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AMIGO and its friends in developing and adult brain

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ACADEMIC DISSERTATION

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for mother
and father

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1 List of original publications

This study is based on the following original publications, which are referred to by Roman numerals:

I   **Kuja-Panula J**, Kiiltomäki M, Yamashiro T, Rouhiainen A, Rauvala H. (2003) AMIGO, a transmembrane protein implicated in axon tract development, defines a novel protein family with leucine-rich repeats. *J Cell Biol* 160:963-73

II   Zhao X, **Kuja-Panula J**, Sundvik M, Chen YC, Aho V, Stenberg T, Panula P, Rauvala H. (2014) Amigo Regulates Development of Neural Circuits in Zebrafish Brain. *J Biol Chem* Epub June 5, 2014

III   Kajander T*, **Kuja-Panula J***, Rauvala H, Goldman A. (2011) Crystal structure and role of glycans and dimerization in folding of neuronal leucine-righ repeat protein AMIGO-1. *J Mol Biol* 413:1001-15

IV   Peltola MA*, **Kuja-Panula J***, Lauri SE, Taira T, Rauvala H. (2011) AMIGO is an auxiliary subunit of the Kv2.1 potassium channel. *EMBO Rep* 12:1293-9.

V   **Kuja-Panula J***, Peltola MA*, Piepponen P, Lauri SE, Taira T,Võika V, Kulesskaya N, Rauvala H. (2014) *Amigo* deficient mice display altered Kv2.1 potassium channel functions and behavioral disturbances related to schizophrenia. **Manuscript in preparation**

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Author's contribution to the studies included in the thesis:
I: The author designed and conducted most of the experiments and analyzed the data and participated in writing the manuscript.
II: The author participated in designing experiments.
III: The author participated in designing experiments, made all molecular biological constructs, produced and purified recombinant proteins, contributed to the data analysis and writing of the manuscript.
IV: The author participated in designing experiments, made all molecular biological constructs, conducted electrophysiological experiments and contributed to the data analysis and writing of the manuscript.
V: The author designed and created the AMIGO deficient mouse line, carried out the electrophysiological experiments, analyzed the data of the electrophysiological experiments and participated in writing the manuscript.

2 Abbreviations

AMIGO   amphoterin induced gene and ORF

CAMs   cell adhesion molecules

CaV   voltage-gated calcium channel

cDNA   complementary DNA

CA1   cornu ammonis 1

CNS   central nervous system

CSCs   chondroitin sulfate chains

DIV   days in vitro

dpf   days post fertilization

ECM   extracellular matrix

FNIII   fibronectin type III domain

GPI   glycosylphosphatidylinositol

HMGB1   high-mobility group B 1

hpf   hours post fertilization

HSCs   heparan sulphate chains

IgSF   immunoglobulin superfamily

KO   knockout

KV   voltage-gated potassium channel

LRR   leucine-rich repeat

LRRTM   leucine-rich repeat transmembrane neuronal

NaV   voltage-gated sodium channel

NCAM   neural cell adhesion molecule

NGF   nerve growth factor

NGL   Netrin-G Ligand

PPI   prepulse inhibition

PSA   polysialic acid

RAGE   receptor for advanced glycation end products

RGD   arginyl-glycyl-aspartic acid motif

SALM   synaptic adhesion-like molecule

ZF   zebrafish

WT   wild type

3 Abstract

The purpose for this PhD research is to find a novel gene induced by neurite outgrowth on amphoterin substrate. The finding was a gene that codes type-I transmembrane protein with six leucine-rich repeat (LRR) motifs and one immunoglobulin domain followed by a short cytoplasmic tail. We named this gene as amphoterin-induced gene and ORF (*Amigo*).

Further characterization of AMIGO protein revealed that AMIGO itself is a neurite outgrowth promoting factor and it is also required for the fasciculation of neurites both *in vitro* and *in vivo*. The mode of these functions was shown to be homophilic, which puts AMIGO in a group of homophilic cell adhesion molecules. We crystallized the extracellular domain of AMIGO to elucidate the mechanism for its functions. AMIGO crystallized as a homophilic dimer where the concave face of the LRR domain was the interface for dimerization. The isolation of *Amigo* allowed us to characterize two other homologous genes called *Amigo2* and *Amigo3* and together they form the novel *Amigo* gene family.

AMIGO protein was found to be an auxiliary subunit of the voltage-gated potassium channel Kv2.1 in adult animals. AMIGO mediates the regulation of the voltage-gating properties of Kv2.1. This AMIGO/Kv2.1 interaction was also studied at the whole animal level by using *Amigo* knockout mice whereby the lack of *Amigo* changed voltage-gated potassium currents and resulted in behavioral problems related to human schizophrenia.

All these findings suggest that AMIGO has two separate roles in the central nervous system (CNS). First, AMIGO is a homophilic adhesion molecule that supports neurite outgrowth and fasciculation of the neurites during development. Second, the AMIGO regulates the properties of the voltage-gated potassium channel Kv2.1 in the mature CNS.

4 Introduction

The human brain is thought to be the most complex structure in the known universe. Our brain contains 100 billion neurons and every neuron is interconnected to another via numerous axons and dendrites, collectively known as neurites. The whole human brain contains 100 trillion synapses. Our knowledge of this intriguing organ has evolved from a mucus producing function (Egyptians) to a blood cooling organ (Aristotle) to a ventricle driven machine (Galen), and finally to the neuron doctrine (Cajal, Waldeyer-Hartz etc.) for which neurons are central to the brain functions.

Neurons are very peculiar cells compared to other cell types seen in multicellular organisms. During development, the nervous system neurons send axons that make contact with other neurons or non-neuronal cells much further than the close vicinity of the cell´s own environment. This process is called neurite outgrowth whereby trophic factors, the extracellular matrix and cell surface adhesion molecules guide neurites to find their right targets. The longest neurites in the contemporary animal kingdom are whale dorsal root ganglion neurites which can be over 20 meters long. How can neurons send signals so far from the cell soma? Diffusion inside or on the membrane surface would be far too slow and even active transport with the aid of motor proteins is just not fast enough. Evolution has solved this problem by producing neurons with another specialized characteristic called electrochemical communication. In electrochemical communication information moves fast enough in active organisms which allows them to act efficiently and cope in changing environments and have perceptions that closely enough match reality.

4.1 Neurite outgrowth

In the early 1950s, Levi-Montalcini together with Cohen (Cohen et al., 1954) pioneered the field of developmental neuroscience when they found the first polypeptide with neurite outgrowth promoting properties which they called nerve growth factor (NGF). This finding opened a new field in neuroscience, and search of novel neurite promoting factors still continues. Neurite outgrowth is a phenomenon in which neurons extend cytoplasmic processes that later polarize into either axons or dendrites. The outgrowth of these neurites is important for the establishment of synaptic connections, which finally leads to brain functions (da Silva and Dotti, 2002). In this introduction, I will discuss major regulatory molecules of neurite outgrowth, with special emphasis upon neuronal leucine-rich repeat (LRR) proteins.

4.1.1 Neurotrophins

Many neurite outgrowth promoting factors have a dual nature because they have both neurite outgrowth and cell survival promoting functions. This is also true in the case of NGF which belongs to the group of molecules called neurotrophins. A common feature of these molecules is that they all share signaling receptors, which belong to two distinct

classes: P75(NTR) and Trk receptor family (Reichardt, 2006). These important molecules have been studied widely over recent decades and many of these molecules or other molecules that are linked to their functions are being adopted by clinics to help to treat various diseases of the central nervous system (CNS) (Schulte-Herbruggen et al., 2007).

4.1.2 Extracellular matrix

The extracellular matrix (ECM) concept is defined in this review to mean only the specific ECM found in the CNS, which is the structure outside the cells that gives both support and guidance for neurons. This matrix is mainly composed of hyaluronic acid, chondroitin sulfate proteoglycans of the lectican family, and tenascin-R. These large molecules combine and form heterogeneous ternary complexes which are then decorated with various other molecules (Wang and Fawcett, 2012). Knowledge of the ECM components in the perineuronal nets and how these molecules modulate neuronal functions is only just beginning to emerge.

4.1.2.1 Proteoglycans

Chondroitin sulfate chains (CSCs) that attach to the protein core of aggrecan and neurocan are important components of the neuronal ECM (Yamaguchi, 2000). It was believed for a long time that the CSCs have a rather homogenous composition but recent accumulating evidence suggest that the CSCs have different compositions during the CNS development (Maeda et al., 2011). This dynamic change in composition might explain why during early CNS development the CSCs have neurite outgrowth promoting functions but later in the adult animal the CSCs can be the main barrier to axonal growth in the form of the glial scar (Silver and Miller, 2004). The other highly and developmentally expressed proteoglycans in the CNS are proteins that contain heparan sulfate chains (HSCs), which bind many growth factors and morphogens. This function was efficiently shown in a study in which the HSC synthesis was conditionally disrupted in the developing mouse CNS and which resulted in malformations and neurite outgrowth defects (Inatani et al., 2003). The HSC mode of function in the developing brain is to enrich and present the neurite outgrowth promoting molecules in their correct places thus forming concentration gradients for the growth cones to follow (Yamaguchi et al., 2010).

4.1.2.2 Laminin

Laminins are major components of the basal lamina, which is the protein layer that supports most cells and organs. Manthorpe et al. (1983) made the original finding that the laminin coated coverslips promote robust neurite outgrowth of chicken and rat neurons *in vitro*. Later on, the integrin receptor family was shown to be involved in the binding of laminin and the promotion of neurite outgrowth (Ignatius and Reichardt, 1988).

4.1.2.3 HMGB1 (Amphoterin)

HMGB1 was isolated as the heparin binding p30 protein, which promoted neurite outgrowth of the rat CNS neurons (Rauvala and Pihlaskari, 1987). Later on the DNA and protein sequence of p30 were discovered and the protein renamed amphoterin (Merenmies et al., 1991) and it was also discovered that the same protein was identified as the nuclear protein called HMG1 (Paonessa et al., 1987). Finally the protein nomenclature was again revised and the protein was called HMGB1 (Bustin, 2001). Even though the first findings were that HMGB1 localization is both extracellular and intracellular, it was first studied more as a nuclear protein specifically a nonhistone architectural chromosomal protein. HMGB1 binds double stranded DNA non-specifically but together with other DNA binding proteins it facilitates nuclear hormone receptors to activate gene expression (Bianchi and Beltrame, 2000). An important finding for extracellular functions for HMGB1 was the discovery of the cell surface receptor for HMGB1 called RAGE (Hori et al., 1995), which has been shown to have a significant role in the neurite outgrowth promoting functions of HMGB1 (Huttunen et al., 1999; Huttunen et al., 2002). Nowadays, the focus of HMGB1/RAGE studies is mostly on inflammatory axis where both molecules have been shown to have important functions in the immune system (Sims et al., 2010).

