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Functional impact of interacting protein on kainate receptors: NeCaB1 and NeTo proteins.

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Ahora que me encuentro escribiendo los agradecimientos, me doy cuenta que esta es posiblemente la única sección de la tesis que no será corregida. De manera que los escribiré tal como soy, tal vez un poco caótico.

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List of abbreviations and acronyms

ACET: S)-1-(2-amino-carboxyethyl)-3-(2-carboxy-5-phenylthiophene-3-yl-methyl)-5-methylpyrimidine-2,4-dione

ACSF: Artificial Cerebro Spinal Fluid

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

APV: R)-2-amino-5-phosphonopentanoate acid

ATD: Amino Terminal Domain

ATPA: 2-amino-3-(3-hydroxy-5-tert-butylisoxazol-4-yl) propionic acid

BAPTA-AM: 1,2-Bis (2-amino-phenoxy) ethane-*N,N,N′,N′*-tetraacetic acid tetrakis (acetoxymethyl ester)

BiFC: Biomolecular Fluoresence Complementation

CA1-3: Cornu Ammonis 1-3

Ca^{2+}: Calcium ion

CAMP: Cyclic Adenosine mono-phosphate

CDNA: Complentary Desoxyribonucleic acid

CNQX: 6-cyano-7-nitroquinoxaline

CNS: Central Nervous System

Con A: Concanavaline A

CRMP2 (-4): Collapsin Receptor mediated protein 2 (-4)

CTD: Carboxy Terminal Domain

DMEM: Dulbecco's Modified Eagle Medium

DNQX: 6,7-dinitroquinoxaline-2,3-dione

DRG: Dorsal Root Ganglion

ECL: Enhanced Chemiluminiscence

$EPSC_{AMPA}$: Excitatory Postsynaptic Currents mediated by AMPA receptors

$EPSC_{KAR}$: Excitatory Postsynaptic Currents mediated by KAR receptors

FCS: Fecal Calf Serum

GABA: γ-Aminobutyric acid

GAPDH: Gliceraldheide Phosphate Deshidrogenase

GFP: Green Fluorescent Protein

GluA1-4: Glutamatergic AMPA receptors 1-4

GluK1-5: Glutamatergic KAR receptors 1-5

GluN1-3: Glutamatergic NMDA receptors 1-3

GRIP1: Glutamate Receptor Interacting Protein 1

GST: Glutathion Sulpho Transpherase

GYKI53655: 1-(4-Aminophenyl)-3-methylcarbamyl-4-methyl-3,4-dihydro-7,8-methylenedioxy-5*H*-2,3-benzodiazepine hydrochloride

H^+: Proton

HEK: Human Embrionary Kidney

HRP: *Horseradish peroxidase*

I_{AHP}: After Hyperpolarization potential mediated current

iGluR: Ionotropic Glutamate receptor

IPTG: Isopropil-β-D-1-tiogalactopiranoside

IUPHAR: International Union of basic and clinical Pharmacology

KAR: Kainate receptor

LB: Lysogeny broth medium

LBD: Ligand Binding Domain

LSB: Loading Sample Buffer

LTD: Long Term Depression

LTP: Long Term Potentiation

MAGUK: Membrane Associated Guanylate kinase

mGluR: Metabotropic Glutamate receptor

mRNA: Messenger Ribonucleic acid

NBQX: 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo quinoxaline

NeCaB1: Neuronal Calcium Binding protein 1

NETO1 (-2): Neurophillin Tolloid-like 1 (-2)

NMDA: N-methyl-D-aspartate

P/S: Penicilyn/Streptomicyn

PBS: Phosphate Buffered Saline

PDZ: Acronism for PDZ-95, DlgA, Zo-1

PICK1: protein Interacting with C kinase 1

PKA: Protein Kinase A

PKC: Protein Kinase C

PLC: Phospho-lipase C

PM: Plasma membrane

PSD-95: Post-synaptic density-95

PTx: Pertusis Toxin

SAP-97 (-102): Synapse Associated Protein-97 (-102)

SEM: Standart Error Media

SNAP-25: Synaptosomal Associated Protein-25

SNP: Single Nucleotide Polymorphism

SUMO: Small Ubiquitin-like molecule

TARP: Transmembrane AMPA Regulatory Protein

TB: Transfer Buffer

TBS-T: Transfer Buffer Supplemented with Tween

TLE: Temporal Lobe Epilepsy

TM: Transmembrane

TMD: Transmembrane Domain

UBP310: S)-1-(2-amino-2-carboxyethyl)-3-(2-carboxy-thiophene-3-yl-methyl)-5-methylpyridimide-2,4-dione

VCT: Venus Carboxy Terminal

VNT: Venus Carboxy Terminal

Zn^{+}: Zinc ion

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Work communications

Poster presentations

Jon Palacios-Filardo, M. Isabel Aller and Juan Lerma. Functional Impact of auxiliary Proteins on Kainate Receptors. *SENC 2011 (Salamanca, Spain).*

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Summary.

Fast excitatory synaptic transmission is mainly mediated by glutamate receptors in the Central Nervous System. This family of receptors comprises three different members: AMPA, NMDA and kainate. Among these, kainate receptors (KARs) are the less understood from a physiological point of view. An attempt to unveil important aspects of KARs physiology is to elucidate the protein interactome around these receptors. In this work we have focus in the interaction of NeCaB1 protein, which was found in a yeast two hybrid screening and Neto1-2 protein interaction.

NeCaB1 binds to the C-terminal domain of GluK5 subunit containing KARs promoting their trafficking and increasing their affinity depending on environmental Ca^{2+}. Thus, NeCaB1 could dynamically determine the kind of KARs at synapses according to synaptic activity, constituting a kind of homeostatic plasticity.

In the other hand, Neto1 and Neto2 functionally interact with and modulate the three main KAR subunits (GluK1-3) but the degree and sign of modulation depend on the type of receptor. In addition, Neto1 or Neto2 set the slow kinetics of KARs, while synaptic targeting relies on GluK5 subunit.

This work clarifies how the complex behavior of kainate receptor might be explained by specific binding with surrounding proteins.

Resumen.

Los receptores de glutamato son los responsables de mediar la transmisión sináptica rápida en el sistema nervioso central. Esta familia de receptores está formada por tres tipos: los receptores de AMPA, NMDA y kainato. Entre estos, los receptores de kainato son los menos conocidos desde el punto de vista fisiológico. Con el objetivo de revelar la función de estos receptores, nosotros estamos interesados en descubrir el interactoma de los receptores de kainato. En este trabajo nos hemos centrado en la interacción con la proteína NeCaB1, la cual se halló en un ensayo de doble híbrido y las proteínas Neto1-2.

NeCaB1 se une al dominio C-terminal de los receptores de kainato que contienen la subunidad GluK5. Esta interacción desplaza los receptores de kainato hacia la superficie celular e incrementa la afinidad de dichos receptores, todo ello operado por la disponibilidad de calcio. Así, NeCaB1 puede controlar la clase de receptores de kainato que se encuentran en la superficie celular en respuesta la actividad neuronal, lo que podría constituir un nuevo modo de plasticidad homeostática.

Por otra parte, las proteínas Neto1 y Neto2 son capaces de interactuar con los tres tipos mayoritarios de receptores de kainato (GluK1-3), pero el grado y signo modulación depende de la composición de los receptores de kainato. También hemos observado que Neto1 y Neto2 son los responsables de las lentas cinéticas de las respuestas sinápticas mediadas por receptores de kainato, mientras que su localización en las sinapsis está mediada por la subunidad GluK5.

Este trabajo pone de manifiesto como las proteínas que interactúan con los receptores de kainato pueden explicar el complejo funcionamiento de estos receptores.

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I. Introduction

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Introduction

Nowadays we know that glutamate is the main excitatory neurotransmitter in the central nervous system (CNS). Almost 60 years ago, Hayashi demonstrated that brief application of glutamate monosodium into monkey and dog cortex produced an increase of neuronal activity (Hayashi, 1956; 1994). More recent and detailed cellular, molecular and electrophysiological studies have demonstrated the role of glutamate as a neurotransmitter in the CNS of vertebrates. First, the presence of an endogenous synthetic machinery and system release for glutamate is present in neurons (Hamberger et al., 1979). Second, the existence of specific glutamate receptors localized at postsynaptic membranes (Evans et al., 1979; Davis et al., 1979), and third, the existence of high affinity glutamate transporters to ensure an active mechanism to terminate the action of glutamate (Logan and Snyder, 1972), lead to conclude that glutamate behaves as a real neurotransmitter in the CNS.

L-glutamate is one of the most abundant aminoacids in the brain and its receptors are widely distributed in vertebrates' CNS. It is accepted that glutamate fulfills several roles in the CNS. Glutamate does not only participate in fast excitatory neurotransmission but also modulates synaptic efficacy (Watkins and Evans, 1981; Mayer and Westbrook, 1987) that has been related with development of synaptic networks and learning processes. On the other hand, an over excitation of glutamate receptors after ischemic episodes or trauma, might provoke cell death (Schwarcz and Meldrum, 1985; Rothman and

olney, 1987). In this way, glutamate receptors play a role in both physiological and pathophysiological conditions in the CNS.

1. Glutamate receptors.

Two big families of glutamate receptors can be differentiated concerning the functional mode of operation: metabotropic glutamate receptors (mGluRs) and ionotropic glutamate receptors (iGluRs). The first class, are formed by subunits which contains 7 transmembrane spanning domains that dimerize to form functional receptors. In the intracellular region of the receptor a trimeric G protein is anchored, which upon glutamate binding is activated, triggering a second messenger cascade. The mGluRs are encoded by 8 different genes (GRM1-GRM8), which give rise to 8 protein subunits, named mGluR1 through mGluR8. Classification of mGluRs is based on sequence homology, pharmacology and functional operation (Nakanishi, 1992). In this way, mGluR1 and mGluR5 form group I, mGluR2, 3 and 6 compose group II and mGluR4, 7, 8 belong to group III.

The mGluRs belonging to type I (group I) are coupled to a G protein which activates phospholipase C that consequently provokes the hydrolysis of diacylglycerol and inositol triphosphate, both seconds messengers with different targets. Type II and III receptors are coupled to G_i protein which inhibits adenylate cyclase preventing the formation of $_c$AMP and further activation of PKA (Pin and Duvoisin, 1995).

Ionotropic GluRs are divided according to their agonist preference: NMDA (N-methyl-D-aspartate), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) and kainate receptors (KARs). These three classes of receptors share a similar structure. The iGluRs are membrane integral proteins formed by combinations of subunits forming a tetramer and a functional ion channel. Each subunit has an approximate molecular weight of 100 kDa (800-900 aminoacids). Four important domains could be defined in iGluRs: the amino terminal (N-terminal) region of the protein lies in the extracellular fluid and encompasses the ATD (amino terminal domain) and LBD (ligand bindind domain). The TMD

(transmembrane domain) is composed by three membrane spanning segments (TM1-TM3) and a fourth domain integrated in the plasma membrane but not crossing it (TM2). This segment forms the pore of the channel. The CTD (carboxy terminal domain) is located in the cytoplasmatic region and thus may interact with a variety of intracellular proteins (Roche et al., 1994, Taverna et al., 1994; Traynelis et al., 2010).

The glutamate binding domain is formed by two extracellular segments, S1 and S2. S1 is placed adjacent to TM1 while S2 connects TM3 and TM4 (Stern-Bach et al., 1994). The tetrameric structure of iGluRs is achieved by a “dimer of dimers” arrangement (Ayalon and Stern-Bach, 2001; Reiner et al., 2012). Dimers are assembled through interactions of the ATDs while tetramers are built by interaction of S2 domain (and possibly TM4 segments) of each dimer structure (Madden, 2002).

Box 1. Evolution of iGluRs.

Aminoacids binding proteins appear in the bacterial kingdom, being glutamate binding proteins present in *Escherichia coli*, named GlnH, which presents conserved glutamate binding domain (GlnH1 and GlnH2) to glutamate binding domains of the human receptor (S1 and S2; Nakanishi et al., 1990). Ion channel activity proteins gated by glutamate had been also found in plants, such as *Arabidopsis thaliana,* GLR genes (Lam et al., 1998). This and vertebrates’ iGluRs genes share important domains that include glutamate binding domains and four transmembrane domains. This might suggest that signaling by glutamate has evolved from a primitive ancestor common to plant and animal linages (Chiu et al., 1999).

Evolutions maintained iGluRs present in cnidaria (*Nematostella vectensis*), ecdysozoans (*Drosophila melanogaster*), lophotrochozans (*Caenorhabditis elegans*) and mammals (*Homo sapiens*; Ryan and Grant, 2009). However, the great divergence of mammalian iGluRs is due to two consecutive whole genome duplications occurred ~500 years ago in the vertebrate linage (McLysaght et al., 2002). This genome duplication provided redundant copies which have evolved in parallel by mutations to make all known iGluRs in vertebrates (Prince et al., 2002).

In mammals, 12 genes encode for iGluRs subunits. Recently a change in iGluRs nomenclature has been produced with the intention of unification. The International Union of Basic and Clinical Pharmacology (IUPHAR) named NMDA receptors subunits as GluN1-3 (previously, NR1-3), AMPA receptor subunits as GluA1-4 (previously, GluR1-4) and kainate receptors as GluK1-5 (previously GluR5-7 and KA1-2, respectively; Collingridge et al., 2009). Among these three types of iGluRs a fourth member, Gluδ receptors historically known as orphan glutamate receptors are considered iGluRs, even though these show weak sequence homology (~25%) with NMDA, AMPA or kainate receptors (Lomeli et al., 1993) and none of the known iGluRs agonists produce measurable currents through these receptors (Schmid and Hollmann, 2008).

1.1. NMDA receptors.

NMDA receptors are the best studied and understood iGluRs mainly by early clonage of subunits forming NMDA and availability of agonists and antagonists. NMDA receptors posses slow activation and deactivation kinetics (Lester et al., 1990) and are permeable to Na^+, K^+ and in particular to Ca^{2+} (Ascher and Nowak, 1998). Structurally are formed by combinations of three subunits (GluN1-3) with several splice variants in each case. GluN1 subunits presents 8 splice variant (Sugihara et al., 1992; Nakanishi et al., 1992), GluN2 subunits has 4 splice variants (Dingledine et al., 1999) and GluN3 appears in 2 splice variants (Cull-Candy et al., 2001). Functional receptors must be formed by a dimer of GluN1 subunits plus dimers of GluN2 or combinations of GluN2/3 subunits (Monyer et al., 1992; Ulbrich and Isacoff, 2008). Activation of NMDA receptors required D-serine or glycine and glutamate simultaneous binding (Johnson and Ascher, 1987). Glycine binding site is located on GluN1 and GluN3 subunits while glutamate binding site is in GluN2 subunits (Furukawa and Gouaux, 2003; Furukawa et al., 2005). Due to different splice variants found in NMDA receptors diverse combinations give rise to different functional receptors with different biophysical properties that have been found at specific neuronal populations (Chazot et al., 1994; Luo et al., 1997; Green and Gibb, 2001; Hansen et al., 2014).

