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Ion channel dysfunction and altered motoneuron excitability in ALS

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Abstract

Dysregulated excitability is a hallmark of Amyotrophic Lateral Sclerosis (ALS) pathology both in ALS research models and in clinical settings. This primarily results from the dysfunction of Na^+, K^+, and Ca^{2+} ion channels responsible for maintaining neuronal thresholds and executing signal transduction or synaptic transmission. The exact dysfunction that each of these ion channel currents display in ALS pathology can vary between different ALS models, mainly induced pluripotent stem cell (iPSC) derived human motoneurons and ALS mouse models. Moreover, results can vary further across ALS mutations and between different developmental periods of these disease models. This review attempts to gather observations regarding ion channel dysfunction contributing to both hyperexcitable and hypoexcitable phenotypes in ALS motoneurons both *in vivo* and *in vitro*, so as to assess their potential as therapeutic targets.

Keywords

ALS; Ion Channels; Electrophysiology; Human

Introduction

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease characterized by the progressive loss of both upper and lower motoneurons leading to voluntary muscle weakness, muscle atrophy, and ultimately respiratory failure [1]. Approximately 5 – 10% of ALS cases are familial, following a Mendelian pattern of inheritance, with 80% of familial patients exhibiting known disease-causing mutations [2]. The vast majority of remaining cases are apparently sporadic in nature, with only 5 – 10% expressing these mutations [3]. Over 20 gene mutations have been linked to ALS pathology, seen in both familial and sporadic cases [4]. The most common gene mutation is found in *C9orf72* (coding for chromosome 9 open reading frame 2), making up 10–15% of total ALS cases. Mutations in

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the gene coding for SOD1 (Super-oxide dismutase 1), a Zn/Cu binding protein responsible for destroying free superoxide radicals, is the most well studied and responsible for 2% of ALS cases. Other commonly studied mutations are found in the genes coding for TDP-43 (TAR DNA-binding protein 43), responsible for 0.9% of ALS cases; and FUS (Fused in Sarcoma), responsible for 0.7% of ALS cases [2, 5], however, many other less common mutations have also been linked to ALS. Exactly how these mutations lead to progressive motoneuron cell death is currently unknown, and the topic is of great interest to researchers.

Cortical hyperexcitability is frequently observed in ALS patients through the utilization of threshold-tracking transcranial magnetic stimulation (TMS) combined with motor electrode potential recording [6–8]. Many studies also have reported axonal dysfunction in ALS patients that is characterized by increased excitability, abnormalities in threshold potential, and a prolonged strength-duration time constant ($_{T}$SD), a parameter which measures the rate of the decline of threshold currents as the stimulus duration is increased, all pointing to hyperexcitability [9, 10]. These abnormalities have been specifically attributed to irregular ionic conductances, more specifically the increased Na^+ and decreased axonal K^+ conductances, a profile that may underlie neurodegeneration and contribute to symptoms, such as fasciculations and muscle cramps found in ALS patients [11–13]. *In vitro* approaches to studying this phenomenon utilizes *SOD1* mouse models, as well as iPSC-derived motoneurons. Hyperexcitability is often seen in these ALS models, typically attributed to altered voltage-gated Na^+ and K^+ channel activity leading to an increased propensity for neuronal firing, and possibly cell death due to increased oxidative stress, intracellular Ca^{2+} build up, followed by mitochondrial dysfunction [13–16]. Dysregulated Ca^{2+} channels may contribute to altered excitability in a similar fashion. More recently, hypoexcitable phenotypes have also been implicated in ALS pathology [17]. Regardless, altered excitability evolves with disease progression and correlates with survival [18, 19], and can hereby serve as a potential therapeutic biomarker in ALS. However, since modulation of excitability in ALS has resulted in significant symptomatic improvement, ion channels and ion homeostasis represents a significant group of therapeutic targets for ALS drug development [20]. Due to the multifactorial nature of ALS pathology, it is likely that this process involves many pathological inputs from surrounding astrocytes, microglia and interneurons, however this review is focused on the intrinsic properties of ALS spinal motoneurons that contribute to the altered excitability.

