XXVII Cycle
PhD Program in Neuroscience
School of Molecular Medicine

Na\textsubscript{v}1.6 As A New Potential Target
In Alzheimer’s Disease

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1. INTRODUCTION

1.1 Alzheimer's Disease

Alzheimer's Disease (AD) is the most frequent neurodegenerative disorder and the most common cause of dementia in the elderly, that leads to severe memory loss and cognitive impairment. Current estimates suggest that AD afflicts more than 5 million individuals in the United States and 24 million people worldwide, with this number projected to double in 20 years.

Pathologically, AD is characterized by the temporal and spatial progression of amyloid plaques arising from extracellular deposition of the fibrillogenic amyloid-beta (Aβ) peptide and other proteins, and intracellular neurofibrillary tangles (NT) due to the hyperphosphorylation of the microtubules-stabilizing protein Tau.

At the cellular level, AD is further associated with progressive dismantling of synapses, neuronal circuits and networks, and eventual neuronal loss within brain regions underlying cognition and memory formation (DeKosky and Sheff, 1990). Loss of synapses is considered the best correlate of cognitive decline in AD, rather than plaques or tangles.

1.1.1 Aβ Peptide And Amyloid Cascade Hypothesis

More than 30 years ago, amyloid peptides were recognized by Glenner (Glenner and Wong, 1984) as a major component of the amorphous plaque-like deposits in the damaged brains of AD patients. Since then, a lot of evidence has demonstrated that Aβ peptide has potential neurotoxic properties (Yankner
et al., 1989). Four other key discoveries have pointed out on the role of this peptide in the pathogenesis of disease. First, Aβ peptide is part of a large type I membrane protein, the Amyloid Precursor Protein (APP), which is encoded by the APP gene on chromosome 21. Second, the APP gene is mutated in a significant fraction of familial AD cases. Third, individuals with Down’s syndrome, who have three copies of chromosome 21 and hence three copies of APP gene, develop clinical and pathological signs of early onset Alzheimer’s. And fourth, mutations of presenil-1 (PSEN1) and presenil-2 (PSEN2) genes, which encode for the catalytic subunit of the γ-secretase activity that liberates the Aβ peptide from the C-terminus of APP, can behave as dominant familial AD genes.

These findings led to the elaboration of a theory of AD known as amyloid cascade hypothesis (Hardy and Selkoe, 2002; Citron, 2004), which best describes the pathogenic events causing Alzheimer neuronal death and leading ultimately to irreversible dementia. Indeed, this hypothesis postulates that in familial AD mutations in either APP or one of the PSEN genes lead to the brain accumulation of a 42-amino acid form of the amyloid peptide that has a tendency to form aggregates. Amyloid aggregates form first small oligomers and finally plaques. The amyloid cascade hypothesis proposes that these Aβ aggregates leads in turn to a series of downstream events such as plaque deposition, tau hyperphosphorylation, inflammation, loss of synaptic structure and function, and death of susceptible neurons (Glenner and Wong, 1984; Tanzi and Bertram, 2005; Walsh and Selkoe, 2004). The hypothesis also proposes that sporadic AD develops when natural history of an individual accelerates a normal age-dependent process of Aβ accumulation. At some
point, sufficient Aβ become deposited that the amyloid cascade is triggered. Subsequently, the sporadic disease follows the same pathway to dementia as the familial form. Therefore, both in familial and sporadic AD the amyloid cascade hypothesis claims that is the excessive accumulation of pathogenic Aβ assemblies in the brain to play a causal role in AD. Indeed, under normal conditions, Aβ is present in a soluble form throughout life, but in Alzheimer’s Disease pathogenesis, Aβ aggregates into higher-order species such as soluble oligomers and insoluble amyloid plaques in a concentration-dependent manner.

In strong support of the pathogenic role of Aβ, neuronal expression of human APP(hAPP) and Aβ in transgenic mice elicits several AD-like abnormalities, including amyloid plaques, neuritic dystrophy, aberrant sprouting of axon terminals, functional and structural synaptic deficits, impairments in learning and memory, and other behavioral alterations (Chin et al., 2004, 2005; Games et al., 1995; Götz et al., 2004; Kobayashi and Chen, 2005; Palop et al., 2003, 2005).

1.1.2 APP processing and Aβ generation

The proteolytic processing pathways leading to the formation of Aβ from the amyloid precursor protein (APP) have been well characterized in a number of cell lines (Selkoe, 2000). APP is a type I transmembrane protein. It is synthesized in the endoplasmic reticulum (ER) and then transported through the Golgi apparatus to the trans-Golgi network (TGN), where is found the highest concentration of APP in neuron at steady-state. From the TGN, APP can be transported in TGN-derided secretory vesicles to the cell surface, where
it is either cleaved by α-secretase to produce a soluble molecule, sAPPα, or re-
internalized via an endosomal/lysosomal degradation pathway.

At least three enzymes are responsible for the processing of APP and
have been called α-, β- and γ-secretases. The processing pathway by α-
secretase, called non-amyloidogenic, cleaves plasma membrane APP within
the Aβ domain in the C-terminal portion of the sequence of this peptide,
producing soluble APPα, which has neurotrophic and neuroprotective effects.
The processing pathway by β- and γ-secretases, called amyloidogenic,
cleaves APP in the N- and C-terminal portions of the Aβ region, respectively,
producing Aβ peptide.

Cleavage of APP by α-secretase precludes Aβ generation as the
cleavage site is within the Aβ domain and releases a large soluble ectodomain
of APP called sAPPα. APP molecules that fail to be cleaved by α-secretase on
the membrane surface can be internalized into endocytic compartments and
subsequently cleaved by β-secretase (BACE) and γ-secretase to generate Aβ.
After α- and β-cleavage, the carboxyl terminal fragments (CTFs) of APP, known
as αCTF and βCTF, respectively, remain membrane associated. αCTF and
βCTF will be further cleaved by γ-secretase generating p83 and Aβ,
respectively. The p83 fragment is rapidly degraded and widely believed to
possess no important function. γ-cleavage can yield both Aβ40, the majority
species, and Aβ42, the more amyloidogenic species, as well as release
intracellular domain of APP (AICD).

Although the majority of Aβ is secreted out of the cell, Aβ can be
generated in several subcellular compartment within the cell, such as ER,
Golgi/TGN and endosome/lysosome. Then, Aβ peptides generated in the Golgi and in recycling compartments can be secreted into the extracellular space (Greenfield et al. 1999). In addition, extracellular Aβ can be internalized by the cell for degradation. The intracellular existence of Aβ implies that it may accumulate within neurons and contribute to disease pathogenesis. The majority of secreted Aβ peptides are 40 amino acids in length (Aβ40), although the smaller fraction (10%) of longer, 42 amino acid species (Aβ42) have received greater attention due to the propensity of these peptides and other derivatives of the amyloid precursor protein to nucleate and drive production of amyloid fibrils (Jarrett et al., 1993). Studies done on familial AD mutations show increases in the ratio of Aβ42/Aβ40, suggesting that elevated levels of Aβ42 relative to Aβ40 is critical for AD pathogenesis, probably providing the core for Aβ assembly into oligomers, fibrils and amyloidogenic plaques.

α-secretase is a membrane-bound endoprotease which cleaves APP primarily at the plasma membrane. In particular, α-secretase is a zinc metalloproteinase. Its activity is constitutive, but it can also be regulated by various factors. Several members of the ADAM family possess α-secretase-like activity and three of them have been suggested as the α-secretase: ADAM9, ADAM10 and ADAM17. Like APP, they are also type-I transmembrane proteins. ADAM17 likely affects regulated, but not constitutive, α-cleavage of APP in various cell lines; in contrast, ADAM10 is the constitutive α-secretase that is active at the cell surface, as demonstrated by the inhibitor effect of ADAM10 dominant-negative form and RNAi of ADAM10 on the endogenous α-cleavage activity in several cell lines. ADAM9 also shows α-secretase activity, but it is involved only in the regulated α-cleavage as ADAM17.
The major β-secretase is BACE1, that is a membrane-bound aspartyl protease. Several studies have confirmed that BACE-1 is the β-secretase involved in APP metabolism; and BACE1 activity is thought to be the rate-limiting factor in Aβ generation from APP. BACE-1 requires an acidic environment for optimal activity; in fact, it is mainly found in the early Golgi, late Golgi/early endosomes, and endosomes that provide an acidic environment. In addition, BACE1 can be found at the cell surface. Several studies have found that BACE1 protein and activity levels are elevated in the regions of the brain affected by AD.

A lot of biochemical evidence has shown that γ-secretase activity resides in a high molecular weight complex consisting of at least four components: presenilin (PS, PS1 or PS2), Nicastrin, anterior pharynx-defective-1 (APH-1), and Presenil enhancer-2 (PEN-2). In mammals, there are two presenilin homologs, PS1 and PS2. Mutations in these two genes, particularly PS1, are causative in the majority of familial AD cases. PSs are multi-transmembrane proteins with an unclear number of transmembrane domains; they possess two highly conserved aspartate residues indispensible for γ-secretase activity and they are the crucial catalytic components of γ-secretase, as confirmed by in vitro assays. (Fig. 1)
Figure 1. APP processing: non-amyloidogenic and amyloidogenic pathways.

1.1.3 Aβ Affects Neuronal Excitability

Excessive accumulation of Aβ is thought to be a causal factor in producing cognitive deficits, but the mechanisms by which Aβ accumulation leads to this deficits is still unclear. Mounting evidence suggests that epileptiform activity may play an important role in the development of AD-related cognitive deficits. In fact, while seizures were previously thought to be secondary to disease progression, aberrant activity and/or seizures may directly contribute to cognitive deficits early in disease progression inducing hippocampal dysfunction and memory deficits.

AD is associated with a 5- to 10-fold increase in seizure incidence (Amatniek et al., 2006; Hauser et al., 1986; Hesdorffer et al., 1996; Lozsadi and Larner, 2006; Mendez and Lim, 2003), and transgenic mouse models of AD exhibit brain-wide aberrant neuronal and epileptiform activity (Hsiao et al., 1995; LaFerla et al., 1995; Moechars et al., 1999; Lalonde et al., 2005; Palop et
Interestingly, the risk of epileptic activity is particularly high in AD patients with early-onset dementia and during the earlier stages of the disease, reaching an 87-fold increase in seizure incidence compared with an age-matched reference population (Amatniek et al., 2006; Mendez et al., 1994). The incidence of epileptic activity is also increased in sporadic AD (Amatniek et al., 2006) but is particularly high in pedigrees with early-onset autosomal dominant AD (Cabrejo et al., 2006; Larner and Doran, 2006; Palop and Mucke, 2009; Snider et al., 2005). Epileptiform activity has been associated with transient episodes of amnestic wandering and disorientation in AD (Rabinowicz et al., 2000).

Recent studies indicate that Aβ peptide can contribute to AD cognitive decline inducing neuronal hyperexcitation and aberrant network activity. Neuronal circuits are smaller assemblies of interconnected neurons within a specific brain region and neuronal networks are larger assemblies of interconnected circuits involving different brain regions. Several recent reports in Alzheimer’s disease-related mouse models suggest that pathologically elevated Aβ destabilizes neuronal activity at the circuit and network levels. In particular, high Aβ could induce aberrant excitatory network activity and compensatory inhibitory responses involving learning and memory circuits, leading to cognitive dysfunction. In fact, Palop et al., 2007 report that transgenic mouse models of AD overexpressing Aβ peptide exhibit altered neuronal activity, spontaneous seizures and epileptiform discharges within the entorhinal-hippocampal circuitry. They propose that the epileptiform activity together with homeostatic responses to this epileptiform activity may contribute
to dysfunction of the circuitry that underlies memory formation because blocking Aβ-induced epileptiform discharges can meliorate cognitive decline and behavior dysfunction in these AD mouse models.

