AMIGO-Kv2.1 Potassium Channel Complex: Identification and Association with Schizophrenia-Related Phenotypes

MARJAANA PELTOLA

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AMIGO-Kv2.1 potassium channel complex:
Identification and association with schizophrenia-related phenotypes

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“If our brains were simple enough for us to understand them, we’d be so simple that we couldn’t”

Ian Stewart
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ABSTRACT

Schizophrenia is a devastating psychiatric illness afflicting approximately 1% of the world’s population. Currently, the disease mechanism is poorly understood and the pharmacological interventions relieve only some of the symptoms. Schizophrenia is highly heritable and genetic factors contribute to about 65-80% of the liability to the illness. However, the genetic etiology is complex and remains largely unknown.

Potassium channels are key determinants of neuronal excitability. Kv2.1 is a widely-expressed voltage-gated potassium channel α-subunit. Kv2.1 channels constitute an essential component of the somatodendritic delayed rectifier current (I\textsubscript{K}) in several neuronal types and regulate excitability, especially during periods of high-frequency firing.

This study outlines the identification and characterization of a novel neuronal transmembrane protein AMIGO, which contains extracellular immunoglobulin (Ig) and leucine-rich repeat (LRR) domains. AMIGO was shown to be widely expressed in cerebral neurons and localized to distinctive clusters in the neuronal plasma membrane, restricted to the cell soma and proximal part of neurites. AMIGO was further identified as an auxiliary subunit of the Kv2.1 potassium channel. AMIGO and Kv2.1 were shown to display extensive spatial and temporal colocalization and association in brain. AMIGO was also shown to modify the voltage-dependent activation of Kv2.1 and neuronal delayed rectifier current (I\textsubscript{K}).

To further understand the physiological role of AMIGO in brain, a mouse line lacking the Amigo gene was created and characterized as part of this study. Absence of AMIGO clearly reduced the amount of the Kv2.1 channel protein in mouse brain and altered the voltage-dependent activation of neuronal I\textsubscript{K}. These changes were accompanied by behavioral and pharmacological abnormalities reminiscent of those identified in schizophrenia. Concomitantly, the rare KV2.1 variant was found to be associated with human schizophrenia. These findings demonstrate the involvement of the AMIGO-Kv2.1 channel complex in schizophrenia-related behavioral domains in mice and establish KV2.1 as a susceptibility gene for schizophrenia spectrum disorders in humans.

In the current study, AMIGO was identified as an integral component of the Kv2.1 channel complex in brain. The convergent findings in humans and mice suggest a role for the AMIGO-Kv2.1 potassium channel complex in the pathophysiology of schizophrenia. Furthermore, these findings suggest AMIGO and Kv2.1 may represent potential new targets for schizophrenia treatment development.
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals. Original publications are reproduced with permission from their copyright holders.

AMIGO, a transmembrane protein implicated in axon tract development, defines a novel protein family with leucine-rich repeats.
Journal of Cell Biology 160(6): 963-973. doi: 10.1083/jcb.200209074

AMIGO is an auxiliary subunit of the Kv2.1 potassium channel.
EMBO Reports 12(12):1293-1299. doi: 10.1038/embor.2011.204

AMIGO-Kv2.1 potassium channel complex is associated with schizophrenia-related phenotypes.

* equal contribution

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The author's last name has changed after publication I.

Author's contribution to the studies included in the thesis:
I: The author performed the studies on temporal and spatial distribution of AMIGO protein, and contributed in writing the manuscript.
II: The author initiated the studies on Kv2.1 by unexpected finding of colocalization of Kv2.1 and AMIGO. The author participated in designing and conducting the experiments, including immunohistochemistry, immunoblotting, colocalization and dispersion studies and electrophysiological data analysis. The author wrote the manuscript with inputs from co-authors.
III: The author participated in designing the experiments and performed the histological and biochemical characterization of the AMIGO KO mice. The author participated in the electrophysiological recordings and assisted in the neurotransmitter analysis. The author initiated the studies of Kv2.1 (KCNB1) in human schizophrenia by identifying the candidate polymorphism. The author wrote the manuscript with inputs from co-authors.
Abbreviations

AF all Finland
DIV days in vitro
DRG dorsal root ganglion
E embryonic day
ER endoplasmic reticulum
eLRR extracellular leucine-rich repeat
FRAP fluorescence recovery after photobleaching
Ig immunoglobulin
I\(_K\) delayed rectifier current
iRNA inhibitory ribonucleic acid
IS internal isolate
K\(^+\) potassium ion
KO knockout
Kv voltage-gated potassium channel
LRR leucine-rich repeat
LRRIG containing leucine-rich repeat and immunoglobulin domains
ODD ordered differential display
OR odds ratio
P postnatal day
PPI prepulse inhibition
PP2B calcineurin
PRC proximal restriction and clustering signal
RT-PCR reverse transcription polymerase chain reaction
RyR ryanodine receptor
S transmembrane segment
SSC subsurface cistern
WT wild-type
1. INTRODUCTION

1.1 Overview

A novel neuronal transmembrane protein, AMIGO, was identified in the early stages of this project (Publication I). AMIGO belongs to the group of LRR proteins, and the extracellular part of AMIGO contains LRR and Ig domains. Both of these domains are important in protein-ligand interactions. Many of the genes encoding extracellular LRRs are expressed in the nervous system (Chen et al, 2006; de Wit et al, 2011). Some of the LRR proteins are involved in cellular processes such as axon guidance, target selection, synapse formation, myelination and growth inhibition (de Wit et al, 2011). Several LRR proteins are implicated in neurological and psychiatric disorders (Aoki-Suzuki et al, 2005; Francks et al, 2007; Kalachikov et al, 2002). However, the binding partners and functions of many of extracellular LRR proteins remain unknown.

The main goal of this study was to examine the properties and the biological function of AMIGO in the nervous system. The distribution of AMIGO was characterized in detail with several methods. Unexpectedly, AMIGO was found to display striking colocalization and association with voltage-dependent potassium channel Kv2.1 (Publication II). Voltage-dependent potassium channels are important regulators of neuronal excitability and signal transduction. Kv2.1 channels constitute an essential component of the somatodendritic delayed rectifier current ($I_K$) in several neuronal types (Baranauskas et al, 1999; Du et al, 2000; Guan et al, 2007; Malin & Nerbonne, 2002; Mohapatra et al, 2009; Murakoshi & Trimmer, 1999). We demonstrated that AMIGO modifies the voltage-dependent activation of Kv2.1 and neuronal $I_K$.

To further understand the physiological role of AMIGO in mouse brain, we created a mouse line lacking $Amigo$ gene (Publication III). Absence of AMIGO clearly reduced the amount of the Kv2.1 channel protein in mouse brain and altered the voltage-dependent activation of neuronal $I_K$. Unexpectedly, AMIGO KO mice displayed several characteristics associated with schizophrenia. Our results in mice clearly identified $AMIGO1$ and $KV2.1$ ($KCNB1$) as candidate genes for human schizophrenia. Consequently, we detected an association of rare $KV2.1$ variant with human schizophrenia and schizophrenia spectrum disorders (Publication III).

This study brings together LRR protein AMIGO, voltage-gated potassium channel Kv2.1 and schizophrenia. An introduction to these subjects is provided in the following chapters.
1.2 Leucine-rich repeat proteins

1.2.1 Characteristics of leucine-rich repeat proteins

Leucine-rich repeats (LRRs) are sequence motifs found in a large number of proteins with diverse structures, locations, and functions in bacteria, fungi, plants, and animals. The primary function of these motifs appears to be to provide a versatile structural framework for the formation of protein-ligand interactions (Kobe & Kajava, 2001).

LRRs are generally 20-29 amino acids in length and are unusually rich in the hydrophobic amino-acid leucine. Each repeat contains an N-terminal conserved part and a C-terminal variable part. The conserved part is defined by a consensus sequence LxxLxLxxNxxL or LxxLxLxxCxxL, where: x can be any amino acid; L is hydrophobic amino acid leucine, valine, isoleucine, or phenylalanine; N is asparagine, threonine, cysteine, or serine; and C is cysteine or serine (Kajava, 1998; Kobe & Kajava, 2001). Structurally, this conserved part of the motif forms a β-strand and a loop region. The C-terminal part of the repeat is more variable in length, sequence and structure. (Enkhbayar et al, 2004; Kajava, 1998; Kobe & Deisenhofer, 1994; Kobe & Kajava, 2001)

LRRs exist in tandem arrays of several repeats (varying from 2 to 52) that together constitute the LRR domain (Matsushima et al, 2005). The arrangement of repeating sequence motifs generates a curved structure with the β-strands stacking to form a β-sheet on the concave surface. This is the defining feature of all LRR domains. The variable parts of repeats form the convex surface of the curved structure. Most LRR domains also have both N-terminal and C-terminal cap regions, which shield the hydrophobic core of the LRR structure (Kobe & Kajava, 2001). In extracellular LRR proteins the capping regions are cysteine-rich motifs with a conserved set of disulfides. The structure of the LRR domain in several LRR proteins is presented in Figure 1.

Many LRR proteins bind ligands with their concave surfaces (Kajander et al, 2011; Kobe & Deisenhofer, 1995; Morlot et al, 2007; Seiradake et al, 2009; Seiradake et al, 2011). The curved structure of the LRR domain and the exposed β-sheet on the concave side form a large binding surface, which makes the LRR domains very effective protein-binding motifs (Kobe & Deisenhofer, 1994; Kobe & Kajava, 2001). Variation in the length and number of repeats, and in secondary structures on the convex side, creates variability in the curvature of the LRR domain in different proteins allowing interactions with a large diversity of ligands.

1.2.2 Proteins with extracellular leucine-rich repeats

There are about 140 human genes encoding proteins with extracellular LRRs (eLRRs) (Dolan et al, 2007). These include secreted, lipid-anchored, and various types of transmembrane proteins. Many of the genes encoding extracellular LRRs are expressed in the nervous system (Chen et al, 2006; de Wit et al, 2011). Because
the LRR domain is an efficient structure for protein-ligand interactions, proteins with extracellular LRR domains are well suited to regulate intercellular communication and cell adhesion. Interestingly, a comparative analysis of eLRR genes has revealed that the eLRR superfamily has greatly expanded in mammals and to a lesser extent in flies. There are 29 eLRR proteins in worms, 66 eLRR proteins in flies, and 135 eLRR proteins in mice (Dolan et al, 2007). The evolutionary need for more molecules involved in adhesion and cell-cell communication arises with the increasing complexity of the organism. In particular, expansion of the eLRR protein superfamily is correlated with complexity of the nervous system (Dolan et al, 2007).

Extracellular LRRs containing proteins have been divided into four subgroups depending on their domain organization (Dolan et al, 2007). The LRR-only class proteins do not contain other recognizable protein domains except
LRRs. The LRR-Ig/FN3 class proteins contain LRRs and immunoglobulin (Ig) and/or fibronectin type 3 (FN3) domains. The LRR-Tollkin class proteins contain LRRs and a cytoplasmic Toll/interleukin 1 receptor domain or cluster with Toll proteins. The LRR-other class consists of proteins that contain LRRs and some other types of domains, e.g. epidermal growth factor (EGF) repeats or a G-protein-coupled receptor domain.

The binding partners and functions of many of eLRR proteins are still unknown. Among eLRR proteins with known binding partners, a large structural variability exists in ligand structure. Well-known LRR proteins in the nervous system include Trk neurotrophin receptors, Nogo receptor (NgR) mediating axonal growth inhibition, and Slit family of extracellular axon-guiding proteins (de Wit et al, 2011; Schwab, 2010). Many eLRR proteins have functions in the innate immune system that are similar in plants and animals (Nürnberger et al, 2004). Many others are involved in various aspects of nervous system development and function (Chen et al, 2006; de Wit et al, 2011). In the nervous system, LRR proteins are involved in cellular processes such as axon guidance, target selection, synapse formation, myelination, and growth inhibition. Several LRR proteins are implicated in neurological and psychiatric disorders, including Alzheimer’s disease, Tourette’s syndrome, night blindness, epilepsy, autism, bipolar disorder, and schizophrenia (Abelson et al, 2005; Aoki-Suzuki et al, 2005; Bech-Hansen et al, 2000; de Wit & Ghosh, 2014; Francks et al, 2007; Kalachikov et al, 2002). Figure 2 represents selected neural eLRR proteins grouped by their associated functions.

1.2.3 LRRIG proteins
Immunoglobulin (Ig) domains are important in protein interactions. An Ig domain is found in many proteins with different functions, including antibodies, cell adhesion molecules, and cell receptors (Williams & Barclay, 1988). These proteins can bind other Ig domain containing proteins or a variety of other molecules, such as antigens and sugars. In cell adhesion molecules, the Ig domain can mediate both homophilic and heterophilic molecular interactions. Structurally, the Ig domain is a sandwich like structure with two antiparallel β-sheets joined together by a conserved cysteine bridge (Bork et al, 1994).

Proteins that simultaneously carry both LRR and Ig domains are called LRRIG proteins/LIG proteins (Homma et al, 2009; MacLaren et al, 2004; Mandai et al, 2009). The domain structure of these proteins combines two versatile binding domains and thus enhances the potential for a wide spectrum of protein-protein interactions.

At least 36 human LRRIG proteins have been identified comprising 13 subgroups: four LINGO proteins, three NGL proteins, five SALM proteins, three NLRR proteins, three Pal proteins, two ISLR proteins, three LRIG, two GPR, two Adlican, two Peroxidasin-like proteins, three Trk receptors, an unnamed protein
Figure 2. Domain organization and function of selected neural LRR proteins. The schematic overview shows the domain organization of selected extracellular LRR proteins with known functions in the nervous system. The proteins are grouped by the cellular processes that they regulate. Protein names are indicated below the diagrams, in red for fly LRR proteins and in blue for mammalian LRR proteins. Domain abbreviations: CT3, cysteine-knot; EGF, epidermal growth factor-like; EPTP, epitempin; FN3, fibronectin type III; GPI, glycosylphosphatidylinositol; Ig, immunoglobulin-like; laminin G, laminin globular; LRRNT and LRRCT, LRR N- and C-terminal flanking domains; PDZ-IS, PDZ interaction site; TIR, Toll/interleukin-1 receptor; TyrK, tyrosine kinase. Reprinted from de Wit et al, 2011. Copyright © 2011, Annual Reviews.
AA11068 and three AMIGO proteins (Homma et al, 2009). Adlican and Peroxidasin are secreted proteins whereas the remaining 11 of these subgroups are membrane associated proteins. Interestingly, most of these proteins-if not all- are expressed in the nervous system (Homma et al, 2009; Kuhnert et al, 2010; Nagasawa et al, 1999). Some are nervous system specific, whereas some are expressed more broadly. Many of these proteins have been associated with neuronal growth modulating functions (Chen et al, 2006).