4.1.3 Cell adhesion molecules

Cell adhesion molecules (CAMs) are cell surface transmembrane proteins that attach the cells to ECM or to other cells. They serve as mechanical links for the cell but they also pass signals into the cell about the surroundings in which it is located. The CAMs are commonly divided in four distinctive families; integrins, cadherins, selectins and immunoglobulin superfamily (IgSF) of which integrins, cadherins and IgSF molecules are important for neurite outgrowth and CNS development. The modus operandi for the CAMs can be heterophilic whereby the CAMs bind to ECM molecules or to different cell surface proteins located on other cells. The modus operandi of the CAMs can also be homophilic, which entails the CAMs binding to the same molecules located on other cells. A common feature for the CAMs is a short cytoplasmic domain, and signaling of the CAMs depends on the interaction with other cytoplasmic signaling molecules/complexes. The outcome of the CAM signaling is often a remodeling of the actin cytoskeleton and a shaping of the cell morphology (Aplin et al., 1999).

4.1.3.1 Integrins

Intergrins form a major CAM family which has 24 receptors for different ligands. Integrins that target laminin or RGD motif containing proteins such as fibronectin are evolutionary ancient and can be found throughout all metazoan. In contrast integrins that target collagens and leukocytes are only found in vertebrates (Hynes, 2002). Integrin signaling is in most cases associated with the actin cytoskeleton through talin protein, which is involved in the unique "inside to out" integrin signaling in addition to the canonical "outside to in" signaling for receptors (Das et al., 2014). Integrins are mostly

expressed during the CNS development and the expression is more modest in the adult brain but recent findings suggest that the integrins could be very potent targets in therapeutic use for CNS injuries because of their very potent neurite outgrowth promoting properties (Eva et al., 2012).

4.1.3.2 Cadherins

Cadherins constitute a large superfamily of transmembrane proteins, and in humans more than 80 members have been found. In the neurite outgrowth function and the CNS development the most important member of the family is called N-cadherin, which belongs to the classical cadherin subfamily. Classical cadherins (referred to as cadherins here) are calcium ion dependent cell-to-cell adhesion molecules, which make homophilic contacts from opposing cells (Tepass et al., 2000). Cadherins have short conserved cytoplasmic tails that interact with beta-catenin, which leads to signaling through the catenin complex to the actin cytoskeleton (Ratheesh and Yap, 2012). N-cadherin is important for mouse embryonic development because homozygous knockout embryos die by day 10 of gestation (Radice et al., 1997), and later on it was shown that N-cadherin is crucial for mouse brain development (Luo et al., 2001). It is thought that N-cadherin together with other cadherins are important guiding molecules for neurite outgrowth and for fasciculation of neurites to bundles and for making anatomically defined areas in the developing and adult CNS (Redies, 2000).

4.1.3.3 Ig-superfamily

The immunoglobulin (Ig) domain is the most frequently used protein domain in vertebrates. The Ig domain is a characteristic sandwich like structure with two antiparallel beta-sheets joined together by a conserved cysteine bridge. This resulting structure forms a versatile domain that can be adapted to bind to other Ig domain containing proteins such as cell receptors, adhesion molecules or a variety of other molecules such as antigens and sugars. In cell adhesion IgSF molecules form homophilic and heterophilic interactions with other IgSF molecules but they can also bind to other protein domains in different CAMs and in the ECM (Barclay, 2003). The IgSF molecules are the most actively used entity in cell-cell adhesion, neuronal migration, axonal guidance and synapse formation in the nervous system.

4.1.3.3.1 NCAM

Neural cell adhesion molecule (NCAM) was the first cell adhesion molecule to be purified and characterized (Brackenbury et al., 1977; Thiery et al., 1977). These early studies formed the basis for future CAM discoveries and there are great number of studies on the NCAM functions. The NCAM´s N-terminal extracellular part consists of 5 Ig domains and 2 fibronectin type III (FNIII) domains. Differential splicing causes three major NCAM forms to exist: GPI-anchored NCAM-120kD, short cytoplasmic tail NCAM-140kD and

long cytoplasmic tail NCAM-180kD. The NCAM function in cell adhesion is homophilic in character and the mode of function is still controversial but homophilic action through the Ig-domains is generally regarded as being important (Kiselyov et al., 2005). Despite the wide expression pattern of the NCAMs during CNS development it is surprising that the NCAM deficient mice are viable and have only minor defects in brain development and behavior (Cremer et al., 1994). Interestingly, mice that only express the soluble extracellular form of the NCAM are embryonically lethal and this result suggested that the NCAM could also have heterophilic interactions (Rabinowitz et al., 1996). One important heterophilic interaction partner was found to be the fibroblast growth factor receptor (FGFR), which was shown to be the signaling molecule for the NCAM induced neurite outgrowth (Williams et al., 1994). The FGFR site involved in binding to NCAM has been mapped to the FGFR Ig2-Ig3 module, whereas the corresponding site in NCAM that binds to FGFR has been mapped to the two FNIII domains (Kiselyov et al., 2003; Kochoyan et al., 2008). Mice deficient of NCAM were found to have decreased phosphorylation of the FGFR in the brain and this diminished FGFR signaling was restored by giving a peptide that mimicks the NCAM binding to FGFR (Aonurm-Helm et al., 2010), which pointed out to the importance of the NCAM/FGFR interplay.

A very interesting feature of NCAM biology is the unique NCAM glycan structure called polysialic acid (PSA). The PSA molecule is a linear chain of α2,8-glycosidically linked N-acetylneuraminic acid residues of different lengths from 8 up to 90 sugar units. PSA comprises about 10% of protein bound neuraminic acids found in the developing brain and almost all is linked to NCAM (Finne, 1982; Galuska et al., 2008). The function of PSA decoration for NCAM is to regulate the adhesiveness of homophilic and heterophilic interactions of NCAM (Hildebrandt et al., 2007).

4.1.3.3.2 RAGE

Receptor for advanced glycation end products (RAGE) is composed of three extracellular Ig domains, a transmembrane domain and a short cytoplasmic tail (Neeper et al., 1992). RAGE is a multiligand receptor that can bind various non-related molecules such as advanced glycation end products (Schmidt et al., 1992), HMGB1 (Hori et al., 1995), S100 (Hofmann et al., 1999), amyloid-beta peptide (Yan et al., 1996) and DNA (Sirois et al., 2013). This binding pattern of many diverse ligands resembles that seen in toll-like receptors, and RAGE is now thought to be one of the pattern-recognition receptors in innate immunity (Ibrahim et al., 2013). RAGE signaling activation has been linked to various pathological situations such as diabetic complications (Matsumoto et al., 2008b; Wendt et al., 2003), cancer (Sasahira et al., 2005; Taguchi et al., 2000) and Alzheimer´s disease (Yan et al., 1996).

The expression of RAGE in adult tissues is generally low though the only exception is the lung where RAGE is highly expressed (Brett et al., 1993). However, during brain development RAGE is seen to be expressed together with its ligand HMGB1. In experiments *in vitro* HMGB1 promotes neurite outgrowth of rat neurons that can be blocked by soluble RAGE or by anti-RAGE antibodies, which suggests that RAGE signaling is able to induce neurite outgrowth (Hori et al., 1995). Later on it was shown that the ligation of RAGE with HMGB1 and also with S100 family proteins induces the

rac/cdc42 signaling (Huttunen et al., 1999; Huttunen et al., 2000). Recently, it has been shown that RAGE can also bind to itself and to promote homophilic cell adhesion, which suggests that RAGE could additionally have more commonplace cell adhesion functions (Sessa et al., 2014).

4.1.3.4 Leucine-rich repeat proteins

The leucine-rich repeat (LRR) motif is a protein structure found in all major branches of living organisms. The evolution of this motif is relatively unknown and it is suggested that the LRRs found in prokaryotes and eukaryotes are an example of convergent evolution during which motif evolved independently. The common feature of LRR motif is the sequence where the hydrophobic amino acid leucine is placed orderly in the amino acid sequence varying from 20 to 30 residues length. All LRR units can be divided into a highly conserved segment and a variable segment. The conserved segment consists of an 11 residue stretch, LxxLxLxxNxL, or a 12 residue stretch, LxxLxLxxCxxL, in which "L" is leucine, isoleucine, valine, or phenylalanine, "N" is asparagine, threonine, serine, or cysteine, and "C" is cysteine, serine or asparagine. One LRR motif is formed from beta-sheet-turn-alpha-helix structure, which is held together by hydrophobic interactions from side chains of mainly leucine but also of valine and isoleucine. The structural LRR domain is formed from 2-45 LRR motifs, which are arranged so that all the beta-sheets and the alpha-helices are parallel to a common axis that results in a nonglobular, horseshoe or arc-shaped molecule. The concave face of the LRR domain is lined with beta-sheets whereas the convex face is made from alpha-helices. The primary function of the LRR domain is to provide a versatile structural framework for protein-protein interactions (Enkhbayar et al., 2004; Kajava, 1998; Kobe and Deisenhofer, 1994; Kobe and Kajava, 2001).
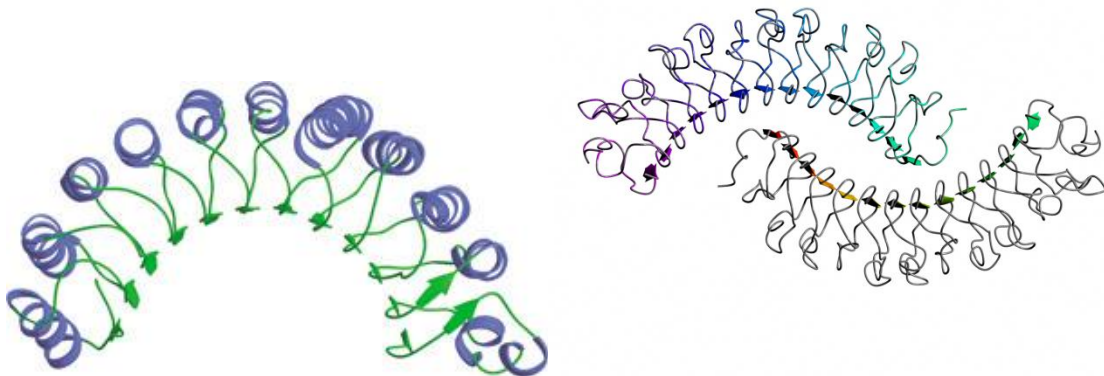

Figure 1. Examples of Leucine-Rich Repeat domains: Rna1p (Hillig et al., 1999) and Decorin dimer (Scott et al., 2004).