Box 2. NMDARs pharmacology.

The fact that NMDA receptors need to be activated simultaneously by two co-agonists makes them unique between iGluRs. GluN1 and GluN3 subunits are able to bind glycine, D- or L- isomers of serine and alanine (Pullanet al., 1987; McBain et al., 1989), even though the mechanism differs between both subunits (Yao et al., 2008). Cyclic and halogentated analogs of glycine, such as D-cycloserine behave as a partial agonist of GluN1 subunits (Hood et al., 1989; Sheining et al., 2001), although, D-serine also activates partially GluN2 subunits (Dravid et al., 2010). The potency of agonist acting on GluN1 is modulated by partner GluN2 variant, been less potent receptors including GluN2A subunit and more potent those containing GluN2D subunit (Kuryatov et al., 1994; Furukawa and Gouaux, 2003; Chen et al., 2008).

GluN2 endogenous agonist may include glutamate and D- or L-aspartate (Benveniste, 1989; Nicholls, 1989). Synthetic GluN2 agonists are mainly formed by cyclic analogs with similar conformational rings, displaying higher affinity than glutamate. (Shinozaki et al., 1989; Schoepp et al., 1991). The affinity of GluN2 agonist varies between splice variants, with higher affinity for GluN2D and lower for GluN2A subunits (Monyer et al., 1992; Erreger et al., 2007).

Selective competitive antagonists block NMDA receptors, sometimes presenting subunit specificity. These include 7-chlorokynurenic acid and its analogs (Birch et al., 1988; Kleckner and Dingledine, 1989) acting on GluN1 subunits and AP-5 or (R)-CPP acting on GluN2 subunits (Davies et al., 1996), amongst others (see Traynelis et al., 2010 for an extensive review). Most potent uncompetitive antagonists are considered to be the open channel blockers, which obstruct the channel pore and therefore ion permeation, such as MK-801, PCP and ketamine (Brackley et al., 1993; Parsons et al., 1995). The first subunit selective antagonist was the phenyl-ethanolamine (ifenprodil), which acts on GluN2B subunits (Williams, 1993; Hess et al., 1998). Chemical modification of ifenprodil generated analogs such as Ro 25-6981 and CP-101,606 (Fischer et al., 1997; Tahirovic et 2008).

Allosteric modulation permits NMDA receptor to exhibit a large variety of responses. Physiological concentrations of Mg^{2+} block NMDA receptors and abolish permeation. The Mg^{2+} unblock requires membrane depolarization above -40 mV (Nowak et al., 1984; Mayer and Westbrook, 1987). Thus, NMDA receptors act as a coincidence detector between glutamate availability and

membrane depolarization to increase intracellular Ca^{2+} concentration. Others NMDA receptor modulators include Zn^{2+} (Peters et al., 1987), protons (Traynelis and Cull-Candy, 1990) both with inhibitory actions on NMDA receptors and polyamines which allosterically potentiate them (Ransom and Deschenes, 1990; Lerma, 1992; Dingledine et al., 1999).

Physiologically, NMDA receptors are located at synaptic and extrasynaptic sites, where they fulfill different roles. Synaptic NMDA receptors are responsible for part of the synaptic transmission and of trigger synaptic plasticity processes, such as long term potentiation (LTP) and long term depression (LTD) (Liu et al., 2004; Berberich et al., 2005). On the other hand, extrasynaptic NMDA receptors may play a major role in neuronal synchronization (Angulo et al., 2004; Fellin et al., 2004). Altogether, NMDA receptors have been implicated in learning and memory (Bliss and Collindge, 1993) at physiological level, while malfunction of NMDA receptors is implicated in several neurological and psychiatric disorders, including neurodenegeration (Arundine and Tymianski, 2003), chronic pain (Woolf and Salter, 2000), Huntington's disease (Milnerwood et al., 2010), Alzheimer's disease (Bordji et al., 2010) and schizophrenia (Lin et al., 2012).

1.2. AMPA receptors.

AMPA receptors are formed by GluA1-4 subunits, which present high homology between them (68-75 %; Boulter et al., 1990). AMPA receptors were the first in which a tetrameric structure was proved (Stern-Bach et al., 1994) and crystallographic studies gave successful results, obtaining detailed structural data on agonist binding domain, paving the way for further analysis on NMDA and kainate receptors (Mayer, 2005).

The functional variety of AMPA receptors may be obtained by different mechanisms. The first of them is related with mRNA editing at the so called Q/R (glutamine/arginine) site. The presence of arginine (R) at TM2 eliminates the Ca^{2+} permeability of the complex, presenting linear or outward rectification in voltage to current relationships. The presence of a glutamine (Q) in this same position allows Ca^{2+} entry and defines strong inward rectification in voltage to current plots (Burnashev et al., 1992). The presence of a R instead of a Q is

generated by editing of the mRNA, a phenomenon which is complete for GluA2 subunits. Thus GluA2 containing AMPA receptors are Ca^{2+} impermeable. GluA2 subunit also influences channel kinetics (Verdoorn et al., 1991) and conductance (Swanson et al., 1997). The second mechanism by which AMPA receptors gain diversity is mRNA splicing. Two splice variants exist in AMPA receptors: forms “flip” and “flop”. These correspond to two different cassettes of 38 aminoacids before TM4 domain which confer the channel different biophysical properties, such as receptor desensitization, “flip” forms desensitizing slower than flop variants (Sommer et al., 1990).

Similar to voltage gated channels, AMPA receptors posses several auxiliary subunits (Transmembrane AMPA receptors Regulatory Proteins, TARPs). There are 8 type of TARPs (TARP γ 1-γ8) all of them with similar structure formed by four transmembrane domains whith both amino and carboxy terminals laying on the cytoplasmatic side. TARPs are widely distributed in a non overlapping manner. Diverse TARP proteins shape AMPA receptor function at different locations (Tomita et al., 2003; Menuz et al., 2007). TARPs have been shown to modulate AMPA receptor gating, pharmacology and trafficking, hence becoming indispensable for its function (see Jackson and Nicoll, 2011 for a review). Beyond TARPs proteins, other auxiliary subunit are able to modulate AMPA receptor function, such as Chornichon homologs-2 and -3 (CNIH-2 and -3; Schwenk et al., 2009), cysteine-Knot AMPA modulating protein (CKAM-44; von Engelhardt et al., 2010) and synapse differentially induced gene 1 (SynDIG1; Kalashnikova et al., 2010).

Box 3. AMPARs pharmacology.

The sequence homology between AMPA and kainate receptors makes difficult to identify specific agonist for both types of receptors. In addition to glutamate (its natural agonist), AMPA, ibotenic acid, willardines and kainate act as agonist on AMPA receptors (Herb et al., 1992; Swanson et al., 1996; Schiffer et al.,1997). Only a few agonists derived from ibotenic acid, such as Br-HIBO, acts on GluA1 and GluA2 over other types of AMPA receptors (Coquelle et al.2000). Interestingly, neither NMDA nor D-aspartate acts have any agonistic activity, making a clear difference with NMDA receptors. This is the reason why, for years, iGluRs have been classified in two groups: NMDA and non-NMDA receptors.

The first known AMPA receptor competitive antagonists were compuds of the family of quinoxalinediones, including CNQX, DNQX and NBQX (Blaque et al., 1988; Honore et al., 1988). Surprisingly, AMPA receptors tethered to auxiliary subunits (TARPs) are partially activated by CNQX or DNQX, but not NBQX (Armstrong and Gouaux, 2000). Conversely, the most effective and specific AMPA receptor non-competitive antagonist are 2,3-benzodiazepines, such as GYKI53665 or its active isomer LY303070 (Paternain et al., 1995; Wilding and Huetter, 1995).

Non-specific AMPA/kainate receptor antagonists include philanthotoxins, which block Ca^{2+} permeable receptors (Jones et al., 1990). Further development of this wasp toxin gave rise to PhTX-56, which presents high selectivity for Ca^{2+} permeable AMPA receptors (Kromann et al., 2002).

1.3. Kainate receptors.

Kainate receptors encompass a poorly understood family of iGluRs. The lack of antagonists to differentiate from AMPA receptors has greatly delayed the physiological knowledge on kainate receptors. This started to change with the identification of GYKI53665 (Paternain et al,. 1995) which paved the way to further understanding of kainate receptors.

1.3.1. Structure of kainate receptors.

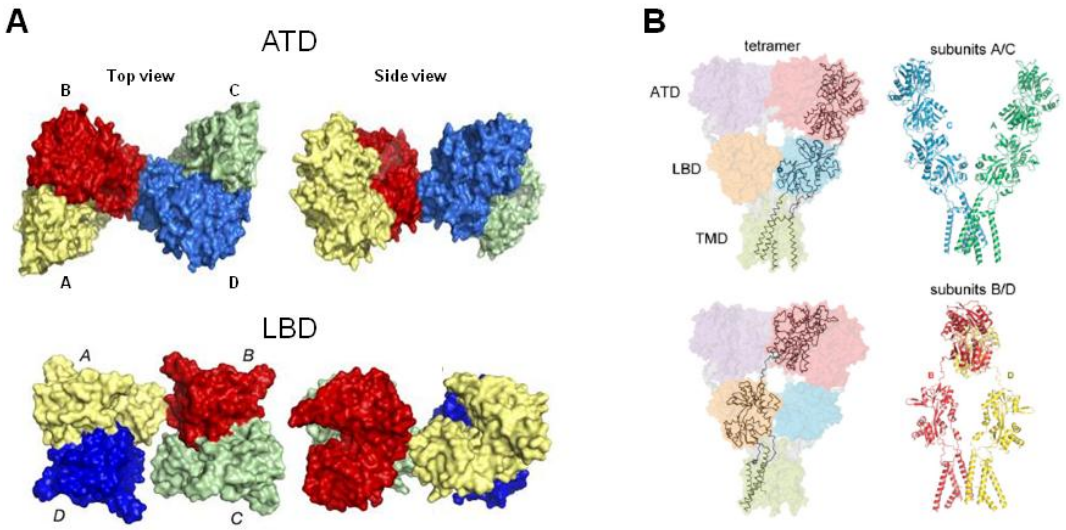

Figure 1. Structure and domain organization of ionotropic glutamate receptors. A, amino terminal domain (top) and ligand binding domain (down) organization of GluK2 kainate receptors, where domains are colored differently (adapted from Das et al., 2010). B, GluA2 AMPA receptor organization, where symmetry mismatch is highlighted (adapted from Sobolevsky et al., 2009).

Kainate receptors are tetramers formed by combinations of 5 subunits (GluK1-5). Kainate receptors are divided into low affinity receptors made by GluK1-3, which are able to form homomeric or heteromeric receptors by themselves and high affinity receptors composed by GluK4-5 subunits, which cannot form functional receptors unless they heteromerize with one of the Glu1-3 subunits (Lerma et al., 2001). Although the tetrameric structure of iGluRs was elucidated some time ago (Rosenmud et al., 1998), the assembly of subunits was recently shown to act as a dimer of dimers (Reiner et al., 2012). Inside the tetrameric structure of kainate receptors, there is a 2-fold symmetry for ATD and LBD, while 4-fold symmetry for TM. This mismatch is caused by different conformation of subunits inside the tetramer, which are known as A/C and B/D subunits (figure 1). In the ATD the most stable interaction are made between

A/B and C/D subunits, while at LBD level interactions are swapped to A/D and B/C subunits (Sobolevsky et al., 2009; Das et al., 2010).

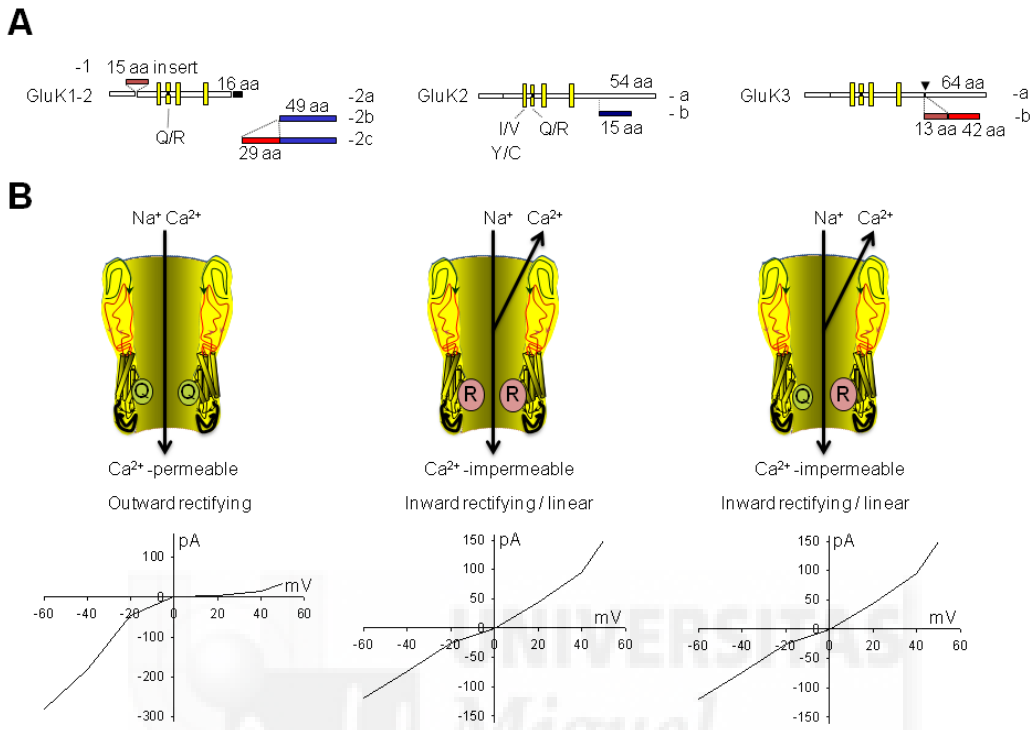

Figure 2. Splicing and editing increase kainate receptors diversity. A, splice variants corresponding to GluK1-3 subunit of kainate receptors (From Lerma 2003 with modifications). B, Q/R edition at the TM2 domain imposes Ca^{2+} permeability and rectification properties of kainate receptors. R edited forms display a dominant effect on kainate receptors

As for AMPA and NMDA receptors, several mechanisms increase functional diversity of kainate receptors, in addition to subunit combinations. First, alternative splicing is found in GluK1-3 but not GluK4-5 subunits (figure 2A). GluK1 subunits coding genes undergo alternative splicing generating different amino and carboxy terminals. At amino terminal, two splice variant referred as 1 and 2 are differentiated by the presence of a 15 aminoacids insertion in variant 1 that variant 2 lacks (Bettler et al., 1992). At carboxy terminal, four different splice variants are known (a-d). Whereas the shortest one (a) presents a premature stop codon which lead 16 aminoacids carboxy tail, variant b is 49 aminoacids longer and, variant c includes an extra 29 aminoacids segment. Variant d presents a different carboxy tail of 44 aminoacids (Bettler et al., 1990; Sommer et al., 1992; Gregor et al., 1993). GluK2 subunit presents two carboxy

terminal isoforms, named a and b (previously GluR6 and GluR6-2), where b variant differs in the last 29 aminoacids (where 15 aminoacids are different and 19 aminoacids are lacking (Egebjerd et al., 1991; Gregor et al., 1993). GluK3 subunit also presents two alternative structures at carboxy terminal (a and b). The form b contains a 13 aminoacids insert which changes the open reading frame generating a premature stop codon generating a C-tail 9 aminoacids shorter than variant a (Bettler et al., 1992; Lerma, 2003; Schiffer et al., 1997).