1.3 AMIGO protein family

1.3.1 Identification of AMIGO protein family
At the early stages of this project, a novel family of three homologous cell adhesion molecules was identified (Publication I). AMIGO (amphoterin-induced gene and ORF) was identified as a gene induced by the neurite outgrowth-promoting protein HMGB1 (amphoterin) in cultured hippocampal neurons. Two other genes were cloned on the basis of their homology to AMIGO. These molecules were named AMIGO2 and AMIGO3. AMIGO proteins contain both immunoglobulin (Ig) and leucine-rich repeat (LRR) domains and thus belong to the group of LRRIG proteins. The domain structure of AMIGO is presented in Figure 8. The identification, characterization and functional studies of AMIGO are described in detail in the Results section of this thesis. The subsequent introductory chapters include the information about AMIGO protein family published following their identification, by our group or by others, which is not included in the Results section of this thesis.

1.3.2 Expression of AMIGO
The distribution of AMIGO mRNA in embryonic and adult mouse tissues was first described by Kuja-Panula et al, 2003 (Publication I). The expression of AMIGO mRNA during development has been studied with in situ hybridization in early (E10) mouse embryos (Homma et al, 2009). In the central nervous system, AMIGO expression was detected in post-mitotic neurons in the developing forebrain, midbrain and hindbrain. In the peripheral nervous system, AMIGO was expressed in all cranial and dorsal root ganglia. Outside the nervous system, AMIGO mRNA was expressed in the inner mesenchyme cells in the branchial arches and limb bud. Mandai et al. (2009) have also detected AMIGO mRNA expression in mouse embryonic (E13.5) dorsal root ganglion neurons.

AMIGO-like immunoreactivity was initially located in central nervous system axonal tracts (Publication I). It has also been reported that AMIGO-like immunostaining is present in multiple brain cell types in adult mouse brain, including neurons, astrocytes, and oligodendrocytes (Chen et al, 2012). According to Chen et al, neuronal AMIGO-like immunoreactivity was mostly restricted to
cell body and dendrites. However, the specificity of AMIGO antibody used in these studies has not been characterized with AMIGO knockout tissue. Cellular and subcellular distribution of specific (knockout tissue-validated) AMIGO immunoreactivity in adult mouse brain is described in the Results (Publication II).

1.3.3 Structure of AMIGO
The crystal structure of the AMIGO ectodomain has been determined (Figure 3) (Kajander et al, 2011). The LRR domain of AMIGO forms a typical curved LRR structure with the β-sheet on the concave surface. The LRR domain contains cysteine-rich N- and C-terminal capping regions with two disulfide bridges in each. The LRR domain is followed by the C-terminal, membrane-proximal C2-type Ig-domain. The crystal structure reveals AMIGO as a dimeric protein with the LRR regions forming the dimeric interface. It is suggested that all three AMIGO proteins form similar dimers, as some key aromatic residue interactions at the dimer interface are conserved in all AMIGO proteins, while the convex surface is not conserved (Kajander et al, 2011). Mutagenesis studies indicate that dimerization is necessary for the proper cell-surface expression of AMIGO (Kajander et al, 2011).

![Figure 3. Structure of AMIGO.](image)

Ribbon diagram of AMIGO monomer (a) and dimer (c). β-strands in pale cyan, helices in red and the Ig domain in blue. The glycan at Asn72 as is shown in stick (gray). (b) Domain structure: the LRRs in monomer fold are colored cyan, C-terminal capping motif is in red, N-terminal capping motif is in blue, and Ig domain is shown in gray (behind the red LRRCT). Reprinted from Kajander et al, 2011, Copyright ©2011, with permission from Elsevier.
1.3.4 Functional role of AMIGO

*In vitro* studies have suggested that AMIGO acts as a homophilic adhesion molecule that induces outgrowth and fasciculation of neurites in central neurons (Publication I). In zebrafish, AMIGO affects the development of neural circuits, and its mechanism is suggested to involve homophilic interactions within the developing fiber tracts (Zhao et al, 2014). Clear defects in corresponding neuronal circuits are not seen in adult AMIGO knockout mice (the Results section, Publication III). However, these circuits have not been studied during development in AMIGO knockout mouse.

AMIGO has also been suggested to regulate dendritic growth and neuronal survival (Chen et al, 2012). Suppression of AMIGO expression with siRNA reduced the number and length of dendrites in cultured cortical neurons (Chen et al, 2012). In a heterologous expression system, SH-SY5Y cells stably expressing AMIGO were more resistant to experimentally induced apoptosis (Chen et al, 2012).

1.3.5 Expression of AMIGO2

The expression of AMIGO2 mRNA during development has been studied with *in situ* hybridization in early (E10) mouse embryos (Homma et al, 2009). AMIGO2 mRNA expression was found primarily in the central nervous system and it was observed only in a small number of post-mitotic cells in the developing forebrain and midbrain. Outside the nervous system, AMIGO2 expression was observed only in the mesonephros.

The expression of AMIGO2 mRNA in adult mouse brain has been studied in detail with *in situ* hybridization (Laeremans et al, 2013). The expression of AMIGO2 was detected in restricted brain areas, including the mitral cell layer of the olfactory bulb, the granular cell layer of the accessory olfactory bulb, preoptic area, habenula, premammillary nuclei, hippocampus, and cerebellum. The expression pattern of AMIGO2 was especially distinct in the hippocampus. AMIGO2 was restricted to specific subfields of the hippocampus including CA2 and CA3a, and the expression was absent from other hippocampal areas. Similar highly restricted expression in the hippocampus has been detected with reporter gene analysis in heterozygous AMIGO2 knockout mice (unpublished observations, Kathleen Gransalke). In addition to the nervous system, AMIGO2 mRNA expression has been detected in adult mouse spleen, lung, kidney, small intestine, and testis with RT-PCR (Kuja-Panula et al, 2003).

The expression of AMIGO2/DEGA has been studied in selected tumor and normal human tissues outside the nervous system (Rabenau et al, 2004). In normal tissues, the strongest expression of AMIGO2 was observed in breast, ovary, uterus, and cervix. Lower expression levels were detected in lung, colon, and rectum.
1.3.6. Functional role of AMIGO2
Following identification of three AMIGOs (Kuja-Panula et al, 2003), AMIGO2 has been identified in two separate studies (Ono et al, 2003; Rabenau et al, 2004). In these studies AMIGO2 was called as Alivin 1 (after “alive” and “activity-dependent leucine-rich repeat and Ig superfamily survival-related protein”) or DEGA (differentially expressed in human gastric adenocarcinomas).

Alivin 1 was identified as a gene whose expression is tightly associated with depolarization and/or NMDA-dependent survival of cerebellar granule neurons (Ono et al, 2003). The study also demonstrated that the expression of AMIGO2/Alivin 1 is dependent on neuronal activity. Furthermore, it was shown that AMIGO2/Alivin 1 promoted depolarization-dependent survival of cerebellar granule neurons in cultures.

DEGA was identified as a gene differentially expressed in human gastric adenocarcinomas (Rabenau et al, 2004). The expression of AMIGO2/DEGA was increased in tumor versus normal tissue in approximately 45% of gastric adenocarcinoma patient samples. Differential expression of AMIGO2 was also detected in thyroid and pancreatic tumors (Rabenau et al, 2004). Suppression of AMIGO2 expression with siRNA in a gastric adenocarcinoma cell line abrogated their tumorigenicity in nude mice, and led to altered adhesion/migration as well as cytogenetic and morphological cell properties (Rabenau et al, 2004).

1.4 Voltage-gated potassium channels

1.4.1 Characteristics of voltage-gated potassium channels
Potassium (K\(^+\)) channels are membrane proteins that form a potassium-selective pore across the membrane. K\(^+\) channels regulate the membrane potential and excitability of neurons and other cell types. They are essential for a wide variety of fundamental physiological processes, including endocrine secretion, T-cell proliferation, muscle contraction, cardiac-rhythm generation, and neuronal signal transduction. Accordingly, potassium channels are important targets of drug development. There are four major classes of K\(^+\) channels: voltage-gated K\(^+\) channels (Kv), Ca\(^{2+}\) activated K\(^+\) channels (KCa), inwardly rectifying K\(^+\) channels (Kir), and two-pore-domain K\(^+\) channels (K2P, “leak” K\(^+\) channels) (Coetzee et al, 1999). A multitude of potassium channel subunits, their post-translational modifications, heterogeneous distribution in the nervous system, and their differential subcellular localization facilitate enormous variability in the electrical properties of neurons (Vacher et al, 2008). The wide variety of possible subunit combinations and accessory proteins extends the diversity of neuronal phenotypes even further.

Kv channels are encoded by 40 genes in humans, which are divided into 12 subfamilies, named Kv1 - Kv12, based on relative sequence homology (Coetzee et
al, 1999; Gutman et al, 2005). For the Kv channel genes, a parallel nomenclature has been developed in official HUGO Human Gene Nomenclature, where they are named KVNx, with a changed fourth letter ‘x’ (Bruford et al, 2008). The original four gene families were assigned the letters A-D (Kv1-Kv4 = KCNA-KCND), and Kv5-Kv12 families have other designations. Kv families Kv5, 6, 8, and 9 encode subunits that act as modifiers (Gutman et al, 2005); these subunits do not produce functional channels on their own. Instead, they form heterotetramers with Kv2 family subunits, increasing functional diversity within this family.

Kv channels are composed of four principal subunits (α-subunits). The four α-subunits are arranged around a central pore as homotetramers or heterotetramers. A single α-subunit is a multi-transmembrane protein containing six transmembrane segments (S1-S6) and a membrane re-entering P-loop between segments S5 and S6. The ion-conducting pore is lined by S5-P-S6 sequences from each of the four subunits. The four S1-S4 segments, each containing four positively charged arginine residues in the S4 segment, act as voltage sensor domains and gate the pore. Schematic representation of the domain structure and the tetrameric organization of Kv channels is presented in Figure 4. Reviewed in (Wulff et al, 2009; Yellen, 2002)

**Figure 4. Domain structure and the tetrameric organization of Kv channels.** Schematic representation of the tetrameric organization of a Kv channel. The right panel represents a single Kv channel α-subunit consisting of six transmembrane segments (S1-S6) and an intracellular NH₂ and COOH terminus. Left panel represents a top view of Kv tetramer in which the four subunits are arranged around a central pore. Reprinted from (Bocksteins & Snyders, 2012). Copyright © 2012, The American Physiological Society.
Voltage-gated potassium (Kv) channels open in response to changes in membrane potential and permit the selective flow of potassium ions across the membrane. Due to the concentration gradient of K⁺ that exists across the cell membrane, the opening of Kv channels results in an efflux of positive charge, which can serve to repolarize or even hyperpolarize the membrane. Activation of Kv channels in excitable cells, such as neurons or cardiac myocytes, thus reduces excitability, whereas channel inactivation has the opposite effect and increases the excitability. In excitable cells, Kv channels are for instance responsible for repolarization after action potential firing. In both excitable and non-excitable cells, Kv channels also play an important role in Ca²⁺ signaling, volume regulation, secretion, proliferation, and migration. Kv channels often form a part of large multimolecular complexes. The function of these complexes may also be influenced by the channel through mechanisms not involving ion-conduction. (Wulff et al, 2009)

1.4.2 Auxiliary subunits of voltage-gated potassium channels
Voltage-gated potassium channels do not exist as independent units merely responding to changes in membrane potential but function as multimolecular complexes able to integrate a variety of signals regulating the channel activity (Li et al, 2006; Pongs & Schwarz, 2010). The channel complex frequently contains auxiliary subunits that are diverse in structure and function. Proteins that associate with K⁺ channels may do so dynamically or they may be constitutively complexed with the channel protein. Auxiliary subunits affect the channel gating as well as the expression, subunit composition, or localization of the channel complex (Li et al, 2006). In addition, auxiliary subunits may link channel function to intra- or extracellular signals, and many of them have been shown to affect the pharmacological properties of the channel (Bett & Rasmusson, 2008; Sesti et al, 2000). The significance of auxiliary subunits is demonstrated in humans and in experimental animals by several associated diseases, such as arrhythmogenesis, hypothyroidism, hypertension, periodic paralysis, sensorineural deafness, and epilepsy (Abbott et al, 1999; Abbott et al, 2001; Brenner et al, 2000; Brenner et al, 2005; Duggal et al, 1998; Roepke et al, 2009; Schulte et al, 2006; Schulze-Bahr et al, 1997; Splawski et al, 1997). So far, auxiliary subunits have been identified only for a portion of the large group of Kv channel α-subunits. The role of KCNE subunits in Kv channel function in mammalian heart and skeletal muscle has been widely demonstrated (Abbott et al, 1999; Abbott et al, 2001; Barhanin et al, 1996; Sanguinetti et al, 1996; Splawski et al, 1997; Tyson et al, 1997). In neurons, the best known auxiliary subunits of Kv channels include the cytoplasmic β-subunits for the Kv1 channels, KchIPs, and DPPLs for the Kv4 channels (An et al, 2000; Nadal et al, 2003; Rettig et al, 1994; Scott et al, 1994).
1.5 Kv2.1

1.5.1 Domain structure of Kv2.1
Voltage-gated potassium channel α-subunit Kv2.1 is a protein of 857 amino acids in humans. The domain structure of Kv2.1 consists of six transmembrane segments (S1-S6) and large cytoplasmic N- and C-terminal domains (Figure 5) (Frech et al, 1989). The membrane-spanning S1–S6 domains comprise approx. 25% of the polypeptide, and form the voltage-sensing and K⁺ ion-selective pore components of the channel.

The Kv2.1 polypeptide is distinguished among K⁺ channels by its unusually long (441-amino acid) cytoplasmic C-terminus. Almost 75% of Kv2.1 protein is cytoplasmic, with the cytoplasmic C-terminus comprising over 50% of the Kv2.1 α-subunit. The cytoplasmic N-terminus contains the tetramerization (T1) domain that is required for the assembly of α subunits into a functional tetrameric channel. The cytoplasmic C-terminus contains the sequence required for specific subcellular localization of Kv2.1 protein (PRC, proximal restriction and clustering signal). The large intracellular regions can mediate interactions with diverse cellular components, and can be targeted by cellular enzymes (e.g. protein kinases and phosphatases) to achieve reversible modification of channel structure and function. (Misonou et al, 2005b)

The only extracellular parts of the Kv2.1 protein are between the transmembrane segments. Kv2.1 amino acid sequence contains a single consensus N-linked glycosylation site on the extracellular S3-S4 linker domain (Frech et al, 1989). However, the native brain Kv2.1 channels and recombinant Kv2.1 channels expressed in heterologous systems, are not N-glycosylated (Shi & Trimmer, 1999).

1.5.2 Kv2.1 gene KCNB1
The human gene coding for Kv2.1 is called KCNB1 and it is located in chromosome 20 at 20q13.2 (Melis et al, 1995). The KCNB1 gene has a simple structure: it contains only a single large (107 kb) intron in the region encoding the beginning of the S1-S6 core domain. De novo mutations in KCNB1 have been identified in epileptic encephalopathy (Torkamani et al, 2014). Single-nucleotide polymorphism in KCNB1 has been associated with increased cardiac left ventricular mass (Arnett et al, 2009). Interestingly, two individuals homozygous for a KCNB1 variation substituting the penultimate amino acid serine 857 with asparagine have been identified in an earlier study on the low voltage alpha EEG trait, and one of the Asn857/Asn857 homozygotes was reported to have schizophrenia and the other had paranoia (Mazzanti et al, 1996). However, the association of the corresponding single nucleotide polymorphism (SNP) rs34280195 with schizophrenia has not been studied before.