Soluble, rather than amyloid plaques, correlates well with the severity of cognitive decline and leads to malfunction of neurons. In fact, the hippocampal region of AD mouse model has an increased proportion of hyperactive neurons prior to the formation of Aβ plaques (Cleary JP, et al. 2005). Moreover, application of Aβ1-42 to the extracellular medium induces CA1 neuron hyperactivity in wild type mice (Minkeviciene et al. 2009). These findings demonstrate that soluble Aβ is involved in the neuronal hyperexcitation, aberrant network activity and cognitive impairment in AD.

However, the molecular mechanisms by which Aβ can contribute to the destabilization of neuronal networks are poorly understood.

1.1.4 Mouse Models Of Alzheimer’s Disease

Various transgenic models of Alzheimer disease (AD) were generated in the last decade in order to advance our understanding of in vivo responses to amyloid insult and the mechanism by which genetic alterations may cause AD. Indeed more features of the human disease are represented in these mice. Moreover, the mice are now being used to test therapeutic agents that may have utility in patients with AD.

The discovery of genes for familial forms of AD has allowed to create transgenic models that reproduce many critical aspects of the disease. Initially, before the discovery of FAD mutations, attempts were made to overexpress wild-type APP in transgenic mice. However, none of these efforts produced
anything that resembled an Aβ plaque or any other recognizable AD-type pathology. After the discovery of FAD mutations in APP, a number of groups turned their attention to making AD models based on the overexpression of transgenes containing FAD mutations using a variety of promoters.

Mutations in APP linked to FAD include Dutch (E693Q), London (V717I), Indiana (V717F), Swedish (K670N/M671L), Florida (I716V), Iowa (D694N), and Arctic (E693G) mutations. To date, more than 160 mutations in PS1 linked to FAD have been discovered. Mutations in a PS2 gene were soon linked to FAD as well. Most of FAD mutations cause aberrant APP processing toward the longer, more amyloidogenic Aβ1-42 species. The Swedish mutation, which is located just outside the N-terminus of the Aβ domain of APP, favors β-secretase cleavage \textit{in vitro} and is associated with an increased level and deposition of Aβ1-42 in AD brain.

Report of the first transgenic mouse to develop a robust AD-related phenotype was published in 1995 (Games D \textit{et al.}, 1995). This line, named PDAPP, overexpresses a human APP transgene containing the Indiana mutation (V717F). After that, in 1996, Karen Hsiao and colleagues created a second mouse line, termed Tg2576, which overexpresses a human APP transgene containing the Swedish mutation (K670N/ M671L). Subsequently, many other transgenic lines were developed with approaches similar to those used to develop PDAPP and Tg2576 mice, characterized by overexpression of one or more human APP mutations alone or combined with mutations of PS1 gene (\textit{Table 1}).
Table 1: mouse models of Alzheimer’s Disease (Schaeffer EL et al. 2011).

Table 1 - Neuropathological features of the main transgenic mouse models of Alzheimer disease.

<table>
<thead>
<tr>
<th>Mouse model</th>
<th>Gene mutation</th>
<th>Intraneuronal Aβ</th>
<th>Parenchymal Aβ plaques</th>
<th>Hypophosphorylated Tau</th>
<th>Neurofibrillary tangles</th>
<th>Neuronal loss</th>
<th>Synaptic loss</th>
<th>CAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>POA-P</td>
<td>APP (V177F)</td>
<td>-</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Tg2576</td>
<td>APP (K670M/671L)</td>
<td>Yes</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
<td>No</td>
<td>-</td>
<td>No</td>
</tr>
<tr>
<td>TgCRND8</td>
<td>APP (K670M/671L, V717F)</td>
<td>Yes</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>APPPS1</td>
<td>APP (K670M/671L)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>APP195</td>
<td>APP (K670M/671L)</td>
<td>-</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Little</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>TgSweD</td>
<td>APP (E693Q, D698E)</td>
<td>-</td>
<td>Yes</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>No</td>
</tr>
<tr>
<td>APPDutch</td>
<td>APP (E693Q)</td>
<td>-</td>
<td>Little</td>
<td>Little</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>APPDutch/PSI</td>
<td>APP (E693Q)</td>
<td>-</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>hAPP-Scn</td>
<td>APP (E693Q, K670M/671L, V717F)</td>
<td>-</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TgArcWe</td>
<td>APP (E693Q, K670M/671L)</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>APPAc</td>
<td>APP (E693Q)</td>
<td>-</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>TgAc-AD</td>
<td>APP (K670M/671L), Tau (P301L)</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>APP+mAD</td>
<td>APP (E693Q, K670M/671L)</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>APP+mPS1</td>
<td>APP (K670M/671L, V717F)</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>APP+mPS1</td>
<td>APP (K670M/671L, V717F)</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>S1/AD</td>
<td>APP (K670M/671L, I716V, V717F)</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

CAA = cerebral amyloid angiopathy; Dash (-) = not reported.

Table 1: mouse models of Alzheimer’s Disease (Schaeffer EL et al. 2011).

Tg2576 have been the most widely studied AD transgenic model because exhibits age-dependent increase of Aβ1-40 and Aβ1-42 levels and Aβ deposition, resulting in senile plaques similar to those found in AD. Aβ plaques were first clearly seen by 11-13 months, eventually becoming widespread in cortical and limbic structures. Aβ deposits were associated with prominent gliosis and neuritic dystrophy, without overt neuronal loss in the hippocampal CA1 field or apparent synapse loss in the hippocampal dentate gyrus. Tg2576 mice exhibited deficits in synaptic plasticity in the hippocampal CA1 field and dentate gyrus, decreased dendritic spine density in the dentate gyrus, and impaired spatial memory and contextual fear conditioning months before significant Aβ deposition, which was detectable at 18 months of age. A spine density decrease was detected as early as 4 months of age, and synaptic dysfunction and memory impairment were observed by 5 months. Moreover, an increase in the ratio of soluble Aβ1-42/Aβ1-40 was first observed at these early ages (4-5 months). Tg2576 mice also showed increased intraneuronal Aβ1-42
accumulation with aging, and this accumulation was associated with abnormal synaptic morphology before Aβ plaque pathology.

1.2 Intrinsic membrane properties of Neurons and Voltage Gated Na\(^+\) Channels

Neurons are highly polarized cells with multiple distinct membrane domains by which they can integrate excitatory and inhibitory synaptic potentials into an output of action potentials. Both, the input/output relation of a neuron and the waveform of an action potential depend on the intrinsic properties of the neuron. These are determined by the neuron’s endowment of voltage- and ion-gated ion channels. In neurons, action potentials (APs) are generated at the axonal initial segment (AIS), and their saltatory conduction occurs via the nodes of Ranvier in myelinated axons.

These processes require a precise distribution of voltage-gated sodium (Nav) channels, which accumulate at high density in these two highly specialized axonal sub-domains and upon depolarization permit the influx of Na\(^+\) ions responsible of the rapid upstroke of action potentials.

1.2.1 Voltage Gated Na\(^+\) Channels Structure

Na\(_V\) channels are integral membrane proteins that are predominantly expressed in excitable cells such as muscle cells and neurons. Expression of voltage gated Na\(^+\) channels was also reported in other cell types such as glial cells ([Chiu et al., 1984] and endothelial cells (Gordienko & Tsukahara, 1994).

Mammalian voltage-gated Na\(^+\) channels (Na\(_v\)) complex is typically a heterodimeric or heterotrimeric structure with a single pore-forming 260 kDa α
subunit associated with one or two accessory β-subunits (33-36 kDa). The α-subunit contains the Na\(^+\) selective pore, the voltage sensor, and the channels activation and inactivation gate. It has been shown that α-subunits alone are sufficient to give rise to a voltage sensitive Na\(^+\) current when expressed in various expression systems (Catterall, 2000). Association of β-subunits modulates Na\(^+\) channel kinetics and voltage dependence of activation and inactivation, and regulate the surface density of Nav1 (Isom, 2001, Isom, 2002, Qu et al., 2001). (Fig. 2)

Figure 2. Topology of voltage-gated Na\(^+\) channel α and β subunits (Brackenbury and Isom, 2011).

A functional voltage gated Na\(^+\) channel α-subunit contains four homologous domains (DI-DIV), each of them consisting of six transmembrane α-helices (S1-S6). Within each domain the S4 segment forms a part of the Na\(^+\) channel’s voltage sensor, which undergoes a conformational change upon depolarization whose movement within the membrane induces in pore opening (Terlau and
Stühmer, 1998). The transmembrane α-helices S5 and S6 form the inner part of the ion channel pore, whereas the linker region between these two segments reaches into the outer leaflet of the membrane's lipid bilayer and forms the outer part of the ion channel pore. The intracellular loop between domain III and IV serves as an inactivation gate that blocks the open channel upon prolonged depolarization (Stühmer et al., 1989, Catterall et al., 2005).

The linkers between the four domains vary in length and have important functions in channel modulation, inactivation, and drug binding as well as the binding sites for the toxins tetrodotoxin (TTX) or μ-conotoxin. Interactions with other proteins also take place within these linker domains. Such interactions are either intracellularly, as the binding to cytoskeleton associated proteins or extracellularly, as binding to Na⁺ channel β-subunits.

β-Subunits of Naᵥ belong to the immunoglobulin superfamily of cell adhesion molecules and associate with α-subunits in two ways: covalently in the case of Naᵥβ2 and Naᵥβ4 subunits through a disulfide bridge in the extracellular domain and non-covalently for Naᵥβ1 and Naᵥβ3 subunits (Patino and Isom, 2010). Naᵥ β-subunit expression is widespread both in excitable and non-excitable tissues (Patino and Isom, 2010).

The Naᵥβ subunits share a similar topology. Structurally, they consist of a single transmembrane domain and larger extracellular than intracellular domains. Naᵥβ-subunits are multifunctional, acting to modify channel gating, regulate channel expression in the plasma membrane; independent of their role in sodium channel association, they serve as cell adhesion molecules in interactions with the extracellular matrix and as well as the cytoskeleton and intracellular signaling molecules (Isom, 2002; Brackenbury and Isom, 2011); in
fact, β-subunit soluble ectodomain and membrane bound C-terminal fragment obtained by their enzymatic cleavage are implicated in the regulation of cell–cell contact and neurite outgrowth (Wong et al., 2005).

1.2.2 Sodium Channel Classification and Nomenclature

The sodium channels are members of the superfamily of ion channels that includes voltage-gated potassium and calcium channels (Yu and Catterall, 2004); however, unlike the different classes of potassium and calcium channels, the functional properties of the known sodium channels are relatively similar.

