers or surface remodeling of GPCR / channel complexes.

Inhibition of VGCCs by phosphoinositides

Direct G $\beta\gamma$ -dependent inhibition of VGCCs is usually fast and requires the activation of Gi/o-coupled receptors. In contrast, initial recordings performed on sympathetic neurons have identified a relatively slow and voltage-independent form of inhibition that is mediated by the activation of Gq-coupled receptors (Gamper et al., 2004). While various studies have ruled out the implication of typical Gq-dependent signaling pathways downstream of phospholipase C β , inositol trisphosphate (IP3), diacylglycerol (DAG) and protein kinase C (PKC), it was proposed that depletion of membrane phosphatidylinositol 4,5-bisphosphate (PIP2) is the mediator of Gq-dependent inhibition of VGCCs (Delmas et al., 2005) (Roberts-Crowley et al., 2009) (Michailidis et al., 2007) (Rodriguez-Menchaca et al., 2012). Consistent with this idea, loss of Ca_v2.2 channel activity typically observed in excised membrane patches can be either reduced by application of PIP2 or in contrast enhanced by depletion of PIP2 (Gamper et al., 2004). In addition, the time course of the slow inhibition of Ca²⁺ currents produced by activation of muscarinic M1 receptors (M1R) in sympathetic neurons nicely correlate with the kinetics of PIP2 hydrolysis, while infusing of PIP2 into the cell via the patch pipette is sufficient to prevent M1R-dependent inhibition of Ca_v2.2 channels (Gamper et al., 2004). More recently, using an exogenous voltage-sensitive 5-phosphatase (VSP) that allows rapid hydrolysis of PIP2 into Pi(4)P, it was shown that membrane depletion of PIP2 suppresses Ca_v1.2, Ca_v1.3, Ca_v2.1 and Ca_v2.2 currents, supporting the idea that depletion of PIP2 is sufficient to mimic the slow inhibition of calcium currents observed under Gq-coupled receptor activation (Suh et al., 2010). However, it is worth

noting that beside VSP-induced PIP2 depletion is similar to the depletion produced by activation of muscarinic receptors, the amplitude of the Ca^{2+} current inhibition is significantly less, suggesting that another signaling pathway might also contribute to the slow Gq-dependent inhibition. Hence, it was proposed that production of arachidonic acid (AA) by the action of phospholipase A2 on PIP2 and other membrane phospholipids elicits modulation of $\text{Ca}_v2.2$ channels (Liu et al., 2001) (Liu and Rittenhouse, 2003). Interestingly, it was also proposed that depletion of membrane PIP2 might reduce $\text{G}\beta\gamma$ -dependent inhibition of $\text{Ca}_v2.1$ channels expressed in *Xenopus* oocytes, suggesting a crosstalk between voltage-dependent and voltage-independent inhibitions (Rousset et al., 2004). Although the molecular mechanism by which PIP2 interfere with $\text{G}\beta\gamma$ regulation is not fully understood, it was proposed that binding of the carboxy-terminal tail of the $\text{Ca}_v2.1$ subunit to membrane phosphoinositides might stabilize a $\text{G}\beta\gamma$ -sensitive state of the channel (Rousset et al., 2004). However, considering that depletion of membrane PIP2 upon $\text{G}\alpha_q$ -dependent activation takes several minutes, it is unlikely that this mechanism account for the fast initial inhibition phase produced by $\text{G}\beta\gamma$ -dimer.

Inhibition of Ca_v2 channels by channel/GPCR complex internalization

Initially proposed by Forscher et al., G-protein-dependent inhibition of VGCCs may require a tight GPCR-channel coupling (Forscher et al., 1986), and evidence exist for direct biochemical interaction between the Ca^{2+} channel and the GPCR. Hence, physical association of $\text{Ca}_v2.1$ channels with metabotropic glutamate receptors mGluR1 has been documented in cerebellar Purkinje neurons as well as in cellular expression systems and involves the direct binding the of carboxy-terminal domain of the $\text{Ca}_v2.1$ -subunit with the carboxy-terminal region of the receptor (Kitano et al., 2003). Similarly, a $\text{Ca}_v2.2$ -ORL-1