For clarity, the name KV2.1 (KCNB1) will be used for the human gene in this study.
1.5.3 Channel assembly of Kv2.1

Four Kv2.1 α-subunits assemble into a tetrameric channel. It has been thought that Kv2.1 α-subunits do not form heteromultimeric channels with the other Kv2 family member, Kv2.2, in mammalian brain, since these two Kv2 α-subunits exhibit contrasting patterns of subcellular distribution in co-expressing cells (Hwang et al, 1993). More recently, however, it has been shown that the long form of Kv2.2 is colocalized with Kv2.1 in a subset of cortical pyramidal neurons and these two proteins are capable of forming functional heteromeric channels (Kihira et al, 2010).

Several studies have suggested that the function of Kv2.1 channels can be diversified through heteromultimerization with the “silent” Kv5, Kv6, Kv8, and Kv9 subunits, which can modify the inactivation, trafficking, drug sensitivity, and expression of Kv2.1 (Bocksteins & Snyders, 2012; Otschytsch et al, 2002; Salinas et al, 1997b). These “silent” subunits do not independently produce electrically functional channels, but in vitro they are shown to interact with the Kv2 subfamily, to form functional heterotetrameric channels and to modulate the Kv2 current (Bocksteins & Snyders, 2012). However, the cellular and subcellular localization of
these subunits in brain is not well characterized (Vacher et al, 2008). Currently, it is unclear how broadly these proteins associate with the Kv2.1 channel complex, since the colocalization of these silent subunits with Kv2.1 in brain has not been demonstrated.

1.5.4 Overall distribution of Kv2.1
Kv2.1 expression and localization has been mostly studied in rodents. It is widely expressed in the central nervous system (Hwang et al, 1993; Klumpp et al, 1995; Muennich & Fyffe, 2004). The localization of Kv2.1 is restricted to neurons, including both principal neurons and interneurons (Du et al, 1998; Hwang et al, 1993; Maletic-Savatic et al, 1995; Trimmer, 1991). In the brain, the Kv2.1 distribution is so broad that Kv2.1 staining pattern resembles that of the Nissl stain in many regions (Vacher et al, 2008). Among interneurons, Kv2.1 is found in the majority of cortical and hippocampal parvalbumin, calbindin, and somatostatin-containing inhibitory interneurons (Du et al, 1998). In spite of the widespread distribution of Kv2.1, certain cells have especially prominent Kv2.1 expression. For example, cortical pyramidal neurons in layers II/III and layer V are especially striking for their high levels of Kv2.1 expression (Hwang et al, 1993; Misonou & Trimmer, 2004; Rhodes et al, 1995). Kv2.1 is also present at high levels in the hippocampus, especially in CA1 pyramidal cells and dentate granule cells (Vacher et al, 2008).

In peripheral nervous system, Kv2.1 is expressed in DRG neurons (Kim et al, 2002). Outside the nervous system, Kv2.1 is also reported to be expressed in cardiac, skeletal and smooth muscle, as well as in pancreatic β-cells (Patel et al, 1997; Van Wagoner et al, 1997; Yan et al, 2004).

1.5.5 Subcellular localization of Kv2.1
The subcellular localization of Kv2.1 in neurons is fascinating. In spite of a broad expression in brain, within individual neurons the localization of Kv2.1 is highly restricted. Kv2.1 is specifically localized to unique micron-sized clusters at perisomal plasma membrane, including cell soma, proximal dendrites and axon initial segment (Figure 6, control conditions). Several studies have demonstrated that Kv2.1 localizes to cell soma and the proximal part of dendrites (Du et al, 1998; Hwang et al, 1993; Maletic-Savatic et al, 1995; Rhodes et al, 1995; Scannevin et al, 1996; Trimmer, 1991), but not to axons and synaptic terminals (Du et al, 1998; Scannevin et al, 1996; Trimmer, 1991). However, more recently Kv2.1 is shown to also be localized in the axon initial segment of several neuronal types (King et al, 2014; Sarmiere et al, 2008).
1.5.6 Kv2.1 clusters

The physiological role of Kv2.1 clusters is still largely unknown. Until now, only the pore forming α-subunits Kv2.1 and Kv2.2 have been localized to these plasma membrane sites in brain (Kihira et al, 2010; Trimmer, 1991). However, Kv2.2 expression in brain is much more restricted than the ubiquitous expression of Kv2.1 (Hermanstyne et al, 2010; Kihira et al, 2010). In cortex, Kv2.2 is expressed only in a subset of pyramidal neurons, where it is shown to colocalize with Kv2.1 (Kihira et al, 2010).

Several studies have addressed the question of what are the possible intra- and extracellular structures associating with Kv2.1 clusters. Kv2.1 clusters at the

Figure 6. Localization and stimulus-induced dispersion of Kv2.1. (a) Glutamate-induced dispersion of Kv2.1. In control conditions, Kv2.1 is localized to large clusters on the plasma membrane of the soma and proximal dendrites in cultured hippocampal neurons. Stimulation with glutamate (10 μM for 10 min) results in translocation of Kv2.1 from clusters to a more uniform distribution on the membrane. Kv2.1 (green) and dendritic marker AP-2 (red). Reprinted by permission from Macmillan Publishers Ltd: Nature Neuroscience 7: 711-718, Misonou et al, 2004, copyright © 2004. http://www.nature.com/neuro/index.html (b) CO2-induced dispersion of Kv2.1. In control conditions, Kv2.1 is localized to large somatodendritic clusters in rat brain (subiculum). Hypoxia/ischemia induced by CO2 inhalation (2 min) results in Kv2.1 translocation such that Kv2.1 staining is uniform on the surface membrane. Republished with permission of Society for Neuroscience, from Misonou et al, 2005a, copyright © 2005; permission conveyed through Copyright Clearance Center, Inc.
plasma membrane are shown with electron microscopy to lie over ER-derived structures called subsurface cisterns (SSC) (Du et al, 1998; Mandikian et al, 2014). These membrane discs are rich in Ca^{2+} releasing channels inositol triphosphate receptors (IP3R) and ryanodine receptors (RyR) and are very closely associated with plasma membrane (Berridge, 1998; Rosenbluth, 1962). Also in cultured hippocampal neurons, Kv2.1 clusters are shown to partly colocalize with clusters of intracellular RyRs (Antonucci et al, 2001; Misonou et al, 2005b). Kv2.1 clusters on the axon initial segment are also found near RyR-rich cisternal organelles (King et al, 2014). Recently, coupling of Kv2.1 channels and RyRs has been studied in more detail (Mandikian et al, 2014). Kv2.1 clusters are found juxtaposed to RyR clusters in neurons in specific brain regions, and this is especially prominent in striatal medium spiny neurons (MSN).

In the extracellular space, Kv2.1 clusters clearly appose cholinergic synapses in spinal motor neurons (Muennich & Fyffe, 2004). However, in cortical and hippocampal neurons Kv2.1 clusters are not associated with synapses (Du et al, 1998; Misonou et al, 2008; Mulholland et al, 2008). In cortical pyramidal neurons, Kv2.1 clusters are shown to reside in extrasynaptic areas (Misonou et al, 2008) and to be faced by astrocytic processes (Du et al, 1998; Misonou et al, 2008).

Interestingly, Kv2.1 localization is regulated so that several stimuli, such as increased neuronal activity and ischemia, are able to induce declustering of the Kv2.1 channel (Figure 6) (Misonou & Trimmer, 2004; Misonou et al, 2005a). Following stimulus, Kv2.1 is diffusely distributed at the neuronal plasma membrane, but the localization remains restricted to the soma and proximal part of neurites. These mechanisms are reviewed in more detail in chapter 1.5.8.

The restricted localization of Kv2.1 is also retained when neurons are cultured. In cultured hippocampal neurons about 20 percent of somal surface was occupied with Kv2.1 clusters at 14 DIV (Fox et al, 2013). Kv2.1 can also form clusters even when heterologously expressed in HEK293 cells or MDCK cells (Mohapatra & Trimmer, 2006; O’Connell & Tamkun, 2005).

A number of studies have focused on defining the determinants of the characteristic subcellular distribution of Kv2.1 (Antonucci et al, 2001; Lim et al, 2000; Scannevin et al, 1996). Deletion analysis of Kv2.1 has revealed a segment of about 25 amino-acids in the C-terminus (amino acids 573-598) that is necessary and sufficient for the proximally restricted and clustered localization (Lim et al, 2000). The segment is termed PRC signal. Within this segment, four residues were found to be especially important for the clustered localization; Ser583, Ser586, Phe587, and Ser 589 (Lim et al, 2000). It is noteworthy that three of these residues are serines, since the phosphorylation and clustering of Kv2.1 are shown to be coupled (Misonou et al, 2004).

The trafficking mechanism of Kv2.1 to distict dendritic subcompartments has been studied recently (Jensen et al, 2014). Kv2.1 channels are sorted into
specific transport vesicles at the Golgi apparatus and subsequently trafficked through a mechanism involving myosin IIB (Jensen et al, 2014).

The mobility of Kv2.1 has been studied in cultured cells with live cell imaging using FRAP (fluorescence recovery after photobleaching) and quantum dot-tracking experiments (Deutsch et al, 2012; O'Connell et al, 2006; Tamkun et al, 2007). Kv2.1 channels are delivered into the cell surface clusters via trafficking vesicles. Following insertion, Kv2.1 is retained in the surface cluster. However, within the surface cluster, the Kv2.1 channel is freely mobile. The clusters themselves are able to move short distances (less than 2 μm in several minutes). Clusters do not make any large-scale movements, remaining roughly within the same membrane area. Clusters are able to fuse to form larger structures, as well as break apart generating smaller structures. Generally, Kv2.1 channels outside the clusters ignore the cluster boundary, readily diffusing through these microdomains. However some non-clustered channels (5% of studied cases) become retained within the cluster. These findings are consistent with the idea that Kv2.1 is retained in the cluster by its association with the underlying subsurface cistern.

It has been suggested that Kv2.1 clusters are insertion platforms for ion channel delivery to the plasma membrane (Deutsch et al, 2012). Deutsch et al. suggest that Kv2.1 clusters function as specialized cell-surface microdomains involved in membrane-protein trafficking.

1.5.7 Function of Kv2.1 in nervous system
Kv2.1 channels constitute an essential component of the delayed rectifier current (I_K) and regulate excitability in several neuronal types (Baranauskas et al, 1999; Du et al, 2000; Guan et al, 2007; Malin & Nerbonne, 2002; Mohapatra et al, 2009; Murakoshi & Trimmer, 1999). Sustained outward potassium current is greatly reduced by intracellular application of the Kv2.1 antibody in cultured hippocampal neurons (Murakoshi & Trimmer, 1999) or by antisense treatment against Kv2.1 in cultured hippocampal slices (Du et al, 2000), indicating that Kv2.1 is a major contributor of the delayed rectifier currents. In particular, Kv2.1 has been shown to regulate excitability during periods of high-frequency firing in hippocampal pyramidal cells or tonic firing in sympathetic neurons (Du et al, 2000; Malin & Nerbonne, 2002). In cortical pyramidal cells, Kv2.1 has been shown to underlie the slowly inactivating potassium current (Guan et al, 2007) and to regulate the firing rate and inter-spike interval during repetitive firing (Guan et al, 2013).

1.5.8 Dynamic modulation of Kv2.1
Kv2.1 has up to 60 putative phosphorylation sites and it is strongly regulated by phosphorylation (Misonou et al, 2006; Murakoshi et al, 1997; Park et al, 2006; Tiran et al, 2003). At least 34 in vivo phosphorylation sites have been identified to date (Park et al, 2006; Trimmer, 2014). Kv2.1 is also modified by SUMOylation
(Plant et al, 2011). These modifications strongly affect the localization and function of Kv2.1.

The phosphorylation, localization, and function of Kv2.1 are coupled. Under normal conditions, Kv2.1 is highly phosphorylated (Misonou et al, 2006) and localized to characteristic somatodendritic clusters (Du et al, 1998; Hwang et al, 1993; Rhodes et al, 1995; Scannevin et al, 1996; Trimmer, 1991). Increased neuronal activity, induced by kainate seizures in vivo or glutamate stimulation in vitro, leads to dephosphorylation of Kv2.1 and dispersion of clustered Kv2.1 to diffuse somatodendritic localization (Misonou et al, 2004; Misonou et al, 2006). These modifications are associated with a large hyperpolarizing shift in voltage-dependent activation of Kv2.1 in vitro and neuronal I_K in vivo, which is able to suppress neuronal activity (Misonou et al, 2004; Misonou et al, 2006; Mohapatra et al, 2009). Additionally, a reduction of neuronal activity, by activity blockade in vitro or with anesthetics in vivo, leads to hyperphosphorylation of Kv2.1, suggesting that the regulation of Kv2.1 is bidirectional (Misonou et al, 2006). Based on these studies, it has been suggested that Kv2.1 acts as an adjustable resistor in neuronal soma, providing a mechanism of homeostatic plasticity (Misonou et al, 2005b; Surmeier & Foehring, 2004).

The major protein phosphatase known to modulate Kv2.1 channel is the Ca2+ and calmodulin-dependent protein phosphatase calcineurin (protein phosphatase 2B, PP2B) (Misonou et al, 2005a; Misonou et al, 2006). Modifications in phosphorylation, localization, and activity of Kv2.1 by glutamate stimulation are thought to be mediated through NMDA receptor activation, followed by elevated cytosolic Ca2+ levels (Figure 7). Increase in intracellular Ca2+ results in activation of calcineurin, which then dephosphorylates Kv2.1 channels. As described above, dephosphorylation of Kv2.1 is coupled to dispersion and alterations in voltage-dependent activation.

Hypoxia/ischemia, produced by CO2 treatment in vivo or chemically-induced experimental ischemia in vitro, elicits a similar dephosphorylation and dispersion of Kv2.1 (Misonou et al, 2005b; Misonou et al, 2008). Consequently, Kv2.1 is suggested to function as a mechanism to suppress pathological hyperexcitability of central neurons during ischemic conditions.

Work performed in cultured HEK293 cells has demonstrated that the insertion of the cytoplasmic C-terminal domain of Kv2.1 to diverse Kv channels is sufficient to transfer Kv2.1-like clustering, and dynamic modulation of localization and voltage-dependent activation to these channels (Mohapatra & Trimmer, 2006). PRC (proximal restriction and clustering) signal was sufficient for the clustered localization, but not for the modulation of clustering (Mohapatra & Trimmer, 2006).
1.5.9 Functions of Kv2.1 unrelated to K+ conductance

Kv2.1 has also been implicated in non-traditional Kv channel functions. Kv2.1 is suggested to play a role in vesicular release (exocytosis) in both neurosecretory cells and sensory neurons (Feinshreiber et al, 2009; Feinshreiber et al, 2010). Kv2.1 binds to both syntaxin and SNAP25 in vitro and thus has been postulated to be directly involved in membrane fusion events (Michaelsvski et al, 2003).

