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COMMENTARY

The road to the brain in Timothy syndrome is paved with enhanced $Ca_V1.2$ activation gating

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Voltage-gated calcium channels are key mediators of calcium entry into electrically excitable tissues such as nerve, muscle, and heart (Zamponi et al., 2015). The mammalian genome encodes 10 different genes that encode different $Ca_V\alpha1$ subunits which fall into three major families Ca_V1, Ca_V2, and Ca_V3, with the former two comprising high voltage-activated channels which typically require co-assembly with ancillary cytoplasmic $Ca_V\beta$ and extracellular membrane anchored $Ca_V\alpha2\delta$ subunits (for review, see Zamponi et al. [2015]). These ancillary subunits add to the functional diversity of the calcium channel family, as does alternative mRNA splicing and RNA editing (Huang et al., 2012; Li et al., 2017). The $Ca_V\alpha1$ subunits are comprised of four homologous transmembrane domains (I through IV), each with six transmembrane helices (S1 through S6) and re-entrant pore loop motifs. The regions comprising S1 through S4 form the voltage sensing domains, with S4 acting as the critical voltage sensors, whereas the regions comprising S5 to S6 form the pore domains and are key structures involved in voltage-dependent inactivation (Zamponi et al., 2015). The cytoplasmic C-terminal regions of Ca_V1 and Ca_V2 channels interact with calmodulin and are important for calcium-dependent inactivation (Zamponi et al., 2015).

Given that calcium ions activate a plethora of cytoplasmic responses that range from gene transcription to the release of hormone and neurotransmitters, voltage-gated calcium channels support many critical physiological functions that depend on specific channel isoforms. It is therefore not surprising that gain or loss of function mutations in several different calcium channel genes have been associated with a wide range of pathologies, including epilepsy, migraine, ataxia, deafness, night blindness, autism, and cardiac arrhythmias (Lory and Mezghrani, 2010; Striessnig, 2021). One puzzling aspect of calcium channelopathies is that although these channels are expressed widely, pathological mutations in some cases functionally manifest themselves only in specific tissues. For example, gain of function mutations in $Ca_V3.2$ T-type calcium channels have been linked to epilepsy, but do not cause a pain phenotype even though these channels are functionally important in the afferent pain pathway (Weiss and Zamponi, 2020). Along these lines, mutations in $Ca_V2.1$ P/Q-type calcium channels can cause epilepsy and ataxia, without strong effects on the neuromuscular junction which relies critically on the activity of these channels (Domitrz et al., 2005, but see Plomp et al., 2000). Equally as intriguing is the notion that different gain of function mutations in a given channel can give rise to highly diverse pathological outcomes. This is exemplified by gain of function mutations in $Ca_V1.2$, which can lead to cardiac arrhythmias that may or may not be accompanied by neurological deficits (Splawski et al., 2004; Burashnikov et al., 2010). An interesting and well-crafted study by Bamgboye et al. (2022) in this issue of the *Journal of General Physiology* begins to shed light into the molecular basis of this divergence.

Timothy syndrome 2 is an autosomal dominant disorder that is due to gain of function mutations in $Ca_V1.2$ L-type calcium channels encoded by the *CACNA1C* gene. The first such mutation identified led to the replacement of glycine 406 with an arginine residue (Splawski et al., 2004). The consequence of this G406R mutation that is localized to the cytoplasmic end of the domain IS6 region of the channel was a massive reduction in both voltage-dependent and calcium-dependent inactivation, in addition to a gain of function in the activation gating properties of the channel. This particular mutation leads to long QT syndrome that is associated with life threatening cardiac arrhythmias, plus a number of systemwide developmental abnormalities such as webbed fingers, and neurological deficits that include neurodevelopmental delay and autism (Splawski et al., 2004). A number of additional gain of function mutations in $Ca_V1.2$ have been linked to Timothy syndrome, and while they universally lead to cardiac pathologies, only some of them are associated with an overt neurological phenotype (Bauer et al., 2021). Bamgboye et al. (2022) addressed this issue by performing a detailed biophysical analysis of a subset of Timothy syndrome