In the standardized nomenclature system, the name of an individual channel consists of the chemical symbol of the principal permeating ion (Na⁺) with the principal physiological regulator (voltage) indicated as a subscript (Naᵥ). The number following the subscript indicates the gene subfamily (currently only Naᵥ1), and the number following the full point identifies the specific channel isoform (e.g., Naᵥ1.1). This last number has been assigned according to the approximate order in which each gene was identified. Splice variants of each family member are identified by lowercase letters following the numbers (e.g., Naᵥ1.1a). In mammals, there are nine different genes, Scn1a to Scn11a, encoding the nine monomeric α-subunits of voltage gated Na⁺ channels, Naᵥ1.1 to Naᵥ1.9 which share about 80% of their sequence (Catterall et al., 2005) (Fig. 3).
Table 1. Mammalian sodium channel α subunits

<table>
<thead>
<tr>
<th>Type</th>
<th>Gene symbol</th>
<th>Chromosomal location</th>
<th>Primary tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na(\alpha).1</td>
<td>SCN1A</td>
<td>Mouse 2 Human 2q24</td>
<td>CNS neurons</td>
</tr>
<tr>
<td>Na(\alpha).2</td>
<td>SCN2A</td>
<td>Mouse 2 Human 2q23–24</td>
<td>CNS neurons</td>
</tr>
<tr>
<td>Na(\alpha).3</td>
<td>SCN3A</td>
<td>Mouse 2 Human 2q24</td>
<td>CNS neurons</td>
</tr>
<tr>
<td>Na(\alpha).4</td>
<td>SCN4A</td>
<td>Mouse 11 Human 17q23–25</td>
<td>SkM</td>
</tr>
<tr>
<td>Na(\alpha).5</td>
<td>SCN5A</td>
<td>Mouse 9 Human 3p21</td>
<td>Uninnervated SkM, heart</td>
</tr>
<tr>
<td>Na(\alpha).6</td>
<td>SCN8A</td>
<td>Mouse 15 Human 12q13</td>
<td>CNS neurons</td>
</tr>
<tr>
<td>Na(\alpha).7</td>
<td>SCN9A</td>
<td>Mouse 2 Human 2q24</td>
<td>PNS neurons</td>
</tr>
<tr>
<td>Na(\alpha).8</td>
<td>SCN10A</td>
<td>Mouse 9 Human 3p22–24</td>
<td>DRG neurons</td>
</tr>
<tr>
<td>Na(\alpha).9</td>
<td>SCN11A</td>
<td>Mouse 9 Human 3p21–24</td>
<td>DRG neurons</td>
</tr>
<tr>
<td>Na(\alpha)</td>
<td>SCN7A</td>
<td>Mouse 2 Human 2q21–23</td>
<td>uterus, astrocytes, hypothalamus</td>
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</tbody>
</table>

Figure 3. Mammalian Na\(\alpha\) subunits (J Physiol 590.11 (2012))

Five sodium channel β subunits have been described so far: Na\(\beta\)1, Na\(\beta\)2, Na\(\beta\)3, and Na\(\beta\)4, encoded by four different genes (SCN1B–SCN4B).

### TABLE 2: The β Subunits

<table>
<thead>
<tr>
<th>Protein</th>
<th>Gene Symbol (Human)</th>
<th>Tissue Location</th>
</tr>
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<tbody>
<tr>
<td>β1</td>
<td>SCN1B</td>
<td>Heart, skeletal muscle, CNS, glia, PNS</td>
</tr>
<tr>
<td>β1A (β1E3)</td>
<td>SCN1B</td>
<td>Heart, skeletal muscle, adrenal gland, PNS</td>
</tr>
<tr>
<td>β2</td>
<td>SCN2B</td>
<td>CNS, PNS, heart, glia</td>
</tr>
<tr>
<td>β3</td>
<td>SCN3B</td>
<td>CNS, adrenal gland, kidney, PNS</td>
</tr>
<tr>
<td>β4</td>
<td>SCN4B</td>
<td>Heart, skeletal muscle, CNS, PNS</td>
</tr>
</tbody>
</table>

Abbreviations: VGSC = voltage-gated Na\(\alpha\) channel; PNS = peripheral nervous system; DRG = dorsal root ganglion.

Figure 4. Classification and distribution of Na\(\alpha\)β subunits (Branckebury et al., 2008)
1.2.3 Voltage Gated Na\(^+\) Channels Functional Expression

The voltage-gated Na\(^+\) channels Na\(_V\)1.1–1.9 are all expressed in excitable tissues. With the exception of Na\(_V\)1.4, that is expressed only in the skeletal muscle, all Na\(_V\)1 subunits are expressed in the nervous system. Na\(_V\)1.1, 1.2, 1.3, and 1.6 are most predominant subtypes in CNS. Na\(_V\)1.5 is the major cardiac sodium channel, whereas Na\(_V\)1.7, 1.8, and 1.9 are important players in nociceptive signaling transduction owing to their presence in peripheral primary sensory afferents. (Catterall et al., 2005).

Na\(_V\)\(\beta\)1, Na\(_V\)\(\beta\)2 and Na\(_V\)\(\beta\)4 are the main Na\(_V\)\(\beta\) subunits expressed in the mammalian brain.

Both the expression of the four predominant Na\(_V\)1 subtypes and the four \(\beta\) subunits are developmentally and spatially regulated in the CNS. Nav1.3 channels are primarily expressed in embryonic and neonatal rodent brain, whereas it is poorly expressed in the rodent adult brain. In contrast, Na\(_V\)1.3 expression remains high in adult human brain (Chen et al., 2000; Whitaker et al., 2001). Nav1.1, Nav1.2, and Nav1.6 display developmentally regulated expression patterns in specialized neuronal subcellular domains. In fact, Nav1.1 is localized primarily in the soma of CNS neurons; it is also found in the dendrites, but it is dominant at the AIS of GABAergic neurons, retinal ganglion cells and in spinal cord motoneurons and nodes of Ranvier. Nav1.6 and Nav1.2 are principally associated with AIS of myelinated and unmyelinated axons, respectively, with Nav1.2 expressed first during development, then being gradually replaced by Nav1.6 concomitantly with myelination (Kaplan et al., 2001). Although greatly diminished, the expression of Nav1.2 might persist in the AIS of adult neurons and is maintained in populations of unmyelinated...
axons (Jarnot and Corbett, 1995; Boiko et al., 2003). The two isoforms coexist in the AIS of L5 pyramidal neurons with a proximal distribution of NaV1.2 and a distal distribution of NaV1.6. Sodium channels in the distal part of the AIS display the lowest threshold, suggesting that this polarized distribution could explain the unique properties of the AIS, including action potential initiation (principally mediated by Nav1.6) and backpropagation (largely supported by NaV1.2). A similar conclusion is drawn in CA1 pyramidal neurons where NaV1.6 sodium channels play a critical role for spike initiation. Nav1.6 is also concentrated nodes of Ranvier in myelinated axons (Schaller and Caldwell, 2000; Boiko et al., 2001; Boiko et al., 2003; Van Wart and Matthews, 2006; Van Wart et al., 2007; Lorincz and Nusser, 2008, 2010), and is found at lower abundance in neuronal soma and proximal dendrites (Krzemien et al., 2000; Lorincz and Nusser, 2010).

Little information is available on β subunit localization at the cellular level, although NaVβ2 may be concentrated at the nodes of Ranvier and NaVβ1 has been detected at the AIS in cerebellar GCs. Sodium channels in the adult central nervous system contain NaVβ1 through NaVβ4 subunits (Isom, 2001).

1.2.4 Types of NaV currents

The voltage gated Na⁺ channels give rise to a prominent transient Na⁺ current (I_{NaT}) and to two smaller Na⁺ currents, namely the persistent Na⁺ current (I_{NaP}) (French et al., 1990) and the resurgent Na⁺ current (I_{NaR}) (Raman & Bean, 1997).

The transient Na⁺ current initiates and mediates the fast rising phase of the action potential. At resting membrane potentials, Nav channels are closed,
requiring depolarization to be activated. A small depolarization of the neuronal membrane potential in response to sensory input or receptor input depolarizes the neuronal membrane potential to the threshold for NaV activation (~ -50 mV). NaV channels activate rapidly (~1 ms to peak) allowing the influx of sodium in an inward direction dictated by the electrochemical gradient and depolarizing the membrane potential further, forming the upstroke of the action potential. NaV channels close within 1–2 ms of opening, a process called fast inactivation that changes the channels to a non-conducting state contributing to the downstroke of the action potential. Channel inactivation persists throughout the depolarizing pulse, thus underlying the action potential refractory period. In addition to fast inactivation, Na+ channels also undergo slow inactivation, which does not primarily depend on the fast inactivation gate. Following hyperpolarization of the membrane potential, the channel recovers from inactivation by returning to the closed, resting state and is re-primed and available again for activation. Recovery from fast as well as slow inactivation is time dependent and leads to a refractory period during which depolarization fails to open the channel pore. Moreover, recovery from inactivation allows the channels to participate in the next action potential and it is required for repetitive firing of action potentials in neural circuits and for control of excitability in nerve and muscle cells.

All the kinetically fast transient channels (NaV1.1–1.7) appear quite similar in functional properties, but in many neurons sodium channels sometimes generate much longer openings as a result of incomplete or defective fast inactivation of Na+ channels and a persistent Na+ (INaP) current can be recorded (French et al., 1990, Crill, 1996, Magistretti & Alon-so, 1999).
$I_{NaP}$ inactivates over a time period of tens of seconds and its amplitude is just a few per cent of that of the transient current at the same potentials, but is still functionally important. The hyperpolarized voltage dependence of activation of persistent sodium currents allows these channels to operate as amplifiers of subthreshold depolarization, because their activation kinetics are fast and they operate over a strategic subthreshold membrane potential range with low potassium channel activation.

In some neurons, sodium channels transiently open upon recovery from inactivation when the membrane potential is repolarized. This transient opening gives rise to a large inward tail current termed \textbf{resurgent current} $I_{NaR}$ (Cannon and Bean, 2010), that may be caused by a temporary block of the channel by an open-state channel blocker that prevents entry of channel into the true inactive state. In fact, this blocker precludes the entry of the inactivation gate into the pore resulting in a temporarily inactive state that is easily reversed by minor hyperpolarization of the cell (Aman and Raman, 2010). The inactivation released upon repolarization is thought not to depend on the inactivation gate of the Na$^+$ channel $\alpha$-subunit itself. One possible mechanism for resurgent current involves the blockade of the channel pore by the C-terminus of NaV$\beta$4 subunit of sodium channels, serving as an additional inactivation gate (Chen \textit{et al.}, 2008 and Aman \textit{et al.}, 2009). This rapidly reversible form of inactivation allows neurons to fire rapidly and repetitively.

\textbf{1.2.5 Molecular basis of Na$V$ currents}

The molecular mechanism by which changes in membrane voltage confer a conformational change on voltage-gated ion channel proteins is
through the movement of modular voltage sensors contained within the S4 segment of domains I–IV (Alabi et al., 2007). Transmembrane segment S4 of each domain contains 3-5 positively charged residues that are essential for the channel response to changes in membrane potential. In response to membrane depolarization, electrostatic interactions between positively charged residues and the depolarized cytoplasm force S4 towards the extracellular surface. This results in a conformational change in S5-S6 that opens the channel pore and allows sodium influx (Yarov-Yarovo Y et al., 2012). S4 segment of domains IV is also implicated in the voltage-dependent coupling of activation to inactivation. In fact, outward movement of this segment is the signal to initiate fast inactivation of the sodium channel by closure of the intracellular inactivation gate (Catterall, 2000).

The short highly conserved intracellular loop connecting homologous domains III and IV of the sodium channel α subunit serves as an inactivation gate. This loop moves into the pore region, binds to the intracellular pore of the channel to inactivate it, preventing any additional current flow. Mutagenesis studies of this region revealed that a hydrophobic triad of isoleucine, phenylalanine, and methionine (IFM) is critical for fast inactivation; moreover, among these three amino acids, phenylalanine residue (F1489) is fundamental for the inactivation because it forms a hydrophobic interaction with an inactivation gate receptor during inactivation (Catterall, 2000).

1.3 Na\textsubscript{v}1.6 Sodium Channel

Voltage-gated sodium channel Na\textsubscript{v}1.6 is encoded by the Scn8a gene, located on distal chromosome 15 in mouse (Burgess et al., 1995b) and on
chromosome 12q13.13 in human (Plummer et al., 1998). Scn8a is a large gene, with 27 exons, and encodes a 1980 residue protein.