It has been suggested that a significant portion of Kv2.1 channels at the cell surface exist in a non-conducting state (Fox et al, 2013; O’Connell et al, 2010), and the non-conducting state depends on the density of Kv2.1 channels (Fox et al, 2013). Kv2.1 is suggested to form insertion platforms for delivery of membrane proteins including other Kv ion channels to the plasma membrane (Deutsch et al, 2012). Kv2.1 has also been suggested to play a structural role in the remodeling of the cortical endoplasmic reticulum (cER) (Fox et al, 2015).
1.5.10 Kv2.1 deficient mice
A study of behavioral and neurological phenotypes of Kv2.1-deficient mice has recently been published (Speca et al, 2014). The study reported that deletion of Kv2.1 leads to neuronal and behavioral hyperexcitability. Kv2.1 deficient mice display reduced body weight compared to wildtypes, but no significant changes in brain anatomy are evident. No alteration in the expression of several related Kv channels in Kv2.1 KO brains is reported. Kv2.1 KO mice are hyperactive and they display impaired spatial learning, failing to improve performance in the Morris water maze. They also exhibit repetitive jumping and rearing when transferred to a new cage.

The effect of Kv2.1 deletion on delayed rectifier current $I_K$ was studied in cultured hippocampal neurons derived from Kv2.1 KO mice. The difference between $I_K$ recorded from KO and WT neurons was surprisingly modest considering the established role of Kv2.1 as a significant component of $I_K$ in several cell types. However, the slowly deactivating component of $I_K$ was shown to be reduced in Kv2.1 KO neurons. (Speca et al, 2014)

Kv2.1 deficient mice experience handling-induced seizures and are susceptible to chemically induced seizures. Specifically, Kv2.1 deficient mice display accelerated seizure progression in response to flurothyl-induced epileptic seizures and are also more susceptible to pilocarpine-induced seizures. In addition, recordings from hippocampal slices revealed increased responses to convulsant bicuculline. (Speca et al, 2014)

Kv2.1 deficient mice also show reduced fasting blood glucose levels and elevated serum insulin levels due to altered glucose-stimulated electrical activity in pancreatic $\beta$ cells (Jacobson et al, 2007).

1.5.11 Physiological and pathophysiological roles of Kv2.1
In the mammalian brain, Kv2.1 is thought to function as a homeostatic suppressor of elevated neuronal activity (Misonou et al, 2004; Misonou et al, 2005b; Misonou, 2010; Mohapatra et al, 2009; Speca et al, 2014; Surmeier & Foehring, 2004) and as a mechanism to suppress pathological hyperexcitability of central neurons during ischemic conditions (Misonou et al, 2005a; Misonou et al, 2008). In DRG neurons, Kv2 downregulation is suggested to contribute to hyperexcitability in chronic pain (Ishikawa et al, 1999; Kim et al, 2002; Tsantoulas et al, 2014).

In addition to regulating excitability in nervous system, Kv2.1 regulates cardiac ventricular repolarization (Xu et al, 1999), insulin secretion by pancreatic $\beta$-cells (Herrington et al, 2006; Li et al, 2013; MacDonald et al, 2002), and hypoxic pulmonary vasoconstriction (Archer et al, 1998; Patel et al, 1997). In cerebral artery smooth muscle, Kv2.1 also regulates myogenic constriction (Amberg & Santana, 2006).
1.6 Schizophrenia

1.6.1 Characteristics of schizophrenia

Schizophrenia is a devastating psychiatric illness producing great suffering for patients and also for their family members. Although general-population incidence estimates vary, it appears to affect 0.5-1% of people worldwide. It is one of the most important public health problems in the world. Heterogeneity is a hallmark of schizophrenia and there is considerable variation between patients (MacDonald & Schulz, 2009; Tandon et al, 2009). In fact, it is now widely accepted that schizophrenia likely includes multiple phenotypically overlapping disease entities or syndromes (Keshavan et al, 2011).

Characteristics of schizophrenia can be divided into three categories: positive symptoms, negative symptoms, and cognitive problems. The term “positive symptoms” refers to symptoms that are in excess of or distortions of normal functions – additions to normal thoughts, emotions, or behaviors (Weiden et al. 1999). These include hallucinations (typically auditory), delusions and thought disorders. The term “negative symptoms” refers to absence or reduction of normal emotions and behaviors. These include, for instance, alogia (poverty of speech), affective flattening (reduction of emotional expressiveness), anhedonia (inability to experience pleasure), avolition (lack of motivation), and apathy (general lack of interest). Cognitive deficits, although not diagnostic criteria, are considered as core features of schizophrenia. These include, for instance, deficits in working memory, attention, verbal learning and memory, information processing, and executive functioning. Mood symptoms can also occur in schizophrenia (Tandon et al, 2009).

Schizophrenia can be described by sequential trajectory, including premorbid phase, prodromal phase, first psychotic episode, repeated episodes of psychosis with inter-episode-remission, and stable phase (Tandon et al, 2009). There is enormous variation in the progression of the illness across patients, however. Psychotic symptoms lead to diagnosis, but patients usually experience other symptoms before the first acute phase of the disorder. These symptoms may exist for a few days or several years. Symptoms preceding the first acute phase are non-specific to schizophrenia, and similar symptoms are experienced in other psychiatric disorders. These include neurotic symptoms (e.g. anxiety), mood symptoms (e.g. depression), cognitive symptoms (e.g. difficulties to concentrate), perceptual symptoms, apathy, sleep disturbances, and behavioral changes (e.g. suspiciousness, social withdrawal) (Yung & McGorry, 1996). The onset of psychotic symptoms is usually during adolescence or early adulthood (MacDonald & Schulz, 2009). The age of onset is earlier in males. The initial decade of illness is generally marked by variable episodes of psychosis and inter-episode remission. Finally, in the stable phase psychotic symptoms are less prominent and negative symptoms and cognitive deficits become increasingly prominent (Tandon et al, 2009). Recovery of varying degrees can occur at any stage of the illness.
Schizophrenia is multifactorial in nature with contributions from multiple susceptibility genes and environmental risk factors. Currently, schizophrenia is widely considered as a neurodevelopmental disorder. However, the disease mechanism is poorly understood.

1.6.2 Genetics of schizophrenia
Schizophrenia is highly heritable and genetic factors contribute about 65-80% of liability to the illness (Lichtenstein et al, 2009; Sullivan et al, 2003). However, the genetic etiology is complex and remains largely unknown. Before the GWAS (genome wide association study) era, linkage analysis, and association studies of candidate genes have revealed a large number of susceptibility regions and genes. Among the most widely studied associated genes are e.g. DISC1 (disrupted in schizophrenia 1), DTNBP1 (dysbindin), NRG1 (neuregulin 1), GRM3 (glutamate receptor, metabotropic 3), and COMT (catechol-O-methyl-transferase) (Chubb et al, 2008; Harrison et al, 2008; Lewandowski, 2007; Li & He, 2007; Munafò et al, 2008). However, even for the most promising genes, there has been a lack of consistency in implicating particular alleles as liable for schizophrenia (Sanders et al, 2008). In recent years, substantial progress in understanding the genetic architecture of schizophrenia has come primarily through large-scale genomic approaches.

Genetic risk of schizophrenia is polygenic, each genetic variant contributing a small increment of risk (Purcell et al, 2014; Ripke et al, 2013). The risk is also heterogeneous across individuals, and the risk variant combinations may differ substantially between affected individuals, especially between families not sharing the same ancestry. Many of the risk-associated genetic variants for schizophrenia also contribute to the risk of other neuropsychiatric disorders, such as bipolar disorder, autism, depression, and attention-deficit hyperactivity disorder (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013; International Schizophrenia Consortium, 2009). Risk variants range in frequency from common to extremely rare (Sullivan et al, 2012). The existing findings account for only a minority of the heritability of schizophrenia, and several factors may contribute to the “missing heritability”, including yet unidentified common or rare variants, gene-gene interactions, and epigenetic factors (Harrison, 2015).

Patients with schizophrenia have an increased burden of large (over 100 kb) rare (frequency less than 1 %) copy number variants (CNVs) compared with controls. They also have an increased frequency of de novo CNVs (Kirov et al, 2012; Xu et al, 2008). CNVs in several distinct genomic regions have been associated with increased risk of schizophrenia. In general, schizophrenia-associated CNVs have large individual effect sizes but are extremely rare in the population. The effect of these CNVs is not specific to schizophrenia. The same CNVs are associated with other neurodevelopmental or psychiatric disorders.
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However, studies on CNVs, especially de novo CNVs, have gained insights into biological processes disturbed in schizophrenia. CNVs are enriched for genes in the postsynaptic density, particularly genes of the NMDA receptor complex, calcium-channel-signaling genes and targets of the fragile X mental retardation protein (FMRP) (Kirov et al, 2012; Szatkiewicz et al, 2014).

The major histocompatibility complex (MHC) region, harboring several genes involved in the immune system and autoimmunity, has been associated with schizophrenia in several studies (International Schizophrenia Consortium, 2009; Ripke et al, 2013; Schizophrenia Psychiatric Genome-Wide Association Study Consortium, 2011; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014; Shi et al, 2009; Stefansson et al, 2009).

The largest GWAS to date was published in 2014 and included almost 37 000 patients with schizophrenia and 113 000 controls from 40 different research centers, together forming the Psychiatric Genomics Consortium (PGC) (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). This was the largest molecular genetic study of schizophrenia, or indeed of any neuropsychiatric disorder, ever conducted. Meta-analysis of these datasets identified 108 genetic loci associated with schizophrenia. These 108 genomic regions included 25 previously reported and 83 novel loci. One associated locus contains the dopamine receptor D2 (DRD2) gene. Other genes especially noted were voltage-gated calcium channels (CACNA1C, CACNA1I, and CACNB2) and many genes involved in glutamatergic neurotransmission and synaptic plasticity (e.g. GRIA1, GRIN2A and GRM3, SRR). In addition to genes expressed in brain, the associations were enriched for genes expressed in tissues involved in immunity, supporting the role of immune dysregulation in schizophrenia. Concerning our study, the most interesting finding was that one schizophrenia-associated locus contained KCNB1 (encoding Kv2.1) and PTGIS genes. In addition, another associated locus contains KCNV1, the gene encoding Kv8.1 potassium channel subunit, which is able to interact with Kv2.1. GWAS only implicate regions, however, not genes. The associated 108 loci contain about 600 genes, and it is unclear which of these genes are the real susceptibility genes for schizophrenia and what are the real causal variants.

Enormous advances in sequencing technology have allowed exome sequencing studies detecting rare variants, SNPs, and small insertions or deletions (indels), which might contribute to schizophrenia (Fromer et al, 2014; Purcell et al, 2014). No single rare allele was associated at genome-wide levels of significance; however, significant enrichments for rare disruptive SNPs and indels were found in proteins affiliated with ARC and NMDAR genes, FMRP-targets, and voltage-gated calcium channels.

The knowledge of underlying genes can help to understand the pathophysiology of schizophrenia and to develop more accurate diagnostic procedures and treatment options. Converging evidence is now emerging from

1.6.3 Environmental risk factors of schizophrenia
Several environmental factors of small effect are associated with increased risk of developing schizophrenia. The environmental risk factors include, for instance, migrant status, older paternal age, Toxoplasmosis gondii antibodies, prenatal infection, famine, cannabis abuse, obstetric and perinatal complications, urbanicity, and winter/spring birth (Brown et al, 2005; Cannon et al, 2002; Davies et al, 2003; Malaspina et al, 2001; McGrath et al, 2004; Mednick et al, 1988; Penner & Brown, 2007; Semple et al, 2005; Susser et al, 1996).

A range of obstetric and perinatal complications have been linked to risk of developing schizophrenia in the offspring. Although the precise mechanisms have not been delineated, fetal hypoxia is most commonly cited as the mediating factor (Zornberg et al, 2000). Predisposing genetic factors might interact with hypoxia in increasing the risk of schizophrenia (Cannon et al, 2000).

1.6.4 Pharmacological treatment of schizophrenia
The introduction of chlorpromazine in the 1950s revolutionized the treatment of schizophrenia (Carpenter & Davis, 2012; Delay et al, 1952). Since then, over 60 antipsychotic drugs have been developed (Tandon et al, 2010). The one pharmacological property shared by all currently-available antipsychotic drugs, except aripiprazole, is their ability to block the dopamine D2 receptor (Carpenter & Koenig, 2008). Although the exact mechanism of antipsychotic drugs is unknown, these drugs are thought to block dopamine receptors in the mesolimbic dopaminergic system to alleviate psychotic symptoms. In addition to D2 receptor, antipsychotic drugs may have varying affinities for other receptors, including D1 receptors, several serotonin receptor subtypes (5-HT2A, 5-HT6, and 5-HT7), α1-adrenergic receptors and muscarinic receptors (Pratt et al, 2012).

Existing medications are most effective for positive symptoms (Leucht et al, 1999; Leucht et al, 2009), but they do have limited ability to improve negative symptoms and cognitive deficits, which often determine the level of functional
impairment (Miyamoto et al, 2012). Despite antipsychotic medication, schizophrenia remains a chronic illness with substantial functional impairments for most cases. In addition, one fifth to one third of all patients with schizophrenia are resistant to antipsychotic drug treatment (Conley & Kelly, 2001). No major differences in efficacy have been observed across various antipsychotic agents in meta-analysis of placebo-controlled studies (Leucht et al, 1999; Leucht et al, 2009). However, clozapine has been shown to be more effective in treatment resistant/refractory cases (Kane et al, 1988; McEvoy et al, 2006).

Antipsychotic medications may cause a range of serious side-effects. In contrast to broadly similar therapeutic effects, antipsychotic agents clearly differ in their propensity to cause different side-effects (Leucht et al; Miyamoto et al, 2012; Tandon et al, 2010). Unwanted consequences of dopamine-receptor blockade are the possible extrapyramidal side effects caused by dopamine blockade in the nigrostriatal system. The extrapyramidal side effects include tardive dyskinesia (involuntary, repetitive movements), Parkinsonism, dystonia, and akathisia (motor restlessness). Other possible side-effects of antipsychotics include, for instance, metabolic adverse effects, cardiac arrhythmias, sedation, prolactin elevation, and related sexual dysfunction, hypotension, and anticholinergic side effects (dry mouth, constipation, blurred vision) (Tandon et al, 2010). A rare but severe adverse effect of clozapine is agranulocytosis, which limits the more widespread use of clozapine (Tandon et al, 2010).

Essentially, the drug treatment for schizophrenia has not advanced substantially in the past 50 years (Carpenter & Davis, 2012). There is clearly a need for more effective and better-tolerated drugs, which would address the various symptom dimensions of schizophrenia.

1.6.5 Endophenotypes of schizophrenia

Reducing complex disorders into components with a clear genetic connection is described as an endophenotype strategy. Endophenotypes or “intermediate phenotypes” are measurable, hereditary characteristics/deficits that are associated with a specific disorder. Endophenotype can be a physiological, biochemical, anatomical, behavioral, or a cognitive feature. They are not seen by the unaided eye and are assessed by experimental, laboratory-based methods rather than by clinical observation. They are assumed to involve the same biological pathways as the disorder but to have simpler genetic architecture compared to the disorder itself. They lie in an intermediate position between genes and clinical symptoms. As such, they should provide substantially more power for finding disease genes. In addition to genetic analysis, endophenotypes are utilized in the development of animal models. (Allen et al, 2009; Gottesman & Gould, 2003; Gould & Gottesman, 2006)
Introduction

By criteria, endophenotype must: 1) associate with illness in the population; 2) be heritable; 3) be state-independent (manifest in an individual whether illness is active or in remission); 4) co-segregate with illness within families; and 5) be present at higher rate in non-affected family members of patients than in the general population (Gottesman & Gould, 2003).