Ca^{2+} channels

Molecular basis of AMPA receptor dysfunction in ALS

Dysregulated calcium homeostasis in motoneurons affected by ALS is a key player in ALS pathology and has been proposed to be a convergent point of major ALS dysfunctional pathways [21]. Increased intracellular calcium levels in affected motoneuron pools can upregulate many signal transduction pathways and promote pathological mechanisms including excitotoxicity, oxidative stress, ER stress, mitochondrial dysfunction, protein misfolding and aggregation and ultimately cell death [21, 22]. The exaggerated Ca^{2+} influx from the extracellular environment by Ca^{2+} -permeable channels is a likely cause of increased intracellular Ca^{2+} concentrations [23, 24]. AMPA receptors (AMPAR) are

ionotropic glutamate receptors composed of multiple subunits primarily responsible for the fast excitatory transmission in the central nervous system (CNS). It is well known that glutamate mediated excitotoxicity is present in ALS pathology, and that it is at least in part mediated by increased intracellular calcium concentrations, some are caused by an exaggerated influx through Ca^{2+}-permeable AMPA-receptors [25, 26]. The GluA2 subunit is the dominant functional determinant for AMPA receptor Ca^{2+} permeability, and its expression is normally increased after early post-natal development, leading to a switch from Ca^{2+} -permeable to Ca^{2+} -impermeable AMPA receptors [27]. In some ALS models, this switch appears to be reversed or inhibited at some point in disease onset or progression. Studies have shown that Ca^{2+}-permeable AMPA receptors lacking the regulatory GluA2 subunit are present and cause the exaggerated influx of Ca^{2+} into the cytoplasm in both *SOD1* ALS and other motoneuron disease models [28–30]. However, other subunits of the AMPA receptors are implicated as well. Some have been found in *SOD1* mouse models where an increased expression of the GluA3 subunit was coupled with a decrease in the GluA2 subunit [31, 32], and that an antisense peptide targeting GluA3 delayed disease onset and progression [32]. This event occurred even though GluA3 isn't the regulatory subunit for AMPAR Ca^{2+} permeability as is GluA2. Similar findings indicated an increase in Ca^{2+}-permeable AMPA receptors in human *C9orf72* iPSC-derived motoneurons, coupled with an upregulation of GluA1 subunits not commonly present in previous mouse model studies [24]. In the same study an increased GluA1 expression was also seen in *C9orf72* post-mortem spinal cord tissue and not in cortical neurons, indicating a motoneuron specific mechanism, as well as validating pathological similarities between the *C9orf72* iPSC derived motoneurons and patient primary motoneurons [24]. However, decreased expression of PICK1, an adaptor protein that regulates trafficking of the GluA2 subunit, was also observed, implying that dysregulated mechanisms involving GluA2 may still underlie the mutant AMPAR phenotype [24]. These results indicate differential mechanisms in which AMAPR components may increase Ca^{2+}-permeable AMPA receptors beyond just the GluA2 regulatory subunit [24]. However, although multiple AMPAR subunits have been implicated in the receptor's disease phenotype, there is little consensus on their role other than a primary role of the regulatory GluA2 subunit.

Aside from alterations in subunit composition, insufficient transcriptional editing of the Q/R site in the GluA2 domain of the AMPAR is also thought to contribute to its Ca^{2+} -permeable phenotype [33]. Q/R editing is a result of edition of GluR2 pre-mRNA in which a gene-encoded glutamine (Q) codon in the channel-forming intramembrane segment is changed to an arginine (R) codon (or A-I conversion in pre-mRNA). This edition is necessary to switch GluA2 from a Ca^{2+}-permeable state to impermeable state during early development stages [34, 35]. Q/R editing in GluA2 is necessary for normal neuron function, where its impairment produces Ca^{2+} -permeable AMPARs resulting in a higher rate of excitotoxicity [36]. This process occurs by the action of the enzyme Adenosine Deaminase Acting on RNA2 (ADAR2), which converts an adenosine at the Q/R site to an inosine (A-I conversion) [36]. The direct correlation between ADAR2 and motor diseases have been shown in knockout mice models with ADAR2 conditionally targeted in motoneurons, resulting in an increase in Ca^{2+} -permeable AMPA activity due to less Q/R editing, followed by a decline in motor function and slow motoneuron death [36]. The same study also found that neurons