Nav1.6 is broadly expressed in the nervous system in a variety of cells including Purkinje cells, motor neurons, pyramidal and granule neurons, glial cells and Schwann cells and is enriched at the nodes of Ranvier (Caldwell et al., 2000; Kearney et al., 2002). Nav1.6 is concentrated at the axon initial segment and nodes of Ranvier in myelinated axons (Schaller and Caldwell, 2000; Boiko et al., 2001; Boiko et al., 2003; Van Wart and Matthews, 2006; Van Wart et al., 2007; Lorincz and Nusser, 2008, 2010), and is also found at lower abundance in neuronal soma and dendrites (Krzemien et al., 2000; Lorincz and Nusser, 2010).

1.3.1 Unique Biophysical Properties Of Na\textsubscript{v}1.6

The electrophysiological properties of Nav1.6 have been characterized in cultured cells and in neurons from mouse. Cell culture studies allow Nav1.6 to be studied in isolation from other channels, which is important for understanding its specific electrophysiological properties. In vivo studies of Nav1.6 have focused on using null mouse mutants to remove this channel in the context of the entire neuron, and provide critical insight into the role of this channel in the complex regulation of neuronal firing.

The role of Na\textsubscript{v}1.6 in regulating neuronal excitability may be related to the following properties: its voltage dependence of activation, its subcellular localization at the axon initial segment (AIS), the site of initiation of action potentials, and its role in persistent and resurgent current. The highest density of the Na\textsubscript{v}1.6 subunit is present in the AISs of all major neuron types of the
neocortex, hippocampus, cerebellum. The Na\textsubscript{V}1.6 is preferred over the other Na\textsubscript{V}α-subunits in the AISs because its activation threshold is shifted to a more hyperpolarized potential (by ~15–25 mV) compared with that of Nav1.2 (Rush et al., 2005) and Nav1.1 subunits (Spampanato et al., 2001), ensuring lower threshold for AP generation. Moreover, Na\textsubscript{V}1.6 shows a reduced use-dependent inactivation at frequencies >20 Hz respect Nav1.2 (Rush et al., 2005) and Nav1.1 subunits (Spampanato et al., 2001) show a robust (Rush et al., 2005), which would allow high frequency firing of nerve cells. Indeed, cerebellar Purkinje cells from mice lacking the Nav1.6 subunit (Raman et al., 1997) showed impaired ability to fire bursts of APs at high frequencies.

The persistent current generated by Nav1.6 is five-fold higher than that generated by Na\textsubscript{V}1.2 (Smith et al., 1998, Rush et al., 2005, Chen et al., 2008). The larger persistent sodium currents of Na\textsubscript{V}1.6 channels maintain depolarization of membrane potential near threshold and thereby permit firing of additional action potentials. The magnitude of persistent current depends on the specific cell type (Rush et al., 2005; Chen et al., 2008), suggesting that this property can be modulated by other factors and not only exclusively attributed to this subunit [Ma et al., 1997].

Resurgent current has been widely associated with the presence of Na\textsubscript{V}1.6. Indeed, in Scn8amed neurons lacking Na\textsubscript{V}1.6 the amplitude of $I_{\text{NaR}}$ is reduced. The resurgent currents of Nav1.6 channels generate inward current after each action potential and permit rapid recovery from inactivation, thereby facilitating high frequency firing (Raman and Bean, 1997; Levin et al., 2006).

Larger relative levels of persistent and resurgent current render membranes containing Na\textsubscript{V}1.6 more excitable than membranes containing the
neuronal channels Na\textsubscript{V}1.1 and Na\textsubscript{V}1.2. Persistent current is important for populations of neurons that undergo repetitive firing and it has been hypothesized that resurgent current contributes to increased excitability leading to spontaneous firing and multi-peaked action potentials.

1.3.2 Regulative Protein Interactions Of Na\textsubscript{V}1.6

Voltage-gated sodium channels are components of large, multi-protein complexes that vary between neurons and at specific subcellular domains. These multiple binding partners regulate gating properties and subcellular localization (Dib-Hajj and Waxman, 2010) of Na\textsubscript{V} channels. Several protein interaction sites have been mapped to the intracellular loops and C-terminus of the channels.

**Microtubule-associated protein Map1b.** Map1b is a cytoskeletal protein that binds microtubules and actin (Riederer, 2007) and contributes to trafficking of several channel and receptor proteins. O’Brien et al. 2012 demonstrated that Nav1.6 is another neuronal protein that is trafficked along the microtubule network to the cell surface by interaction with the light chain of Map1b. Since microtubules extend along the full length of the axon, Map1b could play a role in localization of Nav1.6 to both the AIS and the nodes of Ranvier. The residues 77-80 (VAVP motif) in the N-terminus of Na\textsubscript{V}1.6 are responsible of interaction with the light chain of Map1b. Interestingly, this interaction may be specific to Na\textsubscript{V}1.6 because the VAVP motif is not conserved in the other Na\textsubscript{V} channels; in fact Na\textsubscript{V}1.1 and Na\textsubscript{V}1.2 bind Map1b at a reduced affinity compared to Na\textsubscript{V}1.6 due to the difference in residues of the binding site. Moreover, O’Brien *et al.*, 2012 demonstrated that the interaction with Map1b
increases Nav1.6 peak current density resulting in an increase in current density enhancing trafficking of Nav1.6 to the cell surface without a change in activation or fast-inactivation of the channel.

**Protein kinases.** PKA and PKC have only a small effect on Nav1.6 channel activity (Chen *et al.*, 2008); in fact, the reduction of its current is only 7% by PKC and 8% by PKA, despite multiple predicted PKA and PKC phosphorylation sites (Chen *et al.*, 2008).

Immunohistochemistry of hippocampal neurons demonstrated that the MAP kinase p38 co-localizes with Nav1.6 (Gasser *et al.*, 2010). This stress-activated kinase phosphorylates Na\(_V\)1.6 at serine 553 of the first intracellular loop (Wittmack *et al.*, 2005). This phosphorylation creates a PXpS/TP motif (residues 551-554) that facilitates binding of E3 ubiquitin ligases, Na\(_V\)1.6 internalization and proteasomal degradation (Sudol and Hunter, 2000; Zarrinpar and Lim, 2000; Gasser *et al.*, 2010) inducing Nav1.6 current amplitude reduction, as observed in the ND7/23 cell line (Wittmack *et al.*, 2005) and in hippocampal neurons (Gasser *et al.*, 2010) treated with p38 activator, Anisomycin. Moreover, no effect of p38 on sodium current was observed in Scn8a null (*med*) hippocampal neurons, suggesting that Na\(_V\)1.6 is the predominant sodium channel target of activated p38 (Gasser *et al.*, 2010).

**Ankyrin (Ank).** Ankyrins are adaptor proteins that attach membrane proteins to spectrin components of the cytoskeleton. In the higher vertebrates, three genes encoding for Ankyrins (Ankyrin-R, Ankyrin-B and Ankyrin-G) are been identified: ANK1, ANK2 and ANK3. Direct interaction between ankyrins and voltage-gated sodium channels is well documented (Srinivasan *et al.*, 1988; Davis *et al.*, 1996; Hill *et al.*, 2008). All vertebrate voltage-gated sodium
channels share a conserved ankyrin-binding motif (nine residues) in the second cytoplasmic loop (Nav1.6 residues 1089-1122) (Lemaillet et al., 2003; Gasser et al., 2012), that is necessary for binding to Ankyrin G. It has been recently demonstrated that AnkG binding is essential for targeting and localization of Nav1.6 to the AIS and nodes of Ranvier (Gasser et al., 2012). Interestingly, mutations in conserved residues of the ankyrin-binding motif do not alter the electrophysiological properties of Nav1.6 (Gasser et al., 2012).

**Voltage-gated sodium channel β subunits.** Naᵥβ1 (36 kDa) and Naᵥα subunits are bound in non-covalent manner. In particular, negatively charged residues of extracellular domain and intracellular portion of Naᵥβ1 are involved in the functional interaction with α subunit (McCormick et al., 1998; Spampanato et al., 2004). Biskup et al. 2004 demonstrated that Naᵥβ1 and Naᵥα subunit associate in the endoplasmic reticulum, after that they reach the plasma membrane as a complex; this association is fundamental for the correct delivery to the membrane and localization of Naᵥα subunit in specific neuronal domains.

Interaction between Naᵥβ1 and Nav1.6 is required for function of Naᵥ1.6 at the distal AIS (Brackenbury et al., 2010). In fact, Naᵥβ1 mediate functional association with AIS protein scaffold. Studies of mice null for the β1 subunit (Scn1b-/-) suggest that interaction between β1 and Naᵥ1.6 is required for wild-type expression levels of Nav1.6 at the distal AIS in vitro and in vivo (Brackenbury et al., 2010). A higher proportion of Nav1.1 was observed at the AIS in cultured cerebellar granule neurons and cerebellar Purkinje neuron slices from Scn1b-/- mice (Brackenbury et al., 2010). As a consequence of reduced Naᵥ1.6 at the AIS, slightly reduced levels of resurgent current were
observed in cerebellar granule neuron slices (Brackenbury et al., 2010). Thus, the interaction between β1 and Na\textsubscript{V}1.6 is important for localization and function of the channel.

Sodium channel activity mediate neurite extension during development; in particular, functional reciprocity of Na\textsubscript{V}1.6 and Na\textsubscript{V}β1 is involved in this process. In fact, Brackenbury et al., 2010 demonstrated that transfection of Na\textsubscript{V}β1 subunits into a monolayer of Chinese Hamster Lung cells co-cultured with isolated mouse brain neurons positively affects neurite extension in wild type neurons. Transfection of Na\textsubscript{V} β1 had no effect on Scn8a null neurons in this co-culture system, demonstrating that some sodium channel current-dependent neurite outgrowth is mediated by Na\textsubscript{V}1.6.

Na\textsubscript{V}β4 associates covalently Na\textsubscript{V} α subunit by disulfide bridge; indeed, one of the five cysteine residue of extracellular domain is involved in this bound.

Interaction of Na\textsubscript{V}1.6 and Na\textsubscript{V}β4 has been implicated in the generation of resurgent current (Grieco et al., 2005; Aman et al., 2009). In cultured cerebellar neurons, the β4 subunit is required for generation of resurgent current and contributes to persistent current and repetitive firing (Bant and Raman, 2010). Knockdown of β4 by siRNA in cultured cerebellar granule cells reduced resurgent current from ~9% of transient current in control cells to ~3.7% in treated cells. β4 knockdown resulted in a 7.7 mV hyperpolarizing shift in the voltage dependence of inactivation and a decrease in repetitive firing, changes that are predicted to reduce neuronal excitability. Most, but not all, subpopulations of neurons that have resurgent current express the β4 subunit.
However, full-length β4 is not sufficient to generate resurgent sodium current.

**Calmodulin.** The intracellular concentrations of Ca$^{2+}$ effect gating properties of Na$_V$ channels activating Calmodulin/Calmodulin Kinase II complexes that bind IQ domain of Na$_V$ C-terminus (Deschenes et al., 2002; Maltsev et al., 2008; Mori et al., 2003).

Calmodulin (CaM) is a ubiquitous, small (16.7 kDa) calcium-binding protein that acts as a Ca$^{2+}$ sensor translating changes in cytoplasmic Ca$^{2+}$ into cellular responses by interacting with a diverse group of signaling molecules. Ion channels are prominent targets of CaM, for example L-type voltage-dependent Ca$^{2+}$ channels, but also Na$_V$ channels. All of the voltage-gated sodium channels contain an IQ motif in the C-terminus (Yu and Catterall, 2003; Feldkamp et al., 2011) by which they can associate CaM.