Another source of diversity which increase kainate receptors repertoire is mRNA editing. Editing is only presented in GluK1 and GluK2 subunits. As in the case of AMPA receptors, both GluK1 and GluK2 present Q/R editing at TM2 domain. The Q-to-R substitution abolishes Ca^{2+} permeability while increases Cl^- permeation of the channel (Egebjerg and Heinemann, 1993; Burnashev et al., 1996). In addition, channel rectification properties are also controlled by TM2 edition, in that Q residues accounts for larger conductance and inward rectification, while R residues displayed smaller conductance and linear or slightly outward rectification (Sommer et al., 1991). This phenomenon might be explained by the avoidance of polyamines in the pore when R residue is present (due to an increase of positive charges), which are responsible of rectification properties (Bowie and Mayer, 1995). Ca^{2+} permeability and rectification properties changed in R edition maintain dominant compare to the presence of Q, while Cl^- permeability does not (Swanson et al., 1997; Paternain et al., 2000; figure 2B). In addition to Q/R edition, GluK2 could also be edited at TM1 domain where isoleucine is substituted for valine (I/V) and tyrosine for cysteine (Y/C) in both cases edited forms reduces Ca^{2+} permeability. However it is not well understood the interaction between different edition sites (Kohler et al., 1993).

1.3.2. Pharmacology of kainate receptors.

KARs agonists.

The prototypical agonist for kainate receptors, kainic acid, exhibits a variable affinity for its subunits (kainate receptor agonists and antagonists structures are presented in figure 3A). While kainic acid radioligand binding assays identified two subclasses of kainate receptors: low affinity and high affinty, with dissociation constant (K_d) of ~50-100 nM (Bettler et al., 1992) and ~4-15 nM (Herb et al., 1992), respectively, these values remain far from affinities obtained in hippocampal cultures or after expressing different subunits in recombinant systems (Paternain et al., 1998; Paternain et al., 2000).

Molecules which activate kainate receptors with higher affinity than AMPA receptors include domoate (EC_{50} ~1 µM and 30 µM for native or recombinant receptors, respectively) which produces slow desensitizing currents (Huetner, 1990; Lerma et al., 1993). Subunit selective agonists comprise ATPA and (S)-5-iodowillardine, which show certain selectivity for GluK1 containing kainate receptors and 500-fold more selectivity for kainate than for AMPA receptors. ATPA (and AMPA) also activates heteromeric GluK2/5 formed receptors but with lower affinity (Paternain et al., 2000). Interestingly, ATPA or AMPA elicit different kinetics on GluK1 containing and GluK2/5 heteromeric receptors. While in the first type of receptor this agonist desensitizes responses totally, heteromeric receptors display non-desensitizing currents (figure 3B) (Paternain et al., 2000).

The potent agonist SYM281, which displays 1000-fold selectivity for kainate than for AMPA receptors (but only 200-fold compared with NMDA receptors), also rapidly inactivates kainate receptors, thus becoming used as a functional antagonist because when SYM281 is applied at low concentrations it drives receptors to an inactive state (Jones et al., 1997).

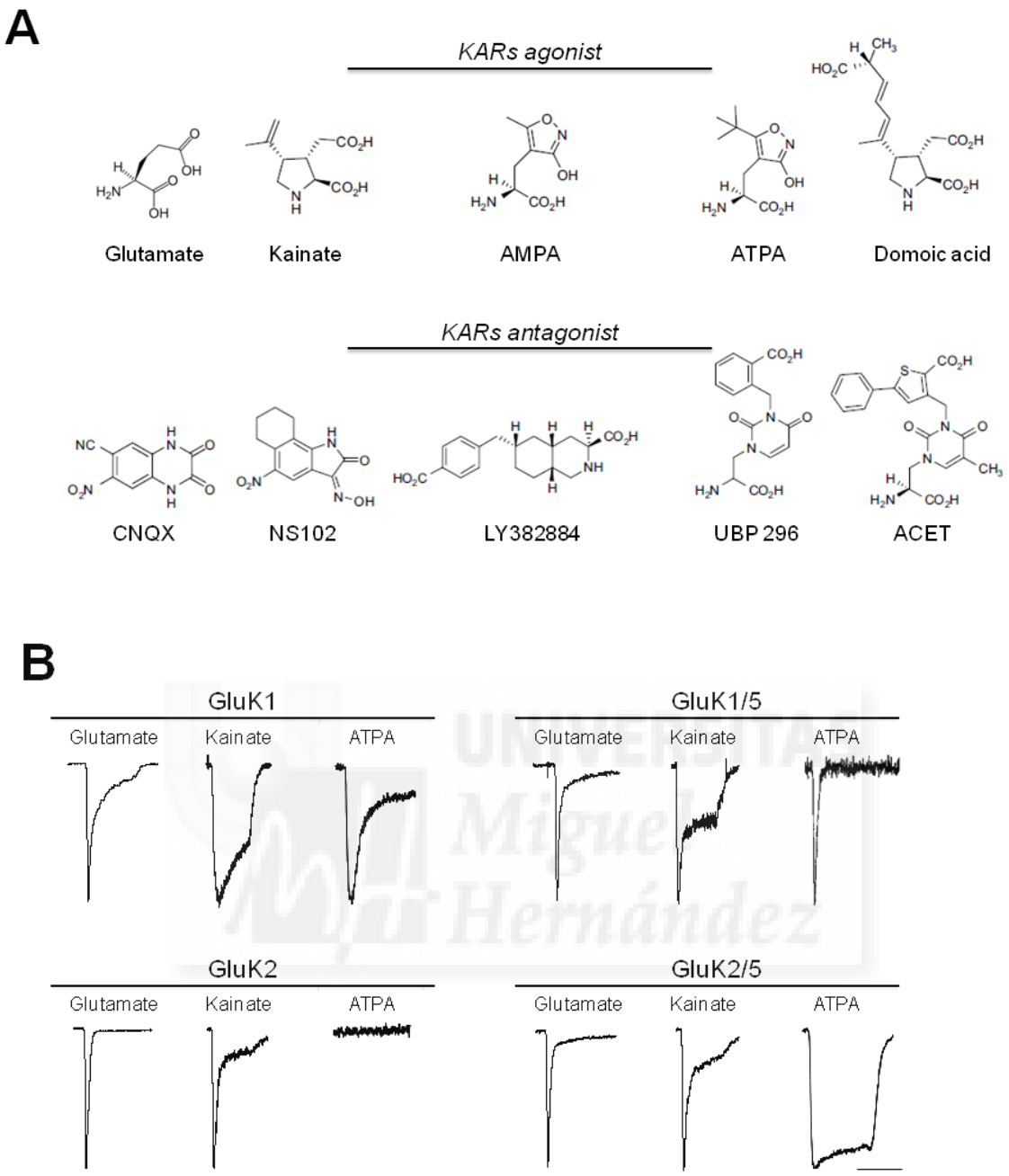

Figure 3.Different pharmacological activation of kainate receptors. A, molecular structure of selected kainate receptor agonist and antagonist. B, pharmacological activation of GluK1, GluK2 and heteromeric receptors with GluK5 subunits, where glutamate, kainate and ATPA evoke different currents. Scale bar 500 ms.

KARs antagonists.

AMPA kainate receptor antagonists, such as CNQX, DNQX and NBQX, also block kainate receptors, although low concentration of NBQX (100-300 nM) show certain degree or selectivity for AMPA over kainate receptors in the hippocampus (Mulle et al., 2000; Perrais et al., 2009). Selective kainate receptor antagonists include NS3763 which acts exclusively on homomeric GluK1 receptors, LY382884 that antagonizes GluK1 containing kainate receptors at any combination (Simons et al., 1998; Christensen et al., 2004), and NS102 that has been used as GluK2 containing kainate receptor antagonist (Verdoorn et al., 1994; Sylwestrak and Ghosh, 2012), although its action is not complete (~ 70%) at its solubility limited (Paternain et al., 1996).

Successful willardine derivatives to block kainate receptors such as UBP286 and UBP302 were found to display more than 100 times more selectivity for GluK1 containing kainate receptors than for AMPA receptors (More et al., 2004; Dolman et al., 2005). Further development of willardine derivatives produced UBP310 and ACET (Dolman et al., 2007; Dragan et al., 2009) with higher specificity for GluK1 receptors. However, UBP310 has been reported to antagonize not only GluK1 but also Glu2/5 and homomeric GluK3 receptors (Perrais et al., 2009; Pinheiro et al., 2013). Moreover, kainate receptors are susceptible of blockage by lanthanides, especially gadolinium (Huettner et al., 1998).

1.3.3. Molecular determinant of kainate receptors channel gating.

Activation of kainate receptors varies depending on subunit composition. As mentioned before, edition at Q/R site reduces channel conductance. The unedited forms of GluK1 recombinant receptors present a conductance of 2.9 pS, being an order of magnitude lower for the edited variant (200 fS). GluK2 receptors exhibit a single conductance of 5.4 fS, which is reduced to 250 fS in the edited variant (Swanson et al., 1996). The combination with GluK5 subunit enhances single channel conductance of unedited variants (Howe, 1996). However, similar to AMPA receptors, kainate receptors also present several subconductances, being ~5, 9 and 14 pS pS for GluK1 and ~8, 15 and 25 pS for GluK2. This might be explained taking in account that AMPA receptors show

several subconductances related with two, three or four agonist molecules bound to the receptor, according to the tetrameric model proposed for iGluRs (Rosenmund et al., 1998; Ayalon and Stern-Bach, 2001).

Glutamate does not only activate iGluRs but also inactivates them upon prolonged exposure to agonist, i.e. undergo desensitization (Jones and Westbrook, 1996; Hansen et al., 2007). As for the other iGluRs, kainate receptors desensitize, which degree depends on subunit composition (Heckmann et al., 1996; Paternain et al., 1998; Paternain et al., 2000). Hence, desensitization is ruled by the physicochemical properties of the bound ligand (Shelley and Cull-Candy, 2010). It is established that receptor desensitization occurs due to the degree of LBD closure upon agonist binding (Armstrong et al., 2006). Thus, reduction of LBD closure decreases desensitization (Weston et al., 2006). It has been proposed that desensitization of kainate receptors depends on subunit occupancy, since glutamate binding to GluK5 is sufficient for activation but not for desensitization, whereas binding to GluK2 fully activates and desensitizes the ion channel (Fisher and Mott, 2011; Reiner and Isacoff, 2014).

Activation and desensitization of kainate receptors depends on agonist concentration and binding efficacy. Hence, a given concentration would activate a certain % of kainate receptor population and then desensitize. Depending on unbinding kinetics, receptors exit desensitization to activate again. The equilibrium formed by receptors which enter and exit desensitization defines the steady-state activation of receptors (Paternain et al., 1998).

1.3.4. Allosteric modulators of kainate receptors.

Allosteric modulators for kainate receptors are able to tune receptor activity.

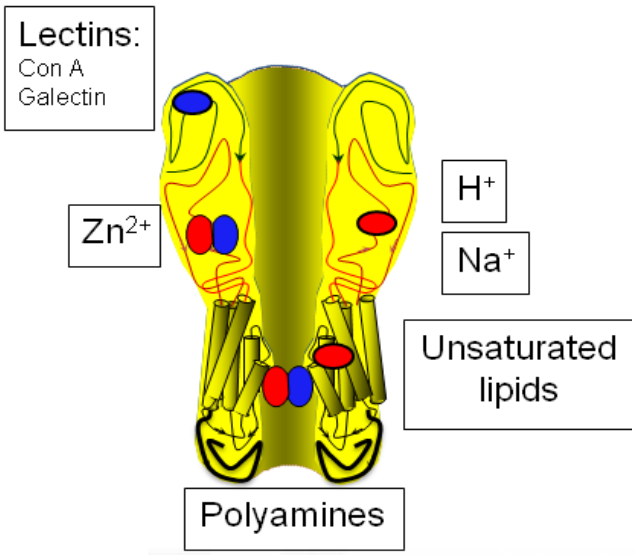

Figure 4. Allosteric modulation of kainate receptors. Different molecules regulate kainate receptor biophysical properties. Increment or decrement of channel gating is plotted by color code and located on specific binding site. Blue stands for potentiation and red for inhibition.

For instance, lectins derived from plant, especially Concanavalin A (ConA) (from *Canavalina ensiformis*), bind to N-glycosyl residues in ATD of kainate receptors (Everts et al., 1997). ConA irreversibly modulates kainate receptor activity by decreasing desensitization and increasing agonist affinity (Huettner et al., 1990; Paternain et al., 1998). It has been shown that ConA keeps receptor activated in the open state (Wong and Mayer, 1993) and thus increases current amplitude. The mechanism of action is not well known, but it might be a decrease in conformational motility of the receptors (Partin et al., 1993; Yue et al., 1995). There are other lectins, such as galectins, which are endogenous of mammalian CNS,that have been shown to modulate AMPA and kainate receptor desensitization (Copits et al., 2014), making it possible that this kind of modulation may happens under physiological conditions. However, there are studies showing that lectins fali to modulate synaptic responses (Wilding and Huettner, 1997).

The binding of different ions to LBD modulates kainate receptor function. Amongst divalent ions, Zn^{2+} was first reported to inhibit kainate receptors (Fukushima et al., 2003) acting as a non-competitive antagonist. Zn^{2+} inhibition of kainate receptors is subunit dependent, the high affinity subunits GluK4-5

being more sensitive than low affinity subunits GluK1-3 (Mott et al., 2008). Zn^{2+} has been proved to stabilize LBD dimer interface of GluK3 subunit (Veran et al., 2012) and the specific pocket for Zn^{2+} allocation within this domain found.

A unique feature of kainate receptors is their requirement on external monovalent ions, such as Na^+ and Cl^- (Paternain et al., 2003; Bowie, 2002. Na^+ is required for channel gating and its reduction greatly diminishes the amplitude of the response and accelerates desensitization (Paternain et al., 2003). Structural studies indicate that two Na^+ flank one Cl^- which increases dimer interface stabilization (Plested and Mayer, 2007). The range of modulation of this ions suggest that it might modulate native kainate receptors according to Na^+ demands, such as its drop after sustained neuronal activity (Herreras and Somjen, 1993) would diminish kainate receptor activity. On the other hand, protons inhibit all types of iGluRs (Christensen and Hida, 1990; Traynelis and Cull-Candy, 1990; Mott et al., 2003). Proton inhibition of kainate receptors does not modify desensitization. It occurs with an EC_{50} of pH 6.9, indicating that at physiological range there is a large pool of receptor affected. However, H^+ modulation of kainate receptors is subunit dependent, since heteromeric GluK2/5 receptors are less sensitive than homomeric ones, while GluK2/4 heteromeric receptors are potentiated by protons rather than inhibited (Mott et al., 2003).