(opioid receptor like-1 also known as nociceptin receptor or NOP) signaling complex has been documented in small dorsal root ganglion (DRG) neurons, and support a tonic agonist-independent G-protein inhibition of the Ca^{2+} channel evidenced by prepulse facilitation (Beedle et al., 2004). Similar observations have been reported for μ - and δ -opioid receptors transiently expressed with $\text{Ca}_v2.2$ channel in tsA201 cells although the existence of these protein complexes remains to be explored in native conditions (Chee et al., 2008) (Evans et al., 2010). Also, a physical interaction exist between $\text{Ca}_v2.2$ channels and dopamine D1 and D2 receptors and requires other channel structural determinants including the II-III intracellular linker (Kisilevsky et al., 2008) (Kisilevsky and Zamponi, 2008) (Weiss, 2009). Although the existence of Ca_v2 -GPCR signaling complexes is unambiguous, the physiological relevance of these interactions is not fully understood. It was proposed that association of the Ca^{2+} channels with GPCRs might control channel density at the plasma membrane, providing an additional level of control of the Ca^{2+} influx. Indeed, activation of ORL-1 receptors triggers an agonist-dependent co-internalization of $\text{Ca}_v2.2$ -ORL-1 complexes into vesicular compartments both in tsA201 cells and DRG neurons (Altier et al., 2006, Evans et al., 2010). However, internalization of $\text{Ca}_v2.2$ channels is not accompanied by a diminution of the membrane Ca^{2+} current, questioning the physiological relevance of this regulation (Murali et al., 2012). In addition, although μ -opioid receptors also physically interact with $\text{Ca}_v2.2$ channels they do not co-internalized indicating that biochemical coupling of the channel with the GPCR is not sufficient to mediate agonist-mediated internalization of the Ca^{2+} channel (Evans et al., 2010). It is also possible that the assembly of Ca_v2 channels and GPCRs provides a mechanism that ensures spatiotemporal regulation of the Ca^{2+} entering synaptic nerve terminals. In addition, tonic channel inhibition mediated by channel-GPCR complexes could also represent a mean to dynamically adjust the Ca^{2+} influx to the electrical

input signal coming to the synaptic ending. Indeed, it was shown that the extent of current facilitation (i.e. current recovery from G-protein inhibition) is dependent on both the duration (Brody et al., 1997) and the frequency of action potentials (AP) (Penington et al., 1991) (Williams et al., 1997). While low frequency AP produces no or little recovery, increasing AP frequency significantly enhances recovery from G-protein inhibition and Ca^{2+} influx and could contribute to short-term synaptic facilitation or depression (Bertram et al., 2003).

Contribution of G-protein modulation to channelopathies

As previously discussed, gating properties of the Ca^{2+} channel significantly affects direct G-protein inhibition, essentially the “OFF” landmarks (i.e. the dissociation of $\text{G}\beta\gamma$ -dimer from the Ca_v2 -subunit). Hence, alteration of channel gating is likely to affect G-protein regulation and synaptic activity. A number of congenital mutations in the gene *CACNA1A* encoding the $\text{Ca}_v2.1$ channel cause familial hemiplegic migraine type-1 (FHM-1), a rare and severe monogenic subtype of migraine with aura, characterize by at least some degree of hemiparesis during aura (Ophoff et al., 1996) (Weiss et al., 2007b)(Ducros, 2013) (Pietrobon, 2013). FHM-1 mutations generally affect structural determinants that are essential for channel gating including the S4 transmembrane segments thought to carry the voltage-sensor controlling channel activation, the S6 transmembrane segment involved in the control of channel inactivation, and the pore-forming loops. Biophysical analyses of channel gating revealed a hyperpolarizing shift of the voltage-dependence of activation, as well as additional effects on channel inactivation kinetics, open probability, and unitary conductance (Hans et al., 1999) (Kraus et al., 1998) (Kraus et al., 2000) (Mullner et al., 2004) (Tonelli et al., 2006) (Tottene et al., 2005). In addition, a knock-in mouse model

expressing the human pathogenic FHM-1 mutation R192Q located in the first S4 segment of the $\text{Ca}_v2.1$ -subunit revealed a decreased neuronal excitability threshold, increased Ca^{2+} influx and cortical spreading depression (i.e. the mechanism underlying migraine with aura (Pietrobon and Moskowitz, 2013)) (van den Maagdenberg et al., 2004), and enhanced excitatory transmission at cortical synapses (Tottene et al., 2009). Similar alterations have been also documented for the S218L mutation (van den Maagdenberg et al., 2010). While intrinsic alteration of $\text{Ca}_v2.1$ channel gating likely contributes to neuronal hyperexcitability, alteration of the G-protein-dependent inhibitory pathway of presynaptic Ca^{2+} channels may also contribute to synaptic hyperexcitability. Consistent with this idea, a decreased G-protein inhibition of R192Q $\text{Ca}_v2.1$ channels was reported (Melliti et al., 2003). Careful analysis revealed that the R192Q mutation does not affect the “ON” landmark but rather favor the dissociation of $\text{G}\beta\gamma$ -dimer following channel activation (“OFF” landmarks), thereby decreasing the inhibitory G-protein pathway (Weiss et al., 2008). Similar results were observed with various other FHM-1 mutations (Weiss et al., 2008) (Garza-Lopez et al., 2012)(Garza-Lopez et al., 2013) indicating that alteration of G-protein regulation of the $\text{Ca}_v2.1$ channel caused by FHM-1 mutations is a common underlying mechanism that certainly contribute to synaptic defects observed during the disease.