The endophenotype concept has had success in schizophrenia, where endophenotypes including impaired prepulse inhibition (PPI, a measure of sensory motor gating deficits), eye-tracking dysfunction, and working memory deficits are already connected to genetic polymorphisms and have enough support to be considered as true endophenotypes of the disorder (Gould & Gottesman, 2006). Other possible schizophrenia endophenotypes include alterations in different electrophysiological measurements of the brain activity, such as event-related potentials P50, P300, and mismatch negativity (MMN) (Turetsky et al, 2007). Many other markers, for instance abnormalities in sustained attention, verbal learning and memory, spatial memory, and brain morphometric features, have been proposed as endophenotypes of schizophrenia (Carpenter & Koenig, 2008).

Prepulse inhibition (PPI) measures sensorimotor gating, which is a process of filtering redundant or unnecessary stimuli in the brain. Normally, an intense sensory stimulus elicits a whole-body startle response in almost all mammals. This rapid, intense sensory stimulus may be sound or light, or it may be tactile, such as an air puff. When a weak stimulus (prepulse) precedes the intense stimulus by 30-300 milliseconds, the startle response is reduced. This phenomenon is called prepulse inhibition. Patients with schizophrenia and their unaffected relatives show deficits in PPI (Braff et al, 1978; Braff et al, 2001; Cadenhead et al, 2000). However, impaired PPI is not specific to schizophrenia. PPI deficits are observed in various other neuropsychiatric disorders including bipolar disorder, obsessive compulsive disorder, and Tourette’s syndrome (Kohl et al, 2013).

1.6.6 Rodent behaviors related to schizophrenia
Animal models are useful tools in defining pathogenesis and treatment development for human disease. Creating adequate animal models of complex neuropsychiatric disorders like schizophrenia represent a particularly difficult challenge. Many of the symptoms, such as hallucinations and delusion, cannot be convincingly ascertained in animals. Nonetheless, several characteristics of schizophrenia, such as abnormal sensory gating, social behavior, working memory, and executive function, have reasonable correlates in mice (Nestler & Hyman, 2010; Powell & Miyakawa, 2006). Table 1 lists mouse behaviors that are considered potentially relevant to signs and symptoms of schizophrenia. Individual rodent behaviors are not animal models of schizophrenia. Rather, they are critical experimental protocols in the development and testing of animal models.
Some measurable abnormalities in schizophrenia can be measured directly in rodents, such as prepulse inhibition of startle (PPI). On the contrary, the positive symptoms of schizophrenia are challenging, if not impossible, to model in mice. As the disturbed dopamine transmission has been connected to psychotic symptoms, these symptoms are traditionally modeled in mice with dopamine-related behavior such as locomotor activity (Lipska & Weinberger, 2000; Pratt et al, 2012). Non-competitive NMDA-receptor antagonists (MK-801, PCP and ketamine) and amphetamine can cause psychosis in humans (MacDonald & Schulz, 2009). In rodents, these psychotomimetic drugs can induce increased locomotor activity, whereas antipsychotic drugs tend to reduce locomotor activity (Bubenikova-Valesova et al, 2008; Yee & Singer, 2013). Although the behavioral consequences of increased dopaminergic activity are different in humans and mice, there may be shared components of underlying biological mechanisms. However, several caveats exist, as the locomotor activity is a nonspecific behavior and the equivalent of locomotor activity in humans is unclear.

Psychotomimetic drugs can exacerbate psychotic symptoms in patients with schizophrenia (Lahti et al, 1995; Lieberman et al, 1987). Increased sensitivity to the locomotor-activating effects of these agents in rodents loosely parallels the increased sensitivity of patients with schizophrenia to these psychotomimetic drugs. Thus, sensitivity to psychotomimetic drugs is considered to be another correlate of positive symptoms (Powell & Miyakawa, 2006).

Several tests for social interaction in rodents have been developed (Powell & Miyakawa, 2006), but many are conducted in a novel environment that invokes exploratory and anxiety-like behaviors in addition to social behaviors complicating the interpretation of results. Social dominance can be measured in rodents by tube test (Powell & Miyakawa, 2006).

One of the most studied cognitive impairments in schizophrenia is impairment in working memory. In rodents, working memory can be measured e.g. in the eight-arm radial maze and delayed matching to place task in Morris water maze (Powell & Miyakawa, 2006). Working memory requires the ability to rapidly form memory traces of unique events and the ability to distinguish currently valid information from older and already-invalid information.
<table>
<thead>
<tr>
<th>Symptom Type</th>
<th>Symptom/sign of schizophrenia</th>
<th>Behavioral changes in rodents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Psychotic symptoms</td>
<td>(Increased locomotor activity)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Increased locomotor activity in response to novelty or stress)</td>
</tr>
<tr>
<td></td>
<td>Sensitivity to psychotomimetic drugs</td>
<td>Enhanced locomotor response to non-competitive NMDA-receptor antagonist (MK-801, PCP, ketamine)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Enhanced locomotor response to amphetamine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increased sensitivity to psychotomimetic drugs in other tests (e.g. increased effect of MK-801, PCP or ketamine in PPI test)</td>
</tr>
<tr>
<td>Negative</td>
<td>Social withdrawal</td>
<td>Decreased interaction with a juvenile conspecific</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Decreased place preference for a caged peer conspecific</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Decreased preference for social novelty</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Altered social dominance on tube test</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Altered aggression behavior on resident intruder assay</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Decreased nesting behavior</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Altered home-cage social interaction</td>
</tr>
<tr>
<td>Cognitive</td>
<td>Anhedonia</td>
<td>Decreased reinforcing properties of drugs of abuse and natural rewards</td>
</tr>
<tr>
<td></td>
<td>Deficits in learning and memory</td>
<td>Impaired alternation in T-maze working memory task</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Impaired performance in 8-arm radial maze working memory task</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Delayed non-match to sample tasks</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Decreased spatial learning in Morris water maze</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Decreased spatial learning in 8-arm radial maze</td>
</tr>
<tr>
<td></td>
<td>Attentional deficits</td>
<td>Decreased latent inhibition</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Impaired 5-choice serial reaction time task</td>
</tr>
<tr>
<td></td>
<td>Executive dysfunction</td>
<td>Decreased set-shifting ability</td>
</tr>
<tr>
<td>Sensory gating</td>
<td>Impaired prepulse inhibition (PPI)</td>
<td>Impaired prepulse inhibition (PPI)</td>
</tr>
</tbody>
</table>

Modified from (Arguello & Gogos, 2006; Nestler & Hyman, 2010; Powell & Miyakawa, 2006)
1.6.7 Existing rodent models related to schizophrenia

It is clear that any animal model of schizophrenia can capture only some aspects of this heterogeneous disorder characterized by abnormalities in uniquely human behaviors. Absence of any single defining symptom or consistent biological marker and limited knowledge of underlying genetic factors hinders the development of animal models for schizophrenia. The value of animal models lies in them being informed approximations of isolated components of the disease. As such, several rodent models have been developed in order to better understand specific aspects of schizophrenia. These can be divided into genetic, developmental, drug-induced, or lesion models (Carpenter & Koenig, 2008). Some models also combine genetic and environmental risk factors.

A large number of genetically modified mice has been developed by modifying schizophrenia associated genes/chromosomal regions or genes involved in biological pathways/processes associated with schizophrenia (Carpenter & Koenig, 2008; Pratt et al, 2012). Such mice provide insight into the neurobiological role of specific genes in terms of behavioral phenotypes, as well as the impact of these specific genes on downstream biological pathways and neuropathological alterations.

Developmental models include e.g. isolation rearing, neonatal immune challenge, maternal malnutrition, and prenatal stress models (Carpenter & Koenig, 2008). The developmental models may be most useful in combination with genetic models as this allows the genetic-environmental interactions to be captured.

Several drug-induced models have been developed. Models based on NMDA receptor antagonists (PCP, ketamine or MK-801) have been widely used in rodents. In addition to producing psychosis or exacerbating psychotic symptoms, administration of NMDA receptor antagonists in humans induces changes closely resembling schizophrenia, also including the negative symptoms of the disease as well as the cognitive deficits (Bubenikova-Valesova et al, 2008). In rodents, both acute and chronic administration of NMDA receptor antagonists has also been successful in modelling cognitive impairment as well as positive and negative symptoms of schizophrenia (Bubenikova-Valesova et al, 2008). In addition to these behavioral changes, chronic administration of NMDA receptor antagonist has been reported to result in a decreased mRNA expression of enzyme GAD67 and reduction of parvalbumin-positive interneurons (Qin et al, 1994; Rujescu et al, 2006). These findings correlate with neuropathological findings in patients with schizophrenia (Benes & Berretta, 2001; Hashimoto et al, 2003; Volk et al, 2000).

Lesion models include, for instance, neonatal amygdalar lesion, neonatal ventral hippocampal lesion, and prefrontal cortical lesion (Carpenter & Koenig, 2008).
2. AIMS OF THE STUDY

A starting point of this study was to identify novel genes induced by the neurite outgrowth-promoting protein HMGB1 (amphoterin). Following the identification of AMIGO, our main goal was to understand the characteristics and function of the AMIGO protein. We were especially interested in the role of AMIGO in Kv2.1 channel complex and the role of AMIGO-Kv2.1 channel complex in mouse behavioral disturbances and human psychiatric disorders. We considered *KV2.1* (*KCNB1*) as a candidate gene for human schizophrenia and schizophrenia spectrum disorders. The specific aims of this study were:

1) To identify novel genes induced by the neurite outgrowth promoting protein HMGB1 (amphoterin)

2) To characterize the expression pattern of the novel gene/protein AMIGO

3) To examine the role of AMIGO in nervous system function with the help of AMIGO KO mice

4) To understand the role of AMIGO in Kv2.1 channel complex

5) To examine the role of the AMIGO-Kv2.1 channel complex in behavioral disturbances

6) To examine the possible association of *KV2.1* (*KCNB1*) variant allele (rs34280195) with human schizophrenia and schizophrenia spectrum disorders
3. EXPERIMENTAL PROCEDURES

The methods used in this study are listed in Table 2. Detailed description of the materials and methods can be found in the original publications, which are referred to by their Roman numerals.

Table 2. Methods used

<table>
<thead>
<tr>
<th>Method</th>
<th>Used in</th>
<th>Author personally involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude brain membrane protein samples</td>
<td>III</td>
<td>III</td>
</tr>
<tr>
<td>Determination of monoamines</td>
<td>III</td>
<td>III</td>
</tr>
<tr>
<td>DNA constructs</td>
<td>I,II</td>
<td></td>
</tr>
<tr>
<td>Electrophysiology of acute hippocampal slices</td>
<td>III</td>
<td>III</td>
</tr>
<tr>
<td>Electrophysiology of cultured neurons</td>
<td>II</td>
<td>II</td>
</tr>
<tr>
<td>HEK293 cell culture and transfection</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>Histological stainings</td>
<td>III</td>
<td>III</td>
</tr>
<tr>
<td>Human genetic studies</td>
<td>III</td>
<td></td>
</tr>
<tr>
<td>Immunocytochemistry</td>
<td>II</td>
<td>II</td>
</tr>
<tr>
<td>Immunohistochemistry (paraffin sections)</td>
<td>I,II</td>
<td>I,II</td>
</tr>
<tr>
<td>Immunohistochemistry (cryosections)</td>
<td>II,III</td>
<td>II,III</td>
</tr>
<tr>
<td>Immunoprecipitation</td>
<td>II</td>
<td></td>
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<tr>
<td>In situ hybridization</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>Knockout mouse production</td>
<td>III</td>
<td></td>
</tr>
<tr>
<td>Lentivirus production and transfection</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>Microscopy and image acquisition</td>
<td>I,II,III</td>
<td>I,II,III</td>
</tr>
<tr>
<td>Mouse behavioral studies</td>
<td>III</td>
<td></td>
</tr>
<tr>
<td>Ordered differential display</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>Primary neuron culture</td>
<td>I,II</td>
<td>II</td>
</tr>
<tr>
<td>Protein samples from brain tissue</td>
<td>I,II</td>
<td>I,II</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>Treatments of primary neurons</td>
<td>II</td>
<td>II</td>
</tr>
<tr>
<td>Western blotting</td>
<td>I,II,III</td>
<td>I,II,III</td>
</tr>
</tbody>
</table>
4. RESULTS

4.1 AMIGO is a novel neuronal LRR protein (I, II)

4.1.1 Identification of AMIGO
A starting point of this study was to identify novel genes induced by the neurite outgrowth-promoting protein HMGB1 (amphoterin). With ordered differential display (ODD), the mRNA expression was compared in cultured hippocampal neurons growing on a surface coated with either HMGB1 or another neurite outgrowth promoting protein laminin (I: Figure 1). A novel gene was found with induced expression on HMGB1 (amphoterin) surface, and was named AMIGO (amphoterin-induced gene and ORF). Subsequently, we have studied the properties and function of AMIGO.

Bioinformatics revealed that the amino acid sequence of AMIGO comprises a small cytoplasmic region, a single transmembrane domain, and an extracellular part containing both immunoglobulin (Ig) and leucine-rich repeat (LRR) domains. The LRR domain of AMIGO is composed of six LRRs (LRR1-6) flanked by N-terminal and C-terminal cap regions (LRRNT and LRRCT). The domain structure of AMIGO is presented in Figure 8.

![Figure 8. Domain structure of AMIGO. Schematic representation of the AMIGO domain structure. (LRRNT) LRR N-terminal cap region, (LRR 1-6) leucine rich repeats 1-6, (LRRCT) LRR C-terminal cap region, (IG) immunoglobulin domain, (TM) transmembrane domain.](image)
AMIGO has five predicted N-glycosylation sites, two in the LRR domain and three in the Ig domain. All of these sites are shown to be at least partially glycosylated in HEK293 cells (Kajander et al, 2011). In mouse brain, the molecular mass of AMIGO was around 80 kDa compared to the predicted molecular mass of AMIGO (56 kDa), suggesting significant post-translational modifications (II: Supplementary Figure S3A). Indeed, the molecular mass of AMIGO in mouse brain samples is reduced following PGNase F treatment, indicating N-linked glycosylation of AMIGO (Chen et al, 2012).

Sequence comparison revealed that AMIGO protein is conserved across species. At the amino acid level, the identity between the rat and mouse AMIGO is 95% and the murine sequences are 89% identical to human AMIGO. Interestingly, the whole transmembrane domain and the cytoplasmic tail are 100% identical between the murine and human AMIGOs. The function of AMIGO has also been studied in zebrafish (Zhao et al, 2014). Zebrafish AMIGO1 shares over 50% identity in its amino acid sequence with AMIGO protein in human, mouse, rat, xenopus, and medaka fish (Zhao et al, 2014).