Kainate receptors can also be modulated by polyamines and fatty acids. Polyamines (spermine and spermidine) potentiate R edited variants while inhibit Q variants (Bowie and Mayer, 1995). Potentiation might occur by relieving proton inhibition (Mott et al., 2003) or while inhibition should be ascribed to acceleration of closing rate and stabilization of closed state (Bowie et al., 1998). Fatty acid modulation also depends on Q/R edition, whereas insaturated fatty acids (such as arachidonic or linolenic acid) inhibit R edited receptors but not Q unedited subunits, been those receptors with at least one edited subunit less modulated than edited ones (Wilding et al., 1998, 2005, 2008).

1.3.5. Distribution of kainate receptors.

In situ hybridization has been revealed as the main technique to study kainate receptor localization since the lack of specific antibodies targeting kainate receptors.

Using different mRNA probes specific for kainate receptor subunits indicate a restricted expression of GluK1 subunit in hippocampus (CA1 area), Cortex, thalamus and hypothalamus (Wisden and Seeburg, 1993; Bahn et al., 1994). GluK1 subunit has also been detected in interneurons of CA1 and CA3 area at both *stratum oriens* and *stratum pyramidale* (Paternain et al., 2000). GluK2 and GluK3 subunit have been localized principally in the cortex and hippocampus, where in this last structure GluK3 showed a larger expression in dentate granule cells. (Wisden and Seeburg, 1993; Bahn et al., 1994). High affinity kainate receptor subunit showed different expression pattern between them. While GluK4 is weakly expressed at the cortex and restricted to granular cells in the dentate gyrus and CA3 area, GluK5 showed a large expression in many nuclei, including cortex, hippocampus and septum (Wisden and Seeburg, 1993; Bahn et al., 1994).

In the cerebellum, Purkinje cells express a combination of GluK1 and GluK4 subunits while granular cells contain GluK2 and GluK5 subunits. GluK3 is expressed at stellate and basket cells of the cerebellum (Wisden and Seeburg, 1993; Bahn et al., 1994).

At the sensory system, kainate receptors have a complex distribution. Kainate receptors have been found in dorsal root ganglion (DRG) neurons (Petralia et al., 1994) and nociceptive spinal afferents (Lucifora et al., 2006).

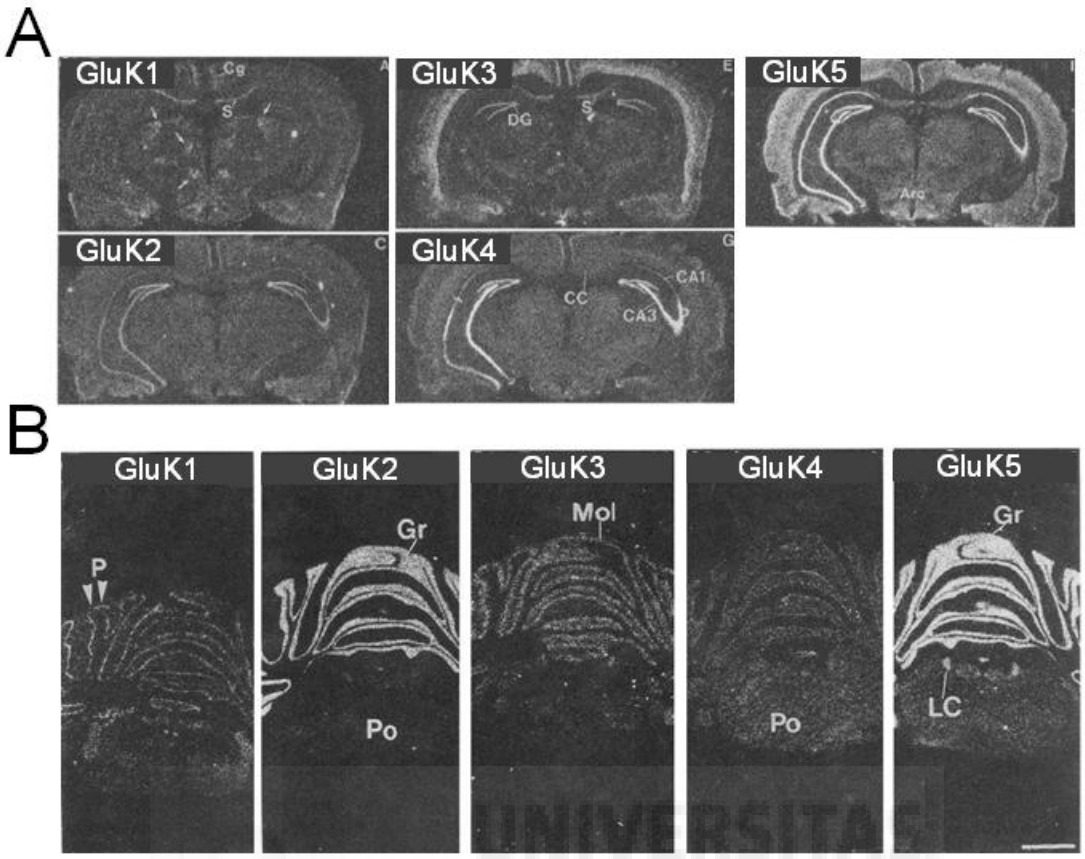

Figure 5. Distribution of kainate receptors in the brain. A, coronal slices showing the distribution of kainate receptor subunits mRNA in the adult mice brain . B, distribution of kainate receptor mRNA at cerebellar coronal slices of adult mice brain (adapted from Wisden and Seeburg, 1993).

1.3.6. Kainate receptors interacting proteins

As for other receptors and channels, interacting proteins have changed our view of kainate receptor function. Indeed, there is a mismatch between recombinant and native kainate receptors that is becoming to be explained by the presence of interacting proteins.

1.3.6.1. Auxiliary subunits on kainate receptors.

The main auxiliary subunits of kainate receptors seem to be Neto1 and Neto2. Since the discovery of Neto2 as an auxiliary subunit for GluK2 containing kainate receptors (Zhang et al., 2008), data indicating the relevance that Neto proteins played on kainate receptor physiology has exponentially increased.

Neto1 and Neto2 are single transmembrane proteins highly conserved. An external N-terminal sequence containing two CUB (complement C1r/C1s, Urchin EGF,Bmp1) domains account for binding the receptor complex (Tang et al., 2011), while the Ldlα (Light Density Lipoprotein α) domain is presumably responsible of functional alterations of channel gating (Zhang et al., 2009). The C-terminal part is less conserved along the family, including a PDZ binding domain in Neto1 that Neto2 lacks (Zhang et al., 2009; figure 6). Both proteins seem to be highly expressed in the cortex and hippocampus, while in the cerebellum Neto1 is not expressed (Michishita et al., 2004).

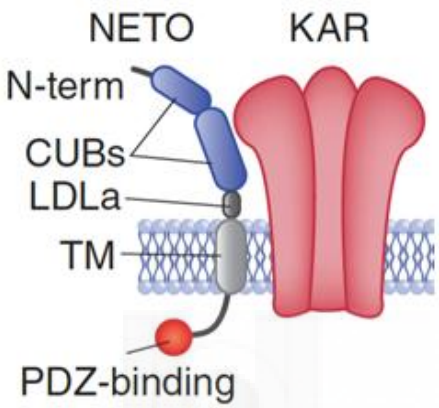

Figure 6. Neto proteins modulate gating and. trafficking of KARs. Neto1 and Neto2 are auxiliary subunits for kainate receptors, which contains two CUB domains and one LDLα domain at the N-terminal. These auxiliary subunits comprise one transmembrane domain and a cytosolic C-terminal, where only Neto1 has a PDZ-binding domain (From Lerma 2011).

Expression of Neto protein into neuronal cultures has demonstrated the ability of Neto1 to associate to GluK2 in kainate receptors (Straub et al., 2011). Neto2 has been suggested to interact GluK1 in synapses in hippocampal cultures (Copits et al., 2011) or GluK2 in the cerebellum (Tang et al., 2012). Genetic ablation of Neto1 in the hippocampus reduces the affinity for kainate of native receptors as well as accelerates deactivation time of kainate receptors EPSCs, rendering kainate receptors mediated EPSCs with similar kinetics to AMPA receptors EPSC (Straub et al., 2011b). The elimination of both Neto1 and Neto2 in mice does not affect any further mossy-fiber to CA3 responses compared to Neto1 null mice (Tang et al., 2011). Hence Neto1 seems to be the partner for synaptic kainate receptors found in CA3 pyramidal neurons. In the cerebellum, where Neto1 is not expressed, Neto2 seems to play a role regulating the amount of receptors at the plasma membrane by linking Neto2 to GRIP1 and thus stabilizing GluK2 containing kainate receptors at the synapses (Tang et al., 2012).

Neto proteins have also been found to partner with other proteins. Neto1 has been described to interact with NMDA receptors (Ng et al., 2008), where it seems to regulate the balanced between GluN2A and GluN2B subunits included in functional NMDA receptors (Wyeth et al., 2014). However, these results are controversial since other groups have not been able to replicate these experimental data (Straub et al., 2011b). In this way, it has been proposed that Neto1 and NMDA receptor interaction is not direct, but through the cytoplasmatic domain of APP protein (Cousins et al., 2013). On the other hand, Neto2 interacts with a neuron-specific K^+-Cl^- cotransporter (KCC2) to maintain the normal abundance of this protein (Ivakine et al., 2013).

1.3.6.2. Transient interacting protein with kainate receptors.

The number of proteins being identified to interact with kainate receptors has increased in the last decade. A large number of proteins regulate kainate receptor trafficking to the cell surface interacting through the C-terminal domain. The proteins 14.3.3 and 4.1 interact with GluK1 and GluK2, increasing forward trafficking (Coussen and Mulle; Vivithanaporn et al., 2006; Copits and Swanson, 2013). PICK1 (protein interacting C kinase) and GRIP1 (glutamate receptor interacting protein-1) co-operate to maintain kainate receptor mediated synaptic responses by stabilizing receptors at the synapses (Hirbec et al., 2003) by the interaction with GluK1 and GluK2 subunits. GluK2 containing receptors may also be promoted to the membrane by the action of cadherin (Coussen and Mulle, 2005) or profillin II (Mondin et al., 2010). On the other hand, heteromeric receptor containing GluK5 subunits may interact with COPI (Vivithanaporn et al., 2006), which diminishes the interaction with 14.3.3 protein, thus avoiding forward trafficking. SNAP-25 interaction with GluK5 containing kainate receptors has been shown to be critical for activity dependent endocytosis of kainate receptors at CA3 pyramidal neurons (Selak et al., 2009). Indeed, internalization of kainate receptors at mossy fiber-CA3 synapse is initiated by PKC activity (Selak et al., 2009) and may also require SUMOylation of GluK2 subunits (Chamberlain et al., 2012). All these evidences point out the importance of

interacting proteins in the control of kainate receptors trafficking to the cellular surface, likely having an impact on synaptic transmission and plasticity.

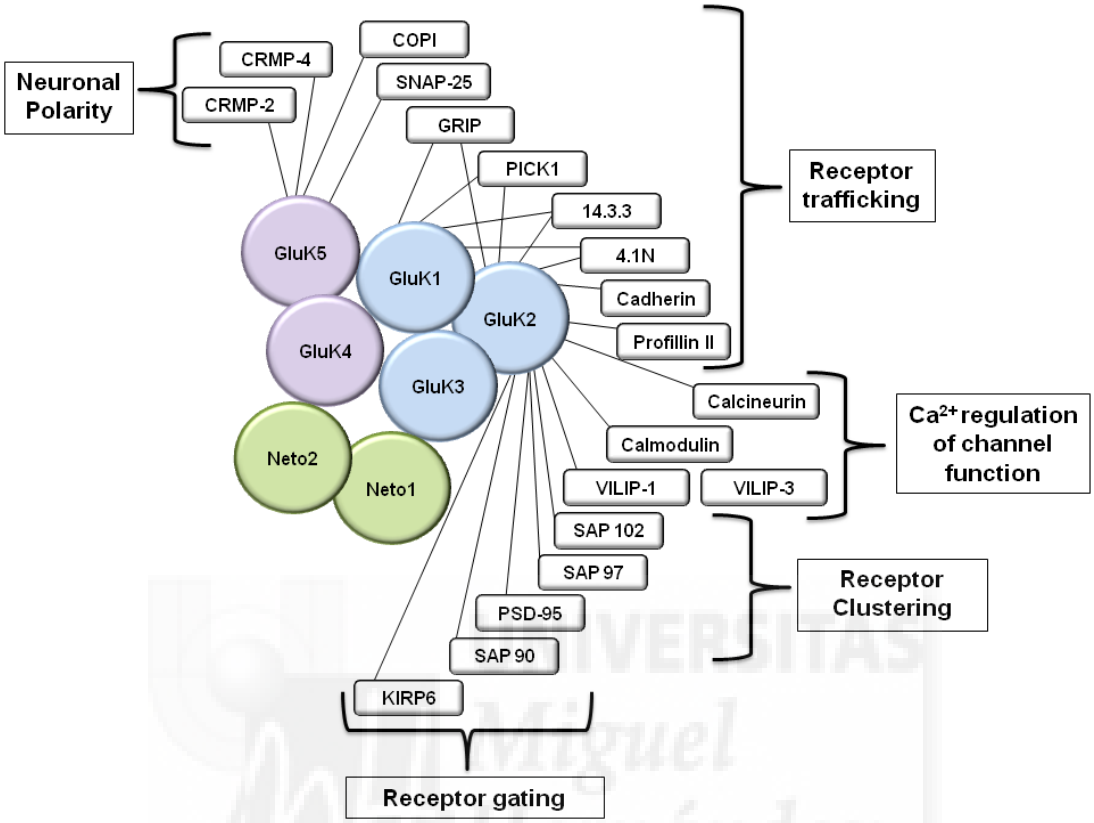

Figure 7. Kainate receptor interactome. Kainate receptor subunits and accessory proteins are shown in circles, where transient interacting proteins are linked with specific binding subunits. Interacting proteins are grouped depending on the role played on kainate receptor physiology.

Beyond the trafficking modulation, kainate receptors interact with a subset of proteins, which cluster receptors in specific plasma membrane domains. Proteins containing one or more PDZ motifs stabilize kainate receptors at the postsynaptic density. Proteins belonging to MAGUK (membrane associated guanylate kinase) family fulfilling this role are PSD-95 (postsynaptic density-95), SAP-90,-97 and -102 (synapse associated protein-90, -97 and -102; Garcia et al., 1998). In addition to cluster kainate receptors at the synapse, interaction with PSD-95 and SAP-90 seems to reduce kainate receptor desensitization (Garcia et al., 1998). Interestingly, a BTB/Kelch family protein (KIRP6) decreases current amplitude and desensitization without altering surface expression of GluK2 kainate receptors (Laezza et al., 2002).