The IQ motif of Na$_V$1.6 is localized between residues 1902–1912 of carboxyl terminus of the channel. The same motif binds apo-CAM, the Ca$^{2+}$-deficient form of calmodulin, and Ca$^{2+}$-bound CAM (Bahler and Rhoads, 2002). Herzog et al., 2003 demonstrated that CaM regulates the current density and the kinetics properties of Na$_V$1.6 currents in a calcium-dependent manner. Indeed, the authors suggest that binding of apo-CAM to Nav1.6 accelerates its inactivation; addition of Ca$^{2+}$ to the system, converting apo-CAM to CAM, slowed Na$_V$1.6 inactivation by ~50% increasing excitability.

**Nedd4.** Nedd4 or Neuronal precursor cell-expressed developmentally downregulated 4 is an E3 ubiquitin ligase responsible of ubiquitination and degradation of protein containing WW domains with PY motif. Na$_V$ channels are target of ubiquitination by Nedd4. Na$_V$1.6 contains two binding sites for...
Nedd4, a PXY motif (residues 1943–1945) at the C-terminus, and the PXpS/pTP motif in the first cytoplasmic loop (residues 551–554) (Abriel et al., 2000; Sudol and Hunter, 2000; Fotia et al., 2004; Ingham et al., 2004), both necessary for internalization and degradation of Na\textsubscript{v}1.6 (Gasser et al., 2010).

1.3.3 Na\textsubscript{v}1.6 Role In Pathophysiological Conditions

Na\textsubscript{v}1.6 appears to be particularly important, because it is the principal channel at axonal sites where action potentials are generated. Juvenile lethality of Na\textsubscript{v}1.6 deficit at postnatal day 21 (P21) suggests that this channel is vital for impulse propagation later in life, when it substitutes Na\textsubscript{v}1.2.

Within the past year, de novo mutations of human SCN8A detected by exome sequencing have revealed a role for Nav1.6 in epilepsy and intellectual disability. Hypoactivity and hyperactivity of Nav1.6 are both pathogenic, but with different outcomes: haploinsufficiency is associated with impaired cognition (Trudeau et al., 2006; McKinney et al., 2008; Rauch et al., 2012) while hyperactivity can result in epilepsy (Veeramah et al., 2012).

Scn8a gene mutations causing Na\textsubscript{v}1.6 loss or disruption result in a variety of recessive neuromuscular phenotypes, including tremor, cerebellar ataxia, dystonia and paralysis, as naturally occurring med mutant Na\textsubscript{v}1.6 (Scn8amed) knockout mice (Meisler et al., 2002, 2004). The tremor and ataxia result from the altered biophysical properties, in particular reduced persistent and resurgent current and diminished spontaneous simple spikes (Levin et al., 2006; Harris et al., 1992; Raman et al., 1997). In addition, prefrontal cortex pyramidal cells and retinal ganglion cells of the homozygous Scn8amed
mutants also show reduced excitability (Maurice et al., 2001; Van Wart, A. and Matthews, 2006).

Na\textsubscript{v}1.6 has diverse and complex roles in epilepsy. Abnormally high levels of persistent Na\textsubscript{v}1.6 current causes neuronal hyperexcitability and leads to epilepsy (Veeramah et al., 2012). Gain of function mutations cause convulsive seizures, as shown in a patient with infantile epileptic encephalopathy (Veeramah et al., 2012). Conversely, Na\textsubscript{v}1.6 loss of function mutations cause absence seizures and are protective against convulsive seizures (Martin et al., 2007). These divergent effects between seizure types are presumably due to independent roles of Na\textsubscript{v}1.6 in separate epileptic networks.

1.4 Voltage-Gated Sodium Channels And Alzheimer’s Disease

Forms of epilepsy are accompanied by cognitive impairment. Elevated incidence of epilepsy has been demonstrated in patients with Alzheimer’s disease, as well in AD-related mouse models that have elevate levels of Aβ exhibit altered neuronal activity and hyperexcitability. Many papers highlight that Na\textsubscript{v} channels can give a strong contribute to the hypersynchronicity of neuronal brain circuits in AD. In fact, altered expression and processing of voltage-gated sodium channels have been described in AD mouse model and patients. Verret et al., 2012 identified alterations in Na\textsubscript{v}1.1 channel in human amyloid precursor protein (hAPP) transgenic mice and AD brains. In particular, they demonstrated a reduction of Na\textsubscript{v}1.1 levels in inhibitory Parvalbumin cells, which prominently express this Na\textsubscript{v} subunit. The authors correlated this reduction to Aβ-induced aberrant network activity and cognitive decline.
because restoring physiological levels of this channel in hAPP mice they are able to enhance inhibitory synaptic currents and decrease network hypersynchronization and memory deficits. Corbett et al., 2013 confirmed the involvement of Na\textsubscript{v}1.1 in AD aberrant neuronal activity; in fact, they proved that surface expression of this Na\textsubscript{v} channel is reduced in inhibitory and excitatory cortical neurons of Tg2576 AD mice model and that Na\textsubscript{v}\textbeta2 is responsible of this reduction. Kim et al., 2007 previously had demonstrated that intracellular domain (ICD) of Na\textsubscript{v}\textbeta2 is able to control the protein expression of Na\textsubscript{v}1 translocating to the nucleus and triggers expression of this Na\textsubscript{v}\alpha subunit. Interestingly, Na\textsubscript{v}\textbeta2 ICD is produced by proteolytic cleavage of BACE1 and \gamma-secretase enzymes, like APP. When Kim et al., 2007 evaluated the consequence of excessive cleavage of Nav\textbeta2 in overexpressing BACE1 transgenic mice, they found an surplus expression of Na\textsubscript{v}1.1. However, Na\textsubscript{v}1.1 is intracellularly retained, doesn’t translocate on the cellular membrane leading to reduced surface levels. Owing to this evidence, Corbett and colleagues demonstrated that AD Tg2576 mice, which express high levels of BACE1, exhibited increased Na\textsubscript{v}\textbeta2 cleavage, intracellularly retention and reduction of surface expression of Na\textsubscript{v}1.1 in cortex of APP mice, like in BACE1 transgenic mice, and spike-wave discharges and abnormal neuronal activity.

In addition to expression changes of one or more Na\textsubscript{v} subunits, it is possible that Aβ can induce neuronal circuit hyperactivity increasing Na\textsubscript{v} current density. In agreement with this possibility, antiepileptic drugs that block sodium channel activity are effective in reducing epileptiform discharges in mouse model of AD (Ziyatdinova et al., 2011). Moreover, it has been demonstrated
that acute (1-2 min) application of soluble Aβ_{1-42} leads to alterations of spontaneous firing in CA1 pyramidal neurons. Moreover, authors proved that acutely exposure to Aβ_{1-42} also increased density of persistent sodium currents and these current are responsible of CA1 pyramidal neuron hyperactivity (Shuan-cheng Ren et al., 2014). Nevertheless, they didn’t investigate the cellular mechanisms by which Aβ_{1-42} can rapidly upregulate persistent sodium currents; they only supposed that Na\textsubscript{V}1.6 could be responsible of this effect.
2. AIM OF THE STUDY

The goal of this work was to determine the role of the Na\(^+\) channel subunit Na\(_V\)1.6 to the Alzheimer’s Disease pathogenesis. In particular, we first sought to determine possible changes in Na\(_V\) current density after the exposure to the A\(_\beta\)_1-42 peptide in mouse wild type and in AD-related Tg2576 hippocampal neurons. Then, to assess the role of Na\(_V\)1.6 subunit, we analyzed the functional contribution of Na\(_V\)1.6 in the same experimental conditions in presence of siRNA direct against Na\(_V\)1.6 mRNA or of Anisomycin that promotes p38 MAP Kinase-mediated Na\(_V\)1.6 endocytosis.
3. MATERIALS AND METHODS

3.1 Drugs And Chemicals

Aβ₁₋₄₂, Poly(D)-lysine Hydrobromide Mol Wt 30,000-70,000 (P7280), Poly(D)-lysine Hydrobromide Mol Wt >300,000 (P7405), Cytosine β-D-arabinofuranoside (Ara-C), Anisomycin and mouse monoclonal anti-β-Tubulin, as well as all other materials for solution preparation, were from Sigma Aldrich (St. Louis, MO, USA). Tetrodotoxin (TTX), rabbit polyclonal anti-Naᵥ1.6 were from Alomone Labs (Jerusalem, Israel). Mouse monoclonal anti-MAP2 was from Sigma-Aldrich (Milan), HBSS, Eagle’s MEM 10X, horse serum (HS), fetal bovine serum (FBS), L-glutamine and phosphate buffered saline (PBS) were purchased from LifeTechnologies (Oslo, Norway). Protease inhibitor cocktail II was purchased from Roche Diagnostic.

3.2 Mice

Animals were kept under standard conditions of temperature, humidity and light, and were supplied with standard food and water ad libitum. Animals were handled in accordance with the recommendations of International Guidelines for Animal Research and the experimental protocol was approved by the Animal Care and Use Committee of “Federico II” University of Naples. All efforts were made to minimize animal suffering and to reduce the number of animal used.

Heterozygous male Tg2576 mice and wild-type littermates, obtained backcrossing male Tg2576 mice with F1 wild-type female, were used for all experiments. Tg2576 mice, purchased from commercial source [B6;SJL-
Tg(APPswe)2576Kha, model 1349, Taconic, Hudson, NY], are well-established AD-related mouse model carrying the human APP Swedish 670/671 mutation (K670N e M671L; Hsiao et al., 1996). F1 wild-type female (B6;SJL) littermates were obtained crossing female C57BL/6 with male SJL; C57BL/6 and SJL mice were purchased from Charles River.

3.2.1 Genotyping: PCR Analysis

Genomic DNA from mouse tails was isolated with salt precipitation method. Tails after the cut were incubated with tail digestion buffer (50 mM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0, 100 mM NaCl, 1% SDS) supplemented with Proteinase K (Sigma Aldrich, Milan, Italy) at a final concentration of 0.5 mg/ml and placed in water bath at 55-60°C overnight with mixing. This step should result in the complete solubilization of the tail fragment.

Genomic DNA from mouse embryonic tissue was extracted with phenol chlorophorm method. Embryonic brain tissue was kept during cerebral dissection and frozen immediately upon collection. After thawing, same volume of Trizol Reagent (Invitrogen) was added to each sample in order to homogenize the tissue and DNA was extracted following manufacturer guideline. DNA concentration and purity of each sample was quantified using Nanodrop Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE,US).

We used following primers to amplify the DNA region with human APP Swedish mutation on both types of genomic DNA: 5’-CTGACCACCTCGACCAGGTCTGGGT-3’ and 5’-GTGGATAACCCCTCCCCAGCCTAGACCA-3’ (Primm, Milan, Italy). 50 ng/µL of DNA were used for PCR reaction. The amplification protocol (30
cycles) was the following: 95°C for 45 s, 55°C for 60 s, 72°C for 60 s. Each 25-
µL reaction contained: 1U of AmpliTaq DNA Polymerase (Lucigen, US) and 0.5
µM of each primer. The amplification products were visualized on agarose (2%)
gel electrophoresis by loading approximately half (10 µL) of each reaction per
lane. The band of 466bp indicated the transgenic genotype, whereas its
absence indicated the wild type genotype.