Mammalian LRR proteins can be found in the reducing environment that exists inside the cells because the self-assembly of the LRR domain does not need cysteine bridges to form. The extracellular LRR domains are usually flanked at both ends by two unique cysteine-rich motifs called the LRR N-terminal (LRRNT) and the LRR C-terminal (LRRCT) domain. These capping domains are needed to bury the hydrophobic core of the LRR domain but in many cases both LRRNT and LRRCT are integral parts of the protein that gives additional surface for interactions (McEwan et al., 2006). Many extracellular LRR domains containing proteins also have Ig and/or fibronectin (FN) domains but in this review proteins are categorized as LRR proteins based on the LRR domain with or without other protein domains.

4.1.3.4.1 Neuronal LRR proteins in cell adhesion

LRR proteins expressed in the CNS are nowadays recognized as a very important family of proteins involved in both the development of the CNS with correct connections and also in the maintenance of an orderly functioning adult CNS. The Slit family (Slit1, Slit2 and Slit3) are a well-studied group of LRR proteins involved in vertebrate brain development. The Slit proteins are extracellular secreted matrix proteins that have four separate LRR domains, of which domain two is needed for the Slit binding to its cell surface receptor Robo (Howitt et al., 2004; Kidd et al., 1999). Slit-Robo signaling was first found in *Drosophila* and this signaling is a highly conserved system in bilaterians in which the axons cross over to the opposite side of the body to find the proper targets. During the CNS development axons travel through the midline and should no longer turn back, and Slit was found to be one of the molecules to make this barrier for "not turning back". The binding of the axonal Robo to the matrix bound Slit repels the growth cone from the midline and thus keeps the axon on the correct side of the body plane. Slit is a soluble extracellular matrix LRR protein. The focus in this review however, is on the cell surface transmembrane proteins that have the extracellular LRR domain with neurite outgrowth promoting functions.

4.1.3.4.1.1 NGL family

The first cloned member of the Netrin-G Ligand (NGL) family was Netrin-G Ligand-1 (NGL-1). NGL-1 was obtained by cDNA library screening in the form of a ligand to a GPI anchored Netrin family member Netrin-G1 (Lin et al., 2003). The NGL family contains three members (NGL-1, NGL-2 and NGL-3) of type-I transmembrane proteins, all of which share a similar structure with an extracellular LRR domain (9 LRR motifs) and one C2 type Ig domain. The short cytoplasmic tails of the family members all have a C-terminal PDZ-binding motif (Kim et al., 2006). It was later shown that NGL-2 binds the second member of the Netrin-G family called Netrin-G2. Interestingly, NGL-1 only interacts with Netrin-G1, whereas NGL-2 only binds Netrin-G2. However, NGL-3 differs from the other NGL proteins in its binding specificity to the receptor tyrosine phosphatases LAR (Woo et al., 2009), PTPdelta and PTPsigma (Kwon et al., 2010). The expression pattern of the NGL family is mainly restricted to the brain, which suggests that these proteins are important for brain development and function. The substrate bound

NGL-1 promotes neurite outgrowth in cell culture and *in vivo* injection of soluble NGL-1 prevents growth of thalamic axons of chick embryo. The only available knockout mouse line regarding this protein family is the NGL-2 deficient mouse. The main findings for this knockout model were disordered synapse formation in the hippocampus and retina along with axonal pathfinding problems in the outer retina (DeNardo et al., 2012; Soto et al., 2013). Much of the interest in the NGL family is centered on their function in synapse formation where they mediate binding with the cytoplasmic PDZ motif to the postsynaptic scaffolding protein PSD-95 (Kim et al., 2006).

4.1.3.4.1.2 SALM family

Synaptic Adhesion-Like Molecule (SALM) family consists of five members of type-I transmembrane proteins characterized by an extracellular LRR domain (5 LRR motifs), one C2 type Ig domain and one FNIII domain followed by a short cytoplasmic tail that ends at a PDZ-binding motif in SALM1-3. However, this PDZ binding motif is missing in SALM4 and SALM5. The expression pattern of the SALM family members suggests their important role in CNS development and function because all members are highly and specifically expressed in brain except SALM3, which is expressed at low levels in some other tissues (Morimura et al., 2006; Wang et al., 2006). Overexpression of SALMs in cultured hippocampal neurons (DIV 4–6) increases neurite outgrowth whereas an antibody directed at the extracellular part of the SALMs suppresses neurite outgrowth (Wang et al., 2008). In adult tissue SALMs are mainly located in the excitatory synapses and SALM1-3 have been shown to interact through their PDZ motifs with the postsynaptic scaffolding protein PSD-95. In synaptogenesis SALM1 can directly interact with the NMDA receptor, whereas SALM2 associates with both NMDA and AMPA receptors (Ko et al., 2006). SALM4 and SALM5 do not have the PDZ motif and thus lack PSD-95 binding properties but they also differ from the other SALMs due to their ability to form homophilic trans interactions between adjacent cells. In contrast, SALM1-3 are able to interact homophilically and also heterophilically between the family members but this interaction only happens in a cis manner on the same cell (Seabold et al., 2008). The *in vivo* functions of the SALM proteins remain to be uncovered in future when the first studies of the SALM deficient mice are available.

4.1.3.4.1.3 Slitrk family

The Slitrk family was named and cloned *in silico* due to its similarity with both the Slit and Trk receptor –families. The Slitrk family consists of six members (Slitrk-1 to Slitrk-6) of type-I transmembrane proteins that have two extracellular LRR domains and each LRR domain is composed of six LRR motifs. The cytoplasmic tail length varies in lenght from 55 to 300 amino acids and the most distal part of the C-terminus contains a region on which a tyrosine residue is flanked by a sequence homologous to the Trk receptor C-terminus. In the Trk receptors this tyrosine residue is phosphorylated which suggests that the Slitrks are similarly regulated. The Slitrk-1 differs from the other Slitrks due to truncated cytoplasmic tail that lacks the homology region to the Trk receptors (Aruga and Mikoshiba, 2003). The Slitrk family is highly and broadly expressed in the CNS and only

Slitrk-6 is additionally expressed in other tissues (Aruga, 2003). Different combinations of the family members are expressed in various brain areas, which suggests that each Slitrk family member has its own role in the development and maintenance of the highly ordered CNS. Overexpression of Slitrk-1 *in vitro* in mouse cortical neurons resulted in enhanced neurite outgrowth (Abelson et al., 2005; Kajiwara et al., 2009) and Slitrk-1 deficient mice displayed anxiety-like behavioral symptoms (Katayama et al., 2010). The Slitrk-6 deficiency in mice leads to diminished neurite outgrowth and innervation of the cochlear in the inner ear (Katayama et al., 2009). The Slitrk-5 knockout mice also have a reduced innervation phenotype specifically in the striatum, which was suggested to lead to obsessive-compulsive–like behaviors in mice (Shmelkov et al., 2010). A very important finding came from the Slitrk-3 deficient mice, which had a decreased inhibitory synapse number and increased seizure susceptibility due to a lack of trans-synaptic interaction between the slitrk-3 and receptor tyrosine phosphatase PTPdelta. That same study showed that all Slitrk family members can bind to PTPdelta, which makes PTPdelta the first known ligand for this protein family (Takahashi et al., 2012).

4.1.3.4.1.4 LRRTM family

Leucine-Rich Repeat TransMembrane neuronal (LRRTM) family was cloned *in silico* due to the similarity with the Slit proteins. The LRRTM family consists of four members (LRRTM1 to LRRTM4) of type-I transmembrane proteins, which have an extracellular LRR domain (10 LRR motifs) followed by a short cytoplasmic tail that ends at the PDZ-binding motif. All LRRTMs are predominantly expressed in the neurons of the CNS, with specific temporal and spatial expression patterns in different brain regions. These expression patterns suggest an important role in the development and maintenance of the CNS (Lauren et al., 2003). Neurite outgrowth and synapse formation are fundamental mechanisms for making a framework for the CNS functions, and the same cell adhesion molecules are often involved in both functions. The neurite outgrowth functions for the LRRTM family have not been studied yet although this protein family has been extensively linked to synapse formation. The first seminal studies of the association of LRRTMs with synapse formation came from unbiased screening for finding synaptogenic proteins, which suggests that all LRRTMs could promote presynaptic differentiation in contacting axons (Linhoff et al., 2009). Further support for synaptic functioning came from the findings that LRRTM1 and LRRTM2 are receptors for important presynaptic proteins called neurexins, and that this interaction cooperately drives the development of the glutamatergic synapses (de Wit et al., 2009; Ko et al., 2009; Siddiqui et al., 2010). More evidence accumulated in studies of LRRTM1 knockout mice, which have reduced hippocampus size and also reduced synaptic density. The LRRTM1 deficiency in mice leads to impaired cognitive functions and claustrophobia-like behavior (Takashima et al., 2011; Voikar et al., 2013). LRRTM3 and LRRTM4 are more homologous to each other than to either LRRTM1 or LRRTM2. An interesting finding was that LRRTM3 and -4 share unique binding properties to the heparan sulfate proteoglycan family glypicans. The glypicans bind to LRRTM3 and -4 through their heparan sulfate chains and this trans-synaptic interaction regulates excitatory synapse development. In the LRRTM4 knockout mice the lack of the LRRTM4 leads to deficient excitatory synapse development and