### 4.1.2 AMIGO protein family

Following identification of AMIGO, two other genes were cloned on the basis of their homology to AMIGO (I: Figure 2A). These molecules were named AMIGO2 and AMIGO3. The three AMIGO proteins encoded by these genes comprise a novel AMIGO protein family belonging to the LRR- and Ig-superfamilies.

All three AMIGOs share a similar domain structure. Sequence homology at the amino acid level between AMIGO and AMIGO2/AMIGO3 is about 50%. The most conserved regions between the three proteins are the LRRs, the transmembrane region, and some parts of the cytosolic tail (I: Figure 2A).

Expression of the AMIGO proteins was studied in mouse tissues with RT-PCR (I:Figure 3A). All of the three AMIGOs are expressed in brain. The expression of AMIGO2 and AMIGO3 in adult mouse tissues is more widespread than the expression of AMIGO. The expression of AMIGO3 mRNA was detected in all tissues studied.

### 4.1.3 Overall distribution of AMIGO

The AMIGO mRNA expression during development was studied with\textit{ in situ}\ hybridization in mouse embryo (I: Figure 3B). A clear AMIGO expression was detected already in the E13 mouse embryo. At this stage, the highest expression was found in the dorsal root ganglia and in the trigeminal ganglion, with some expression in the central nervous system.
Results

With RT-PCR analysis from different adult mouse tissues, AMIGO mRNA was shown to be primarily expressed in the nervous system, including cerebrum, cerebellum, and retina (I: Figure 3A). We have also studied the expression of Amigo gene with lacZ reporter gene in tissues of heterozygous AMIGO knockout mice. These studies confirmed wide expression of the Amigo gene in the nervous system, including cerebrum, cerebellum, pons, spinal cord, dorsal root ganglia, and sympathetic chain ganglia (data not shown). According to preliminary analysis with NeuN and GFAP antibodies, lacZ reporter expression was limited to neurons and was not present in astrocytes (data not shown).

In situ hybridization studies demonstrated that the mRNA of AMIGO was prominently expressed in adult brain, where the most intense signal was detected in the hippocampus (I: Figure 3B). The localization of AMIGO protein in mouse cerebrum was further defined with immunohistochemistry. Immunostaining of AMIGO was widely detected in mouse cerebrum (II: Supplementary Figure S2A and C). AMIGO seemed to be present in practically all cerebral neurons. Intensity of staining varied between different brain areas and different neuronal cell types. Especially strong immunoreactivity was present in cortical structures. In neocortex, the most prominent AMIGO staining was detected in the large pyramidal neurons of layer V (II: Supplementary Figure S2E).

4.1.4 Subcellular distribution of AMIGO
Although wide in tissue level, immunostaining of AMIGO had a restricted subcellular localization. Staining was clearly restricted to the soma and to proximal part of neurites (II: Supplementary Figure S2E and G). In the large pyramidal cells of the neocortex, the staining extended along the apical dendrite more distally from the soma than in any other neurons studied (II: Supplementary Figure S2E and G). At higher magnification, immunostaining revealed a distinct, punctate pattern (II: Supplementary Figure S2G). AMIGO appeared in clusters associated with neuronal plasma membrane.

We also studied the localization of AMIGO in neuronal primary cultures. Similar distinctive subcellular distribution of AMIGO was preserved in cultured hippocampal neurons (II: Figure 1C and Supplementary Figure S5A). Staining was restricted to the peri-somatic portion of cultured neurons and the staining was highly clustered in nature in most of the cells.

4.1.5 Temporal expression of AMIGO protein in brain
The expression of AMIGO protein during brain development was studied with immunoblotting using mouse brain lysates from several postnatal stages (II: Supplementary Figure S3A). AMIGO was detected with western blotting techniques at low level after birth. The amount of AMIGO increased gradually,
with clear increase in protein amount around two weeks after birth. The amount of AMIGO continued to increase toward adulthood.

A similar increase in AMIGO protein amount toward adulthood was seen in rat-brain lysates, commencing around two weeks after birth (I: Figure 4C). The rat brain samples also demonstrated down-regulation during postnatal stages P6-P10, which was not evident in mouse samples. The expression of AMIGO protein was also studied during embryonic development with rat brain samples. The AMIGO protein was detected in embryonic brain samples starting from E14 stage.

4.2 AMIGO is a component of Kv2.1 potassium channel complex (II)

4.2.1 Spatial and temporal co-expression of AMIGO and Kv2.1 potassium channel
One of the key questions in this study was to understand the distinct (wide, but subcellularly restricted) localization of AMIGO in the nervous system. We were fascinated to find out that similar localization has been extensively demonstrated for the voltage-gated potassium channel Kv2.1. While Kv2.1 is widely expressed in mouse cerebral neurons, its subcellular localization is highly restricted to large clusters on the soma and proximal dendrites (Du et al, 1998; Hwang et al, 1993; Rhodes et al, 1995; Scannevin et al, 1996; Trimmer, 1991). We therefore compared the spatial and temporal expression of AMIGO and Kv2.1. Indeed, a highly similar staining pattern was seen for Kv2.1 and AMIGO in successive histological sections of mouse cerebrum (II: Supplementary Figure S2). The staining patterns were identical in every brain location studied. For both proteins, the staining was clustered in nature. In addition, western blotting revealed a parallel increase in expression of AMIGO and Kv2.1 proteins during postnatal brain development (II: Supplementary Figure S3).

4.2.2 Colocalization of AMIGO and Kv2.1 potassium channel
Since the spatial and temporal expression of AMIGO strongly resembled the expression of Kv2.1 potassium channel, double immunohistochemical staining was performed to determine their localization. Confocal microscopy revealed a striking colocalization of AMIGO and Kv2.1 (II: Figure 1A and B). Both proteins were localized to the same distinct clusters at the neuronal perisomal membrane. Localization of AMIGO in mouse cerebrum overlapped with that of Kv2.1 in all brain areas studied.
Results

AMIGO and Kv2.1 are both expressed natively in cultured neurons (Kuja-Panula et al, 2003; Maletic-Savatic et al, 1995). In addition to brain sections, colocalization of AMIGO and Kv2.1 was studied in rat primary neurons. AMIGO and Kv2.1 also colocalized extensively in cultured hippocampal neurons (Figure 9 and II: Figure 1C). In cultured cells, many of the neurons displayed clustered staining similar to staining in neurons in vivo.

As neurons were grown in culture, the AMIGO and Kv2.1 stain became gradually stronger. Both proteins were detected in characteristic clusters in the plasma membrane around 14 days in vitro. Even during this in vitro development, AMIGO and Kv2.1 were detected simultaneously and in the same location (data not shown).

In addition, we transfected HEK293 cells with AMIGO and Kv2.1. Transfected Kv2.1 is reported to form large clusters in HEK293 cells like those seen in neurons (Mohapatra & Trimmer, 2006; O'Connell & Tamkun, 2005). When expressed together in HEK293 cells, AMIGO and Kv2.1 colocalized significantly (II: Figure 1D). Both proteins were localized to ring-shaped structures. These structures resembled the clusters seen in neurons. When transfected separately, Kv2.1 was not recognized by AMIGO antibody, and vice versa (II: Supplementary Figure S1)

Figure 9. Colocalization of AMIGO and Kv2.1. Double-immunostaining of cultured hippocampal neuron with AMIGO (red) and Kv2.1 (green) antibodies. The overlay of images is presented in the right panel (merged). AMIGO and Kv2.1 exhibited striking colocalization. Both proteins localized to clusters in the neuronal soma and the proximal part of neurites.
4.2.3 Association of AMIGO and Kv2.1 potassium channel

We next asked whether AMIGO associates with Kv2.1 using immunoprecipitations with AMIGO and Kv2.1 antibodies. Immunoprecipitation from mouse brain lysates demonstrated the association of AMIGO and Kv2.1 proteins (II: Figure 2 and Supplementary Figure S4). The interaction was prominent in DSP-crosslinked tissue material, but it could be detected even without crosslinking (II: Supplementary Figure S4).

As a control, the association of AMIGO with Kv1.2 in mouse brain lysates was also assessed. Kv1.2 is another widely expressed Kv channel α-subunit in brain, but the localization of Kv1.2 is predominantly axonal. No association was seen between AMIGO and the Kv1.2 protein (II: Figure 2).

4.2.4 Stimulus-induced relocalization of AMIGO and Kv2.1

The phosphorylation stage, localization, and activity of Kv2.1 are known to be strongly regulated. Under normal conditions, Kv2.1 is heavily phosphorylated and its localization is clustered (Du et al, 1998; Hwang et al, 1993; Misonou et al, 2006; Trimmer, 1991). Several stimuli are able to induce a dramatic dephosphorylation of Kv2.1 and dispersion of Kv2.1 from clusters. Dephosphorylation and diffusion can be induced in rat brain by kainate-induced seizures or in cultured neurons by glutamate stimulation (Misonou et al, 2004). Similar changes are induced by CO2 treatment in rat brain or by a chemical ischemia model in cultured neurons (Misonou et al, 2005a; Misonou et al, 2008). We were interested in the localization of AMIGO and the interaction of AMIGO and Kv2.1 upon stimulation induced change in Kv2.1 localization.

In cultured hippocampal neurons, a short glutamate treatment has been shown to induce Kv2.1 dephosphorylation and dispersion of Kv2.1 from clusters (Misonou et al, 2004; Misonou et al, 2006). We studied the localization of AMIGO and Kv2.1 in cultured hippocampal and cortical neurons after treatment with glutamate (10 μM glutamate for 15 min) or with glutamate and NMDA receptor blocker AP-5 (II: Supplementary Figure S5). In control conditions, a large proportion of neurons displayed clustered staining of Kv2.1 and AMIGO. After treatment with glutamate, most of the neurons displayed diffuse staining of Kv2.1, as reported previously. Importantly, the glutamate treatment also dramatically decreased the clustered staining of AMIGO. NMDA receptor antagonist AP-5 was able to reduce the diffusion of AMIGO and Kv2.1 from clusters. In all conditions, AMIGO and Kv2.1 colocalized in all neurons studied, whether the staining pattern of the neuron was clustered, diffuse, or a combination of clustered and diffuse.

We also studied the distribution of AMIGO in mouse brain in response to CO2 treatment. Distribution of both AMIGO and Kv2.1 was diffuse in cortical neurons of CO2-treated mice (II: Figure 2D). However, the staining of both proteins remained restricted to the soma and the proximal part of the neurites. We
concluded that localization of AMIGO displayed a similar, dramatic change in response to CO\textsubscript{2} treatment, as was previously reported for Kv2.1. AMIGO and Kv2.1 colocalized in brains from CO\textsubscript{2} treated animals as well as in brains obtained without hypoxia.

4.2.5 AMIGO associates with Kv2.1 following stimulus-induced relocalization
AMIGO and Kv2.1 were both diffusely distributed over the proximal neuronal plasma membrane in mouse brain after CO\textsubscript{2} treatment. It was unclear, however, whether the interaction of AMIGO and Kv2.1 was retained in diffuse localization or whether both proteins were independently dispersed over the membrane. To address this question, we immunoprecipitated AMIGO and Kv2.1 also from brain lysate of CO\textsubscript{2}-treated mice (II: Figure 2). With western blot we confirmed the decreased size of Kv2.1 after CO\textsubscript{2} treatment, previously shown to be due to dephosphorylation of the channel protein (Misonou et al, 2005a). Immunoprecipitations demonstrated that AMIGO and Kv2.1 were also associated when Kv2.1 was dephosphorylated, and both proteins were diffusely localized to neuronal membrane.

4.2.6 AMIGO alters voltage-dependent activation of Kv2.1
To examine the effects of AMIGO on Kv2.1 channel current, we recorded ionic currents in HEK293 cells expressing Kv2.1 either alone or together with AMIGO. Transfected HEK293 cells have been widely used to study the Kv2.1 channel, since the properties of the channel in transfected HEK293 cells resembles the properties of the Kv2.1 channel in neurons (Mohapatra & Trimmer, 2006; O'Connell & Tamkun, 2005). Transfected Kv2.1 forms large clusters and is extensively phosphorylated, as in neurons (Mohapatra & Trimmer, 2006; O'Connell & Tamkun, 2005). In contrast, when expressed in Xenopus oocytes, Kv2.1 is reported to be in a non-phosphorylated form (Misonou et al, 2005b). As we have shown, AMIGO and Kv2.1 colocalize extensively in transfected HEK293 cells (II: Figure 1D).

Kv2.1 current is activated by membrane depolarization. No significant difference was seen in current amplitudes at maximal membrane depolarization (+100 mV) when the Kv2.1 was present alone or with AMIGO. However, the Kv2.1 current was activated at lower (more hyperpolarized) membrane potentials, when AMIGO was expressed with Kv2.1 (II: Figure 3).

4.2.7 AMIGO alters voltage-dependent activation of neuronal I\textsubscript{K}
Kv2.1 is an essential component of delayed rectifier currents (I\textsubscript{K}) in hippocampal neurons and several other neuronal types (Du et al, 2000; Murakoshi & Trimmer, 1999). We therefore studied the effect of AMIGO on neuronal I\textsubscript{K} in cultured hippocampal neurons. The expression of AMIGO was inhibited with iRNA and the I\textsubscript{K} currents were measured with whole-cell patch-clamp recordings. Following
inhibition, a clearly reduced amount of AMIGO protein in cell lysates was confirmed with Western blotting (II: Supplemental Figure S1C). Consistent with our data from the heterologous expression system in HEK293 cells, the currents were similar in response to strong membrane depolarization, but inhibition of AMIGO significantly decreased the current at the threshold of activation (II: Figure 4). The I_K current was activated at higher (more depolarized) membrane potentials, when AMIGO was inhibited.

4.3 AMIGO KO mice display reduced amount of Kv2.1 protein and altered electrophysiological properties of neurons (III)

In this study, we have generated mice that lack the Amigo gene (III: Supplementary Fig. 1). To further understand the physiological role of AMIGO in mouse brain, we have studied the molecular, electrophysiological, morphological and behavioral properties of these knockout (KO) mice.

4.3.1 Brain structure of AMIGO KO mice appears normal
AMIGO KO mice develop and breed normally. Their brain morphology was studied in detail using histological staining approaches. Luxol fast blue/cresyl violet staining revealed no gross morphological differences between AMIGO KO and WT brain (III: Figure 1A). Immunostainings for markers of neurons and glial cells (NeuN, neurofilament M, GFAP, CNPase) were essentially similar in all areas studied (data not shown).

4.3.2 Decreased amount of Kv2.1 channel in AMIGO KO mouse brain
We compared the amount of Kv2.1 protein in membrane protein samples prepared from AMIGO KO and WT brains (III: Figure 1B). We detected a 45% reduced amount of Kv2.1 protein in AMIGO KO brains as compared to WT. Similar results were obtained from total brain samples (data not shown).

Kv1.2 antibody was used as a control (III: Figure 1B). Kv1.2 is another widely expressed Kv channel α-subunit in brain, but the localization of Kv1.2 is predominantly axonal. The amount of Kv1.2 was unaltered in AMIGO KO mice.