Alteration of channel gating can also be caused by change in regulatory subunits. Hence, an epileptic lethargic phenotype in mouse resulting from the loss of expression of the $\text{Ca}_v\beta_4$ -subunit is accompanied by a $\text{Ca}_v\beta$ -subunit reshuffling (Burgess et al., 1997). Considering that $\text{Ca}_v\beta$ -subunits significantly affect G-protein inhibition of Ca_v2 channels in a $\text{Ca}_v\beta$ isoform-dependent manner (Weiss et al., 2007a), it is likely to contribute to the altered excitatory synaptic transmission observed in those animals (Hosford et al., 1999) (Caddick et al., 1999).

Concluding remarks and perspectives

Since the first description of the phenomena by Dunlap and Fischbach in the late seventies (Dunlap and Fischbach, 1978), great advances have been made in our understanding of the underlying molecular regulation of neuronal VGCCs by GPCRs and its importance in physiology. In this review, we provided an appreciation of its tremendous complexity, arising not only from the numerous molecular channel and G-protein determinants involved in the regulation, but also from the channel subunit composition, GPCR subtype, interactions with synaptic proteins and other intracellular signaling pathways, and likely many more factors that have not been yet characterized. Although numerous channel / G-protein binding determinants have been described, the molecular mechanism by which G $\beta\gamma$ -dimer mediates inhibition of the Ca²⁺ current remains incompletely understood. It is likely that more discrete interactions that have not been yet characterized support this inhibition, and the recent structural information obtained from structurally similar channels will certainly help to find out the molecular basis of G-protein inhibition. In addition, the use of small molecules and peptides to selectively disrupt interaction of G-protein $\beta\gamma$ -dimer with some effectors has been demonstrated *in vitro* and *in vivo* on various models of heart failure and morphine tolerance (Bonacci et al., 2006) (Mathews et al., 2008) (Casey et al., 2010). A deeper biochemical and functional characterization of G $\beta\gamma$ -channel interaction will certainly provide important information to identified molecules targeting G-protein inhibition of VGCCs with potential therapeutic benefits. From a more physiological point of view, although the most evident outcome of G-protein regulation of presynaptic Ca²⁺ channel is a reduction of the Ca²⁺ influx entering nerve terminals (“ON” landmark), the observation that “OFF” landmarks might play an important role in fine tuning synaptic

strength, possibly contributing to short-term synaptic facilitation / depression, represents an interesting concept in molecular neuroscience which certainly merits to be further investigated. In addition, the notion that G-protein regulation is altered by pathological mutations in the Ca²⁺ channel complex not only contributes to our understanding of the pathogenesis of neuronal Ca_v channelopathies, but also emerges as an important signaling pathway for potential new therapeutic strategies.

Finally, merely twenty GPCRs from over nearly 1000 estimated from the sequencing of the human genome (including many orphan receptors) (Fredriksson et al., 2003) (Vassilatis et al., 2003) (Zhang et al., 2006) have been described for modulating VGCCs. While investigations into this extraordinary field continue, it is likely that many new GPCRs can underlie modulation of not only neuronal VGCCs but also channels expressed in other tissues like heart and skeletal muscle, and certainly represent a considerable source of potential therapeutic targets for the treatment of channelopathies in general.

Authorship contributions

Wrote or contributed to the writing of the manuscript: JP and NW

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Footnotes

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Legends for figures

Fig. 1. Structural organization and diversity of voltage-gated Ca²⁺ channels. VGCCs are integral membrane proteins composed of a Ca_v pore forming-subunit surrounded by Ca_vβ, α₂δ and in some cases γ auxiliary subunits. **A.** Putative membrane topology of the Ca_v pore forming-subunit. It consists of four repeat homologous domains (I to IV) linked by large intracellular loops (loops I-II to III-IV), each composed of six transmembrane segments (S1 to S6). The S4 segment of each of the four domains, rich in positively charged residues, presumably constitute the voltage-sensor while the P loops between segments S5 and S6 form the pore selectivity filter. **B.** Schematic representation of the VGCC macromolecular complex embedded in the plasma membrane in association with typical auxiliary subunits. **C.** Overview of the diversity of VGCCs. High-voltage-activated (HVA) channels are composed of Ca_v1 (L-type) and Ca_v2 (P/Q-, N- and R-types) subfamilies, while low-voltage-activated (LVA) channels are only composed by Ca_v3 channels, so-called T-type channels.