3.3 Mouse Hippocampal Cell Cultures

Primary neuronal cultures were prepared from hippocampi of embryonic day
(E) 16 Tg2576 and wild-type littermate mice. Embryonic age (E) was calculated
by considering E0.5 the day when a vaginal plug was detected. Briefly, pregnant animals were anesthetized and sacrificed by cervical dislocation.
Hippocampal tissues from embryos were dissected in ice-cold dissecting
medium (HBSS supplemented with 27 mM glucose, 20 mM sucrose, 4 mM
sodium bicarbonate), centrifuged, and the resulting pellet was mechanically
dissociated with a fire polished glass pipette. Cells were resuspended in plating
medium consisting in Eagle’s MEM (MEM, Earle’s salts, supplied bicarbonate-
free) supplemented with 5% fetal bovine serum, 5% horse serum, 2 mM L-
glutamine, 20 mM glucose, 26 mM bicarbonate, and plated on 35mm culture
dishes or onto 18 mm glass coverslips (Glaswarenfabrik Karl Hecht KG, Sondheim, Germany) coated with 100 µg/ml poly(D)-lysine at a density of one
embryo hippocampi/1 ml. Three days after plating, non-neuronal cell growth
was inhibited by adding 10µM of cytosine arabinofuranoside. 24 hours after this
treatment, the planting medium was replaced by growth medium (Eagle’s
Minimal Essential Medium with 20 mM glucose, 26 mM NaHCO3
supplemented with 2mM L-glutamine and 10% horse serum. Neurons were
cultured at 37°C in a humidified 5% CO₂ atmosphere. All the experiments were performed between 8-16 days in vitro DIV.

3.4 Aβ Oligomers Preparation And Cellular Treatments

Aβ₁₋₄₂ oligomers were made according to the method described by Pannccione et al. (2012). Briefly, lyophilized Aβ₁₋₄₂ synthetic peptides were dissolved in PBS to obtain a 1 mM stock solution, incubated for 48 hours at 37°C to pre-aggregate the peptide, and stored at -20°C (Lorenzo and Yankner, 1994). Before all experiments, we tested a pre-aggregated preparation of the Aβ₁₋₄₂. SDS-PAGE was performed using monoclonal antibody 4G8 (Sigma Aldrich, Milan, Italy), which recognizes an epitope within residues 1-17 of human Aβ. Results showed that the oligomers between 18 and 32 kDa were the major species of Aβ₁₋₄₂ peptide in the preparation (data not shown).

Aβ₁₋₄₂ exposure was carried out in growth medium at the final concentration of 5 μM. When we performed time-course experiments, the Aβ₁₋₄₂ was added to culture medium at above mentioned concentration for 10 minutes, 1, 24, 48 and 72 hours and kept throughout the experiment.

3.5 Anisomycin Treatment

Tg2576 and wild type hippocampal neurons grown on glass coverslips for 12 and 15 DIV were pretreated for 30 minutes at 37°C in a humidified 5% CO₂ atmosphere with 10 μg/ml Anisomycin (Sigma Aldrich), a specific activator of MAP p38α. For electrophysiological experiments, at the end of the pre-treatment the culture medium was replaced with the bath solution. Same protocol was used for immunocytochemistry analysis.
3.6 Western-blot analysis

Hippocampal neurons from Tg2576 and wild-type mice were washed thoroughly to remove medium using PBS. To obtain total lysates for immunoblotting analysis, neurons were scraped in ice-cold RIPA buffer (50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 0.25% sodium deoxycholate, 150 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 0.1% SDS, Protease Inhibitor Cocktail II), sonicated, incubated for 1 hour on ice and centrifuged at 12000 g at 4 °C for 30 min. The total protein content of resulting supernatant was determined using the Bradford reagent.

Hippocampal tissues from Tg2576 and wild-type were homogenized in a glass teflon grinder (10 strokes at 500 rpm in about 1 min) using a lysis buffer containing (in mM): 250 sucrose, 10 KCl, 1.5 MgCl₂, 1 EDTA, 1 EGTA, 1 dithiothreitol, 20 HEPES, pH 7.5, (Angulo et al. 2004) and completed with Protease Inhibitor Cocktail II. Tissue suspensions were then sonicated and incubated for 1 hour on ice. After centrifugation at 12,000 g at 4 °C for 5 min, the supernatants were collected. The protein content of resulting supernatant was determined using the Bradford reagent.

70 μg of proteins were mixed with a Laemmli sample buffer; then, they are applied and resolved on 10% SDS-PAGE polyacrylamide gels. Following transfer onto nitrocellulose membranes (Hybond-ECL, Amersham Bioscience, UK), non-specific binding sites were blocked by incubation for 2 hrs at 4°C with 5% non-fat dry milk (Bio-Rad Laboratories, Milan, Italy) in TBS-T buffer; subsequently, incubated with primary antibodies overnight at 4°C. After three 10-min washes with TBT-T, the membranes were incubated 1 h with the appropriate secondary antibody. Excessive antibodies were then washed away.
three times (10 min) with TBS-T. Immunoblots were visualized by enhanced chemiluminescence (ECL) (Amersham-Pharmaccia-Biosciences, UK). Films were developed using a standard photographic procedure and the relative levels of immunoreactivity were determined by densitometry using ImageJ Software (NIH, Bethesda, MA, USA).

Primary antibodies used were: rabbit-polyclonal antibody anti-Na\(_v\)1.6 and rabbit-polyclonal antibody anti-Na\(_v\)β1 (1:1000; Alomone Labs); rabbit-polyclonal anti-p38α antibody (1:1000; Santa Cruz Biotechnology); mouse monoclonal anti-tubulin (1:3000; Sigma Aldrich).

### 3.7 Electrophysiological recordings

Transient Na\(^+\) currents (I\(_{\text{Na}}\)) were recorded, by patch-clamp technique in whole-cell configuration, in the following groups of hippocampal neuronal cultures: (a) wild-type, (b) wild-type+\(\text{Aβ}_{1-42}\), (c) wild-type+Anisomycin (d) Tg2576, (e) Tg2576+Anisomycin. Hippocampal neurons were plated on 25-mm glass coverslips for recording. Currents were filtered at 5 kHz and digitized using a Digidata 1322A interface (Molecular Devices). Data were acquired and analyzed using the pClamp software (version 9.0, Molecular Devices).

All recordings were performed at room temperature (20-21°C). The total inward Na\(^+\) current was measured by applying, from a holding potential of -70 mV, depolarizing voltage steps of ...-ms duration ranging from -70 to +50 mV. These were preceded by conditioning pulses at -100 mV lasting for .. s to allow full recovery from \(I_{\text{Na}}\) inactivation. Possible changes in cell size occurring after specific treatments were calculated by monitoring the capacitance of each cell.
membrane, which is directly related to membrane surface area, and by expressing the current amplitude data as current densities (pA/pF). The capacitance of the membrane was calculated according to the following equation: \( C_m = \tau_c \cdot I_0 / \Delta E_m (1 - I_\infty / I_0) \), where \( C_m \) is the membrane capacitance, \( \tau_c \) is the time constant of the membrane capacitance, \( I_0 \) is the maximum capacitance current value, \( \Delta E_m \) is the amplitude of the voltage step, and \( I_\infty \) is the amplitude of the steady-state current.

The neurons were perfused with External Ringer solution contained (in mM): NaCl 126, NaHPO4 1.2, KCl 2.4, CaCl2 2.4, MgCl2 1.2, glucose 10 and NaHCO3 18, TEA 20, and nimodipine 10 \( \mu \)M (pH 7.4). The pipette solution contained (mM): Kgluconate 145, Mg-ATP 1, and 0.1 CaCl2, 2 MgCl2, 0.75 EGTA, HEPES 10, pH 7.3. TEA was included to block delayed outward rectifier K+ components; nimodipine (10 \( \mu \)M) was added to external solution to block L-type Ca\(^{2+}\)-channels. TTX (50 nM) was added to the bath solution to isolate components of Na\(^+\)-currents flowing through TTX-sensitive Na\(^+\) channels.

### 3.8 Immunocytochemistry in hippocampal neurons

Hippocampal neurons obtained from Tg2576 and wild type mice were washed in cold PBS and fixed in 4\% (w/v) paraformaldehyde in PBS for 40 minutes at room temperature. After four 10-min washes in PBS, the cells were first pre-incubated in PBS containing 3\% (w/v) bovine serum albumin (Sigma, Milan, Italy) for 60 minutes and then with the primary anti-Na\(_V\)1.6 polyclonal antibody (1:1000) and primary anti-MAP2 monoclonal antibody (1:1000) at 4\(^\circ\) C overnight. Next, cells were washed in PBS and, for double
immunofluorescence, were instead incubated in a mixture of fluorescent labeled secondary antibodies (Alexa 488- or Alexa 594- conjugated anti-mouse or anti-rabbit IgGs) for 1h at room temperature. Cell nuclei were stained with Hoechst (Sigma, Milan, Italy). After the final wash, cells were mounted and coverslipped with Vectashield (Vector Labs, Burlingame, CA). Slides were analysed with a confocal microscope (Zeiss, Nikon Instruments, Florence, Italy) equipped with a CCD digital camera (Coolsnap-Pro, Media Cybernetics, Silver Springs, MD, USA) and Image Pro-Plus software (Media Cybernetics, Silver Springs, MD, USA).

3.9 Na\textsubscript{v}1.6 Silencing RNA Transfection

Small interfering RNA (siRNA) against Na\textsubscript{v}1.6 and the validated nonsilencing AllStars negative control siRNA that has no homology to any known mammalian gene were purchased from Qiagen (Milan, Italy). Two different predesigned siRNAs directed against mouse SCN8a transcript (GenBank accession number NM_001077499; Entrez Gene ID 20273) were tested: Mm_Scn8a_5 Flexitube siRNA (Cat.No. SI02671277) and Mm_Scn8a_6 Flexitube siRNA (Cat.No SI02713956), which bound two different coding sequences on Na\textsubscript{v}1.6 mRNA downstream of the transcription start site. The siRNAs were transiently transfected using HiPerFect Transfection Reagent (Qiagen) at a final concentration of 50 nM in serum free OptiMEM medium for 5 hours, at the end of which OptiMEM was replaced by growth medium.

Wild type hippocampal neurons were used for siRNA transfection experiments; the gene-silencing efficiency of siRNA was determined 48 hrs after transfection by electrophysiological measurements.
3.10 Statistical Analysis

Statistical analysis were performed with ANOVA followed by Newman test or Student t-test. Differences were considered to be statistically significant at \( p<0.05 \).
4. RESULTS

4.1 Aβ_{1-42} exposure raised Na\textsubscript{V} functional activity in hippocampal neurons

We first examined whether Aβ_{1-42} peptide effected sodium current density e/o kinetics in hippocampal neurons recording sodium currents by patch-clamp technique in whole-cell configuration. To this purpose, we exposed hippocampal neurons to Aβ_{1-42} peptide for different time, (10 minutes, 1, 24, 48, and 72 hours. Time-course experiments revealed that the exposure to 5 μM Aβ_{1-42} didn’t immediately modify Na\textsubscript{V} channels activity, as observed from electrophysiological recordings after 10 minutes exposure. On the other hand, 24 and 48 hrs of Aβ_{1-42} treatment induced a significant increase of Na\textsuperscript{+} current density, followed by a return to basal levels at 72 hours (Fig. 5A and 15). These currents were completely inhibited by the extracellular application of the selective sodium channel blocker TTX (50 nM) in control conditions and after exposure to 5 μM Aβ_{1-42} (Fig. 5D) indicating that this Na\textsuperscript{+}-influx is mediated by TTX-sensitive voltage-gated sodium channels.