in the nuclei of oculomotor nerves were resistant to the mutation despite showing decreased editing, which is consistent with ALS disease progression [36]. Although this suggests a correlation between ADAR2 activity and motoneuron disease, reduced RNA editing of the Q/R site is not found in the majority of familial ALS models or patients [37, 38]. However, it is a strong pathological marker of sporadic ALS cases making it a target for sporadic ALS therapy, and giving insight into possible disease mechanisms [24, 37]. In fact, decreased R/Q editing has been demonstrated in the majority of sporadic ALS cases regardless of clinical manifestation and is absent in other motoneuron disease cases, implying its disease-specific role in ALS [37, 39]. In addition, reduced ADAR2 activity in sporadic patients is almost exclusively coupled with decreased nuclear TDP-43 and increased abnormal cytoplasmic TDP-43 fragments (a common observation in both familial and sporadic ALS), suggesting a molecular link between these two ALS-specific molecular defects [37, 40].

Molecular basis of voltage-gated calcium channels in ALS

In addition to glutamate receptors, Ca^{2+} flux into motoneurons can occur through voltage gated calcium channels (VGCCs). VGCC activation results from a depolarizing current, and is responsible for depolarizing the neuron, regulating excitability, release of neurotransmitters, and influx of calcium as a secondary messenger modulating gene expression and cell behavior. Because AMPAR receptor targeting has not translated well into therapies for ALS, another source of intracellular Ca^{2+} overload may result from defects in VGCCs [41], as VGCC activity has been shown to modulate motoneuron excitability [23, 41, 42]. N-, P/Q-, L-, and R-type Ca^{2+} channels are seen in wildtype motoneurons, although the proportion of the inward current responsible for each type is varied [23, 43]. Research regarding VGCC defects in ALS pathology is not common, however a study by Chang and Martin [23] was done comparing VGCC activity between cultured spinal motoneurons from healthy and G93A-*SOD1* mice. The study found that N-type high voltage-activated (HVA) Ca^{2+} currents were increased in the *SOD1* mouse model, possibly due to transcriptional upregulation of VGCC subunits verified by RT-PCR, and increased localization of Ca1B (the subtype of $\alpha 1$ subunit in Calcium channels for N-type VGCCs) on the plasma membrane. The mechanism responsible for this was speculated to be a change in the redox state of the receptor due to mutant SOD1 induced oxidative stress. Interestingly, this observation was limited to large alpha-motoneurons. The high metabolic load of large alpha-motoneurons is consistent with the previously proposed theory that oxidative stress attributes to VGCC over-activity. Persistent Ca^{2+} currents (PC_{Ca}) are important in generating plateau potentials, amplifying synaptic inputs and modulating motoneuron excitability [23]. Additionally, abnormally increased PC_{Ca} amplitude, regulated by the L-type Ca^{2+} channel, is also observed in *SOD1* mouse motoneurons [23]. High PC_{Ca} may increase motoneuron excitability in response to an excitatory post-synaptic potential, contributing to the hyperexcitability phenotype. However, VGGCs may not play a pathological role in all ALS subtypes as seen by a study in which the Ca^{2+} channel blocker lomerizine was found to be a successful neuroprotective agent for *SOD1* but not for *TDP-43* ALS mutants [42].

To date there has been little success specifically targeting either AMPAR or VGCC in clinical settings. However, the drug Riluzole, the most successful ALS therapeutic drug, acts

in part as an AMPAR antagonist by possibly blocking the exaggerated influx of Ca^{2+} from the above pathological mechanisms.