Fig. 2. GPCR activation cycle. In the inactive state of the receptor (**A**), the GDP-bound Gα-subunit and the Gβγ-dimer are associated together on the receptor. Conformational change of the receptor upon agonist binding (**B**) catalyze the exchange of GDP for GTP on the Gα-subunit and GTP-bound Gα-subunit and Gβγ-dimer complex dissociates (**C**) and activates downstream signaling. Intrinsic hydrolysis of GTP to GDP, potentially stimulated by RGS proteins, allows reassociation of the Gβγ-dimer with GDP-bound Gα-subunit (**D**), which terminates G-protein signaling.

Fig. 3. Putative kinetic model illustrating landmarks of $G\beta\gamma$ -dependent regulation of Ca_v2 channels. This scheme relies on a single conducting state where dissociation of the $G\beta\gamma$ -dimer has occurred while the channel remains open. The transition from $G\beta\gamma$ -bound closed channel (**B**) to $G\beta\gamma$ -unbound open conducting channel (**D**) requires a transition through an additional transient $G\beta\gamma$ -bound open non-conducting state of the channel (**C**) characterized by whole cell current inhibition (**G**) (“*ON*” landmark). This transition state produces a time-dependent current recovery from $G\beta\gamma$ -dependent inhibition, at the origin of the slowing of activation / inactivation kinetics of the whole cell current (**H**) and an apparent depolarized shift of the voltage-dependence of activation (**I**) (“*OFF*” landmarks). Dissociation of the $G\beta\gamma$ -dimer from the Ca_v channel-subunit is intrinsically independent of the membrane potential and is rather triggered by conformational changes of the Ca_v -subunit upon channel opening.

Fig. 4. Schematic representation of the Ca_v molecular determinants involved in $G\beta\gamma$ -mediated regulation of VGCCs. **A.** Schematic representation of the location of the important Ca_v channel molecular determinants of $G\beta\gamma$ -dependent regulation identified on the basis of functional evidence. Several $G\beta\gamma$ determinants are present within the I-II loop including the region S2 containing the QXXER motif and the G-protein interaction domain (GID) (in red). The alpha-interaction domain (AID) of the $Ca_v\beta$ -subunit on the I-II loop is shown in blue. Furthermore, additional molecular determinants located within the amino- and carboxy-terminal regions of the $Ca_v2.1$ and $Ca_v2.2$ subunits have been functionally documented. The EF-hand, IQ (Ca^{2+} -binding domain) and pre-IQ domains, and Ca^{2+} -binding domain (CBD), which constitute the carboxy-terminal Ca^{2+} -binding domains, are shown in blue. $G_q\alpha$ and $G_o\alpha$ binding sites are shown in purple. **B.** Schematic representation of a

presumable G-protein $\beta\gamma$ binding pocket within the Ca_v2 -subunits involving the different Ca_v molecular determinants functionally and biochemically characterized, and likely responsible of the variable sets of $\text{G}\beta\gamma$ -mediated modulation of the channel (“*ON*” and “*OFF*” landmarks). The carboxy-terminal region of the Ca_v2 -subunits also contributes in some cases to the biochemical coupling of the channel with the GPCR.

Fig. 5. Functional role of the $\text{Ca}_v\beta$ -subunit in $\text{G}\beta\gamma$ -dependent modulation of VGCCs. In this scheme, the presence of a $\text{Ca}_v\beta$ -subunit does not affect the binding of the $\text{G}\beta\gamma$ -dimer to the channel and thus does not alter the maximal extend of inhibition (“*ON*” regulation). In contrast, $\text{Ca}_v\beta$ -mediated inactivation of the channel delimitate a temporal window during which the $\text{G}\beta\gamma$ -dimer can dissociate from the open channel before it inactivates, letting Ca^{2+} flowing into the cell. Hence, a “slow inactivating” channel produced for example by the $\text{Ca}_v\beta_2$ -subunit (**A**), presents a large temporal window for $\text{G}\beta\gamma$ unbinding ($\text{G}\beta\gamma$ -unbound open channel state) and current recovery evidenced by an increased “*OFF*” regulation landmarks (slowing of activation / inactivation kinetics) and apparent decreased “*ON*” regulation (apparent decreased current inhibition). In contrast, a “fast inactivating” channel produced by $\text{Ca}_v\beta_3$ -subunit (**B**) rather undergoes inactivation, decreasing the occurrence of a $\text{G}\beta\gamma$ -unbound open channel state, producing an apparent increased current inhibition (“*ON*” regulation) while preventing “*OFF*” regulation.

Tables

Table 1. Neurotransmitters- and receptors-mediated G-protein modulation of Ca_v2 channels. Abbreviations: SCG, Superior Cervical Ganglion; DRG, Dorsal Root Ganglion.