impaired excitatory transmission in the dentate gyrus granule cells (de Wit et al., 2013; Siddiqui et al., 2013). A more detailed picture of this important protein family will eventually emerge when detailed studies of the LRRTM2 and LRRTM3 deficient mice are available.
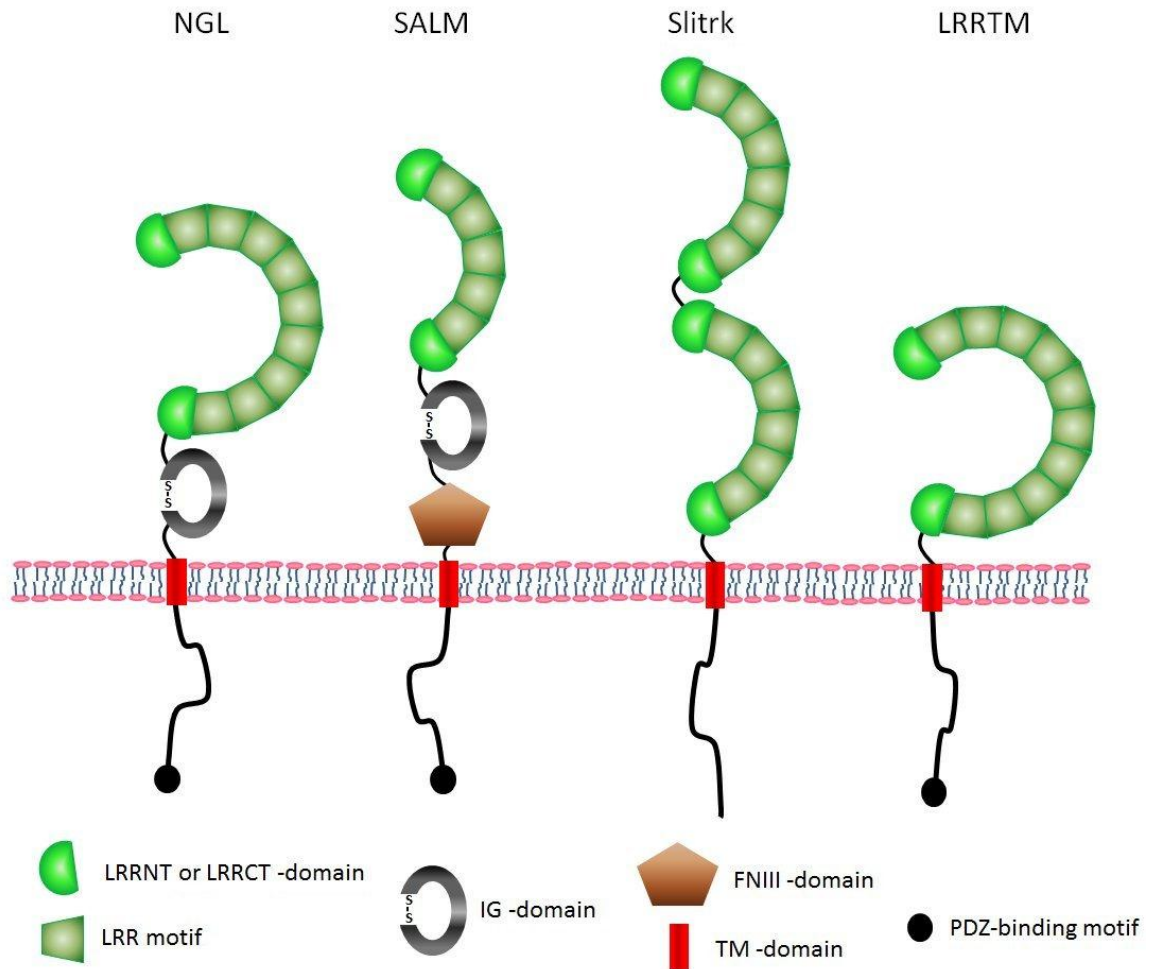

Figure 2. Schematic view of neuronal LRR protein families. LRRNT; leucine-rich repeat N-terminal capping domain, LRRCT; leucine-rich repeat C-terminal capping domain, LRR; leucine-rich repeat motif, IG-domain; immunoglobulin domain, FNIII; fibronectin type III domain, TM – domain, transmembrane domain.

4.2 Electrochemical communication

In electrochemical communication two kinds of signaling worlds are combined together and the CNS is the place where this shift from electricity to chemistry (and vice versa) has evolved in superiority. The electrical signal for communication travels through neurites in a form of ionic flow through specific pores called "voltage-gated ion channels" on the cell membrane. When electrical signal travels to the site called the synaptic cleft that is located between two separate neurons, the signal is changed to a chemical in the form of neurotransmitters, which travel to the oppose side of the cleft by diffusion. On the adjacent cell the transmitter binds to a specific receptor that in turn propagates the signal in electric or chemical form depending on the receptor type the cell expresses. The focus in this review is on ion channels that are activated by voltage and are named as voltage-gated ion channels.

Voltage-gated ion channels are plasma membrane spanning proteins that make a regulated pore for ions to travel according to their electrochemical gradient. Regulation of the pore opening for ions is driven by specialized protein structures, which sense the voltage difference across the plasma membrane.

4.2.1 NaV

Voltage gated sodium currents were first discovered by Hodgkin and Huxley in their pioneering work using the voltage clamp method (Hodgkin and Huxley, 1952). Voltage gated sodium channels (NaVs) are responsible for the initiation and propagation of the action potentials and are vital for normal neuronal signaling. The NaVs are made of two distinctive polypeptides, which are the alpha and the auxiliary beta subunits. In mammals the NAV alpha subunit protein family consists of nine members, and each alpha unit is a large polypeptide that contains four homologous transmembrane spanning regions. On the plasma membrane the alpha subunit assembles to form a specific pore for the sodium ions and this unit also contains the necessary structures for regulation of the pore opening. The auxiliary beta subunit is a single-pass type-I transmembrane protein that regulates the alpha subunit´s gating properties, protein expression profile and linking of the alpha subunit to the cytoskeleton and the ECM (Catterall, 2000a). One interesting finding regarding the beta subunits is that they are heavily glycosylated with negatively charged sialic acids. This negative charge brought by the beta subunit is needed for optimization of NaVs functioning to sense differences in the membrane potentials. The alpha unit itself also contains sialic acids for tuning the gating properties (Johnson and Bennett, 2006; Johnson et al., 2004). The NaVs can also be modulated through the phosphorylation of specific residues on the alpha subunit, which takes place after the activation of the cytoplasmic protein kinases. The phosphorylation effect on different alpha subunit members can vary from an increase to a decrease of the current amplitude (Chahine et al., 2005). Various knockout mouse models are available to study different NaV alpha and beta subunits. A common feature for these knockout models is a susceptibility to epileptic seizures, which is thought to be caused by discoordinated firing patterns of the neurons. This indicates the importance of the NaVs in normal CNS functions.

4.2.2 CaV

Voltage-gated calcium channels (CaVs) differ from other voltage-gated ion channels because they are less involved in the propagation of action potentials but they do have a unique role in bridging the electrical signal its chemical counterpart. Calcium ion (Ca^{2+}) functions in biology as an important second messenger in a myriad of different cellular events. The Ca^{2+} concentration inside the cytosol in neuronal tissue is one-thousandth that of the outside. This large electrochemical concentration difference is utilized by the CaVs which open due to the polarization of the membrane during the action potential. This leads to the initiation of the synaptic transmission in the synaptic terminals through the second messenger Ca^{2+} (Catterall, 2011). The main structural component of the CaVs is the pore forming alpha unit. In vertebrates there are nine different CaV alpha unit genes, which are expressed differentially in various tissues. The structure for the CaV alpha unit shares a similar evolutionally related structure to that of the NaVs, whereby the CaV alpha unit is formed from a large polypeptide with four homologous repeats each of which have six transmembrane helices. Self-assembly of this polypeptide on the cell membrane forms a specific Ca^{2+} permeable pore that opens depending on voltage (Catterall, 2000b). The CaV functions are also regulated by auxiliary subunits that belong to three separate protein families called: CaVbetas, alpha2deltas and gammas. The functions of these auxiliary subunits vary from the regulation of CaV alpha unit surface expression to gating properties (Arikkath and Campbell, 2003). Many CaV alpha or auxiliary subunit deficient mouse models are available in which many neurological human diseases related to the CaVs are recapitulated. A common feature of CaV deficiency is ataxia and a susceptibility to seizures (Benarroch, 2010).

4.2.3 KV

The canonical view for voltage gated potassium channel (KV) currents is that they are the counterpart for the voltage gated sodium currents during action potential propagation. The KVs opens to repolarize the membrane potential closer to potassium ion equilibrium and further away from the action potential firing threshold upon the action potential depolarization. This function renders the KVs as the primary regulators for the patterns of action potential firing. Forty members of the KV protein family have evolved in mammals, which indicates the importance of these ion channels for the correct functioning of the CNS. The principal alpha subunits of the KVs are coded by genes from 12 subfamilies, KV1 through KV12 (Gutman et al., 2005). The kinetics of the voltage-gated properties between the KV members varies considerably and the combination of the KV members expressed by the cell shapes the action potential frequency and patterning in that particular cell type. The KVs are formed from four individual subunits in each of which contains six transmembrane helices. The N- and C-termini face the cytoplasm. These subunits then self-assemble to form the voltage sensing structure and the regulated pore for the potassium ions. Interestingly, the Kv1-Kv4 subfamilies can form either homomeric or heteromeric, functional tetrameric channels which increase further the possible KV current repertoire of the cell. This membrane-spanning segment structure in KVs is

homologous to the NaVs and the CaVs. The KVs are formed from four separate proteins, whereas in the NaVs and the CaVs these four separate parts have merged into one entity/gene. This difference between the KVs and the NaVs/CaVs suggest that the KVs are evolutionally older and, the NaVs and the CaVs have evolved later from the KVs (Anderson and Greenberg, 2001).

When neuronal voltage-gated potassium currents are recorded one can find two distinct currents: Fast transient peak current I_A is fast activating and fast inactivating, whereas the delayed rectifier current I_K is more slowly activating and very slowly inactivating or non-inactivating (Mathie et al., 1998). Both I_A and I_K counteract the NaV currents by repolarizing the action potential back to the resting potential. The fast transient I_A currents play an important role in controlling the action potential durations, and both I_A and I_K regulate how often and at which frequency the neuron fires the action potentials (Yuan et al., 2005). The exact *in vivo* role of the single channel type has been difficult to verify because one neuron can express many different KV alpha units with overlapping voltage-gating kinetics. A big hindrance is the lack of specific pharmacological tools to specifically block certain KVs. For example, *in vivo* studies that use mouse knockout lines have the problem of potential compensation for the loss of one KV by altered expression of a related KV (Foehring, 2008).