Na^+ channels

Altered Na^+ currents in *SOD1* mouse models

The regulation of Na^+ currents is also often dysfunctional in ALS models, leading to altered neuronal excitability in both *in vivo* and *in vitro* cases [44–47]. The hyperexcitability of these cells reflects both intrinsic motoneuron hyperexcitability and increased/altered excitatory inputs derived from interneurons and/or modulating astrocytes [48, 49]. Hyperexcitable phenotypes are predominantly found in embryonic or early postnatal *SOD1* ALS models [45, 47, 50, 51]. For this reason, most excitability studies in mouse models utilized this developmental stage. This may indicate that hyperexcitability affects vulnerable neuron populations before disease onset. Embryonic ALS mouse studies have revealed an early hyperexcitability phenotype, attributed to an increase in persistent inward Na^+ current amplitudes (PIC_{Na}) [45]. This current, in contrast to the fast inactivating Na^+ current, is noninactivating or persistent. It is responsible for regulating membrane potential in the subthreshold range, and dendritic depolarizations, facilitating repetitive firing and enhancing synaptic transmission [52]. Increased PIC_{Na} amplitude may allow for increased repetitive firing or synaptic potentials leading to hyperexcitability. An increase in repetitive firing can be seen in multiple ALS mouse model studies as well as in iPSC-derived neurons [53, 54]. In other studies, the recovery from fast-inactivation after action potential propagation rather than increased Na^+ current amplitude is accelerated in ALS models, which increases depolarization frequency [55]. Altered Na^+ currents coupled with hyperexcitability has also been seen in neonatal *SOD1* mouse models [47, 49, 56]. Increased PIC_{Na} currents coupled with increased spontaneous transmission are seen in this developmental period, also altering the rate of motoneuron firing to injected current relationship (f-I relationship) [47, 49, 54]. However, one study asserted that motoneurons affected in ALS do not exhibit intrinsic hyperexcitability at this stage [56]. Clinically, prolongation of T_{sp} has been consistently identified in sporadic ALS and linked to neurodegeneration [9], as well as in familial forms of ALS linked to mutations in *SOD1* and *C9orf72* [57]. It is proposed that increased T_{SD} (strength duration time constant, a measure for axonal excitability) that presents in clinical ALS pathology is due to an exaggerated PIC_{Na} current that facilitates depolarization towards threshold potentials, although reduced K^+ current was also identified in some cases [9, 57, 58].

Different Na^+ channel isoforms are expressed in motoneurons at different developmental stages. Embryonic motoneurons express predominantly the isoforms Nav1.2 and Nav1.3, followed by a decrease in Nav1.3 expression and persistent Nav1.1, Nav1.2, and Nav1.6 expression in the adult CNS [59, 60]. More recently, it has been shown that one of the most common mutations occurring in ALS patients, SOD1A4V, increased peak Na^+ amplitudes from Nav1.3 and induced a hyperpolarizing shift in the voltage dependence of the Na^+ channel in embryonic ALS mouse motoneurons [44]. Since Nav1.3 is not commonly present in adult mice, it is hard to speculate on its role in disease onset and progression. In adult humans Nav1.3 is also rare, however axonal transection in a dorsal

root ganglia model caused an increase in its expression [61]. This implicates axotomy could be a mechanism that de-differentiates neurons to an “embryonic state” that leads to the re-expression of Nav1.3. It would be intriguing to find out whether this could be the case in traumatic brain injuries, which is speculated to be a risk factor for ALS [62, 63]. In contrast to embryonic models, the majority of electrophysiology studies in adult ALS mouse models both in vivo and in vitro exhibit no hyperexcitability but a slight hypoexcitable phenotype [50, 51]. However, ventral-root recordings of adult SOD1 mice displayed a lessened depression of compound action potentials (APs) evoked by dorsal root stimulation compared to control, indicating an increased tendency to fire [46]. Although intrinsic hyperexcitability is not observed in adult *SOD1* mouse models, an increase in beta3 protein (a voltage-gated Na^+ channel subunit) is seen in presympotmatic adult mice and an increase in levels of both transcript and total protein is seen in symptomatic adult mice [64]. This indicates that mutant SOD1 may facilitate the transport, folding, and assembly of the beta3 protein in presympotmatic mice, before an increase in transcript is observed. This beta3 protein subunit was found to directly affect persistent Na^+ currents, altering the neurons excitability near firing threshold [65, 66]. The dysregulation of beta3 protein and the electrophysiological implications correlate with observations in embryonic and neonatal models, but it does not correlate with observations in adult mice, suggesting the presence of some other mechanism(s). These results suggest that increased Na^+ current and intrinsic hyperexcitability may play a more critical role in pre-symptomatic stages rather than during disease onset and progression.