The reduced amount of Kv2.1 was also visible in immunohistochemical stainings, where the intensity of Kv2.1 immunostaining was clearly lower in AMIGO KO brain compared to WT (III: Figure 1C).

4.3.3 Localization of Kv2.1 is not altered in the AMIGO KO mouse brain
A number of studies have focused on defining the determinants of the distinct subcellular distribution of Kv2.1 (Lim et al, 2000; Mohapatra & Trimmer, 2006; Scannevin et al, 1996). The striking colocalization of AMIGO and Kv2.1 prompted us to consider the possible role of AMIGO in Kv2.1 localization. We
therefore examined whether the localization of Kv2.1 was altered in AMIGO KO brain (III: Figure 1C). Although the intensity of Kv2.1 immunohistochemical staining was clearly weaker in KO brain, the localization of Kv2.1 protein was unaltered. Kv2.1 remained localized to the soma and proximal part of neurites, and the staining was clustered in nature.

4.3.4 Voltage-dependent activation of neuronal I\textsubscript{K} is altered in AMIGO KO mice

Since Kv2.1 is an essential component of the I\textsubscript{K} current in hippocampal neurons (Du et al., 2000; Murakoshi & Trimmer, 1999), and the inhibition of AMIGO alters the voltage-dependent activation of the I\textsubscript{K} current in cultured hippocampal neurons (II), we compared the neuronal I\textsubscript{K} currents in hippocampal slices from WT and AMIGO KO mice (III: Figure 1D and E). In agreement with our result in cultured neurons, we found a decreased current density in the KO mice at the threshold of activation. The I\textsubscript{K} current was activated at higher (more depolarized) membrane potentials in AMIGO KO mice.

4.4 AMIGO KO mice display several schizophrenia-related features (III)

The behavioral phenotype of the AMIGO KO mice was studied in detail (III: Table 1). AMIGO KO mice performed normally in a number of behavioral tasks. However, the KO mice displayed many schizophrenia-related behavioral abnormalities. Modeling psychiatric disorders in animals is obviously challenging. However, several characteristics of schizophrenia have correlates in mice such as: abnormal social behavior, impaired working memory, and defective prepulse inhibition (PPI) (Lipska & Weinberger, 2000; Powell & Miyakawa, 2006). Psychotic symptoms are traditionally modeled in mice by studying dopamine-related behavior such as locomotor activity (Lipska & Weinberger, 2000; Pratt et al., 2012).

4.4.1 AMIGO KO mice display increased locomotor activity

AMIGO KO mice displayed increased locomotor activity. In an open field, the KO mice traveled significantly more than the WT littermates (III: Table 1, Figure 2A and B). The increased locomotor activity of AMIGO KO mice was also evident in the elevated plus maze (III: Table 1). The locomotor activity of the AMIGO KO mice in the open field was reduced by the application of the antipsychotic drug clozapine (1 mg/kg), which could not be observed in the WT mice (III: Figure 2C and D).
4.4.2 AMIGO KO mice display sensitivity to psychotomimetic drug
We then tested the effects of the psychotomimetic drug MK-801 in the AMIGO KO and WT mice (III: Figure 2E and F). MK-801 is a non-competitive NMDA receptor antagonist that produces psychosis in humans. Non-competitive NMDA antagonists are used to model several aspects of schizophrenia in mice and have been shown to exacerbate symptoms in schizophrenic patients (Lipska & Weinberger, 2000). The AMIGO KO mice were more sensitive to the locomotor-activating effect of MK-801 than WT mice. In the WT animals, a low dose of MK-801 (0.2 mg/kg) slightly increased the distance travelled, but the effect was not significantly different from saline. In the KO animals, the same dose significantly increased the distance travelled.

4.4.3 AMIGO KO mice display reduced prepulse inhibition
The acoustic-startle response was measured in the AMIGO WT and KO mice. The AMIGO KO mice displayed reduced levels of acoustic startle (III:Table I). After baseline acoustic startle was established, we measured sensorimotor gating ability in the form of prepulse inhibition (PPI). PPI is a widely-used endophenotype of schizophrenia (Turetsky et al, 2007). Several studies have demonstrated impaired PPI in schizophrenic patients (Gottesman & Gould, 2003; Keshavan et al, 2008). AMIGO KO mice demonstrated a reduced PPI compared to WT littermates, which was seen in all prepulse intensities studied (III: Figure 2G).

Antipsychotic drugs improve the impaired PPI in schizophrenic patients and in animal models of schizophrenia (Kumari & Sharma, 2002). We found that the antipsychotic drug haloperidol (1 mg/kg) improved the reduced PPI in the AMIGO KO animals (III: Fig. 1H).

4.4.4 AMIGO KO mice display altered social behavior
When studying social behavior with the resident-intruder test, there was no significant difference between AMIGO KO and WT mice regarding time that the animals spent in social or nonsocial activity (III: Table 1). However, this test can be affected by increased activity of the AMIGO KO mice. Social dominance, measured using the tube test (III: Table 1), demonstrated that AMIGO KO mice were significantly more submissive than WT. The WT mice won in 73% of trials whereas the KO mice won only in 25%.

4.4.5 AMIGO KO mice have impaired cognitive function
We also detected a specific cognitive deficit in the AMIGO KO mice. At the outset, no difference from the WT littermates was seen in Morris water maze (data not shown). Further testing in the IntelliCage platform, which enables automated monitoring of spontaneous and learning behavior in a home cage-like
environment, also demonstrated no difference in many of the behavioral parameters, including group learning and relearning in corner preference task. AMIGO KO mice did, however, demonstrate a significantly impaired performance in the IntelliCage patrolling task (III: Table 1), where the mouse has to learn that the “correct” corner is changed in a clockwise manner after every “correct” visit to that drinking corner. The task is comparable with the eight-arm radial maze: both of them test behavioral flexibility and working memory (Too et al, 2013; Weyer et al, 2011).

4.4.6 Other behavioral properties of AMIGO KO mice
Nociception (Hot plate) and motor coordination (Rota-Rod, Beam walking test) of the AMIGO KO mice were normal (III: Table 1). The circadian activity of the AMIGO KO mice was not altered (data not shown). AMIGO KO mice displayed no abnormalities in spontaneous alternation in Y-maze and IntelliCage (III: Table 1). From the two tests modeling anxiety-related behavior, the light/dark test suggested that the AMIGO KO mice are more anxious (III: Table 1). However, the elevated plus maze suggested that the AMIGO KO mice are less anxious (III: Table 1). The discrepancy between these two tests may be due to the hyperactivity of the AMIGO KO mice and dependency of the used tests on general locomotor activity.

4.4.7 Neurotransmitter analysis of AMIGO KO mice
Several transmitter systems are suggested to be altered in schizophrenic brains, including dopamine, glutamate, GABA, and serotonin (Keshavan et al, 2008). To determine whether there are alterations in monoamine neurotransmitter and their metabolite levels in AMIGO KO mice, we performed HPLC analysis of brain samples from different areas (III: Supplementary Table 1). The serotonin concentration in prefrontal and temporal cortex of the KO mice was higher than in WT mice, whereas the 5-HT turnover (5-HIAA/5-HT ratio) was unchanged.

4.5 Association of KV2.1 variant allele with human schizophrenia (III)
We found altered amount and function of Kv2.1 channel and several schizophrenia-related abnormalities in the AMIGO KO mice. Our results clearly identified \( AMIGO1 \) and \( KV2.1 (KCNB1) \) as candidate genes for human schizophrenia and related psychiatric disorders. We were especially interested in the \( KV2.1 (KCNB1) \) variation substituting the penultimate amino acid, serine 857 with asparagine (Ser857Asn). Two individuals homozygous for Asn857 have been identified in a study addressing a possible association of the variant allele with an EEG trait (Mazzanti et al, 1996). Interestingly, one of the Asn857/Asn857
homozygotes was reported to have schizophrenia and the other had paranoia. However, the association of the corresponding single nucleotide polymorphism (SNP) rs34280195 variant with schizophrenia has not been studied before.

We hypothesized that the allele Asn857 of variation rs34280195 would comprise a rare, high-impact genetic risk factor for schizophrenia. We analyzed three nonsynonymous SNPs of KV2.1 (KCNB1) in a Finnish schizophrenia family sample comprising of 3335 individuals (1209 with schizophrenia spectrum disorder) from families with multiple cases of schizophrenia (III: Table 2). According to the hypothesis, the minor allele of Rs34280195, corresponding to Asn857, associated significantly with schizophrenia and schizophrenia-spectrum disorders (p = 0.0019 and p = 0.0087, respectively; Bonferroni-corrected p-values 0.01 and 0.05). When we studied the association further we found that the association was strongest in a subpopulation of our study (p = 1.2 x 10^{-5} and p = 2.2 x 10^{-5}, for schizophrenia and schizophrenia spectrum disorders, respectively), which includes families from an internal isolate (IS) with high prevalence of schizophrenia and a very limited number of founder chromosomes (Hovatta et al, 1997). In the families that were not from the internal isolate, but from all around Finland (AF families) there was a tendency for association with schizophrenia-spectrum disorders (p = 0.035, not corrected for multiple testing). No significant association was evident for rs34467662 and rs112735799 with schizophrenia or schizophrenia-spectrum disorders in either set of families.

The Ser857Asn variation (rs34280195) was enriched in the IS families. The allele frequency in the IS families was 0.078. The allele frequency in families with schizophrenia that were not from the isolate was 0.034. In 1000 Genomes database, the allele frequency for this variation in Finns is 0.012 (N=93) and in all reported populations 0.005 (N=1089). Thus, the coding sequence variant rs34280195 associated highly significantly with schizophrenia and schizophrenia-spectrum disorders in families from an internal isolate of Finland, and the disease-associated allele was particularly enriched in these families.
5. DISCUSSION

5.1 Localization of AMIGO
Several different localizations have been described for AMIGO protein in adult rodent brains by us and by others with immunohistochemistry using different antibodies (Chen et al, 2012; Kuja-Panula et al, 2003; Peltola et al, 2011). However, only the antibody used in publication II in this study has been validated with AMIGO KO mouse tissue. With this antibody we have defined the wide distribution of AMIGO in cerebral neurons and the localization of AMIGO in clusters in soma and proximal part of dendrites. AMIGO is also shown to colocalize with Kv2.1. More recently, the localization of AMIGO to clusters with Kv2.1 has been replicated with a different antibody, by an independent group, in a study addressing the coupling of Kv2.1 channels and ryanodine receptors (Mandikian et al, 2014). The specificity of a monoclonal AMIGO antibody (Neuromab clone L86A/37) used by Mandikian et al. has also been validated with AMIGO KO mouse tissue. In conclusion, despite the discrepancy in published data about AMIGO localization, we are confident that AMIGO localizes to clusters at neuronal perisomatic plasma membrane together with Kv2.1. This localization has been defined with two different antibodies, which are both validated with AMIGO KO mouse tissue.

5.2 AMIGO as a component of Kv2.1 potassium channel complex
We have identified AMIGO as an integral component of Kv2.1 channel complex in mouse brain. The association and extensive colocalization of AMIGO and Kv2.1 in mouse brain and in cultured cells has been demonstrated in this work. We have shown that AMIGO modifies the activation of Kv2.1 and neuronal I\textsubscript{K} current. In AMIGO KO mice, the amount of Kv2.1 protein is clearly reduced. These findings define AMIGO as function-modulating auxiliary subunit of Kv2.1 and thus provides novel insights into regulation of neuronal excitability.

One of the most striking findings of this study was the colocalization of AMIGO and Kv2.1 proteins: an impressive colocalization was demonstrated in both mouse brain and cultured hippocampal neurons. In mouse brain, AMIGO and Kv2.1 colocalized in all anatomical areas examined and the overall distribution of these two proteins were highly similar. In addition, heterologous expression of both proteins in HEK293 cells resulted in corresponding colocalization. Colocalization has rarely been demonstrated as extensively for any other potassium channel \( \alpha \) subunit and its auxiliary subunit. This might partly reflect the unavailability of antibodies working efficiently in immunohistochemistry. Similarly, this might partly reflect the ability of auxiliary subunits to associate with several different \( \alpha \)-subunits and the cell type specific regulation of ion channel properties.
by differential combination of α- and auxiliary subunits. Importantly, our observation that Kv2.1 is in complex with AMIGO implies that the full understanding of the properties and function of Kv2.1 channel in brain requires experimental systems where AMIGO is present. Moreover, the wide presence of AMIGO and Kv2.1 in cerebral neurons indicates that the channel complex contributes to fundamental properties of neurons.

Two other auxiliary proteins (KChAP and MiRP2) have been suggested to interact with Kv2.1 based on in vitro binding assay or on immunoprecipitation from rat brain (McCrossan et al, 2003; Wible et al, 1998). Based on in vitro findings, KCNE5 is also suggested to modulate Kv2.1 (David et al, 2015). Currently, it is unclear how broadly these proteins associate with the Kv2.1 channel complex in brain, since the colocalization of native proteins has not been demonstrated.

Auxiliary subunits of ion channels may have several roles in a channel complex. They are shown to affect the subunit assembly, trafficking, protein stability, conduction properties, localization, and pharmacological properties of the channel (Li et al, 2006; Pongs & Schwarz, 2010). In this study, AMIGO is shown to affect the voltage-dependent activation of Kv2.1, enabling Kv2.1 activation at lower membrane potentials. Convergent evidence is obtained from three experimental systems. In HEK293 cells, Kv2.1 current is activated at lower membrane potentials, when AMIGO is present. In cultured hippocampal neurons, I_K current, which is shown to be largely produced by Kv2.1 (Du et al, 2000; Murakoshi & Trimmer, 1999), is activated at higher membrane potentials when the expression of AMIGO is inhibited. Also, the I_K current is activated at higher membrane potentials in the acute hippocampal slices of AMIGO KO mice than in WT mice. AMIGO thus contributes to neuronal excitability through modulation of Kv2.1 and I_K currents.

Some of the auxiliary subunits of ion channels affect the surface expression of the principal subunit. Nonetheless, Kv2.1 has been reported to lack intracellular accumulation indicating regulated trafficking, as has been demonstrated for some other Kv channels. (Manganas & Trimmer, 2000; Shi et al, 1996; Shibata et al, 2003). In all cells examined, Kv2.1 channels are efficiently trafficked through the endomembrane system and expressed on the cell surface (Misonou et al, 2005b). In AMIGO KO mice, we have not detected any intracellular accumulation of Kv2.1. This implies that AMIGO might not contribute to the surface expression of Kv2.1. This is supported by the fact that AMIGO does not affect the maximal amplitude of Kv2.1 current in HEK293 cells. Nevertheless, levels of Kv2.1 protein were reduced to half in AMIGO KO mice. Interestingly, AMIGO expression is also reduced in various brain regions in mice lacking Kv2.1 (Cobb et al, 2013). It seems that both AMIGO and Kv2.1 are required for the full expression of Kv2.1 channel complex.