Neurotransmitter	Receptor	Ca _v channel	Tissu / Species	Reference
Ach	M4	Ca _v 2.2	SCG / Rat	(Bernheim et al., 1991)
	M2	Ca _v 2.1 & Ca _v 2.2	SCG / Mouse	(Shapiro et al., 1999)
Adenosine	A1	Ca _v 2.2	Ciliary ganglion / Chicken	(Yawo and Chuhma, 1993)
		Ca _v 2.2	DRG / Chicken	(Kasai and Aosaki, 1989)
		Ca _v 2.1 & Ca _v 2.2	Cerebellum / Rat	(Dittman and Regehr, 1996)
		Ca _v 2.2 & Ca _v 2.3	Hippocampus (CA3-CA1) / Rat	(Wu and Saggau, 1994)
ATP / ADP	P2Y	Ca _v 2.2	SCG / Rat	(Brown et al., 2000) (Filippov et al., 2000a)
Dopamine	D2	HVA	DRG / Chicken	(Marchetti et al., 1986)
Endocannabinoids	CB1	Ca _v 2.1	SCG / Rat	(Garcia et al., 1998a)
		Ca _v 2.1, Ca _v 2.2 & Ca _v 2.3	Cerebellum	(Brown and Russell, 2004)
GABA	GABA B	Ca _v 2.1 & Ca _v 2.2	DRG / Rat	(Dolphin and Scott, 1987)
			DRG / Chicken	(Deisz and Lux, 1985) (Grassi and Lux, 1989)
			Cerebellum/ Rat	(Dittman and Regehr, 1996)

		Ca _v 2.2 & Ca _v 2.3	Hippocampus (CA3-CA1) / Guinea pig	(Wu and Saggau, 1995)
		Ca _v 2.2	SCG / Rat	(Filippov et al., 2000b)
Galanin	GalR1	Ca _v 2.2	Hypothalamus / Rat	(Simen et al., 2001)
Glutamate	mGluR1	Ca _v 2.2	SCG / Rat	(Kammermeier and Ikeda, 1999)
LHRH	LHRH-R	Ca _v 2.2	SCG /Bullfrog	(Elmslie et al., 1990) (Boland and Bean, 1993) (Kuo and Bean, 1993)
Noradrenaline	α_2 -adrénergique	Ca _v 2.2	SCG / Bullfrog	(Bean, 1989)
		Ca _v 2.2	SCG / Rat	(Garcia et al., 1998b)
		Non-L	NG108-15	(Docherty and McFadzean, 1989, McFadzean and Docherty, 1989)
NPY	Y2	Non-L	SCG / Rat	(Plummer et al., 1991)
		Ca _v 2.2	SCG / Rat	(Toth et al., 1993)
Opioids - Enkephalins	μ	Ca _v 2.2	NG108-15	(Kasai, 1992)
Opioids - Dynorphins	κ	Non-L	DRG / Rat	(Bean, 1989)
Serotonin	5HT-1A	Non-L	Spinal neuron / Lamprey	(Hill et al., 2003)
Somatostatin	SS-R	Ca _v 2.2	DRG / Rat	(Ikeda and Schofield, 1989b)
			SCG /Rat	(Ikeda and Schofield, 1989a)
Substance P	NK1	Ca _v 2.2	SCG / Rat	(Shapiro and Hille, 1993)
VIP	VIP-R	Ca _v 2.2	SCG / Rat	(Zhu and Ikeda, 1994)