Many KV alpha units undergo post-translational modification including phosphorylation and glycosylation and are regulated by auxiliary units. The phosphorylation effect for the KVs can be manifest as a decrease in current amplitude (Macica et al., 2003) to a depolarizing shift in gating kinetics (Schrader et al., 2006; Winklhofer et al., 2003). The KV alpha units are usually decorated with negatively charged sialic acids, which change the KV gating properties to more negative values (Schwetz et al., 2010; Schwetz et al., 2011; Thornhill et al., 1996). Such a sialic acid function resembles equivalent changes seen in the NaVs. Various intracellular and transmembrane proteins associate as auxiliary subunits with the KV alpha units. The best known auxiliary subunits are the KV beta subunit family together with the KCNE family. The KV beta subunits are intracellular proteins that regulate the KV1 family of alpha units. The effect of certain beta subunits can be subtle or very profound, such as in the case of Kv1.1 whereby the binding of the KV beta1 changes the channel from the I_K current to I_A. The KCNE family members are type 1 transmembrane proteins that regulate the current density and gating kinetics of the KV alpha unit by interacting with alpha units through the transmembrane domain (Li et al., 2006; Sun et al., 2012).

4.2.3.1 Kv2.1

Kv2.1 was cloned in 1989 from the rat brain as the gene that encodes the mammalian counterpart of the *Drosophila* Shab channel (Wei et al., 1990). When this cDNA was expressed in a *Xenopus* oocyte the result was a typical delayed rectifier I_K current (Frech et al., 1989) Histology analysis revealed the channel to be localized specifically to neurons for which the channel had a unique punctate staining on the cell soma (Trimmer, 1991). When another evolutionally related KV was cloned (Hwang et al., 1992) it was evident that in the mammalian brain two similarly functioning I_K channels exist that are now called Kv2.1 and Kv2.2 (Maletic-Savatic et al., 1995).

The structure of Kv2.1 is unique compared to other KV alpha units due to its large cytosolic N- and C-termini that have 123 possible residues for phosphorylation. The phosphorylation status of Kv2.1 has been under extensive investigation during recent years. Kv2.1 was shown to be heavily phosphorylated both *in vitro* in heterologous COS-1 cells and *in vivo* in rat brain membranes. Interestingly, Kv2.1 attained the high phosphorylation status rapidly during pulse chase labeling, which suggests that this phosphorylation pattern is important for properly functioning Kv2.1 (Shi et al., 1994). Shortly after a study (Murakoshi et al., 1997) showed that the phosphorylation stage of Kv2.1 regulated the voltage-gated properties of the channel because dephosphorylation of Kv2.1 led to a hyperpolarizing shift in the channel opening. Surprisingly, the characteristic staining pattern of the Kv2.1 was also regulated by phosphorylation. Kv2.1 localizes on cell plasma membrane in ring like clusters which are above specialized endoplasmic reticulum structures called subsurface cisterns (Du et al., 1998). When Kv2.1 is dephosphorylated *in vitro* and also *in vivo* due to certain treatments that mimick ischemia or hyperexcitability, the Kv2.1 clusters dissociate and the Kv2.1 channels diffuse across the plasma membrane (Misonou et al., 2005a; Misonou et al., 2004). These dephosphorylation changes both in gating and localization of Kv2.1 are suggested to be important for the Kv2.1 function as a homeostatic regulator of neuronal excitability. When a neuron fires action potentials at high frequency the Kv2.1 role is to inhibit the possibility for the action potential reoccurrence, and simultaneously the Kv2.1 activity reduces the firing rate. Dephosphorylation of Kv2.1 happens during the repetitive firing of the action potentials, and this leads to a hyperpolarizing shift in the Kv2.1 gating properties, which makes Kv2.1 an even more potent suppressor of the high frequency firing. The cell "remembers" the high activity state of the past because the dephosphorylated state of Kv2.1 persists for a while and this phenomenon resembles homeostatic plasticity (Misonou et al., 2005b).

5 Aims of the study

The aim of the study was to find a novel gene that is specifically expressed in the CNS that is involved in neurite outgrowth and CNS development, then to study the role of that novel gene on molecular and whole animal levels:

1. to find and clone a novel gene in rat hippocampal neurons that is induced by HMGB1-promoted neurite outgrowth

2. to characterize the expression pattern of the novel gene/protein (later named AMIGO)

3. to reveal the *in vitro* and *in vivo* role of AMIGO by cell culture methods and by creating AMIGO deficient mice

4. to resolve the exact structure of the extracellular part of the AMIGO protein

5. to find interaction partners for AMIGO

6 Experimental Procedures

More detailed information about methods and materials used can be found in the original publications.

Table 1. *Methods used in the thesis where author was personally involved.*

Method	Publication
Acute hippocampal slice preparation	IV
Antibody production	I, IV
Bioinformatics	I, III
Cell culture and transfection	I, III, IV
Chemical cross-linking	IV
Co-immunoprecipitation	I, III, IV
DNA constructs	I, III, IV, V
Electrophysiology	IV, V
Immunocytochemistry	I, IV
Knockout mouse production	V
Lentivirus production and transfection	IV
Microscopy	I, IV
Neuronal cell culture	I, IV
Ordered differential display	I
Recombinant protein production	I, III,
RT-PCR	I, V
Southern blotting	V
Western blotting	I, III, IV, V

6.1 Animals

Mice that were deficient of the *Amigo* gene were created by using the standard homologous recombination method described by Folger et al. (1982). Briefly, homologous arms around the mouse *Amigo* locus were cloned into a targeting vector so that the AMIGO protein coding sequence was replaced with the LacZ gene. After electroporation of the targeting vector into the mouse embryonic stem (ES) cells, targeted clones were found with the help of Southern blotting and PCR techniques. The *Amigo* targeted ES cell clone was introduced into the mice by using an aggregation method, which resulted in

chimeric mice that were mated with WT mice to get the first heterozygous *Amigo* targeted mice. The *Amigo* deficient mice were subsequently backcrossed to C57BL/6JOlaHsd background at least 8 rounds before all experiments.

6.2 Primary neuronal cultures

Rat hippocampal or cortical neurons were dissociated from E17/E18 rat embryos and were cultivated in Neurobasal supplemented by B27, L-glutamine and antibiotics. 40 000 hippocampal neurons per 1 cm^2 or 80 000 cortical neurons per 1 cm^2 were plated on different substrates as detailed in each experiment.

7 Results and Discussion

7.1 Discovery of AMIGO and its structure (I, II, III)

7.1.1 Cloning of a novel neuronal cell adhesion molecule

The aim of this study was to clone novel genes involved in neurite outgrowth on HMGB1 (Amphoterin) substratum (Rauvala and Pihlaskari, 1987) compared to the more commonly used neurite outgrowth-promoting substrate, laminin (Manthorpe et al., 1983). The method chosen for finding genes involved in the HMGB1-promoted neurite outgrowth was ordered differential display (ODD) as described by Matz et al. (1997). This method enabled us to clone a gene whose expression was induced on the HMGB1 substrate, and interestingly this induction was also seen on anti-RAGE substratum. RAGE is a known cell surface receptor for HMGB1 therefore this result suggested that the induction of the gene of interest was due to HMGB1-induced RAGE signaling. The full-length transcript was revealed by using the 5´RACE method (Matz et al., 1999), which showed that the gene was novel and unpublished. We named the gene Amphoterin-Induced Gene and ORF (*Amigo*). The sequence suggested that the *Amigo* gene encodes a type-I transmembrane protein with a putative signal sequence and a transmembrane region. The N-terminus of the AMIGO protein faces outside the cell whereas the C-terminus is located in the cytosol. A closer analysis of the protein revealed that the extracellular part of the protein is formed from a putative LRR-domain (with 6 LRR motifs) and a single Ig domain. The short cytosolic tail (99 amino acids) did not show any known protein motifs but interestingly this cytoplasmic part has been well conserved during mammalian evolution. The human AMIGO cytosolic part displayed at least 96% similarity with *Placential* and around 90% similarity with *Marsupial* AMIGO sequences, which suggests some still unknown important function for the AMIGO cytosolic tail. The LRR domain of the AMIGO resembles their counterparts seen in Slit (Whitford et al., 2002) and the Nogo receptor (Fournier et al., 2001) protein families. In contrast, the Ig domain of the AMIGO showed a similarity to PFAM software defined immunoglobulin V-set motifs that can be found in T-cell receptors and adhesion junction molecules (Punta et al., 2012). The upregulation of the AMIGO during neurite extension combined with the deduced AMIGO protein sequence with the LRR and Ig domains suggest a role in cell adhesion and possibly in neurite outgrowth.

7.1.1.1 AMIGO defines a novel protein family

When we used the AMIGO sequence information we also cloned two other novel genes homologous to AMIGO, and for convenience we named them: AMIGO2 and AMIGO3. The general layout of the AMIGO2 and -3 is the same as in AMIGO and the similarity of the amino acid sequences between the AMIGO family members is 50-55%. Certain regions in the sequences such as the transmembrane segments, the LRR domains and some sequence motifs in the cytosolic tails are more highly conserved. Gene expression analysis of the AMIGO family in adult mouse tissues demonstrated that the AMIGO gene

is more specifically expressed in the CNS, whereas AMIGO2 and especially AMIGO3 expression are more widely distributed (Fig. 3A in publication I).