Interesting, when we analysed the I–V-relationships for endogenous sodium currents in control and Aβ_{1-42}-treated hippocampal neurons, we observed that Aβ_{1-42} exposure not only increased the peak current but also modified the kinetics of Na\textsubscript{V} channels. In fact, 24 hrs Aβ_{1-42} treatment caused a significant leftward shift in the voltage dependence of activation: in fact, the peak current of Na\textsuperscript{+} currents occurred at -20 mV in the Aβ_{1-42}–exposed neurons respect -10 mV in the control (Fig. 5C). Interesting, in accordance with the
time-course experiments, at 48 and 72 hours Aβ1-42 exposure we observed that the progressive reduction of the peak current was associated with no significant changes in the voltage dependence of activation (Fig. 5C).

Figure 5. Aβ1-42 exposure raised NaV functional activity in hippocampal neurons. (A) I_{Na} traces recorded under control conditions and after exposure to Aβ1-42 for different time. (B) Quantification of I_{Na} represented in A. (C) I–V-relationships for endogenous sodium currents in control and Aβ1-42-treated hippocampal neurons. (D) I_{Na} traces recorded under control conditions and after Aβ1-42 exposure in presence of TTX and relative percentage of TTX-induced I_{Na} inhibition. All values are expressed as mean ± SEM of current densities in 3 independent experimental sessions (* p<0.05 versus respective controls).

4.2 Aβ1-42 fails to increase Na⁺ currents in NaV1.6 silenced mouse hippocampal cultures

In order to test which is the contribution of NaV1.6 activity to the Aβ1-42 effects on NaV currents, we explored the effects of selective knockdown of this subunit on the sodium currents recorded after the exposure to beta-amyloid peptide.

To this aim, we performed silencing experiments of NaV1.6. Firstly, we tested the silencing efficiency of two different NaV1.6 siRNAs, the Mm_Scn8a_5
and Mm_Scn8a_6, both at the concentration of 50 nM, transfecting wild type hippocampal neuronal cultures. Patch clamp experiments revealed that Mm_Scn8a_6 siRNA was more effective than Mm_Scn8a_5 to knock down Na\textsubscript{v}1.6 as revealed from the significant reduction of the Na\textsuperscript{+} currents (Fig. 6A and 6B). Then, we used Mm_Scn8a_6 siRNA to silence Na\textsubscript{v}1.6 expression before the exposure to Aβ1-42. Interestingly, knocking down of this sodium channel subunit before Aβ1-42-treatment (24 hours) counteracted the Na\textsubscript{v} currents increase (Fig. 6D and 6E).

**Figure 6** Aβ1-42 fails to increase Na\textsuperscript{+} currents in Na\textsubscript{v}1.6 silenced mouse hippocampal cultures. (A) Na\textsubscript{v}1.6 protein levels under control conditions and after siRNA transfection (B) I_{Na} traces recorded under control conditions and after siRNA transfection. (C) Quantification of I_{Na} represented in B. (D) I_{Na} traces recorded under control conditions and after Aβ1-42 exposure after siRNA transfection or Anisomycin treatment. (E) Quantification of I_{Na} represented in D. All values are expressed as mean ± SEM of current densities in 3 independent experimental sessions (* p<0.05 versus respective controls).
4.3 pp38-mediated internalization of Na\textsubscript{v}1.6 counteracts A\textbeta- induced I\textsubscript{Na}\textsuperscript{+} increase

To confirm that the increased density of I\textsubscript{Na} in the wild-type hippocampal neurons exposed to A\textbeta\textsubscript{1-42} for 24 hours is mainly due to Na\textsubscript{v}1.6 α-subunit, we added Anisomycin (10μg/mL) for 30 minutes at culture medium hippocampal neurons before recording sodium currents. Anisomycin is an antibiotic that is routinely used to activate p38 MAP kinases (Cano and Mahadevan, 1995). Wittmack et al. (2005) have been demonstrated that Anisomycin-activated α-pp38 phosphorylates Na\textsubscript{v}1.6 at a single serine residue (Ser553) within the sequence motif Pro-Gly-Ser553-Pro in loop 1 (Nav1.6/L1). This phosphorylation reduces selectively Na\textsubscript{v}1.6 current density because decreases the number of available channels by internalization of this channel.

In agreement with results of silencing experiments, patch clamp recordings revealed that Anisomycin-induced activation of p38α was able to prevented I\textsubscript{Na} increase in wild-type hippocampal neurons exposed to A\textbeta\textsubscript{1-42} peptide. More interestingly, as pp38α promoted only Na\textsubscript{v}1.6 endocytosis, the reduction observed of total Na\textsuperscript{+} current density indicated that Na\textsubscript{v}1.6 significantly contributes to A\textbeta\textsubscript{1-42}-induced I\textsubscript{Na}\textsuperscript{+} enhancement (Fig 6D and 6E).

4.4 Increased Na\textsubscript{v} Activity In Hippocampal Neurons Of AD Mouse Model Tg2576

To understand whether sodium-current densities are also increased in AD, hippocampal neurons obtained from a mouse model of Alzheimer’s disease, Tg2576, were used to record total sodium currents. Time-course recording experiments at different days in vitro revealed that hippocampal neurons from Tg2576 transgenic-mice displayed an early increase of Na\textsuperscript{+}.
current densities respect wild-type hippocampal neurons. In fact, \( \text{Na}_V \) activity was significantly increased already at 8 DIV in culture, reached the maximal value at 12 DIV, and showed a less pronounced, but still significant, increase at 15 DIV (Fig. 7A and 7B) in the transgenic neurons respect wild-type. These results are in agreement with those obtained in the hippocampal neurons exposed to \( \text{A}_{\beta 1-42} \).

Intriguingly, analysis of the I–V-relationships for endogenous sodium currents in wild-type and Tg2576 hippocampal neurons revealed that 12 DIV in culture Tg2576 neurons displayed a significant leftward shift in the voltage dependence of activation compared to wild-type neurons, with current peak at -30mV rather than at -10 mV of wild-type neurons (Fig. 7C), suggesting that the Tg2576 hippocampal neurons have an increased number of channels open at a given voltage, consistent with an increase in the activity of the \( \text{Na}_V \) channels.

![Figure 7](image)

**Figure 7.** (A) Increased \( \text{Na}_V \) Activity In Hippocampal Neurons Of AD Mouse Model Tg2576. (A) \( I_{\text{Na}} \) traces recorded in wild type and Tg2576 hippocampal neurons at different time
in culture. (B) Quantification of $I_{\text{Na}}$ represented in A. (C) $I-V$-relationships for endogenous sodium currents in wild type and Tg2576 hippocampal neurons. All values are expressed as mean ± SEM of current densities in 3 independent experimental sessions (* p<0.05 versus respective controls).

4.5 The Anisomycin-stimulated $\text{Na}_\text{V}1.6$ endocytosis prevents increase of $I_{\text{Na}}^+$ currents in hippocampal neurons of Tg2576 mice

To examine whether also in the hippocampal neurons of the Tg2576 mice the increased density of $I_{\text{Na}}$ is mainly due to $\text{Na}_\text{V}1.6$ α-subunit, as in the hippocampal neurons exposed to $\text{A}_\beta_1-42$, we exposed control and Tg2576 hippocampal neurons to Anisomycin (10µg/mL) for 30 minutes before recording sodium currents. We observed that Anisomycin induced a significant reduction of sodium currents in Tg2576 hippocampal neurons respect untreated transgenic neurons, comparable to those recorded in untreated wild type neurons (Fig. 8A and 8B), indicating that the increase of $\text{Na}_\text{V}1.6$ peak currents mainly contribute to the increased sodium currents in transgenic hippocampal neurons.

We also examined the effects of Anisomycin on steady-state biophysical properties of $\text{Na}_\text{V}$ currents. Interesting, the analysis of the $I/V$ relationship of $\text{Na}^+$ currents recorded in Anisomycin-pretreated Tg2576 hippocampal neurons revealed that the treatment counteracted the leftward shift in the voltage dependence of $\text{Na}_\text{V}$ activation. In fact, as in the wild-type hippocampal neurons, the current peak occurred at -10mV rather that at -30mV, further supporting the hypothesis that $\text{Na}_\text{V}1.6$ is responsible of the increased sodium current density in the transgenic hippocampal neurons (Fig. 8C).
Figure 8. The Anisomycin-stimulated Na\textsubscript{V}1.6 endocytosis prevents increase of $I_{\text{Na}}^+$ currents in hippocampal neurons of Tg2576 mice. (A) $I_{\text{Na}}$ traces recorded in wild type and Tg2576 hippocampal neurons under control conditions and after treatment with Anisomycin. (B) Quantification of $I_{\text{Na}}$ represented in A. (C) $I$–$V$-relationships for endogenous sodium currents in wild type and Tg2576 hippocampal neurons under control conditions and after treatment with Anisomycin. All values are expressed as mean ± SEM of current densities in 3 independent experimental sessions (* p<0.05 versus respective controls).

4.6 Overexpression of Na\textsubscript{V}1.6 channel subunits in Tg2576 hippocampal neurons as a neuroprotective mechanism

Because Na\textsubscript{V}1.6 activity was significantly up-regulated in Tg2576 hippocampal neurons, we measured the expression levels of this subunit in
these neurons. Immunoblot analysis performed with a Na\textsubscript{V}1.6-specific antibody on protein extracts from wild-type and Tg2576 hippocampal neurons at 12 DIV revealed one band at 260 kDa that corresponds to subunit. Densitometric analysis showed that this band was considerably more intense in hippocampal neurons of Tg2576 than in the controls (Fig. 9A).

Immunocytochemical analysis, performed with the same anti-Na\textsubscript{V}1.6 antibody, confirm raised expression of Na\textsubscript{V}1.6 in Tg2576 hippocampal neurons respect in wild type. More interestingly, Nav1.6 immunosignal pattern was more different between wild type and Tg2576 cultured hippocampal neurons; in fact, we observed a plasma membrane localization and a punctuated staining pattern mostly confined throughout the neuropil in wild type hippocampal neurons, whereas, in Tg2576 hippocampal cells, the Na\textsubscript{V}1.6 immunosignal was more confined to the soma plasma membrane (Fig. 9B).

Moreover, to confirm that the reduction of total sodium current observed in Tg2576 hippocampal neurons after the treatment with Anisomycin is due to decreased levels of Na\textsubscript{V}1.6, immunocytochemical experiments were performed in Anisomycin-treated wild type and Tg2576 hippocampal neurons. Interestingly, Na\textsubscript{V}1.6 immunosignal is significantly reduced in both neuronal cultures, confirming that pharmacological stimulation of Na\textsubscript{V}1.6 endocytosis abolishes sodium current increase. Moreover, Anisomycin-induced Na\textsubscript{V}1.6 internalization had different effects in wild type and Tg2576 hippocampal neurons. In fact, Anisomycin rapidly induced numerous beads in most neurites of transgenic hippocampal neurons compared with wild type cells, in which no morphological changes were observed. The appearance of focal bead-like swellings in the dendrites and axons as a consequence of Na\textsubscript{V}1.6 sodium
channel density reduction at the plasma membrane reflected neurite damage and indicated that most likely overexpression of this sodium channel subunit could be a protective mechanism against Aβ1-42 toxic effects (Fig. C).

![Figure 9. Na\textsubscript{1.6} and Na\textsubscript{β1} Auxiliary Subunit expression in Tg2576. (A) Immunoblotting analysis of Na\textsubscript{1.6} and Na\textsubscript{β1} and densitometric analysis in Wild Type and Tg2576 hippocampal neurons. (B) Immunocytochemical analysis of Na\textsubscript{1.6} signal in Wild Type and Tg2576 hippocampal neurons, without or after treatment with Anisomycin. (C) Quantification of neuritic beading observed in B. All values are expressed as mean ± SEM of optic densities in 3 independent experimental sessions (* p<0.05 versus respective controls).]