Altered Na^+ currents using iPSC-derived motoneurons from ALS patients

The use of a more recent model, iPSC-derived neurons from human ALS patients, it has been reported that there exists the same discrepancy in the hyperexcitability vs hypoexcitability phenotypes, and their role in disease progression. Studies utilizing *TDP-43* and *C9orf72* mutant iPSC-motoneurons reported hypoexcitability phenotypes [53], whereas those using *SOD1* mutant iPSC-motoneurons reported clear hyperexcitability [67]. One recent study by Devlin et al. (2015) found a way to link both phenotypes [68]. The intrinsic properties of *TDP-43* and *C9orf72* iPSC-motoneurons were studied, as in previous reports, however monitoring over a longer time period resulted in an overall switch from a hyperexcitable phenotype to a hypoexcitable phenotype. This observation was coupled with a decrease in fast-inactivating Na^+ currents. Further studies in *SOD1* and *FUS* motoneurons revealed strong hypoexcitability correlated with attenuated sodium channel expression levels and reduced Na^+ current peak amplitudes [53]. Due to iPSC-derived motoneurons being a relatively recent advancement, data comparing multiple ALS mutants are limited. Further studies need to be carried out comparing multiple mutants in parallel, to elucidate mutant-specific molecular mechanisms affecting excitability. It is worth noting that in these studies, it is difficult to determine whether late stage hypoexcitability is due to disease pathological progression or long-term culture conditions, even with reports of hypoexcitability while maintaining cell viability [68].

Regardless of the phenotype, alteration in Na^+ channel function regulates excitability in ALS pathology and has been employed as a therapeutic target. Riluzole, the only successful drug available for ALS treatment, blocks TTX-sensitive Na^+ channels. Although this drug’s

therapeutic effect most likely proceeds by a number of different mechanisms, ranging from ion channel activity to other cellular processes, Riluzole treatment *in vitro* decreased PIC_{Na} with a concurrent decrease in excitability to levels of control [54]. This indicates that the therapeutic action of Riluzole, in part, may be due to regulating Na^+ channel activity and decreasing intrinsic excitability. Na^+ channel blockage by other compounds such as Flecainide (currently in clinical trials) has been seen to stabilize peripheral nerve excitability [69]. However, this mechanism of action does not explain the growing amount of data implicating hypoexcitability in ALS. However, the molecular foundation behind Na^+ channel alteration in ALS is not very well understood. Oxidative stress resulting from mutant SOD1 may directly affect Na^+ channel function via oxidation [45], and more specifically, oxidative stress can activate kinases such as protein kinase C (PKC) [45]. PKC is known to phosphorylate the alpha unit of certain Na^+ channels, and PKC levels are elevated in the *SOD1* mouse model [44]. Other indirect mechanisms may include increased or decreased axonal trafficking of ion channels. Investigation of the molecular link between ALS gene mutations and ion channel dysfunction may provide better insight on strategies targeting these channels for ALS treatment.

K^+ channels

K^+ channel dysfunction in ALS

Voltage-gated K^+ channels are responsible for opposing the inward current created by voltage-activated Na^+ channels during AP propagation. Due to their direct action on membrane excitability, K^+ channels are also implicated in pathological excitability changes in ALS motoneurons. In fact, a decrease in protein expression of delayed rectifier K^+ channels is specifically seen in the ventral roots of post-mortem sporadic ALS patient spinal cords [70]. Hypermethylation and downregulation of K^+ channels is also found in ALS postmortem spinal cords [71]. This evidence in human ALS cases further solidifies the idea that molecular dysfunction of K^+ channels occurs in disease pathology and should somewhat be reflected in ALS models. One study has observed a decrease in protein expression of the voltage-activated ion channels Kv1.6 and Nav1.6 in rat motoneurons exposed to the cerebral spinal fluid from sporadic ALS patients [72]. However, although most embryonic and post-natal ALS mouse studies do exhibit some amount of hyperexcitability, evidence supporting specific K^+ current dysfunction in SOD1 mouse model *in vitro* studies is sparse, with most studies focusing on the previously mentioned Voltage-activated Na^+ currents.