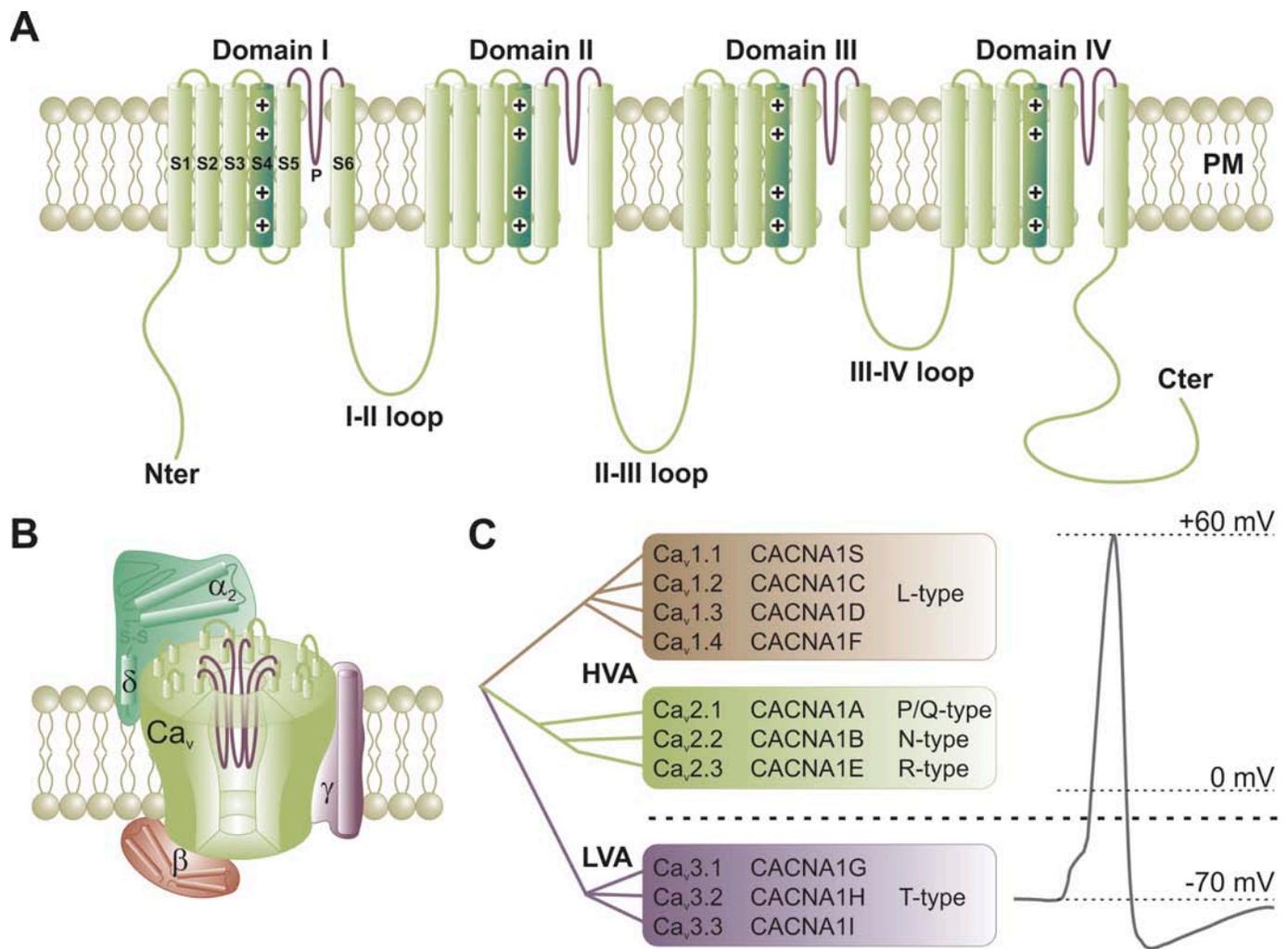


Figure 1