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mutations that resided either at the distal ends of the various S6 transmembrane regions (L762F, I1166V, I1166T, and I1475M), or within an EF hand motif in the C-terminal region of the channel (E1496K). While all of these regions have been associated with channel inactivation, some of these mutations have multisystem effects, whereas some mediate pathologies that are cardiac selective. The authors created mutant $Ca_V1.2$ cDNAs and heterologously co-expressed them in tsA-201 cells along with $Ca_V\beta1b$ or $Ca_V\beta2a$, and $Ca_V\alpha2\delta$ subunits, followed by patch clamp analysis. Only one mutation (I1166T, which causes multisystem effects) altered the voltage-dependence of activation via a 20-mV hyperpolarizing shift and thus a gain of function, whereas both mutations in position 1166 mediated a slowing of deactivation kinetics (again consistent with a gain of function). L762F and E1496K slowed voltage-dependent inactivation, without affecting calcium-dependent inactivation, whereas the other mutations selectively affected calcium-dependent inactivation. The authors then did a deeper dive into the molecular mechanisms of the mutations-induced changes in calcium-dependent inactivation and by using calcium uncaging methods and single channel recordings, they convincingly demonstrated an intrinsic change in the maximum amount of calcium-dependent inactivation that could not be secondarily attributed to alterations in the open probability of the channel. Finally, the authors applied physiologically relevant action potential stimuli to cells expressing the various variants, and they found that the I1166T mutant and the originally described G406R variant (which also causes a multisystem disorder) both exhibited an action potential train-induced decrease in current amplitude. This was accompanied by an increase in the half width of the action potential–induced calcium current, suggesting an overall mix of gain and loss of function in calcium entry during an action potential train. In summary, the biophysical analysis performed by the authors reveals that mutations that produce a gain of function by reducing inactivation (whether the voltage- or calcium-dependent) or enhancing activation lead to a cardiac phenotype, whereas only those mutations that enhance activation lead to a neurological deficit (Fig. 1). Therefore, the authors were able to attribute the clinical presentation of distinct pathologies to specific alterations in gating parameters of the channel, which is remarkable indeed. Without taking away from the significance of these findings, it is important to note that while the work of Bamgboye et al. (2022) constitutes very strong correlative evidence, other factors may potentially also be at play, as we will discuss below.

First, different types of electrically excitable tissues are known to have different resting membrane potentials, and different action potential shapes and firing rates. In particular, neuronal action potentials are much shorter than those in the heart, such that alterations in the inactivation rates of $Ca_V1.2$ may perhaps be more tolerated than changes in activation range and deactivation kinetics. This in turn may affect to what extent overall calcium entry is affected by a given mutation. In some ways, a putative effect of resting membrane potential would be reminiscent of the fact that clinically administered L-type calcium channel blockers can exhibit selectivity for vascular L-type channels simply based on state dependence arising from a more depolarized resting membrane potential (Sun and Triggle, 1995). Second, different tissues may express differing amounts of $Ca_V1.2$ channel protein along with other L-type calcium channel isoforms, and thus a gain of function may accordingly manifest itself more or less severely. Third, different tissues may express a different complement of interacting proteins such as BK potassium channels whose activity is potently regulated by L-type calcium channel-mediated calcium entry (Plante et al., 2021), or ryanodine receptors which are differentially coupled to $Ca_V1.2$ in neurons and cardiac cells (Rougier and Abriel, 2016; Hiess et al., 2022). Finally, there is evidence that some pathogenic mutations in other types of calcium channels such as $Ca_V2.1$ and $Ca_V3.2$ only manifest themselves biophysically when present in specific splice isoforms of the channel (Adams et al., 2009; Powell et al., 2009). As $Ca_V1.2$ and also $Ca_V1.3$ channels are subject to regulation by alternative splicing and RNA editing (and often in a tissue and developmental stage specific manner), it is conceivable that certain mutations may exhibit pathogenic effects only in tissues that express a particular splice variant. This last point is particularly relevant to Timothy syndrome with regard to the G406R mutation. This residue (along with Glycine 402 which can also be altered in Timothy syndrome patients) is located within a mutually exclusive exon which leads to only a subset of channels being affected (Bauer et al., 2021). Although the variants studied by Bamgboye et al. (2022) are present in constitutive exons and thus present in all copies of the channel, alternative splicing at other sites could potentially affect the severity of the biophysical effects via allosteric mechanisms. These above considerations are very similar to those used to explain the discrepancy between the pathogenic effects of some calcium channel mutants in the absence of major biophysical alterations observed in transient expression studies that has been reported for a number of calcium channelopathies (e.g., Heron et al. [2007]).

With all of that being said, Bamgboye et al. (2022) have clearly identified a key parameter that determines whether mutations in $Ca_V1.2$ channels are likely to give rise to a neurological abnormality. Going forward, it will be very interesting to see if this correlation can be further strengthened by the analysis of additional variants, including perhaps known mutations in $Ca_V1.2$ that have been identified in patients with Brugada syndrome a channelopathy that appears to be cardiac specific (Burashnikov et al., 2010). It will also be important to determine how the various variants examined here alter the firing properties and output of neurons transfected with the various mutant constructs. Moreover, it has been shown that the mutant G406R affects neurite outgrowth in a voltage-dependent and calcium-independent manner via a RhoA pathway, and it would therefore be informative to know whether other mutant constructs display such an effect (Krey et al., 2013). As the authors allude to, it will be interesting to see if transfection of neurons with different mutant constructs leads to distinct alterations in gene expression in these cells. Finally, in their study, the authors showed that the effects of the L-type calcium channel agonist BAYK8644 are blunted in several of the variants, likely because the mutations result in a gating mode switch that already favors a higher open probability. It will therefore be worthwhile assessing to what extent the mutations alter the interactions with dihydropyridine antagonists, and other known calcium channel inhibitors such as phenylalkylamines. Such studies, along with that by Bamgboye et al.