Calmodulin, calcineurin, Villip-1 and -3 have been shown to interact with kainate receptors and modulate receptor function depending on intracellular Ca^{2+} concentration (Coussen and Mulle, 2005). Furthermore, several activity dependent plasticity events require coordination of calcium sensors and interacting proteins. As mentioned before, SNAP-25 mediated endocytosis of kainate receptors involve the interaction with PICK1 (Selak et al., 2009), which has been shown to act as a calcium sensor in the hippocampus (Jo et al., 2008). One more example of how calcium sensors trigger kainate receptors plasticity is provided by CaMKII (Calcium calmodulin kinase II), which phosphorylates GluK5 receptors decreasing interaction with PDS-95 and decreasing kainate receptors-mediated synaptic responses (Carta et al., 2012).

Recently, the role played by kainate receptors in neuronal polarity (Tashiro et al., 2003) has been clarify by the identification of CRMP2 and CRMP4 (collapsin response mediator protein -2 and -4). In detail, the non-canonical signaling of kainate receptors triggers CRMP2 phosphorylation to modulate cytoskeleton dynamics, thus controlling neurite outgrowth (Marques et al., 2013).

In addition, other post-translational modifications of kainate receptors might also impact receptor function. These issues are depicted in box 4.

Box 4. Post-translational modifications on kainate receptor C-terminal domain.

The long C-terminal of GluK1-2b may be phosphorylated by PKC at serines 879 and 885, triggering receptor endocytosis (Rivera et al., 2008). GluK2 subunit may also be phosphorylated by PKC at serine 846 and 868, producing receptor internalization (Nasu-Nishimura et al., 2010) or PKA activity at serines 846, 856, 868 and threonines 894 and 905. In this case PKA activity is linked to potentiation of GluK2 responses (Kornreich et al., 2007). GluK5 is also bidirectionally regulated by phosphorylation, where PKC acts on serines 833, 836 and 840 potentiating GluK2/5 heteromeric receptors (Rojas et al., 2012. CaMKII activity, phosphorylating serines 859, 892 and threonine 976, uncouples GluK5 from PSD-95, increasing receptor motility, moving them away from the synaptic zone and thus reducing kainate mediated synaptic responses (Carta et al., 2013).

Other types of modifications include palmitoylation and SUMOylation, which have been found in the C-terminal domain of GluK2 subunit. Palmitoyation of cystein 858 and 871 seems to have a negative effect on phosphorylation at nearby serines (positions 856 and 868; Pickering et al., 1995). SUMOylation has been found at lysine 886 and regulates GluK2 internalization which engages modulation on synaptic transmission and plasticity (Martin et al., 2007; Chamberlain et al., 2012).

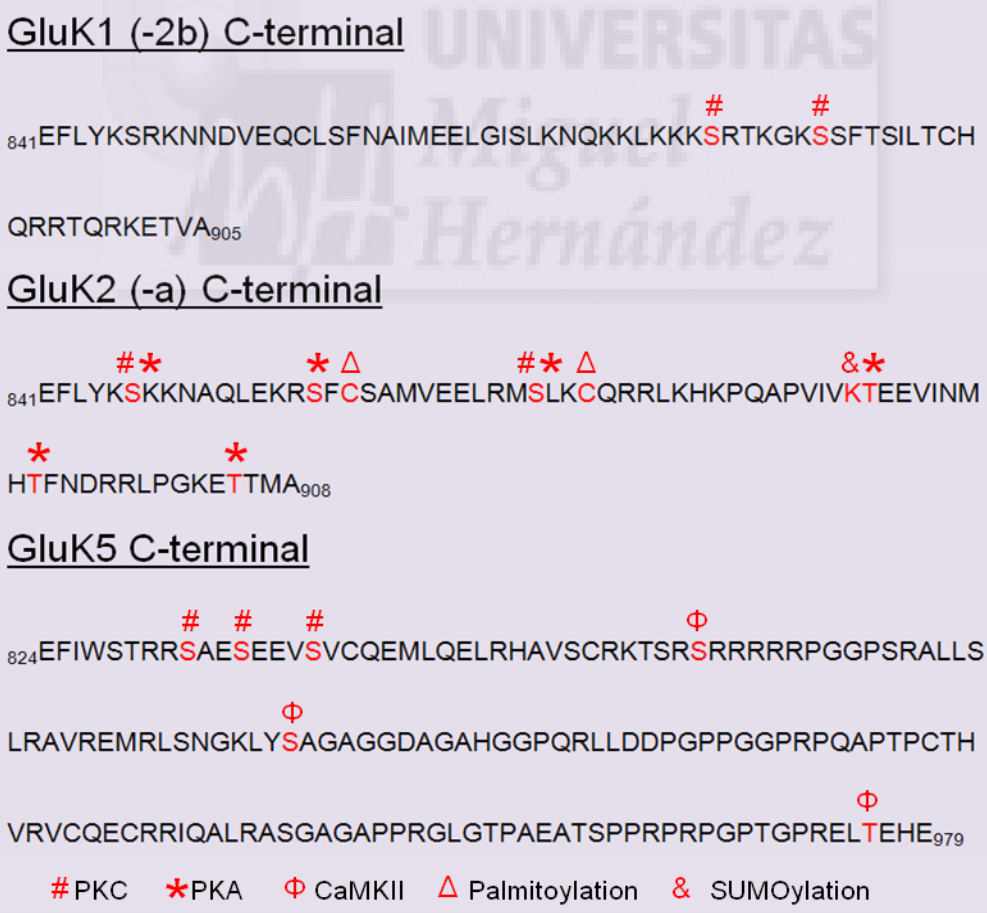

Figure 8. Among different subunits composing kainate receptors, only GluK1, GluK2 and GluK5 had been identified as target for post-translational modifications. The aminoacids modified are shown in red and the enzyme which modifies them is indicated by a symbol above.

1.3.7. Physiological roles of kainate receptors.

1.3.7.1. Role of kainate receptors in development.

Kainate receptors are highly expressed during development (Bahn et al., 1994). Kainate receptors regulate growth cone motility at hippocampal cultures in a bidirectional manner, increasing growth cone motility at low receptor stimulation and decreasing it upon higher induced activity (Tashiro et al., 2003; Ibarretxe et al., 2007). Moreover, development of DRG neurons is also controlled by kainate receptor action. Low kainate concentration activates G_o protein (refer to box 5), which enhances neurites outgrowth. On the other hand, high kainate concentration causes inward depolarization and decreases neurites elongation (Marques et al., 2013). These might influence synaptic connectivity, which has been proven at hippocampal development. Indeed, while maturation of mossy fibers contact with CA3 pyramidal cells normally occur at postnatal day 6-9, GluK2 Knock-out mice show a delay (Lanore et al., 2012).

Kainate receptors seem to modulate network activity during hippocampal development. At CA1 pyramidal cells kainate receptors keep the probability of release low (Lauri et al., 2006). This is produced by tonic activation of GluK1 containing kainate receptors. At mossy fiber synapses, kainate receptors facilitates glutamate release onto CA3 pyramidal cells (Lauri et al., 2005), which allows network bursting. One more case shows I_{AHP} inhibition by tonic activation of kainate receptors on CA3 interneurons at the first postnatal week (Segerstrale et al., 2012).

1.3.7.2. Role of kainate receptors as mediators of synaptic transmission.

Excitatory post-synaptic currents mediated by kainate receptors ($EPSC_{KAR}$) were demonstrated only after the specific AMPA receptor antagonism by GYKI53655 was ideintified (Paternain et al., 1995). From the beginning it was clear that unlike AMPA and NMDA receptors, kainate receptors were not localized in all synapses (Castillo et al., 1997; Vignes et al., 1997). Excitatory inputs of mossy fiber onto CA3 pyramidal cells elicit $EPSC_{KAR}$ with

small amplitude and slow onset and decay (Castillo et al., 1997; Vignes et al., 1997). Synapses in which kainate receptor responses have been found include: Schaeffer collaterals on CA1 interneurons (Cossart et al., 1998; Frerking et al., 1998), in the basolateral amigdala (Li and Rogawski, 1998), thalamocortical connections (Kidd and Isaac, 1999), in the dorsal horn neurons of the spinal cord (Li et al., 1999) and parallel fibers and Golgi cells in the cerebellum (Bureu et al., 2000). High affinity kainate receptor subunits seem to be required for ionotropic activity, since the removal of GluK4-5 abolish kainate receptor mediated responses in CA3 pyramidal neurons (Fernandes et al., 2009)., The characteristic slow onset and decay kinetics of kainate receptor synaptic responses provide kainate receptor with integrative capacities of synaptic information (Frerking and Ohliger-Frerking, 2002; Straub et al., 2011).

Box 5. Activation of trimeric G proteins through kainate receptors.

Involvement of G protein activation on kainate receptor action was first seen at pyramidal CA1 inhibitory synapses (Rodriguez-Moreno and Lerma 1998). Using DRG neuron cultures it was possible to dissect G protein activation, which was showed to require GluK1 subunits and its action was ion flux independent (Rozas et al., 2003). This signaling mechanism differs from channel activity and was described as a non-canonical signaling of kainate receptors.

Molecular evidences showing a direct interaction between kainate receptor and G protein has not been well substantiated. However, different subunits have been postulated to be required for G proteins activation: GluK1 (Rozas et al., 2003; Lauri et al., 2005; Rivera et al., 2007; Caiati et al., 2010; Segerstrale et al., 2010), GluK2 (Melyan et al., 2002; Fisahn et al., 2005) and GluK5 (Ruiz et al., 2005). In all cases, the non-canonical signaling seems to be pertusin toxin (PTx) sensitive (Rodriguez-Moreno et al., 1997; Rozas et al., 2003; Lauri et al., 2005; Rivera et al., 2007; Caiati et al., 2010; Segerstrale et al., 2010), which indicates involvement of $G_{o/i}$ protein. Hence, signaling cascade comprises activation of phosphor-lipase C (PLC), opening of intracellular Ca^{2+} stores by IP3 formation and activation of protein kinase C (PKC) (Rodriguez-Moreno et al., 1997; Rozas et al., 2003; Rivera et al., 2007). Although some data indicate that kainate receptor may also activate protein kinase A (PKA) (Negrete-Diaz et al., 2006; Gelsomino et al., 2013).

1.3.7.3. Role of kainate receptors modulating network excitability.

At the postsynaptic level, kainate receptors are able to inhibit voltage-dependent K^+ channels responsible of the slow afterhyperpolarization current (I_{AHP}; Melyan et al., 2002). The relieve of slow and medium I_{AHP} inhibition causes an increase of firing frequency at postsynaptic cell, which affect network excitability (Ruiz et al., 2005; Chamberlain et al., 2013).

The modulation of I_{AHP} requires activation of a G_o protein and it has been shown to occur upon synaptic or pharmacological activation of kainate receptors (Melyan et al., 2002, 2004; Ruiz et al., 2005; Chamberlain et al., 2013). Interestingly, I_{AHP} inhibition through kainate receptors is independent of channel gating, since activation of kainate receptors at postsynaptic CA1 pyramidal neurons display I_{AHP} inhibition (Melyan et al., 2004), although no synaptic kainate receptors have been found at this synapse (Castillo et al., 1997; Vignes et al., 1997). In addition to CA1 neurons, mossy fiber to CA3 pyramidal neuron synapses also exhibit I_{AHP} inhibition (Ruiz et al., 2005; Fisahn et al., 2005). Most of the studies implicate GluK2 subunit in the inhibition of I_{AHP} (Melyan et al., 2002; Ruiz et al., 2005; Fisahn et al., 2005). Although GluK5 subunit was shown to play a role in his signalling (Ruiz et al., 2005), these results were recently challenged since the double GluK4-5 knock-out mice displayed normal modulation of I_{AHP} (Fernandes et al., 2009).

1.3.7.4. Role of kainate receptors in the modulation of synaptic transmission.

At the presynaptic level, kainate receptors have been shown to modulate neurotransmitter release at both inhibitory and excitatory synapses.

Modulation of inhibitory synapses.

Pharmacological activation of kainate receptors inhibits GABA release in the hippocampus (Rodriguez-Moreno et al., 1997, 1998; Vignes et al., 1998). Kainate receptors exert this action through the non-canonical signaling involving the GluK1 subunit, which activates a PTx-sensitive G protein, PLC and PKC

(Rodriguez-Moreno et al., 1997, 1998; Vignes et al., 1998). GABA release inhibition has also been observed in other structures, such as neocortex (Ali et al., 2001), amygdala (Braga et al., 2004), striatum (Jin and Smith, 2007) and hypothalamic supraoptic nucleus (Bonfardini et al., 2010). However, some controversy has been shown at hippocampus, where inhibition of GABA release has been ascribed (at least to some extent) to endocannabinois signaling through CB1 receptors (Lourenço et al., 2010, 2011).

Kainate receptors may also facilitate GABA release from hippocampal CA1 interneurons (Mulle et al., 2000; Cossart et al., 2001), hypothalamus (Liu et al., 1999), neocortex (Mathew et al., 2008) and hypothalamic supraoptic nucleus (Bonfardini et al., 2010). In this last study, the authors described the mechanism of action by which kainate receptors depress or facilitates GABA release. While depression of GABA release requires PLC activity and thus it could be ascribed to the non-canonical signaling, facilitation on GABA release is blocked by philantotoxin, a blocker of Ca^{2+}-permeable channels, involving the ionotropic activity of kainate receptors (Bonfardini et al., 2010).

Modulation of excitatory synapses.

A change from low to high frequency stimulation increases to a great extent mossy fiber to CA3 neurotransmission, a process where presynaptic kainate receptors have been implicated (Schmitz et al., 2001; Contractor et al., 2001). This effect is blocked by either kainate receptor antagonists (Schmitz et al., 2001) or philantotoxin (blocker of Ca^{2+}-permeable receptors; Lauri et al., 2003), implicating a Ca^{2+} raise at the presynaptic sites, likely induced by ionotropic kainate receptors, although Ca^{2+} increases from intracellular stores may also be necessary (Lauri et al., 2003; Scott et al., 2008). However, some controversy came from the fact that facilitation of glutamate release might reflect an over activation of recurrent CA3 network (Kwon and Castillo, 2008). Nevertheless, pharmacological blockage of postsynaptic kainate receptors (Pinheiro et al., 2013) or GluK4/5 ablation (which abolishes synaptic kainate receptor component) does not alter facilitation of glutamate release from mossy fiber terminals, indicating that at least in part, kainate receptors are responsible of presynaptic facilitation.

Facilitation of glutamate release from mossy fiber terminal has been obtained applying low concentration of kainate (Schmitz et al., 2000; Kamiya and Ozawa, 2000). On the other hand, application of high concentration of kainate depresses synaptic transmission (Schmitz et a., 2000). Glutamate release inhibition from mossy fiber terminal involves the non-cannonical pathway, since the effect is blocked by G protein inhibitors (Frerking et al., 2001). Similarly, tonic activation of kainate receptors in the globus pallidus depresses glutamate release, which involves PKC activation (Jin and Smith, 2007). At the Schaffer collateral to CA1 synapse, the inhibition of glutamate release seems to be indenpent of protein kinases but on the action of G protein βγ-subunits , which inhibit presynaptic Ca^{2+} channels (De Waard et al., 1997).