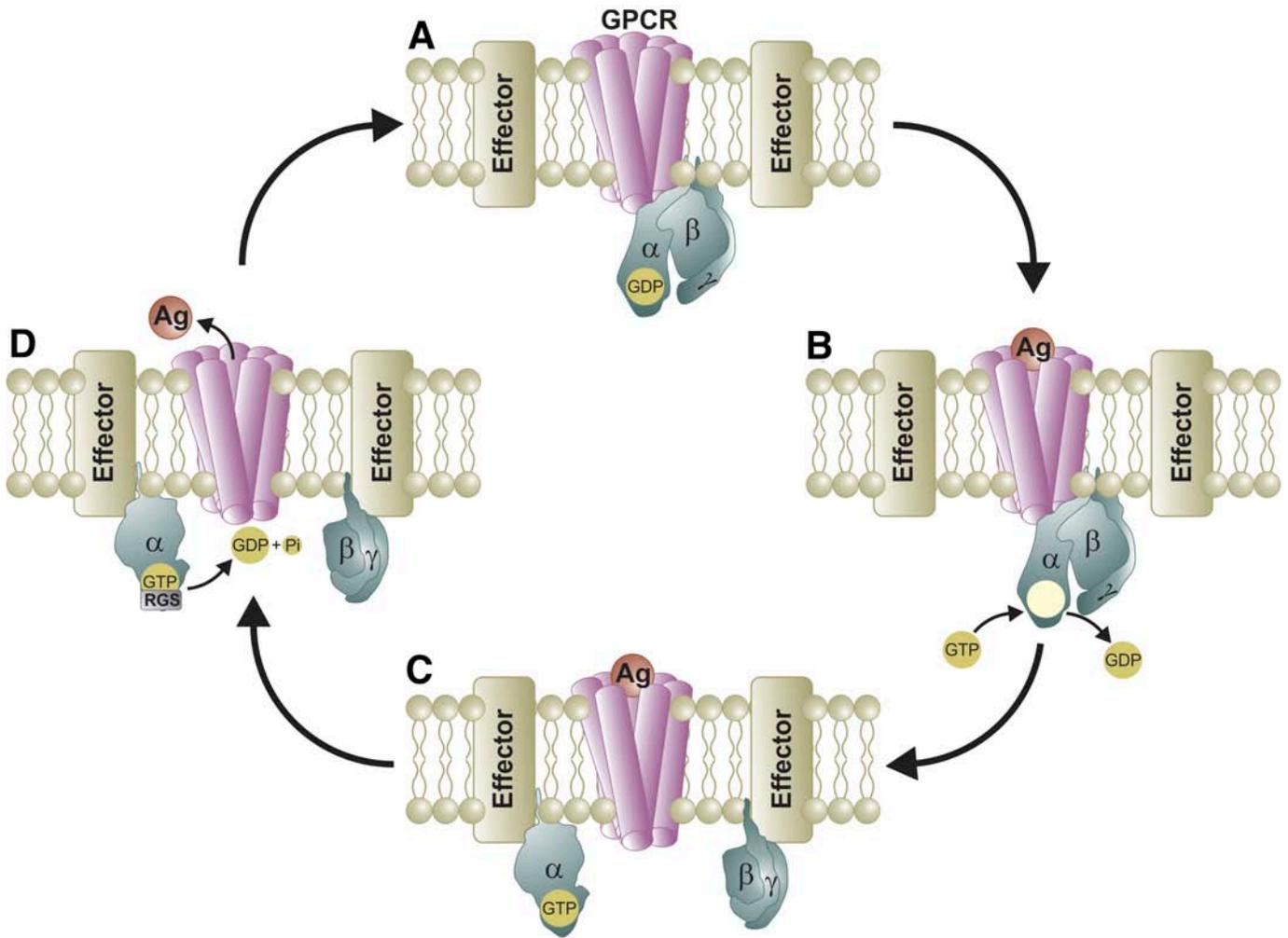
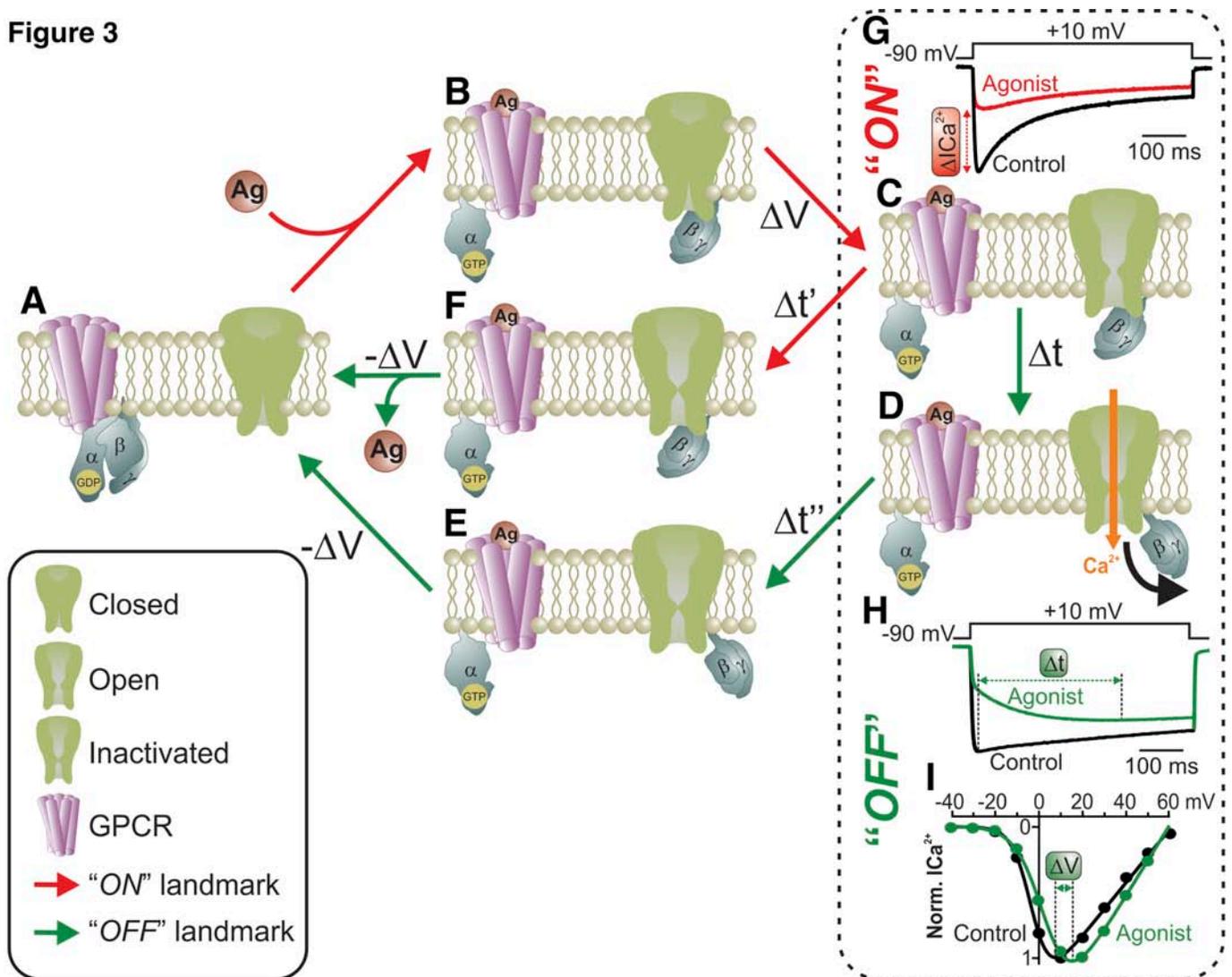