K^+ channel dysfunction in iPSC-derived motoneurons from ALS patients

However, in iPSC-derived motoneurons, altered K^+ channel currents are directly correlated with cell excitability in which both hyper- and hypoexcitability is observed [67, 68]. In one such study, decreased delayed rectifier K^+ channel currents were found to be solely responsible for the hyperexcitable phenotype independent of Na^+ channel activity [67]. In the same study, Retigabine, an anti-convulsant used to treat seizures, pharmacologically activated the voltage-activated K^+ channel Kv7, lowering its firing rate and decreasing hyperexcitability to control levels in *C9orf72*, *SOD1*, *Fus*, and *TDP-43* iPSC-derived mutant motoneurons [67]. In contrast to the previous study, a shift from early stage hyperexcitability to late stage hypoexcitability reflected a decrease in persistent voltage-gated K^+ currents in

C9orf72 and *TDP-43* iPSC-derived motoneurons [68]. However, this study did not explore the ion currents responsible for this early hyperexcitability, and the observation was also coupled with a decrease in Na^+ currents indicating that a general loss of ion channel function may result in hypoexcitability, rather than a specific loss of either the K^+ or Na^+ current. Others have seen a specific elevated K^+ current responsible for reduced Na/K ratios in hypoexcitable FUS mutants, where both elevated K^+ current and attenuated Na^+ current were responsible for the same observation in hypoexcitable *SOD1* mutants [53]. Differential K^+ channel alterations across these different studies likely reflect mutation specific mechanisms, as well as different developmental stages likely responsible for this hyper-/hypoexcitability discrepancy. Although evidence suggesting the role of altered K^+ currents in excitability changes in ALS is lacking, compared to Ca^{2+} and Na^+ channels, axonal excitability studies in ALS patients indicated direct abnormalities in Na^+ and K^+ channels correlating to an observed hyperexcitable phenotype [9].

Summary and Future Directions

Research to date suggests that ion channel dysfunction in motoneurons likely contributes to altered intrinsic excitability in ALS pathology. Most electrophysiology studies in early ALS mouse models implicate dysregulated Na^+ currents as the most impactful factor in a hyperexcitable phenotype [44, 45, 47, 49–51, 56], whereas in iPSC-derived motoneuron models, both Na^+ and K^+ channel dysfunction are implicated in altered electrophysiology [53, 67, 68]. Ca^{2+} channels may also contribute to altered excitability by raising the membrane potential closer to threshold. However, exaggerated influx of extracellular calcium most likely contributes to altered excitability due to mitochondrial dysfunction, oxidative damage, and altered cellular transport rather than directly affecting membrane potential properties [21]. Although the debate on whether hyper- or hypoexcitability contributes to cell death and denervation is far from settled, the *SOD1* mouse model seems to primarily have pre-symptomatic hyperexcitability [44, 45, 47, 49, 50] followed by late stage normal or hypoexcitability [50, 51]. This is consistent with clinical manifestations in which patients express increased peripheral nerve excitability months before disease onset [9]. Studies in the iPSC-derived motoneurons are much more variable, with many observing either hyper- [53, 68] or hypoexcitability [67, 68]. This may also be due to a pathological switch between the two phenotypes during disease progression [68]. Clinically, both hyper- and hypoexcitability are observed in ALS patients, while axonal hyperexcitability seems to be more prominent [9, 73]. Overall, significant progress has been made concerning the mechanism of hyperexcitability in ALS and its potential as a therapeutic target. However, debates will continue regarding the roles and mechanisms of hyper- or hypoexcitability in ALS pathology, such as which one is more responsible for denervation, or whether one is a compensatory mechanism or pathological subsequent of the other, or both are simply pathological phenotypes representing different disease stages or different ALS subtypes.

Although there is still question about the role of altered excitability in disease onset and progression, it is an attractive target for ALS therapy development. The potential for pharmacological action targeting ion channels is evidenced by the antagonistic action of Riluzole on AMPA receptors and voltage-gated Na^+ channels, as well as other compounds including the Na^+ channel blocker Flecainide [69] and the K^+ channel agonist Retigabine

[67]. Edavarone, a new antioxidant drug approved for ALS, has also displayed therapeutic potential by reducing oxidative damage caused by increased intracellular calcium [74]. Novel therapeutics targeting these ion channels may be used in conjunction with other therapeutic methods to limit excitotoxicity in motoneurons, while employing regenerative techniques such as neural grafts and stem cell therapies to enhance recovery. Also, with the advent of iPSC technology, human-derived motoneuron models expressing different ALS mutations are being widely employed for the study of ALS. Studying pathological differences in channel activity between different mutants, as shown in the above review, may lead to more efficient patient-specific therapies.

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