When this research work was in progress the *Amigo2* gene was shown to be induced by neuronal activity and the expression of the AMIGO2 protein promoted depolarization-dependent survival of cerebellar granule neurons (Ono et al., 2003). The mRNA expression of *Amigo2* in mouse CNS has been studied in more detail and *Amigo2* was shown to be specifically expressed in the CA2/CA3 area of the hippocampus (Laeremans et al., 2013). *Amigo2* has been also shown to be a differentially expressed gene in gastric adenocarcinoma tumors (Rabenau et al., 2004). AMIGO3 has recently been shown to be a co-receptor of NgR1/p75 complex that is involved in myelin-induced axon growth inhibition (Ahmed et al., 2013).
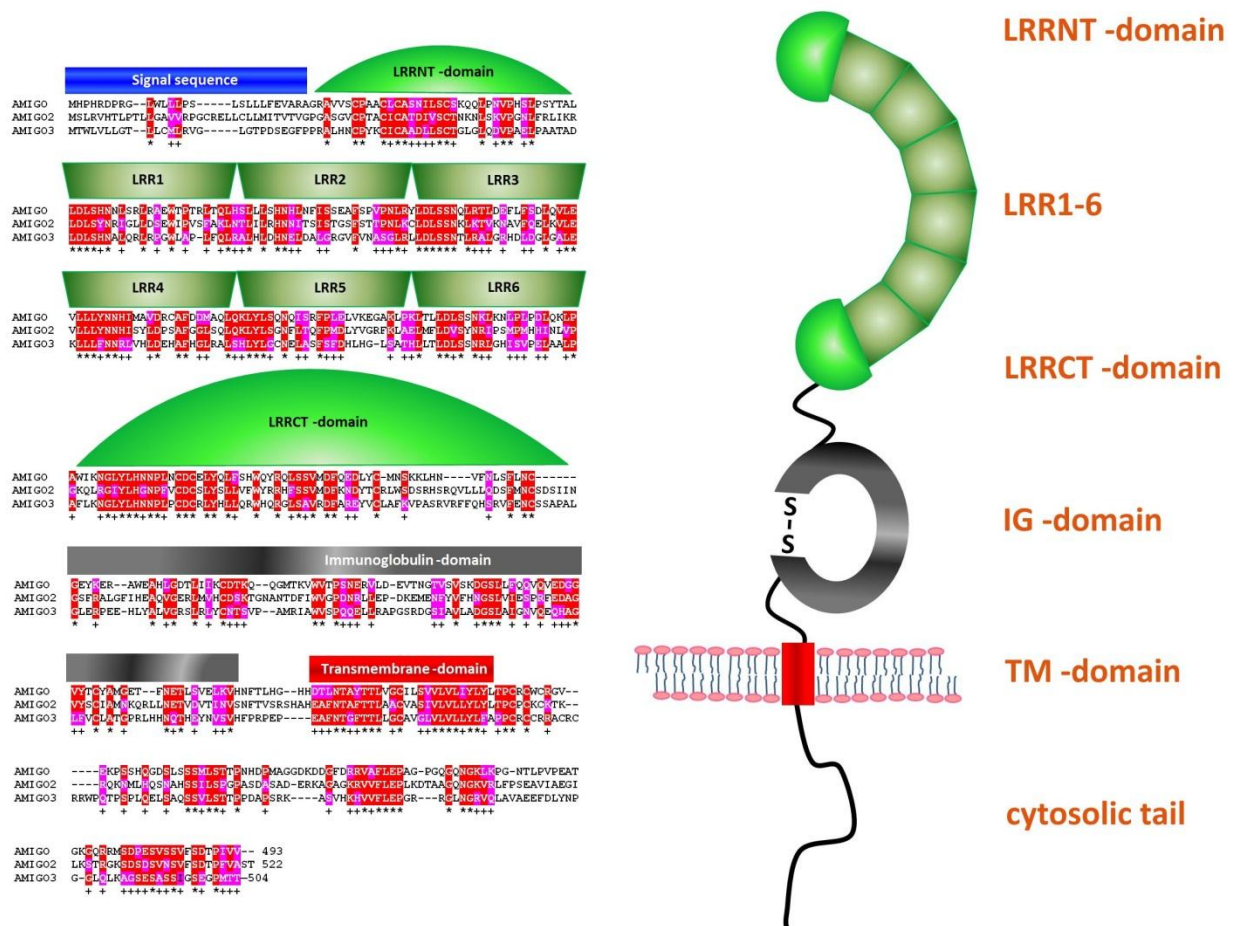

Figure 3. Alingment and schematical view of the AMIGO family members

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7.1.2. AMIGO crystal structure

Recombinant mouse AMIGO extracellular domain was produced in large quantities in *Drosophila* Schneider 2 cells (Schneider, 1972) and after a series of optimizations AMIGO crystals were formed in 0.1 M 4-morpholineethanesulfonic acid, pH 6.5, and 1.6M $MgSO_4$. The quality of the AMIGO crystals allowed us to determine the AMIGO extracellular structure at 2 Å resolution. The structure of the AMIGO ectodomain followed the prediction made *in silico* by sequence homology with one LRR domain and one Ig domain. The striking feature was that AMIGO crystallized as a dimer such that the concave face of the LRR domain was responsible for the dimer interface.
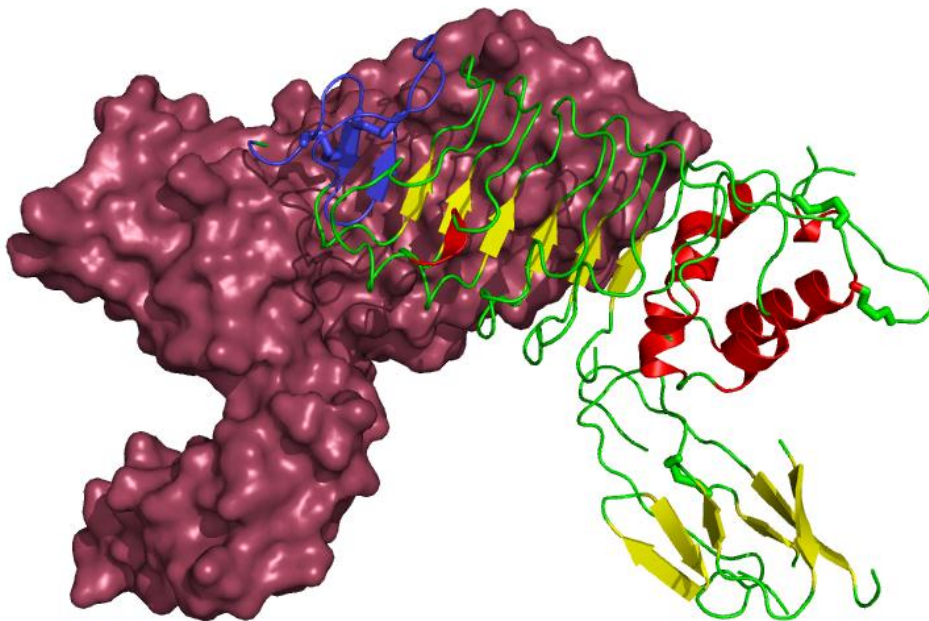

Figure 4. AMIGO dimer where the two AMIGO proteins depicted are interacting through the LRR domain. One AMIGO is pictured as a space filling model and the other as a ribbon model.

7.1.2.1 Dimer formation is needed for AMIGO secretion

The exact structure of AMIGO allowed us to pinpoint the important amino acid residues that interact on two separate AMIGOs to form a dimer. Eight residues where chosen from the dimer interface for mutational analysis and for control purpose one residue was chosen outside the area. The idea was to produce these recombinant proteins with residues mutated to alanine and study the possible dissociation constant (Kd) value differences between these mutants. We found to our surprise, that the mutation of all eight important dimer interface residues led to impaired secretion of the recombinant AMIGO protein, whereas the control mutation close to the interface did not result in any effect (Fig. 7 in publication III). The AMIGO interface mutants were presumably detected by the quality control machinery of the endoplasmic reticulum (ER) and were thus prevented from exiting the ER (Ellgaard and Helenius, 2003). This result suggests that correct dimer

interface formation is needed for the proper folding of the AMIGO dimer, and without this dimerization AMIGO is misfolded and discarded from the pathway that directs AMIGO to the plasma membrane.

7.1.2.2 AMIGO is heavily glycosylated

The analysis of the AMIGO sequence predicted (Lutteke et al., 2006) five possible N-glycosylation sites, and indeed some of these were already occupied by N-glycosylation in the AMIGO crystals. The AMIGO crystals were formed from recombinant protein expressed in *Drosophila* therefore we had to study the N-glycosylation of AMIGO in mammalian cells to prevent the possible effects of N-glycosylation differences between insects and mammals from obscuring our results (Altmann et al., 1999; Tomiya et al., 2004). We mutated the five possible N-glycosylation sites predicted in the mouse AMIGO with alanine (N72 and N269 on LRR domain, N315, N348 and N359 on Ig domain). These mutations together with the wild type (WT) AMIGO were transiently expressed in HEK293 cells and the total cell lysates were subjected to Western blotting using an anti-AMIGO antibody. The result showed that all the five predicted N-glycosylation sites of the AMIGO were occupied (Fig. 3 in publication III). The exact role of the N-glycosylation on the AMIGO protein surface is not yet known but interestingly four of the N-glycosylation sites (N269 on the LRR domain, N315, N348 and N359 on the Ig domain) have been conserved during the evolution from teleost (zebrafish) to mammals. The Ig domain has three N-glycosylation sites, and we suggest that these sugars help to orientate the LRR domain properly on the plasma membrane. Further, the N-glycosylated AMIGO was found to contain complex glycans with negatively charged sialic acids (Juha Kuja-Panula unpublished result).

7.1.2.3 All AMIGO family members are dimers

The AMIGO structure allowed us to model the structure of the other members of the AMIGO family. The modelling together with the analysis of gel filtration and small-angle X-ray scattering (SAXS) results suggested that both AMIGO2 and AMIGO3 are dimers. The sequence alignment of the ectodomains of the three AMIGOs showed that the site of the dimer interface on the AMIGO surface was conserved between the AMIGO family members, which also gave additional support for the dimer formation for the AMIGO2 and -3 (Fig. 4 in publication III). The SAXS data also revealed that AMIGO2 differs from AMIGO and AMIGO3 in having a more elongated dimer, which is possibly due to a longer linker sequence between the LRR and the Ig domain. The dimer interface conservation between the AMIGO family members suggests that the AMIGOs could form dimers with each other.