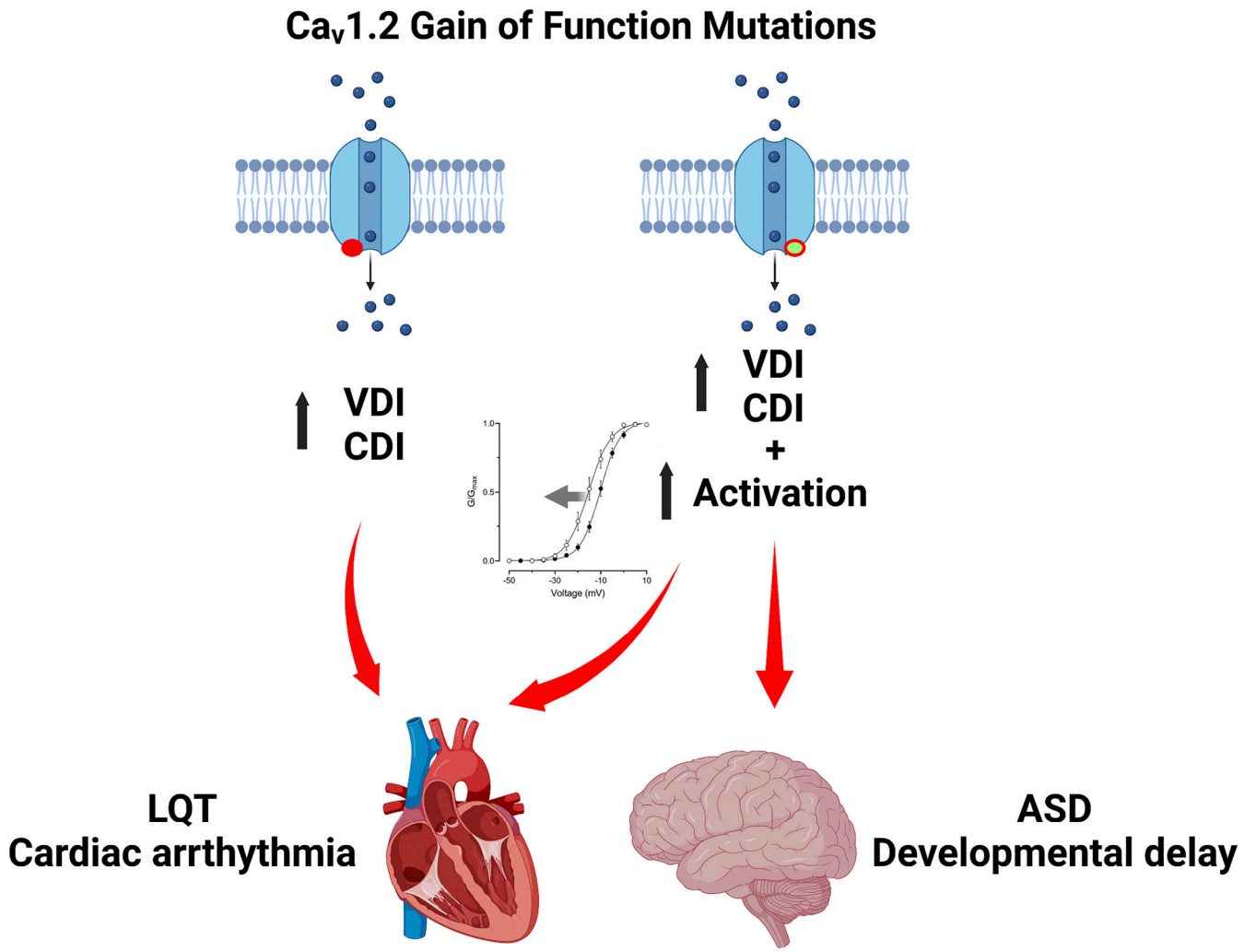

Figure 1. **Schematic representation of the brain and heart and its impact by different types of gain of function mutations in Timothy syndrome.** Figure created with BioRender.com.

(2022), will constitute an important step to not just understanding the pathology of Timothy syndrome, but also towards future strategies for therapeutic intervention .

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