1.3.8. Kainate receptors in CNS disorders.

Genetic manipulation of animal models and genetic analysis of human diseases have indicated that kainate receptors might be involved in several neurological and psychiatric diseases. Thus, pharmacological intervention at kainate receptors has been postulated as a potential possibility for certain CNS diseases.

1.3.8.1. Neurological diseases.

Kainate injections are a well stabilized model for epileptogenic activity, which reproduces several features of human temporal lobe epilepsy (TLE). Kainate applications have been shown to reduce GABA release (Clarke et al., 1997; Rodriguez-Moreno et al., 1997), avoiding recurrent inhibition onto pyramidal cells in the hippocampus and triggering epileptogenic activity (Rodriguez-Moreno et al., 1997). Interestingly, removal of *Grik2* gene reduces kainate sensitivity to produce seizures (Mulle et al., 1998). Moreover, GluK4 and GluK5 subunits have also been implicated in epilepsy, since mRNA for these subunits have been found altered in epileptic patients (Grigorento et al., 1997; Mathern et al., 1998). Pharmacological attempts to block epileptogenic activity include two GluK1 receptor antagonists, LY37770 and LY382884, which have been shown to prevent electrical or pilocarpine-induced seizures in vivo (Smolders et

al., 2002). However, a GluK1 receptor agonist (ATPA) has been reported to have an antiepileptic effect, supposedly by enhancing interneuron activity (Khalilov et al., 2002).

As pointed out before, kainate receptors are strongly expressed in DRG and dorsal horn neurons, where kainate was demonstrated to depolarize primary afferents (Agrawal and Evans, 1986). Moreover, GluK1/5 heteromeric kainate receptors seem to be the only iGluRs in DRG neurons (Bahn et al., 1994; Rozas et al., 2003). Although pain perception might not be considered a disease, persistent or neurophatic pain is associated with several disorders. In a primate model of neuropathic pain, a GluK1 antagonist (LY382884) induced a reduction of nociceptive responses of spinothalamic neurons (Palecek et al., 2004). There is a study in humans using LY293558, a mixed kainate/AMPA receptor antagonist, to diminish dental pain (Gilron et al., 2000), presumably through GluK1 antagonism.

Grik2 gene has also been linked to mental retardation, since mutations of this gene were identified from a genetic familiar study in which several members suffered from mental retardation (Motazacker et al., 2007). This idea has been recently supported by the observation of a delay in the functional maturation of mossy fiber pathway in the GluK2 knock-out mice (Lanore et al., 2012).

Neurophaty	Epilepsy	GluK1	GluK2	—	GluK4	GluK5
	Neurophatic pain	GluK1	GluK2	—	—	—
	Mental retardation	—	GluK2	—	—	—
Psychiatric disorders	Autism	—	GluK2	—	—	—
	Schizophrenia	GluK1	GluK2	GluK3	GluK4	—
	Bipolar	GluK1	GluK2	GluK3	GluK4	—
	Depression	—	—	GluK3	GluK4	—

Figure 9. Summary of disorders where different kainate receptor subunits have been implicated (adapted from Bowie et al., 2008).

1.3.8.2. Psychiatric diseases.

There is an emergence of data indicating a link between kainate receptors and psychiatric conditions. However, the fact that multiple psychiatric disorders share several clinical manifestations complicates the diagnosis. Indeed, a polygenic basis for psychiatric diseases is commonly found, such as, schizophrenia, bipolar disorder or depression (Ripke et al., 2001; Sklar et al., 2011). This is becoming true for kainate receptors, which different genes have been implicated in schizophrenia and bipolar disorders. In detail, it has been found a reduction of *Grik1* and *Grik2* in limbic cortices of schizophrenic and bipolar patients (Beneyto et al., 2007). Single nucleotide polymorphism (SNP) accounts for alteration on *Grik3* gene to produce susceptibility for depressive disorders (Schiffer and Heinemann, 2007) and schizophrenia (Begni et al., 2002). It has been proposed that anxiety and depression treatment are associated with *Grik1* and *Grik4* genes (Paddock et al., 2007), implicating these genes in mood disorders. Certainly, a 14 pb deletion/insertion in the 3' UTR region of Grik4 gene has been associated with bipolar disorder, whereas 14 bp deletion increases mRNA and protein levels of GluK4 in hippocampus and cortex, which confers protection against bipolar disorder (Pickard et al., 2008; Knight et al., 2012).

GriK2 gene has been associated with autism (Jamain et al., 2002; Shuang et al., 2004). However, these findings remain under debate, since other studies failed to find a positive association between GriK2 and autism (Dutta et al., 2007). It is unlikely that these psychiatric diseases originate from a kainate receptor dysfunction rather than a general alteration of glutamate neurotransmission (Wilson et al., 2006; Pickard et al., 2006).

II. Aim

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Kainate receptors are widely distributed in the brain. However their physiological role is poorly understood. Moreover, kainate receptors have been revealed to be important in the modulation of neuronal activity during development and in the adult brain. The function of kainate receptors depends in their location. Presynaptic and postsynaptic receptors have been described and they can be also extrasynaptic sites. Similarly, their properties depend on their interacting proteins at these subcellular localizations.

The main objective of this work was to understand how kainate receptors interacting proteins, especially those interacting with GluK5 subunits, shape receptor function at the synapse by imposing new properties. Among other subunits of kainate receptors, GluK5 contains a much longer C-terminus which might explain their differential trafficking, targeting and selective interaction with other proteins.

Work carried out in the lab, established the interaction between NeCaB1 and GluK5 protein by means of two-hybrid screening. One of our goals was to understand the role played by NeCaB1 on GluK5 containing kainate receptors. For that purpose we studied:

1. – The properties of the interaction between NeCaB1 and GluK5 containing kainate receptors.

2. – The modulation of NeCaB1 and GluK5 interaction by Calcium (Ca^{2+}).

3. – The functional consequences of NeCaB1 binding to GluK5 containing kainate receptors.

Neto proteins are auxiliary subunits of kainate receptors that had been shown to interact with native kainate receptor and modulate receptor gating. We wanted to further determine the impact of Neto1 and Neto2 on kainate receptors function and for that reason we studied:

4. – The functional interaction between Neto proteins and kainate receptors. In particular we addressed the study of their impact on properties such as:

i) Current amplitude.

ii) Desensitization kinetics

iii) Agonist affinity.

v) Sodium dependence channel gating.

5. – The trafficking of kainate receptors to the cell surface.

We found that hippocampal cultures lack synaptic kainate receptors. We decided to use this model to:

6. Determine which subunit of kainate receptors or interacting proteins were key for the synaptic targeting of these receptors.

III. Material and methods

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III. Material and methods

1. Biological models.

Cell lines. Human Hembrionary Kidney (HEK293) cell line was chosen due to its ability to express exogenously introduced plasmids in a reproducible way. HEK293 cells were used for molecular biology and for electrophysiological experiments. HEK cells were originally bought from Sigma A frozen stock was kept in liquid nitrogen (-200 ºC) and defrosted when necessary to refresh old HEK cells. These cells were grown in DMEN (Invitrogen) with 10% fetal calf serum (FCS, Invitrogen) and 1% Penicillin/Streptamicin (P/S, Sigma). HEK cells were kept in an incubator at 37ºC under 95%O_2/5%CO_2 atmosphere for 6-10 divisions and then refreshed with new cells from frozen stock.

Hippocampal primary cell cultures. Cultures were made as described previously (Banker and Goslin, 1998). A graphic cartoon can be seen in figure 10. Prior to the culture, astrocytes were placed in a cell culture flask (M24, Invitrogen) and allowed to grow in DMEN plus 10% FCS and 1% P/S, until a monolayer was obtained. Three days before the culturing neurons, DMEN medium was replaced by Neurobasal (Invitrogen) plus 1% Glutamax, 2% NS21 medium supplement and 1% P/S, (i.e. complete medium) where hippocampal neurons were grown.

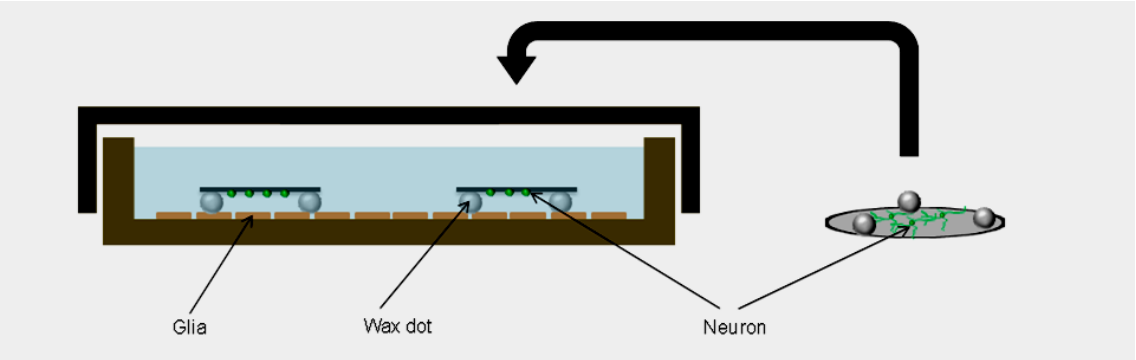

Figure 10. Cartoon illustrating Banker culture technique. On the right, neurons are seeded first on coverslips with wax dots and 4-5 hours later transferred to glia containing well, on the left.

Hippocampal cells were mechanically dissociated from P0 mice by treatment with trypsin (Type II-S, Sigma; 0.12 mgr/ml, 30 min at 37ºC). Then, cells were placed on coverslips with DMEN plus 10% FCS and 1% P/S that had been previously treated with laminin (4 mg/ml; Sigma) and poly-D-lysine (5 mg/ml; Sigma) during 8 hours in successive steps. Five hours after seeding, coverslips were moved to M24 flasks containing an astrocytic monolayer growing in complete medium and placed in that way where astrocytes and hippocampal neuron faced each other. Flasks were incubated at 37ºC under 95%/5% O_2/CO_2 atmosphere until use, 17-20 days later. The medium was partially (40%) replaced at days 3, 7, 11 and 15.

2. $_C$DNA constructs.

All plasmids were transformed in bacteria (*Escherichia Coli* DH5α) and purified $_C$DNA products stored at -20 ºC dissolved in TE buffer. In table 1, plasmids used are shown with indication of the vector in which they were cloned and the procedures in which they were used.

3. Transfections.

Introduction of exogenous $_C$DNA into neurons was performed by Lipofectamine 2000 (Thermo). Growing cells attached to a coverslip or plate were first washed with PBS and changed to a medium containing DMEN without FCS or P/S. To start with the transfection, $_C$DNA was mixed with Optimen reagent (Invitrogen) for 20 minutes and then added to the cells in DMEN. After 4 hours, cells were washed and fresh DMEN in FCS and P/S added. Twenty-four to 48 hours later, transfected cells showed a regular expression of gene of interest.

Protein expressed	Vector	Experimental use
GFP	pCDNA 3	El, PD, TA
GluK1-2a	pCDNA 3	BiFC, El, IP, TA
GluK2	pRK5	El, IP
GluK3	pCDNA 3	El, IP
GluK5-VNT	pCDNA 3	BiFC
GluK5-$\Delta C^{825-959}$-VNT	pCDNA 3	BiFC
GluK5-ΔC^{859}-VNT	pCDNA 3	BiFC
GluK5-ΔC^{891}-VNT	pCDNA 3	BiFC
GluK5-ΔC^{941}-VNT	pCDNA 3	BiFC
GluK5-ΔC-VNT	pCDNA 3	BiFC
GST	GPEX-6.1	PD
GST-$GluK5_{C\text{-terminal}}$	GPEX-6.1	PD
Myc-GluK1-2b	pCDNA 3	IP, TA
Myc-GluK5	pCDNA 3	EL, IP, TA
NeCaB1	pCDNA 3	IP
NeCaB1-GFP	pCDNA 3	El, PD, TA
NeCaB1-VCT	pCDNA 3	BiFC
Neto1-GFP	pCDNA 3	El, TA
Neto2-GFP	pCDNA 3	EL, TA

Table 1. cDNA used to express desired proteins. Protein expressed, cDNA vector and the experiment in which was used is shown. BIFC, Bioflurescence complementation; El, electrophysiology; IP, immunoprecipitation; PD, Pull-down; TA, Trafficking Assay.

4. Electroporations.

Electroporation was used to introduce genes of interest into HEK cells to performed electrophysiological experiments. $_C$DNA was electroporated at

different ratios (8 µg of cDNA total) in a suspension of HEK293 cells (Gene pulser; Bio-Rad, Hercules, CA). Afterwards, cells were seeded in Petri dishes coated with poly-D-lysine (25 mgr/ml) and cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin, maintained in a humidified incubator at 37°C (CO_2 5%). Electrophysiological experiments were carried out the next day

5. Protein analysis.

Protein extraction. Brain homogenates were prepared from C57 strain 20-21 days old mice. First, mice were anesthesiated with Isofluorane (Esteve) and sacrificed by decapitation. Then, the brain was extracted and washed in cold SHEEP solution, removing the cerebellum and brainstem. Remaining brain tissue was introduced in a glass homogenizer with 1 ml of MKM buffer with inhibitors of proteases. Homogenized solution was incubated for 1 hour at 4 ºC and then centrifuged at 1000 x g at 4 ºC. The supernatant was isolated and protein concentration measured by the Bradford method by using a spectrophotometer (Nanodrop, ND-100). Protein samples were used immediately or frozen at -20 ºC for later use.

In the case of cell cultures, cells were rinsed with PBS and detached with lysis buffer plus proteases inhibitors. Then, similar to brain homogenates, the samples were incubated in lysis buffer, centrifuged and measured for quantification.

SDS-PAGE (Electrophoresis). Protein samples already boiled with LSB (which contains SDS), were loaded into polyacrylamide gel lines and run at 35 mA per gel using a power supply (BioRad) during 3 hours at 4ºC. As a marker for protein migration, a visible protein ladder (Precision Plus Protein Dual Color Standard, Biorad) was used.

Protein transfer. Proteins separated in the polyacrilamide gel were transferred into a nitrocellulose membrane (Protran BA85, GE Healthcare) in a wet electroblotting transfer tank (Hoefer TE 22 Mini Tank Transfer Unit, Amersham)

in TB buffer. Protein transference was carried out using 350 mA during 4 hours at 4 ºC.

Antibody tagging. Ponceau S (Sigma) was used to visualize protein bands in nitrocellulose membrane and evaluate transfer efficacy. Then, nitrocellulose membranes were washed in water for 5 minutes and blocked with 5% milk powder dissolved in TBS-T at run temperature for 1 hour. Specific proteins were tagged to corresponding primary antibody (see table 2 for reference information) dissolved in 5% milk powder in TBS-T and incubated with nitrocellulose membrane overnight at 4 ºC. Then, membranes were washed three times in TBS-T buffer for 5 minutes and incubated with specific secondary antibodies (modified with peroxidase enzyme, HRP) for 60-90 minutes at room temperature. Afterwards, membranes were washed again three times in TBS-T for 5 minutes and treated with the peroxidase substrate ECL (ECL Plus, Pierce), which reacted with the modified secondary antibody to emit chemoluminiscense.