7.2 Role of AMIGO in developing CNS (I, II, III)

The expression pattern of the *Amigo* gene was studied in more detail in mouse embryos (embryonic day 13; E13) and in adult mouse brain sections by using mRNA *in situ* hybridization. In the E13 embryos the *Amigo* gene was expressed strongly in the developing dorsal root ganglia and in the trigeminal ganglion. In the adult mouse brain, the expression was widely distributed (Fig. 3B in publication I). The same protein family was also found in zebrafish (ZF, *Danio rerio*) which suggests that the ancestral AMIGO protein evolved 450 million years ago before early tetrapods diverged from the teleost linage (Soltis and Soltis, 2012). The ZF AMIGO family differs from its mammalian equivalent in that the ZF family lacks the AMIGO2 counterpart instead it has two variants of AMIGO3 called -3a and -3b (publication II). *Amigo* is the predominant AMIGO family transcript in the whole ZF larvae. Its expression rises after two days post fertilization (dpf) and continues to rise during early development (Fig. 1B in publication II). AMIGO protein staining could be clearly seen in the larval brain at 28 hour post fertilization (hpf). The AMIGO protein was found to be localized both in the neuronal progenitor cell layers and in the early developing fiber tracts. The staining in the fiber tracts could be seen on the leading front and along the fascicles on the medial longitudinal fascicle (MLF) and on the post-optic commissure (POC).

7.2.1 AMIGO in neurite outgrowth and fasciculation

The original method for finding AMIGO and its expression pattern suggested a role in neurite outgrowth. Indeed, when rat hippocampal neurons were plated *in vitro* onto the AMIGO extracellular matrix neurite outgrowth was evident after 24 hours compared to the control situation (Fig 6 in publication I). When *in vitro* hippocampal neurons were plated on poly-L-lysine matrix for four days, neurites started to form bundles in a phenomenon called fasciculation (Rutishauser and Edelman, 1980) whereby neurites grow alongside each other by using pioneer axons as the substratum for their growth cones. Interestingly, the addition of the AMIGO ectodomain into the culture medium inhibited the formation of the neurite bundles which suggests that AMIGO has a role in neurite fasciculation (Fig. 7 in publication I). The same role of the AMIGO in fasciculation was also revealed *in vivo* in the developing zebrafish CNS where the knockdown of endogenous AMIGO or the dominant negative approach using the AMIGO ectodomain resulted in disturbed fasciculation of the MLF and POC fiber tracts, which are also the places where AMIGO is higly expressed during ZF CNS development (publication II).

7.2.2 AMIGO family proteins display homophilic interactions

The results from both neurite outgrowth and fasciculation assays guided us to study possible mechanisms of AMIGO in these two phenomena. We carried out co-immunoprecipitation assays in heterologous HEK293T cells with two differentially tagged variants of AMIGO (GFP- or V5-tag). The same procedure was also used for AMIGO2 and AMIGO3. An unrelated transmembrane protein RAGE was tagged with the V5 sequence for control purposes. The HEK293T cells were transiently transfected with all

possible combinations of the GFP and V5 tagged proteins, and were then subjected to co-immunoprecipitation with anti-GFP or anti-V5 antibodies. Western blotting of the samples showed that the AMIGO-GFP could be pulled down by the AMIGO-V5 variant, which suggests that the AMIGO molecule possess homophilic interaction capabilities. The same was also true for AMIGO2 and AMIGO3, which also proved to be homophilic although no interaction was seen with the control protein RAGE. Interestingly, the AMIGO family members display heterophilic binding properties with each other. The homophilic interaction of the AMIGOs was further characterized by using a bead aggregation assay (Fig 8 in publication I). These findings along with those of the crystal structure of the AMIGO suggest that the defasciculation seen with a dominant negative approach in both mice and zebrafish are due to the inhibition of homophilic binding of the AMIGOs between adjacent neurites. These results allow the categorization of the AMIGO family members as cell adhesion molecules (CAMs), which are a group of proteins whereby many members possess homophilic interaction properties.

7.2.3 Could AMIGO make trans-homodimers between cells?

The AMIGO matrix promoted neurite outgrowth and the soluble AMIGO was able to prevent fasciculation of the neurites. Could these results implicate that AMIGO works as a homophilic cell adhesion molecule between two separate cells in trans orientation? The AMIGO dimer as seen in the crystal structure was released from the ER in cis orientation to the plasma membrane, and this dimer should be opened to allow two AMIGO molecules to meet each other in trans-orientation and to dimerise. The conclusion from the SAXS data (publication III) suggested this possibility and thus accordingly a role for AMIGOs as homophilic cell adhesion molecules.

7.2 Role of AMIGO in adult CNS (II, IV, V)

7.2.1 AMIGO associates with voltage-gated potassium channel Kv2.1

7.2.1.1 AMIGO co-localizes and interacts with Kv2.1

When adult mouse CNS and mature *in vitro* hippocampal neurons were stained by specific anti-AMIGO antibody the staining pattern was restricted to the cell soma and to the proximal part of the neurites. A closer look of the immunostaining pattern showed that AMIGO was concentrated in punctate/ring like clusters (Fig. 1 in publication IV). This peculiar staining pattern resembled that seen for the voltage-gated potassium channel Kv2.1 (Trimmer, 1991), which belongs to the group of delayed rectifier channels. We then double-stained both those neurons *in vitro* and *in vivo* with AMIGO and Kv2.1, and they showed striking co-localization (Fig.1 in publication IV). The temporal expression pattern of AMIGO and Kv2.1 were also similar, and when both were expressed in a heterologous system the co-localization was evident. The cell surface localization of Kv2.1 is

extensively regulated by phosphorylation whereby dephosphorylation leads to the diffusion/dispersion of Kv2.1 around the cell membrane (Misonou et al., 2004; Misonou et al., 2005b). Interestingly, when neurons were treated both *in vitro* and *in vivo* to induce the dephosphorylation of Kv2.1, AMIGO diffused from the cell surface clusters. This suggests that AMIGO and Kv2.1 are intimately coupled. This interplay was further studied by co-immunoprecipitation experiments in the adult mouse brain and indeed AMIGO and Kv2.1 readily pulled down each other whereas the control protein Kv1.2 did not show any interaction with AMIGO (Fig. 2 in publication IV).

We also studied the possibility that the AMIGO-Kv2.1 interaction is evolutionarily conserved from fish to mammals. In early the ZF CNS (28 hpf) the co-localization was not detected, whereas in the adult ZF CNS, the AMIGO and Kv2.1 association was evident in both tissue co-localization and in co-immunoprecipitation experiments (Publication II). When these results were compared to those of the mouse they suggested that AMIGO belongs to the Kv2.1 channel complex and this interaction has evolved at least 450 million years ago.

7.2.1.2 AMIGO changes voltage gating properties of Kv2.1

The results presented above raise the possibility that AMIGO could change the electrophysiological characteristics of the Kv2.1 channel. Consequently, we address to this question when we used a heterologous system by transiently transfecting HEK293 cells together with AMIGO and Kv2.1. A non-related type-I transmembrane protein NCAM was also used to transfect the HEK293 cells as a control. An important finding was that the voltage-dependent activation of the Kv2.1 shifted to more negative values when AMIGO was present, whereas the control protein NCAM did not have any effect on the HEK293 cells (Fig. 3 in publication IV). This result was studied further to find the important parts of the AMIGO protein that were needed for changing the Kv.21 voltage gating. A series of fusion proteins were used whereby the AMIGO extracellular, transmembrane or cytosolic domains were swapped with their NCAM counterparts. The hyperpolarizing effect of AMIGO on Kv2.1 was totally abolished by using an AMIGO variant with the NCAM transmembrane domain. Reduced AMIGO potency was evident in variants when the extracellular or cytoplasmic parts were changed to the NCAM counterparts (Suppl. Fig 6S in publication IV). These effects suggest that the interaction sites for AMIGO and Kv2.1 are located within/on their transmembrane domains whereas both the extracellular and cytoplasmic domains contribute to the electrophysiological effect through an unknown mechanism that remains to be elucidated.

7.2.1.3 Inhibition of AMIGO in cultured neurons changes voltage-gated potassium currents

After the heterologous overexpression studies we then investigated what happens to voltage-gated potassium currents when endogenous AMIGO is inhibited. We used the short hairpin RNA method in mature 14-day old *in vitro* cultured hippocampal neurons. Even though hippocampal neurons can contain many different voltage-gated potassium

channels, the inhibition of AMIGO significantly decreased the delayed rectifier current I_K at negative membrane potentials, around the action potential threshold (Fig 4 in publication IV). When this same protocol was applied to neurons treated by the specific Kv2.1 blocking agent GxTX-1E (Herrington et al., 2006), the voltage-gated potassium currents were significantly changed, which revealed that our experimental setup partially addressed voltage-gated potassium currents originating from Kv2.1. Those results with neurons lacking AMIGO contradicted those of the overexpression situation and this fits the hypothesis that AMIGO is indeed an auxiliary unit of the voltage-gated potassium channel Kv2.1.

7.2.2 AMIGO deficiency in mice leads to schizophrenia like symptoms

7.2.2.1 AMIGO regulates Kv2.1 protein expression and voltage-gated potassium currents

We produced a mouse line that was deficient in the *Amigo* gene to study the *in vivo* functions of AMIGO in mammals. Those mice bred normally without any obvious signs of problems in the normal caged environment. Moreover, when the brains of the adult mice were studied under Luxol fast blue/creysyl violet staining there were no differences between the knockout (KO) and wild type (WT) mice. Immunohistochemical staining with common markers for neurons and glia also found no obvious phenotypes in these cell types in the *Amigo* KO mice. Histological staining of the adult mouse brain sections with the anti-Kv2.1 antibodies however revealed that the intensity for Kv2.1 was clearly reduced in the KO mice. We then studied this phenomenon more closely by using Western blotting with samples made from adult mouse brain membranes. The reduction of the Kv2.1 band intensity in the samples was 45% in the *Amigo* KO mice compared to their WT littermates. The same samples were stained with Kv1.2 as a control, and did not show any difference between the genotypes. Similar diminished expression of Kv2.1 was also seen in developing ZF CNS (3 dpf) when AMIGO expression was inhibited using morpholino oligos (Fig. 5A in publication II). The finding that the Kv2.1 expression is reduced in the situation when AMIGO is missing supports the idea that the functions of AMIGO and Kv2.1 are intimately associated.

The electrophysiological findings in an earlier publication (IV) motivated us to see whether there is a difference in voltage-gated potassium currents between the *Amigo* KO and the WT mice. Measurements were made in samples of CA1 pyramidal neurons by using acute hippocampal slices obtained from both genotypes. The findings from this setup were in agreement with our previous *in vitro* electrophysiological results. We could see a similar reduction in the voltage-gated potassium currents at negative membrane potentials around the action potential threshold in the *Amigo* KO mice compared to their WT littermate controls. Even though pyramidal neurons simultaneously express many different voltage-gated potassium currents, we suggest that the difference seen in the KO mice was due to a lack of AMIGO/Kv2.1 interaction which led to an improperly functioning Kv2.1 channel.