4.7 Reduced Expression Of Na\textsubscript{β1} Auxiliary Subunit In Tg2576 Hippocampal Neurons

To investigate which mechanism could be responsible of leftward shift in the voltage dependence of Na\textsubscript{V} current activation, we measured the expression
levels of Na\textsubscript{V}β1 auxiliary subunit because it has been demonstrated that β1 subunit has modulatory effects on the kinetics and gating of Na\textsubscript{V}1.6 sodium channels, in particular co-expression of Na\textsubscript{V}1.6 and Na\textsubscript{V}β1 determines depolarizing shift in the midpoint potential for channel activation (Bingjun He and David M. Soderlund, 2014). To this aim, we analyzed Na\textsubscript{V}β1 protein levels in cellular lysates of wild-type and Tg2576 hippocampal neurons at 12 DIV. Western blotting analysis revealed a significant decrease in the optical density of the 36kDa (Na\textsubscript{V}β1) specific bands in hippocampal neurons of Tg2576 respect wild type (Fig. 9A).
5. DISCUSSION

The results of the present study provide the first evidence that Na\textsubscript{V}1.6 channel subunit is involved in AD etiopathogenesis. In hippocampal neurons Aβ\textsubscript{1-42} induced a time-dependent upregulation of Na\textsuperscript{+} currents with a peak at 24 hours. Similarly, Tg2576 hippocampal neurons displayed a Na\textsuperscript{+} current upregulation with a peak at XII DIV. This enhancement was selectively mediated by Na\textsubscript{V}1.6 channel isoform. In fact, the removal of Na\textsubscript{V}1.6 isoform either by NaV1.6 silencing or the pharmacological pretreatment with Anisomycin, as well known activator of stress-related p38 MAPK (X. Guo et al. 2011), prevented this increase. Moreover, Na\textsubscript{V}1.6 augmented activity was due to the reduction of its auxiliary subunit β1 protein expression. By contrast, in the late phase, at 72 hours after Aβ\textsubscript{1-42} or in hippocampal neurons at XV DIV Tg2576, the Na\textsuperscript{+} currents decreased and were comparable to the respectively controls.

It is well known that Na\textsubscript{V}1.6 is the major isoform at nodes of Ranvier in myelinated axons and, additionally, is distributed along unmyelinated C-fibers of sensory neurons and is expressed in presynaptic and postsynaptic membranes of the neocortex and cerebellum (Caldwell et al., 2000). This pattern of expression implies that Na\textsubscript{V}1.6 sodium channels play important roles in both electrical and chemical signaling in the brain. Na\textsubscript{V}1.6 currents might significantly impact axonal conduction and may significantly contribute to the pathophysiology of the injured nervous system such as multiple sclerosis (Black et al. 2007; Craner MJ, 2004 and 2005). Recently, several mutations in Na\textsubscript{V}1.6 encoding gene are been identified in patients affected by epilepsy (Estacion M et al., 2014).
It has been suggested an involvement of Na\textsubscript{V}1.6 channel subunit in AD since Mukhamedyarov MA \textit{et al}. (2009) found that A\textsubscript{β}1-42-induced depolarization is driven by increased Na\textsuperscript{+}-influx to muscle fibers through TTX-sensitive Na\textsuperscript{+}-channels but the exact molecular mechanism is yet unknown. Interestingly, in the present study we observed a time-dependent modulation of Na\textsuperscript{+} currents. In the early phase of AD we observed an enhanced activity of Na\textsubscript{V} channels. In fact, we observed an upregulation of Na\textsuperscript{+} currents both in hippocampal neurons exposed to A\textsubscript{β}1-42 or Tg2576 hippocampal neurons with a peak at 24 hours or at XII DIV, respectively. Moreover, this Na\textsuperscript{+} current upregulation was associated with a significant negative shift in the peak of activation of the Na\textsuperscript{+} currents of -10 mV in A\textsubscript{β}1-42 exposed hippocampal neurons or of -20 mV in Tg2576 hippocampal neurons. By contrast, in the late phase, at 72 hours after A\textsubscript{β}1-42 or in hippocampal neurons at XV DIV Tg2576, when the Na\textsubscript{V}1.6 currents abruptly decreased returning at control values, no negative shift in the peak of activation was observed. It is well known that distal end of the AIS is the preferred site for action potential initiation in cortical pyramidal neurons because of its high Na\textsubscript{V} channel density. In particular, low-threshold Na\textsubscript{V}1.6 and high-threshold Na\textsubscript{V}1.2 channels are preferentially accumulate at the distal and proximal AIS, respectively. Patch-clamp recording in neurons revealed a high density of Na\textsuperscript{+} current and a progressive reduction in the half-activation voltage (up to 14 mV) with increasing distance from the soma at the AIS. Moreover, recent evidence show that distal Na\textsubscript{V}1.6 promotes action potential initiation, whereas proximal Na\textsubscript{V}1.2 promotes its backpropagation to the soma (Hu W, \textit{et al} 2009). On this basis it was possible to speculate that the upregulation of Na\textsuperscript{+} currents that occurred in our experimental conditions could
be due to an increased activity of \( \text{Na}_V \)1.6 channel subunits. Interestingly, the removal of \( \text{Na}_V \)1.6 channel subunits by silencing directed against \( \text{Na}_V \)1.6 was able to prevent the upregulation of \( \text{Na}^+ \) currents observed in hippocampal neurons exposed to \( \text{A}\beta_{1-42} \) such as in Tg2576 hippocampal neurons. Moreover, the pretreatment with Anisomycin, as well known activator of stress-related p38 MAPK (Guo X. et al. 2011 Neuroscience) was able to counteract the upregulation of \( \text{Na}_V \)1.6 activity both in hippocampal neurons exposed to \( \text{A}\beta_{1-42} \) and in Tg2576 hippocampal.

It is well known that phosphorylation provides a fast post-translational modification of proteins that has been shown to regulate the acute response of cells to a variety of stimuli and that phosphorylation of \( \text{Na}_V \) channels has been shown to produce rapid modulation of \( \text{Na}^+ \) currents. This phenomenon seems closely mediated by mitogen-activated protein kinases (MAPK). MAPKs are expressed in neurons and are activated in several pathological conditions including AD. In mammalian cells, three principle MAPK pathways, including ERK, JNK, and p38 MAPK, have been identified. In particular, p38 MAPK pathway is especially relevant to the response of environmental stress and inflammatory stimuli (Saklatvala, 2004 and Kumar et al., 2003). Moreover, in AD brain, increased levels of activated p38 MAPK are detected and associated with neuropil theads, and neurofibrillary tangle-bearing neurons (Hensley et al., 1999 and Sun et al., 2003). In AD transgenic mice, p38 MAPK is significantly activated in microglia, astrocytes and neurons, around and distant from the plaques, which indicates the possible involvement of stress-related signaling pathways during the pathogenesis of AD (Hwang et al., 2004, Hwang et al., 2005 and Giovannini et al., 2008). In addition, it has been demonstrated that
the activator of stress-related p38 MAPK, anisomycin, could induce Aβ overproduction by transcriptional activation of APP, BACE1, and PS1 genes through DNMT-dependent hypomethylation and histone H3 hyperacetylation suggesting the involvement of epigenetic mechanism by which oxidative stress contributes to the pathogenesis of AD (Guo et al. 2011). Interesting, sequence analysis shows that Na⁺V1.6 contains a putative MAPK recognition module in the cytoplasmic loop. Moreover, it has been demonstrated that Na⁺V1.6 channels and p38 MAPK colocalize in rat brain tissue and that activated p38 phosphorylates Na⁺V1.6, specifically at serine 553 (S553) significant and selective reducing peak Na⁺V1.6 current amplitude (Wittmack et al. 2005).

The immunoblot and immunocytochemical analysis, performed with the selective anti-Na⁺V1.6 antibody, confirmed that this enhanced Na⁺V1.6 activity was associated to Na⁺V1.6 protein expression upregulation. In particular, immunocytochemical analysis revealed a pronounced perikaryal staining intensely confined to the soma plasmamembrane in Tg2576 hippocampal neurons whereas in wild type neurons this signal was a punctuated staining pattern mostly confined throughout the neuropil and less intense. Moreover, in the presence of Anisomycin Na⁺V1.6 immunosignal was significantly reduced in Tg2576 hippocampal neurons in the presence of p38 activator.

The pathophysiological role played by Na⁺V1.6 enhanced activity is almost controversial. Thus from one site the increase of Na⁺V1.6 activity is considered neurodetrimental since cause an increase in axonal intracellular sodium through a persistent Na⁺ currents increase that leads to membrane depolarization and further activation of Na⁺V channels. On the other hand, the increase in axonal intracellular sodium promotes the reversal of Na⁺/Ca²⁺

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exchanger that plays a neuroprotective action during AD in the early phase (Pannaccione et al. 2012). Immunocytochemical analysis performed in the presence of Anisomycin showed that the internalization of Na\textsubscript{v}1.6 seemed to play a detrimental role. In fact, Tg2576 hippocampal neurons exposed to Anisomycin displayed an enhanced number of neurites with beading. Neuritic beading, focal bead-like swellings in the dendrites and axons, is a neuropathological sign in epilepsy, trauma, ischemia, aging, and neurodegenerative diseases such as AD (Hideyuki Takeuchi, Tetsuya Mizuno, Guiqin Zhang, Jinyan Wang, Jun Kawanokuchi, Reiko Kuno, and Akio Suzumura 2005 JBC Vol. 280, No. 11, Issue of March 18, pp. 10444 –10454). Several previous studies report that neuritic beading is a reversible response to neurotoxic stimuli independent of neuronal death. By contrast, a recent study demonstrate that dendritic beading correlates with disease severity in experimental autoimmune encephalomyelitis rat spinal cord (Zhu, B., Luo, L., Moore, G. R., Paty, D. W., and Cynader, M. S. (2003) Am. J. Pathol. 162, 1639-1650), suggesting that beading paralleled neuronal damage. Furthermore, the mechanisms underlying neuritic bead formation are completely unknown. Our results seemed to suggest that Na\textsubscript{v}1.6 upregulation could be involved in a neuroprotective mechanism.

Finally, western blot analysis showed that the band at ~260 kDa, corresponding to Nav1.6, was significantly higher in Tg2576 hippocampal neurons than in wild type neurons suggesting that the upregulation observed was due to an upregulation of Na\textsubscript{v}1.6 isoform. Interestingly, immunoblot analysis performed with a specific antibody against β1 auxiliary subunit of Na\textsubscript{v}1.6 isoform revealed that the band at ~36 kDa corresponding to β1
auxiliary subunit was downregulated in Tg2576 hippocampal neurons when compared to controls suggesting that this augmented activity was due to the reduction of the protein expression of β1 auxiliary subunit. It has been reported that Na\(_{V}\)1.6 isoform and β1 auxiliary subunit in many brain regions coassemble in heteromultimeric complexes. The reciprocal interaction between the Na\(_{V}\)1.6 and β1 subunits identifies a specific functional association between these two subunits that promotes neurite outgrowth, determines sodium channel localization and activity. In fact, β1 subunit modulates the kinetic and gating properties of Na\(_{V}\)1.6 (Bingjun He, David M. Soderlund 2014). In particular, the voltage dependence of Na\(_{V}\)1.6 activation, recorded in the absence of β1 subunits, was shifted in the direction of hyperpolarization compared to Na\(_{V}\)1.6 recorded in the presence of β1 (Burbidge et al., 2002; He and He and Soderlund 2011; Bingjun He, David M. Soderlund 2014).

Collectively, these results seems to demonstrate that the upregulation of Na\(_{V}\)1.6 activity may be interpreted as a survival strategy activated by neurons in an attempt to defend themselves from the death messages triggered by this peptide during the early phase of exposure.
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