Figure 2

Figure 3



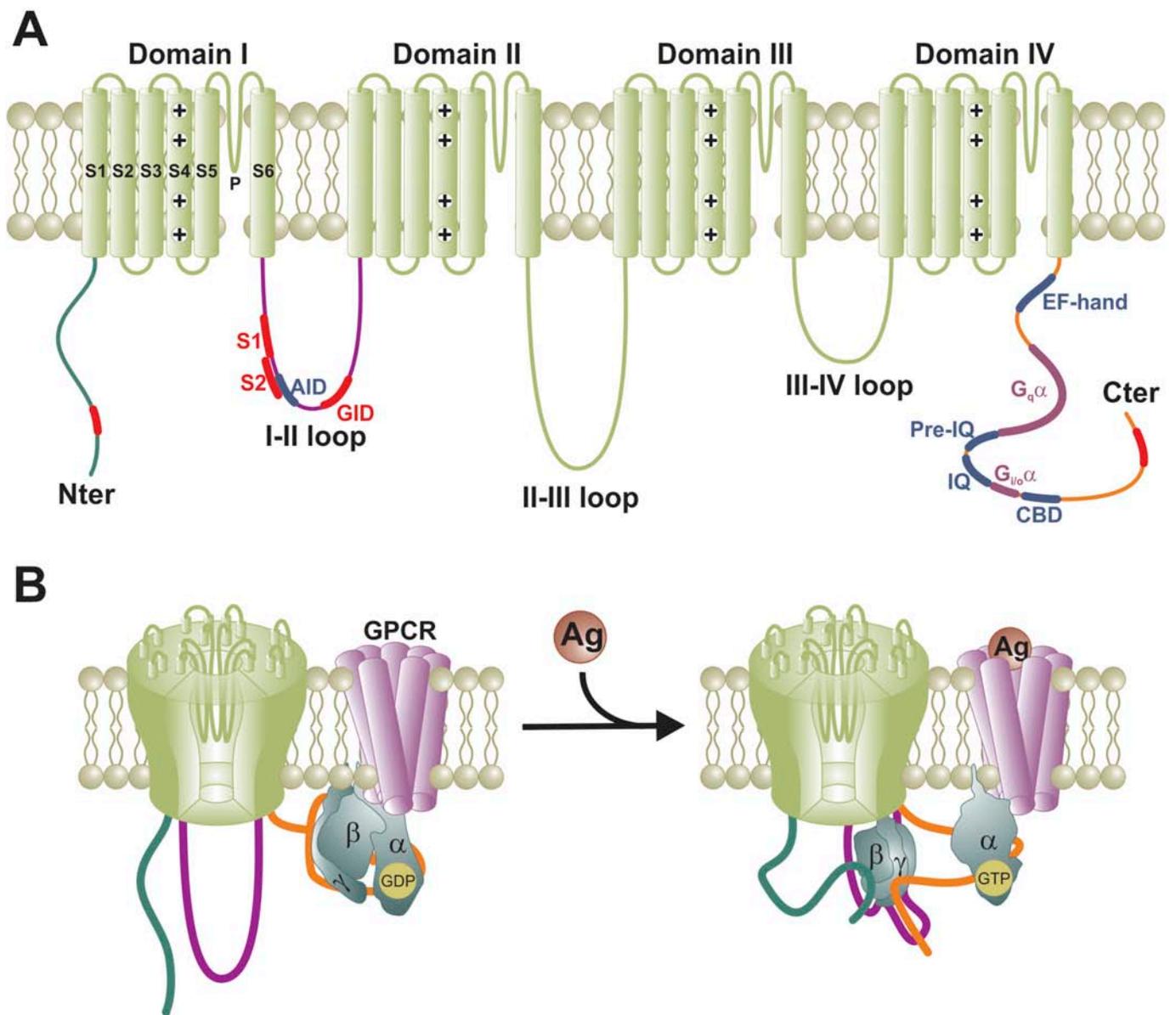


Figure 4