Some of the auxiliary subunits of ion channels affect the localization of the principal subunit. The subcellular localization of Kv2.1 is fascinating. Kv2.1 is
restricted to the soma and proximal part of neurites, and the localization is highly clustered (Du et al, 1998; Lim et al, 2000; Scannevin et al, 1996; Trimmer, 1991). Due to striking colocalization of AMIGO and Kv2.1, we considered the possible role for AMIGO in regulating the Kv2.1 localization. However, it seems that AMIGO is not essential for the perisomaly restricted localization of Kv2.1, or for the formation of Kv2.1 clusters, since this characteristic Kv2.1 localization is preserved in AMIGO KO mice. Kv2.1 is also able to form clusters when expressed in HEK293 cells (Mohapatra & Trimmer, 2006; O’Connell & Tamkun, 2005), in which no AMIGO is expressed endogenously (II, Supplemental Figure S1). The nature of Kv2.1 clusters remains largely unknown. Until now, only the pore forming subunits of Kv2.1 channel complex have been localized to these plasma-membrane sites in brain (Kihira et al, 2010; Trimmer, 1991). Clustering of Kv2.1 illustrates that neurons possess specialized membrane domains that are still poorly understood. Hopefully, identification of AMIGO in these sites will facilitate the understanding of these membrane domains in neurons.

The extracellular parts of Kv2.1 protein are very small. The presence of AMIGO brings an additional extracellular component to the Kv2.1 channel complex. AMIGO is also significantly glycosylated, whereas the extracellular parts of Kv2.1 are not glycosylated (Chen et al, 2012; Kajander et al, 2011; Shi & Trimmer, 1999). The extracellular part of AMIGO contains LRR- and Ig-domains and thus provides a motif for protein interaction and cell adhesion. Cell adhesion molecules are well known as auxiliary subunits for sodium channels. Beta subunits of voltage-gated sodium channels are transmembrane proteins with an Ig-domain and several glycosylation sites (Isom et al, 1992). AMIGO is the first Ig-domain-containing auxiliary subunit associated with a potassium channel. Beta subunits (β1 and β2) of sodium channels are multifunctional. They serve both as modulators of channel gating and as cell-adhesion molecules, affecting cell-cell interaction and binding extracellular matrix and cytoskeletal proteins (Brackenbury et al, 2008). Kv2.1 clusters at the plasma membrane lie over ER-derived structures called subsurface cisterns (Du et al, 1998). These membrane discs are rich in inositol triphosphate receptors and ryanodine receptors and are very closely associated with plasma membrane (Berridge, 1998; Rosenbluth, 1962). Kv2.1 clusters are shown to reside in extra-synaptic areas and to be faced by astrocytic processes in pyramidal neurons (Du et al, 1998; Misonou et al, 2008). In motor neurons, Kv2.1 clusters are strongly associated with cholinergic synapses (Muennich & Fyffe, 2004). It is tempting to speculate whether AMIGO contributes to localization of Kv2.1 in respect to extra- or intracellular structures associated with Kv2.1 clusters, such as subsurface cisterns or astrocytic processes.

Several stimuli can induce dispersion of Kv2.1 from clusters. Diffusion is induced in rat brain by kainate-induced seizures or in cultured neurons by glutamate stimulation (Misonou et al, 2004; Misonou et al, 2006). Similar changes are induced by hypoxia/ischemia produced by CO2 treatment in rat brain or by a
chemical ischemia model in cultured neurons (Misonou et al, 2005a). All of these stimuli are related to excitotoxicity and it is unclear whether the diffusion is associated only with pathological situations or also with normal physiological function. Mechanisms of dispersion are described and discussed extensively elsewhere (Misonou et al, 2004; Misonou et al, 2005a; Misonou et al, 2006; Misonou et al, 2008; Mohapatra & Trimmer, 2006; Mohapatra et al, 2008; Mulholland et al, 2008). In this study, we would like to emphasize the observations that the association and colocalization of AMIGO and Kv2.1 is preserved during dramatic stimulus-induced changes in Kv2.1 channel localization. These observations emphasize the role of AMIGO as an integral component of the Kv2.1 channel complex.

The AMIGO protein family contains two other homologous proteins: AMIGO2 and AMIGO3. It is possible that these proteins interact with Kv2.1 or with a related potassium channel α-subunit, Kv2.2. According to our studies, AMIGO2 and AMIGO3 are expressed in tissues outside the nervous system more widely than AMIGO. In the nervous system, the expression of AMIGO2 is detected in restricted brain areas (Laeremans et al, 2013). It is likely that further studies, and especially the development of novel antibodies, will clarify the detailed distribution and function of the other AMIGOs in the future. Similarly, the possible colocalization and association of AMIGOs with the other Kv2 family member, Kv2.2, will be an interesting subject for future studies. In contrast to the ubiquitous expression of Kv2.1, the expression of Kv2.2 in rat brain is relatively limited and restricted to specific brain areas (Hermanstyne et al, 2010; Kihira et al, 2010).

5.3 AMIGO-Kv2.1 channel complex in schizophrenia-related phenotypes
AMIGO KO mice display several alterations that are used to model schizophrenia in rodents. These include increased locomotor activity, sensitivity to the psychotomimetic drug MK-801, altered social behavior, and impaired PPI and cognitive function. In addition, the pharmacological response of the AMIGO KO mice suggests similarity to human disease, since the antipsychotic drugs are able to relieve the hyperactivity and the impaired PPI. Several transmitter systems are implicated to be altered in schizophrenic brains, including dopamine, glutamate, GABA, and serotonin (Keshavan et al, 2008). In this study, increased serotonin concentrations were detected in the prefrontal and temporal cortex of AMIGO KO brains. Functional alterations in these brain areas are associated with human schizophrenia (Keshavan et al, 2008). It is quite intriguing to find this many schizophrenia-related abnormalities in a mouse line lacking a single gene. It is especially noteworthy that behavioral abnormalities relevant to all three major
symptom clusters of schizophrenia are present in the AMIGO KO mice. Even the mouse models created by deleting known schizophrenia-associated genes do not generally recapitulate the breadth of the schizophrenia clinical profile (Carpenter & Koenig, 2008).

The behavioral disturbances of the AMIGO KO mice are likely to be mediated through altered function of the Kv2.1 potassium channel. In AMIGO KO mice, the amount of Kv2.1 protein is clearly decreased and voltage-dependent activation of neuronal I_K, largely attributable to Kv2.1, is altered. Our results are supported by the recent finding that the Kv2.1 (alias Kcnb1) -deficient mice are strikingly hyperactive (Speca et al, 2014), clearly resembling the hyperactivity phenotype of the AMIGO KO mice found in our study. Kv2.1-deficient mice also exhibit defects in spatial learning. However, the behavioral phenotype of the Kv2.1 deficient mice has not been previously compared to schizophrenia. Furthermore, occurrence of AMIGO as a part of the Kv2.1 channel complex is conserved across species and was also recently demonstrated in adult zebrafish brain (Zhao et al, 2014). Due to our findings on mice, behavioral characteristics and the expression of Kv2.1 were studied also in zebrafish. Indeed, knockdown of amigo in zebrafish essentially abolishes the expression of the Kv2.1 protein and leads to behavioral alterations characterized by hyperactivity and defective escape responses. Interestingly, the behavioral alterations in the amigo knockdown morphant can be rescued by injection of kv2.1 mRNA. Knockdown of amigo in zebrafish also leads to early developmental defects in fiber pathways of the brain. However, these developmental defects are not rescued by kv2.1 mRNA (Zhao et al, 2014). Therefore, it seems that behavioral regulation by Amigo in zebrafish critically depends on Kv2.1, whereas the role of Amigo in fiber-pathway development does not depend on Kv2.1.

After our study was completed, but not yet published, KV2.1 (KCNB1) emerged as a candidate gene for schizophrenia also in a large international GWAS meta-analysis, as common variants located at chromosome 20q13.13 in the vicinity of KCNB1 and PTGIS genes were found to associate modestly with schizophrenia (OR = 1.07) (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Only about one third of the heritability of schizophrenia has been estimated to be captured by the common variations included in genotyping platforms used in the large-scale genome-wide analyses, and relatively rare, high-impact variations have been suggested to contribute to the missing heritability of schizophrenia (Ripke et al, 2013; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Variants are typically distributed in varying frequencies among different populations. The unique population history, distinctive pattern of rare functional variants (Lim et al, 2014), and presence of regional isolates with high risk for schizophrenia (Perälä et al, 2008), makes Finns an ideal population to target these rare disease-causing variants for schizophrenia, as was recently demonstrated for population from Northern Finland (Stoll et al,
We have now been able to detect an association of a rare coding variant of \textit{KV2.1} (\textit{KCNB1}) with schizophrenia and schizophrenia spectrum disorders. We have thus established the role of \textit{KV2.1} (\textit{KCNB1}) as a schizophrenia susceptibility gene.

Interestingly, in addition to identifying \textit{KV2.1} (\textit{KCNB1}) as a candidate gene for schizophrenia, the largest GWAS study of schizophrenia also implicated locus containing \textit{KCNV1} gene (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). \textit{KCNV1} encodes “silent” Kv8.1 potassium channel subunit, which fails to produce electrically functional channels alone, but is able to interact with Kv2.1 and modify the Kv2.1 channel activity (Chiara et al, 1999; Ottschytsh et al, 2002; Salinas et al, 1997a).

Kv2.1 is strongly regulated by NMDA receptors, calcium signaling, and calcineurin (PP2B). All of these mechanisms are also connected to schizophrenia. (Fromer et al, 2014; Gerber et al, 2003; Kirov et al, 2012; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014).

Kv2.1 regulates excitability during periods of high-frequency firing and is suggested to function as a component of homeostatic plasticity (Du et al, 2000; Surmeier & Foehring, 2004). Altered Kv2.1 channel complex is thus expected to lead to a situation where neurons are more prone to high-frequency firing without proper homeostatic control. Since Kv2.1 channels are widely expressed in different brain regions, and present in pyramidal neurons as well as inhibitory interneurons, altered Kv2.1 activity could have complex effects on overall brain function.

Organized neuronal firing is crucial for brain function. Neural oscillations are linked to several important brain functions disturbed in schizophrenia, such as consciousness, attention, memory, and sensory processing (Uhlhaas & Singer, 2010). Since potassium channels play a central role in neuronal synchronization, the schizophrenia-like behavior resulting from AMIGO/Kv2.1 disruption could at least partially be accounted for by disturbed synchrony and cortical oscillations associated with schizophrenia (Uhlhaas & Singer, 2010).

The phosphorylation, localization, and activity of Kv2.1 are shown to be coupled (Misonou et al, 2004; Misonou et al, 2005b). In this respect, it is relevant that the Ser857Asn variation replaces a phosphorylation target serine with asparagine. Disrupted Kv2.1 phosphorylation could affect the localization and activity of the channel and thus alter the neuronal excitability.

Hypoxia-ischemia-related fetal and neonatal complications are associated with increased risk of schizophrenia (Cannon et al, 2000; Zornberg et al, 2000). Predisposing genetic factors might interact with hypoxia in increasing the risk of schizophrenia. Interestingly, the phosphorylation, localization and activity of Kv2.1 are strongly regulated by hypoxia/ischemia (Misonou et al, 2004; Misonou et al, 2005a; Misonou et al, 2008). Kv2.1 is suggested to function as a mechanism to suppress pathological hyperexcitability of central neurons during ischemic
Discussion

conditions (Misonou et al, 2005a). Disrupted Kv2.1 phosphorylation could affect the channel responsiveness to hypoxia and thus predispose to ischemic injury.

Our convergent findings in humans and mice suggest a role for the AMIGO-Kv2.1 potassium channel complex in pathophysiology of schizophrenia. Including our study and recent findings on other channels, there is now emerging evidence that voltage-dependent potassium channels and their interaction partners may contribute to the pathophysiology of schizophrenia and related psychiatric disorders. In addition to GWAS findings mentioned above, Kv11.1 gene (KCNH2) is linked to an increased risk of schizophrenia and shown to affect cortical physiology, cognition, and antipsychotic treatment response (Apud et al, 2012; Huffaker et al, 2009). The gene encoding Caspr2 (CNTNAP2), an adhesion protein interacting with Kv1 potassium channels, has been associated with schizophrenia and epilepsy (Friedman et al, 2007). Kv7.2 gene (KCNQ2) has been associated with bipolar disorder (Borsotto et al, 2006). Additionally, it has been shown that the amount of Kv3.1 channels is reduced in patients with untreated schizophrenia (Yanagi et al, 2013). The finding is interesting since AMIGO KO mice exhibit a reduced amount of Kv2.1.

Some studies have observed that potassium channel activators have antipsychotic effects (Akhondzadeh et al, 2002; Sotty et al, 2009; Vukadinovic & Rosenzweig, 2012). It is also suggested that the current antipsychotic drugs could mediate part of their therapeutic actions by affecting potassium channels since variation in Kv11.1 gene (KCNH2) modulates antipsychotic treatment response in patients with schizophrenia (Apud et al, 2012). Additionally, it has been shown that the amount of Kv3.1 channels is reduced in patients with untreated schizophrenia and normalized with antipsychotic drugs (Yanagi et al, 2013). Our findings emphasize the role of potassium channels as attractive targets for treatment development for schizophrenia, and identify two novel drug target candidates; AMIGO and Kv2.1. Based on the murine studies presented here, agents modifying the function of AMIGO-Kv2.1 channel complex might have broader effects on schizophrenia symptoms than traditional antipsychotics. From these two molecules, AMIGO has a significantly larger extracellular part and is therefore more accessible as a drug target. Additionally, the crystal structure of the extracellular part of AMIGO is solved (Kajander et al, 2011), facilitating structure-based drug design. From the viewpoint of treatment development, it is good to bear in mind that Kv2.1 channels play role in several physiological processes outside the nervous system. Kv2.1 is shown to function in cardiac ventricular repolarization (Xu et al, 1999), insulin secretion by pancreatic β-cells (MacDonald et al, 2002), and hypoxic pulmonary vasoconstriction (Archer et al, 1998; Patel et al, 1997). The detailed expression profile of AMIGO outside the nervous system remains to be determined. However, based on our study the expression of AMIGO is more restricted to the nervous system. AMIGO therefore appears as a potentially more specific target for drug development.
6. CONCLUSIONS

- A novel neuronal transmembrane protein, AMIGO, has been identified. AMIGO defines a novel LRR protein family together with AMIGO2 and AMIGO3.

- AMIGO is predominantly expressed in the central nervous system and widely present in cerebral neurons. At the subcellular level, AMIGO localizes to distinct clusters at the neuronal plasma membrane of cell soma and proximal part of neurites.

- AMIGO is an integral component of Kv2.1 potassium channel complex in brain. AMIGO fine-tunes the electrophysiological properties of the Kv2.1 channel.

- The phenotypic characteristics found in the AMIGO knockout mice demonstrate the involvement of AMIGO-Kv2.1 channel complex in schizophrenia-related behavioral disturbances in mice.

- \( KV2.1 (KCNB1) \) is a susceptibility gene for schizophrenia and schizophrenia spectrum disorders in humans.

- Our convergent findings in humans and mice suggest a role for AMIGO-Kv2.1 potassium channel complex in the pathophysiology of schizophrenia. Our results define AMIGO and Kv2.1 as potential new drug targets for schizophrenia.
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AMIGO-Kv2.1 Potassium Channel Complex: Identification and Association with Schizophrenia-Related Phenotypes

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