Primary antibodies					
Antibodies	Supplier	Host	Type	[Antibody] mg/ml	Dilution
α-Bassoon	Synaptic Systems	Mouse	Monoclonal IgG	n.d.	1:1000
α-GAPDH	Abcam	Rabbit	Polyclonal IgG	n.d.	1:2000
α-GFP	Santa Cruz	Mouse	Monoclonal IgG	0.2 mg/ml	1:500
α-GluK2/3	Millipore	Rabbit	Monoclonal IgG	n.d.	1:1000
α-GluK5	Millipore	Rabbit	Polyclonal IgG	0.4 mg/ml	1:500
α-IgG	Molecular probes	Mouse	Monoclonal IgG	8 mg/ml	1:10000
α-Myc	Santa Cruz	Mouse	Monoclonal IgG	0.4 mg/ml	1:1000
α-NeCaB1	Abnova	Mouse	Polyclonal IgG	0.5 mg/ml	1:500
α-NeCaB1	Dr. T. Südholf	Rabbit	Polyclonal IgG	n.d.	1:2000
α-PSD95	Neurolab	Mouse	Monoclonal IgG	1 mg/ml	1:5000
α-SNAP25	millipore	Goat	Polyclonal IgG	0.4 mg/ml	1:2000

Secondary antibodies					
Antibodies	Supplier	Host	Type	[Antibody] mg/ml	Dilution
α-Goat HRP	Molecular probes	Donkey	Monoclonal IgG	1 mg/ml	1:1000
α-Mouse Alexa 555	Molecular probes	Goat	Monoclonal IgG	1 mg/ml	1:1000
α-Mouse Alexa 647	Molecular probes	Goat	Monoclonal IgG	1 mg/ml	1:1000
α-Mouse HRP	Molecular probes	Goat	Monoclonal IgG	1 mg/ml	1:8000
α-Rabbit Alexa 488	Molecular probes	Donkey	Monoclonal IgG	1 mg/ml	1:1000
α-Rabbit Alexa 555	Molecular probes	Donkey	Monoclonal IgG	1 mg/ml	1:1000
α-Rabbit HRP	Molecular probes	Goat	Monoclonal IgG	1 mg/ml	1:15000

Tabla 2. Primary and secondary antibodies used during this thesis.

Protein quantification and data analysis. Western blot quantification was made with the Phosphoimager (Science Lab, 2001) using Image Gauge software. Data was extracted in a ".tif" file and protein bands were quantified with the Quantity One software (BioRad, 2003).

6. Pull Down experiments.

GST-fusion purification. The plasmid pGEX-6P (Amershan-Pharmacia) was used to express GST protein. The C-terminal region of GluK5 subunit was cloned into the C-terminal of the GST This vector was introduced in E. coli (BL-21 strain, for a better yield in protein expression), where pGEX-6P encoding protein synthesis (GST-fusion protein) was induced by adding Isopropil-β-D-Thiogalactoside (IPTG) after 12-16 hours.

Bacteria cultures expressing pGEX-6P were grown in LB at 37 ºC until 80% of maximal confluence was obtained and then GST-fusion protein was induced with IPTG. At this point temperature was changed to 25 ºC for better protein production. After 12-15 hours, the culture was centrifuged and the pellet (bacteria) resuspended in PBS with proteases inhibitors (Protease Inhibitor Cocktail tablets, complete EDTA-free; Roche). Membrane disruption was carried out by sonication (10 pulses of 15 seconds in ice-cold environment)

obtaining cytosolic extract. This extract was directly used in next step or frozen at -80 ºC for a later use.

Pulling down protein complexes. Bacterial pellets expressing GST-fusion protein were incubated with Glutathione-sepharose beads (Thermo) for specific binding in buffer A during 4 hours at 4ºC. Three washing steps in buffer A ensured the specific binding and purification of GST-fusion protein in Glutathione-sepharose beads. Purified GST-fusion protein in beads was mixed with cellular lysates to permit interaction of proteins. This reaction was performed during 8 hours at 4 ºC in buffer B. Afterwards, beads carrying purified GST protein were washed, retaining protein complexes formed between GST-fusion and surrounding proteins in the cellular lysates. Pellets were resuspended in LSB for posterior boiling at 95 ºC, separating proteins and sepharose beads.

7. Protein immunoprecipitation.

HEK cells transfected with desired $_C$DNA and growing at a maximal confluence of 80% were lysated by adding lysis buffer and incubated for 1 hour at 4 ºC for cellular disruption. After then, a centrifugation step at 4000 x g was used to separate the nucleus and big cellular assemblies (in the pellet) from solubilized protein fraction.

Antibodies were immobilized in 50% protein G-Sepharose slurry (Amersham Bioscience) at 4 ºC to eliminate nonspecific binding. These beads were mixed with solubilized proteins to bind antibody to target protein at 4ºC during 4 hours. Multiple steps of centrifugation, decantation and washing in lysis buffer allowed to clear sepharose beads for solubilized proteins, remaining antibody target protein and interacting proteins stuck to the beads. Bound proteins were eluted from beads by adding 15 µL of LSB and boiling at 95 ºC. One more step of centrifugation drove beads to the pellet and protein fraction (which included the light and heavy chain of antibody plus antibody specifically tagged proteins) in the soluble fraction.

8. Protein biotinylation experiments.

Protein biotinylation was used to mark and then precipitate plasma membrane proteins, obtaining a pool of proteins separated from the whole set of cellular proteins.

Protein biotinylation. HEK cells growing in plastic dishes covered with poly-lysine were transfected at 75% of maximal confluence and used 36 hours later, when the confluence reached 100%. Cells were then washed with cold PBS three times and incubated with Sulpho-NHS-SS-Biotin (Thermo) at 4 ºC during 15 minutes. This compound bound plasma membrane proteins by tagging their lysine residues. After then, cells were washed three times with quenching solution (192 mM glycine, 25 mM TRIS in PBS) and incubated for 10 minutes at 4 ºC. Finally, cells were washed again in cold PBS and lifted by adding cold lysis solution (which includes proteases inhibitors; Protease Inhibitor Cocktail tablets, complete EDTA-free; Roche). Cells were resuspended in 0.5 ml lysis buffer and incubated at this solution for 60-90 minutes in a wheel at 4 ºC.

Separation of biotinylated proteins. To reserve a pool of total protein, 25 µl were taken from protein lysates (5% of the total amount, serving to quantify the amount of membrane fraction proteins). The rest of the proteins were mixed with 50 µl of 50% slurry Streptabiding-Agarose beads (Sigma-Aldrich) during 90-120 minutes at 4 ºC. This mixture allowed biotinylated proteins to bind Streptavidin beads which was isolated by centrifugation, producing a precipitation similar to that described in Co-inmunoprecipitation assay. The difference with this last technique lied in that biotin-streptavidin binding substitutes antibody-target binding, making possible to isolate tagged proteins. In this case, plasma membrane proteins were tagged by biotin. Successive washing steps with lysis buffer produced a purified membrane protein sample that was resuspended in 40 µl of lysis buffer plus LSB (at 50% each) to separate bound proteins from agarose beads by boiling at 90-95 ºC for 5 minutes.

9. Immunocytochemistry.

This protocol was used equally used for immunocytochemistry in HEK cells or hippocampal primary cultures.

Cells growing in coverslips were rinsed in PBS and then fixed with 4% paraformaldehyde plus 90 mM sucrose for 15 minutes and washed again in PBS. Cells were then permeabilized in PBS containing 0.02% Triton-X for 10 minutes at room temperature. BSA 3% was used as blocking solution to avoid non specific interaction between antibodies and hydrophobic proteins(room temperature, 90 minutes). Primary antibodies were diluted in blocking solution and used as mentioned in table 2 at 4 ºC overnight. Afterwards, coverslips were washed in blocking solution and secondary antibodies (conjugated with alexa-fluorophores) were added during 90 minutes in agitation at room temperature. The coverslips were washed in blocking solution and mounted with Vectashield (Vector Laboratories, Burlingame) for nuclear staining (DAPI) and bleaching prevention. Observations were performed using an upright Laser Confocal microscope (Leica SPII).

Membrane protein immunocytochemistry. To label specifically the membrane fraction of target protein, the above protocol was modified in that cells were slightly fixed for 5 minutes in paraformaldehyde (4% in sucrose) and not permeabilized to prevent access to intracellular protein fraction.

10. Bimolecular Fluorescence Complementation (BiFC).

We used an optimized version of YFP venus which was split in two halves: the amino-terminal (VNT) encompassing aminoacids 1-154 (T153M, which reduced self-assembly; Saka et al., 2007) and carboxy-terminal (VCT) encompassing residues 155-239 (these constructs were kindly provided by James C. Smith from University of Cambridge, UK). VNT half was fused to NeCaB1 through its N-terminal part with a linker of 12 amino acid residues (GSAGSTGSGSSG). VCT was fused to GluK5 (or GluK5 C terminal deletions) through its C-terminal cytoplasmatic region with the same linker as the VNT

(VCT chimeras were cloned by Dra. Isabel Aller). The BiFC technique consists in the reconstruction of vYFP which yield in fluorescence recovery and thus used as a marker for protein interaction (Kerppola, 2006).

HEK cells transfected with NeCaB1-VNT and GluK5-VCT chimeras plus GluK1 to make kainate receptors functional, were transfected in HEK cells, which were fixed after 15-20. Interaction between NeCaB1 and GluK5 was quantified using the reconstruction of vYFP fluorescence as readout (excitation at 488 nm and emission 495-515 nm), corrected by the sum of NeCaB1-VNT and GluK5-VCT expression (BiFC index). To that end, GluK5 was detected with a commercial Myc antibody (detected by Alexa 555, excitation at 555 and emission window 565-595 nm) and NeCaB1 with the NeCaB1 commercial antibody (detected by secondary Alexa 647 nm, excitation at 633 nm and emission window 655-700 nm (see table 2 for specifications).

11. Real time PCR (RT-PCR).

Quantitative RT-PCR. Reverse transcription was performed with 5 reverse SuperScript double stranded cDNA systhesis Kit (Invitrogen). Real-Time qPCR was performed (10 min at 95 °C, 15 s at 95 °C, and 1 min at 60 °C; 40 cycles) using a SYBR green Master Mix (Appied Biosystems) in a StepOne software (Applied Biosystems). A linear concentration–amplification curve was established by diluting pooled samples. Quantified results for individual cDNAs were normalized to cyclophilin expression level.

Each experiment was performed in triplicate and the primers used were as follows:

Neto1 forward 5’-GATATAATTTCACACCTGATCCCG 3’

Neto 1 reverse 5’-CGCCCATCTCAAACTCACACGCTGGC-3’;

Neto 2 forward 5’- CCCATTCCAGATTGCCAGTTTGA-3’

Neto 2 reverse 5’- GGCCAGGCTTTGTTTTCTCTTCT 3’

cyclophilin forward, 5_-AGGTCCTGGCATCTTGTCCAT- 3’

cyclophilin reverse, 5_-GAACCGTTTGTGTTTGGTCCA-3’.

Semiquantitative RT-PCR. Total RNA from P0 hippocampus and DIV 19 hippocampal cultures was isolated using RNeasy mini kit (Qiagen). Reverse transcription was performed with 500 ng of total RNA using a SuperScript Double-stranded cDNA Synthesis Kit (Invitrogen). Results were normalized with GAPDH expression. Each experiment was performed in triplicate and the primer sequences used were as follows:

Neto1 reverse : 5'GTTGTATTCAGATTCATGTTTG3';

Neto1 forward: 5'CATCACTGTGGATCCCAACTGTC3' giving a band of 310 pb.

Neto2 forward: 5' :GAAGCAGTGCTATCGAAAATCT 3';

Neto2 reverse: 5'CTTAGTGAAAATAGTTCATAATG 3' giving a band of 500 pb;

GADPH forward: 5' TGCTGAGTATGTCGTGGAGTCT 3';

GADPH reverse: 5'GGTCCAGGGTTTCTTACTCCTT3' giving a band of 730 pb.

These experiments were performed by Dra. Isabel Aller.

12. Electrophysiological recordings.

HEK cells were recorded after 24-48 hours post transfection. Hippocampal primary cultures were recorded after 16 days to ensure synaptic connections between neurons. The whole cell configuration of the patch clamp technique was used. Borosilicate (WPI) pipettes (3-5 MΩ) were generated by a horizontal puller (mod. P-87, Sutter Instruments). Cells were perfused using a fast perfusion system (Lerma et al., 1998) consisting in seven tubes which allowed rapid change between different bathing solutions. Perfusion speed was assessed using the change in the pipette open tip potential upon jumping from a ACSF and 10% diluted ACSF, reporting a rise time (20-80%) value of 0.5 ms (Figure 11).

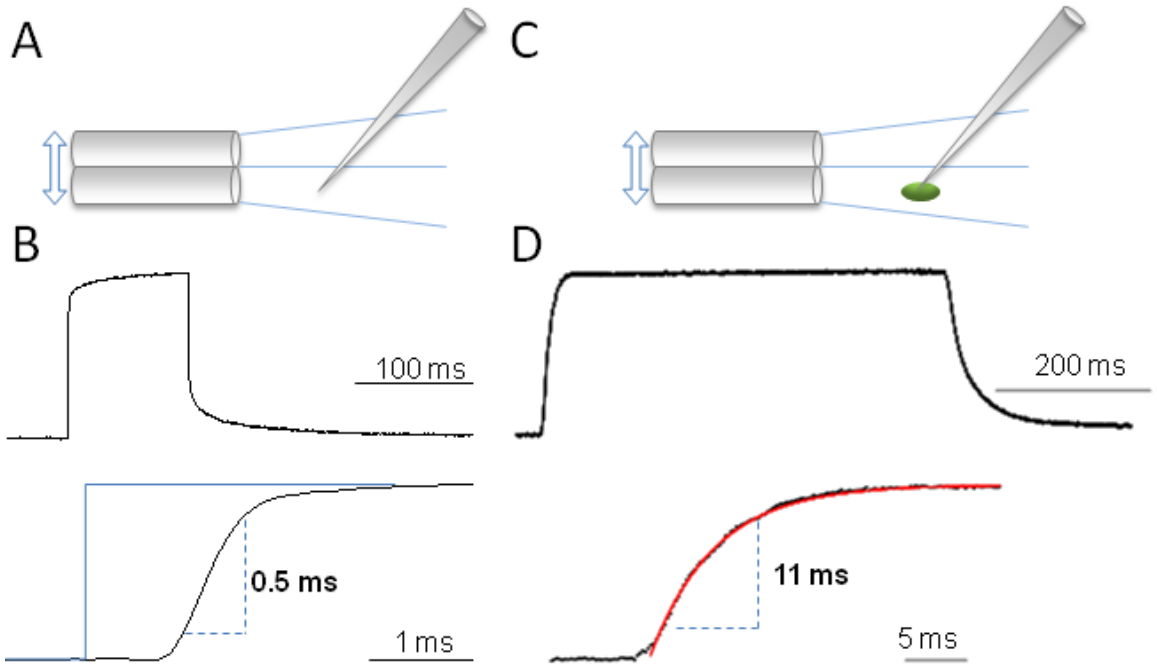

Figure 11. Fast perfusion system used in electrophysiological experiments. A, cartoon of two contiguous tubes, which moved to change bathing solution around pipette tip. B, a 100 ms pulse caused a current drop with an onset rise time (20%-80%) of 0.5 ms. C, same as in A but patching a HEK cell which allowed evaluation of speed for solution replacement around the cell. D, a pulse of 500 ms; onset rise (20%-80%) time of 11 ms.