7.2.2.2 *Amigo* knockout mice have disrupted complex behavioral traits

We subjected the *Amigo* KO mice and the WT littermate to an extensive battery of behavioral tests in order to elucidate the possible role of AMIGO *in vivo*. The *Amigo* KO mice were normal for tests that measured the nociception and motor co-ordination, which supported the notion that the *Amigo* KO mice could not be separated behaviorally from their WT controls in routine tests. However, test patterns for studying more complex behavioral traits found differences between the KO mice and their WT littermates.

When mice were placed in the open field test arena it was obvious that the *Amigo* KO mice traveled more (158%) during the test period, which indicated that the *Amigo* KO mice were hyperactive. Interestingly, when the *Amigo* KO mice and their WT littermates were subjected to a low dose of the psychotomimetic drug, MK-801, the hyperactivity phenotype was significantly increased in the KO mice, whereas the MK-801 effect was not evident in the WT mice. A similar hyperactive phenotype was also seen in AMIGO deficient ZF larvae (3dpf), which suggests that a lack of AMIGO leads to a disruption of an evolutionarily related neuronal function (Fig. 9A in publication II).

Sensorimotor gating is a phenomenon in which the animal automatically filters out non-related information from important information, which allows the animal to cope in a complex environment. This system can be found in fish (Burgess and Granato, 2007) and humans (Adler et al., 1982), thus the measurements of the sensory gating are done similarly across all the species using prepulse inhibition (PPI). The PPI measures the startle response of the animal in a test chamber in which a weak prestimulus inhibits the startle caused by a stronger stimulus. The *Amigo* KO mice showed significantly reduced PPI when compared to WT littermates. Reduced PPI is a common feature found in the schizophrenia patients which can be relieved by administration of the antipsychotic drug haloperidol (Kumari and Sharma, 2002). When the *Amigo* KO mice were treated with haloperidol we noted a clear rescue effect and their PPI responses shifted to more normal values.

Two different experiments were used in the social behavior testing which were: the resident intruder test for the social stress paradigm and the tube test to determine dominant and subdominant behavior. In the resident intruder test the *Amigo* KO mice did not differ from the WT littermates, which suggests normal coping by the *Amigo* KO mice for social stress. In the tube test the WT littermates won significantly more trials than the *Amigo* KO mice, which suggests that an AMIGO deficiency leads to subdominant behavior.

The memory consolidation of the *Amigo* KO mice was initially studied using the Morris water maze and the two genotypes were found not to differ from each other. We studied memory more closely, by placing the *Amigo* KO and WT mice in IntelliCage (Endo et al., 2011) to measure many spontaneous and memory behaviors in homecage-like environment. The versatile analysis properties of IntelliCage method enabled us to find memory deficiency in the *Amigo* KO mice in the task called "patrolling". In this paradigm, a mouse has to learn that the "correct" corner is changed in a clockwise manner after every "correct" visit. This memory task is similar to the 8-arm radial maze and both tests measure behavioral flexibility and working memory (Too et al., 2013; Weyer et al., 2011).

7.2.2.3 Behavioral changes in *Amigo* knockout mice resemble schizophrenia

Schizophrenia is one of the most common human mental disorders and it affects about 0.7 % of the world's population. It is generally believed that this sickness has been disrupting the lives of people through the history of mankind even though it was first diagnosed as recently as as 1887 by Kraepelin (Engstrom and Weber, 2005). The symptoms for schizophrenia are classically divided into two categories: positive and negative symptoms. The positive symptoms could be called "gain of function" symptoms whereby patients have extra feelings and behaviors such as hallucinations and delusions. In the negative symptoms, such as asociality, apathy and cognitive problems, patients lack behaviors and feelings that usually are present (Kay et al., 1987) in healthy human beings. Even though the numbers of studies on schizophrenia are enormous the real understanding of the disease mechanisms is still lacking.

An important tool that reveal the disrupted systems of the schizophrenic CNS have been the various knockout mouse models available (Jones et al., 2011; Nestler and Hyman, 2010). Modeling of complex human cognition related sickness in rodents is challenging but certain behavioral features have been linked to the symptoms of human schizophrenia and these include prepulse inhibition, memory deficiency, hyperactivity and social problems (Gainetdinov et al., 2001; Lai et al., 2014; Papaleo et al., 2012; Powell et al., 2012).

When the behavioral problems of the AMIGO deficient mice are surveyed together one notes that the disrupted behavioral domains are also found in well-established mouse models of schizophrenia (see table I). The listed domains in such models are hyperactivity, PPI deficit, social deficit and memory deficit. An important finding is that the *Amigo* KO mice recapitulate all these human schizophrenia related behavioral disturbances. This result strongly suggests that the human *AMIGO* gene should be taken as a potential candidate gene for schizophrenia. We speculate that in the adult human CNS AMIGO is needed to fine tune the Kv2.1 voltage-gating properties and a loss of AMIGO could cause a situation where the neurons are excessively prone to firing action potentials, which lead to an overactive CNS. Support for neuronal hyperactivity in schizophrenic brains have come from many studies where the overactivation could be located in many distinct brain areas and functions (Del Pino et al., 2013; Huffaker et al., 2009; Suh et al., 2013). The common feature is that even though the disrupted functions of the brain can vary they all are processed at brain level as disrupted neuronal synchrony that leads to schizophrenia like symptoms (Uhlhaas and Singer, 2010). The AMIGO-Kv2.1 relationship was clearly seen in zebrafish for which AMIGO deficiency resulted in hyperactivity and defective sensorimotor functions. Those behavioral phenotypes seen in zebrafish were rescued by ectopic expression of Kv2.1. Interestingly, a recent study on mice deficient in Kv2.1 also show a similar hyperactive phenotype compared to that seen in the *Amigo* KO mice (Speca et al., 2014). We suggest that behavioral changes seen in the AMIGO deficient mice are due to misbehaving Kv2.1 channels and AMIGO´s role in the adult CNS is mediated through regulation of Kv2.1 channels.

Table 2. *Behavioral problems reported in mouse models of schizophrenia and their existence in AMIGO KO mouse*

mouse models of schizophrenia	hyper-activity	PPI deficit	social deficit	memory deficit
BACE1 KO (Savonenko et al., 2008)	✔	✔	✔	✔
Calcineurin KO (Miyakawa et al., 2003)	✔	✔	✔	✔
Del22q11mouse model (Paylor et al., 2001)		✔		✔
Corticotropin releasing factor overexpression (Dirks et al., 2002)		✔		
DBA/2 mouse (Stevens et al., 1998)		✔		
DISC1 DN mouse (Hikida et al., 2007)	✔	✔		
Disheveled 1 KO (Lijam et al., 1997)		✔	✔	
DAT KO (Giros et al., 1996; Morice et al., 2007; Ralph et al., 2001)	✔	✔	✔	
ErbB4 conditional KO (Del Pino et al., 2013)	✔	✔	✔	✔
GABA A alpha3 receptor KO (Yee et al., 2005)	✔	✔		
GAP-43 KO (Metz and Schwab, 2004)	✔	✔		
GDI1 KO (D'Adamo et al., 2002)			✔	✔
Grin1(D481N) mutant (Kew et al., 2000; Labrie et al., 2008)			✔	✔
Heterozygous Nurr1 KO (Rojas et al., 2007)	✔			✔
Heterozygous Reeler mouse (Barr et al., 2008; Krueger et al., 2006)		✔		✔
Homer 1a KO (Szumlinski et al., 2005)		✔	✔	✔
mGluR1 KO (Brody et al., 2003)		✔		
mGluR5 KO (Gray et al., 2009)		✔		✔
NCAM-180 KO (Wood et al., 1998)		✔		
Neuregulin 1 hypomorph (Stefansson et al., 2002)	✔	✔		
Neurexophilin 3 KO (Beglopoulos et al., 2005)		✔		
NMDA NR1 hypomorph (Fradley et al., 2005; Mohn et al., 1999)	✔	✔	✔	
nPAS 1/3 KO (Erbel-Sieler et al., 2004)	✔	✔		✔
Proline Dehydrogenase KO (Gogos et al., 1999)		✔		
SREB transgenic mouse (Matsumoto et al., 2008a)		✔	✔	✔
STOP KO (Begou et al., 2008; Fradley et al., 2005)	✔	✔	✔	✔
D2 receptor overexpression (Kellendonk et al., 2006)				✔
Type III Nrg1 KO (Chen et al., 2008)	✔			✔
AMIGO KO (publication V)	✔	✔	✔	✔

8 Concluding remarks

The construction of the human brain with its complex neuronal circuits and electrochemical functions is a marvelous product of half billion years of evolution. This work provides novel information as to how one gene called *AMIGO* from the pool of ca. 20 000 human genes participates in developing the functional neuronal circuitry of brain. The remarkable small number of genes that when expressed constitute such a complex organism like a human being, necessitate many genes having pleiotropic roles and this seems to be also true in the case of *Amigo*.

The conclusions of this work comprise two major parts:

A. Discovery of the AMIGO family and the role in the developing CNS

1. AMIGO defines a novel cell adhesion molecule family together with AMIGO2 and AMIGO3.
2. AMIGO functions as a homophilic cell adhesion molecule during CNS development, which guides/supports neurite outgrowth through fasciculation.
3. AMIGO crystallization as a dimer and structural studies suggest that dimerization has been conserved throughout the evolution of the AMIGO family.

B. Role of AMIGO in adult CNS

1. AMIGO is an auxiliary unit of the voltage-gated potassium channel Kv2.1 that fine-tunes the electrophysiological properties of the channel in the adult brain.
2. Mice lacking AMIGO show reduced expression of Kv2.1 protein associated with changes in voltage-gated potassium currents.
3. Deficiency of AMIGO leads to schizophrenia-related behavioral disturbances, which suggest that AMIGO should be considered as a candidate gene for human schizophrenia.

Future work is warranted to reveal possible binding partners for the AMIGO cytosolic tail, which will reveal the cytosolic signaling properties of AMIGO alone in neurite outgrowth and together with the Kv2.1 complex in regulation of neuronal excitability. The mechanism of how AMIGO shifts the voltage-gating properties of Kv2.1 is an intriguing question for future studies.

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