Electrophysiological recordings were carried out with an EPC-7 amplifier (List). Signals were filtered at 2 KHz and acquired into a personal computer at a sampling rate of 2-5 KHz (LAB-Master card) using he pCLAMP software (Axon Instruments). All the experiments were performed at room temperature.

To avoid changes at tip potential, a homemade salt-agar bridge (125 mM NaCl with 2 % agarose) was used as the reference electrode. Both, reference and recording electrodes were chlorinated with HCl 1 M. Pipettes were filled with internal solution containing (in mM): 117 $CsMeSO_3$, 9 NaCl, 10 HEPES, 10 TEA and 0.3 EGTA at pH 7.4 and 300 mOsm. External solution (ringer or ACSF), consisted of (in mM): 160 NaCl, 10 HEPES, 15 glucose, 2.5 KCl, 1.8 $CaCl_2$ and 1 $MgCl_2$ at pH 7.4)

Used agonists and antagonists were prepared the same day. Table 3 shows further details of these compounds.

HEK and hippocampal cultures			
Compound	Action	Concentration	Supplier
APV	NMDAR antagonist	50 µM	Abcam
ATPA	KARs agonist	From 1 mM to 10 µM	Tocris
BAPTA-AM	Ca2+ chelator	5 µM	Abcam
CNQX	AMPAR/KAR antagonist	20 µM	Tocris
Glutamate	Glutamate Receptors agonist	From 30 mM to 1 µM	Sigma-Aldrich
GYKI	AMPAR antagonist	25 µM	ABX
Ionomicin	Ca^{2+} selective ionofore	1 µM	Sigma-Aldrich
Kainate	KARs agonist	50, 300 (µM)	Abcam
LCCG-I	mGluRII agonist	10 µM	Tocris
Picrotoxin	GABAA antagonist	50 µM	Sigma-Aldrich

Tabla 3. Compounds used in this study in HEK cell and hippocampal primary cultures.

14. Solutions and buffers.

- **Bacteria grow medium.**

LB:

10 gr Bacto-Triptone, 5 gr Bacto-yeast-extract, 10 gr NaCl for 1 L.

- **DNA handle solutions.**

Electroporation solution:

Sucrose 270 mM, HK_2PO_4 5.6 mM, H_2KPO_4 1.4 mM, $MgCl_2$ 1 mM; pH 7.4: 300 mosm

TE buffer:

10 mM TRIS, 1 mM EDTA; pH 8

- **Electrophysiological solution.**

ACSF:

160 mM NaCl, 10 mM HEPES, 15 mM glucose, 2.5 mM KCl, 1.8 mM $CaCl_2$ and 1mM $MgCl_2$; pH 7.4

Recording solution:

117 mM $CsMeSO_3$, 9 mM NaCl, 10 mM HEPES, 10 mM TEA and 0.3 mM EGTA; pH 7.4; 300 mOsm

In the case where 100 nM free Ca^{2+} want to be obtained, 0.741 mM EGTA is balanced with 0.740 mM $CaCl_2$

- **Protein Analysis.**

Electrophoresis solution (SDS-PAGE):

25 mM TRIS, 192 mM bicine, 0.1 % Sodium Dodecyl Sulfate; pH 8.3

LSB:

50 mM TRIS, 12.5 mM EDTA, 10 % glycerol, 2 % Sodium Docecyl Sulfate, 1 % β-mercaptoethanol, 0.02 % bromophenol blue

MKM buffer:

20mM MOPS, 150 mM KCl, 1 % Triton X-100; pH 7.4

PBS:

137 mM NaCl, 2.7 mM KCl, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM; pH 7.3

Polyacrilamide gells:

Stacking gel*:* 6 % Acrylamide/Bis (37:5:1) Rotipherase, 125 mM TRIS pH 6.8, 1 % SDS, 1 % ammonium persulfate, 0.2 % TEMED

Running gel: 10 % Acrylamide/Bis (37:5:1) Rotipherase, 375 mM TRIS pH 8.8, 1 % SDS, 1 % ammonium persulfate, 0.2 % TEMED

Quenching solution: 192 mM glycine, 25 mM TRIS in PBS

SEEP buffer: *320 mM sucrose,* 10 mM HEPES, 1 mM EGTA, 0.1 mM EDTA; pH 7.4

Transfer Buffer (TB):

10 mM CAPS (pH 11), 10% methanol

Transfer Buffer-tween (TBS-T):

100 mM TRIS, 1.5 M NaCl, 0.2 % Tween; pH 7.5

- **Pull-down experiments.**

Buffer A (GST Binding buffer): 20 mM TRIS, 250 mM NaCl, 2 mM EGTA, 2 mM EDTA; 1 % Triton X-100; pH 7.4

Buffer B (Protein Binding buffer): 20 mM TRIS, 250 mM NaCl, 0.02 % Tween; pH 7.4

IV. Results

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IV. Results I. NeCaB1 on GluK5 containing KARs

Using GluK5 C-terminal region as bait, a mRNA library from rat DRG neurons was screened by Yeast two hybrid method. In this experiment, NeCaB1 (Neuronal Calcium Binding protein 1) was identified as a possible interacting protein. With this in mind, we began to characterize NeCaB1 and GluK5 interaction to unravel the role played by NeCaB1 on GluK5 containing kainate receptors function.

NeCaB1 belongs to a neuronal Ca^{2+} binding protein family, which is composed of three members (NeCaB1-3). The three members share structural homology, been composed by an EF-hand domain at the N-terminal, which unlike other Ca^{2+}-binding proteins only posses a single Ca^{2+} binding site, a central NeCaB homology region and a putative antibiotic biosynthesis monooxigenase domain at the C-terminal (Sugita et al., 2002).

NeCaB1 is the less understood member of this family. It was isolated from brain extracts as an interactor of C2A domain of synaptotagmin I (Sugita et al., 2002). In the other hand, NeCaB2 has been shown to interact with adenosine A_{2A} receptor (Canela et al., 2007) and with mGluR5 receptor (Canela et al., 2009). NeCaB2 binding to either adenosine A_{2A} or mGluR5 receptor is disfavored by Ca^{2+}. It has been described a complementary expression pattern for NeCaB1 and 2, both in the hippocampus and spinal cord (Zimmermann et al., 2013; Zhang et al., 2014). NeCaB3, has been shown to be expressed in the brain and muscle and was first identified as XB51 (Sumioka et al., 2003) an interactor of XL11 protein amino terminal domain, which is related with APP intracellular metabolism, suppressing β-amyloid (A β) formation (Tomita et al., 1999; McLoughlin et al., 1999).

1. Space and time localization of NeCa1 and GluK5.

While studying two protein interactions, it is essential to check if both proteins are expressed in the same place and time. To clarify this point, we checked the subcellular localization of NeCaB1 and GluK5 by subcellular fractioning and western blot analysis using brain tissue of adult mice. First, we tested subcellular localization of NeCaB1 and GluK5 at three different compartments. GluK5 showed expression in the plasma membrane (PM) and synaptosomal fraction, while NeCaB1 showed expression in those two compartments plus in the soluble fraction of the cell. As a control, SNAP-25 expression was checked. Whereas this protein was not localized in the soluble fraction, it appeared in plasma membrane and synaptosomal fractions (Figure 12 A, these experiments were done by Dr. Rocio Rivera).

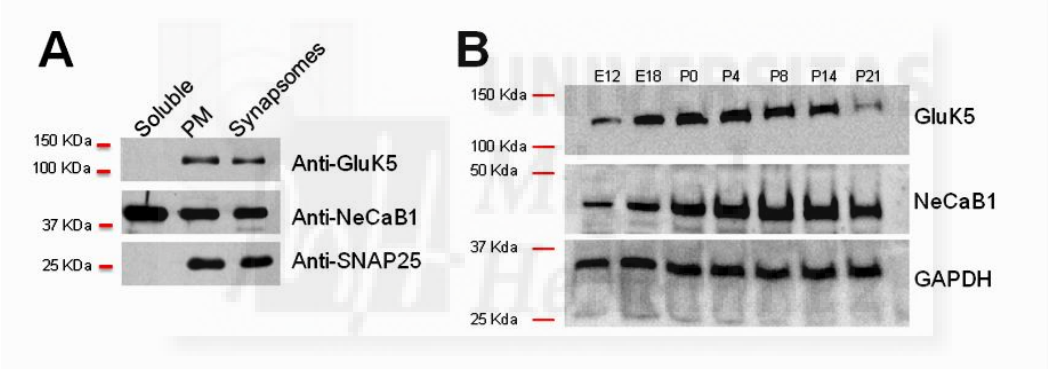

Figure 12. NeCaB1 subcellular and developmental expression. A, three different subcellular compartment were isolated, whereas NeCaB1 is described as a soluble protein that is present at plasma membrane (PM) and synaptosomal fraction. B, NeCaB1 showed expression as early as embryonic stage 12 days (E12), similar to GluK5 subunit of KARs. GAPDH was used as loading control.

Regarding the expression time of NeCaB1 and GluK5, different time points were checked in mice brain. GAPDH enzyme was used as a loading control to normalize expression at different time points. As it can be seen in figure 12 B, NeCaB1 and GluK5 were expressed at the same time in the brain, starting from E12 or before until adulthood. Unfortunately, we could not perform a double immunohistochemistry due to the fact that both antibodies were raised in rabbit and we could not avoid crosstalk between them.

These results show that NeCaB1 and GluK5 are expressed at the same compartments within the brain cells and this may occur at the same time.

2. NeCaB1 interacts with GluK5 containing KARs.

To confirm NeCaB1 and GluK5 interaction, HEK cells were used to reconstruct the functional kainate receptor by either transfecting GluK1 or GluK2 together to GluK5, and co-transfecting NeCaB1. Different experiments were designed to probe co-immunoprecipitation of NeCaB1 by kainate receptor subunits.

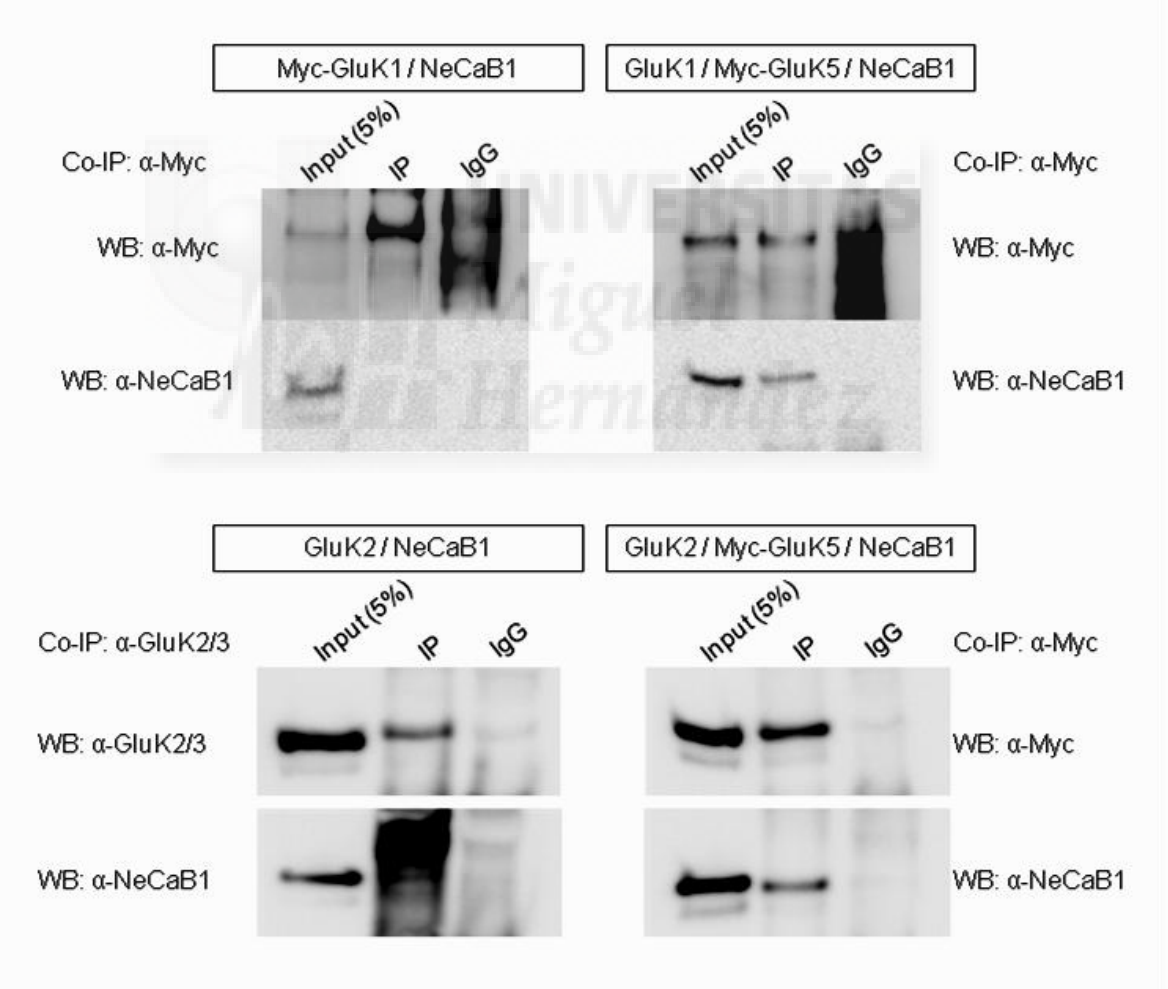

Figure 13. NeCaB1 protein interacts with GluK5 subunit containing KARs. Validation of NeCaB1 & GluK5 interaction by Co-Inmunoprecipitation. HEK cells expressing GluK1 or GluK2 and the heteromeric forms with GluK5 subunit plus NeCaB1 were used to co-immunoprecipite NeCaB1 protein. Different epitopes tagged to KARs subunit were used for Co-inmunoprecipitation in each case. Mouse IgG was used as a control of co-immunoprecipitation.

First, Myc tagged GluK1 was expressed with NeCaB1 and we observed that NeCaB1 was not co-immunoprecipitated (Figure 13). In the second experiment, myc-tagged GluK5 protein was expressed together to GluK1. In this case, NeCaB1 was co-immunoprecipitated by myc antibody. The third experiment probed the ability of myc-GluK5 to co-immunoprecipite NeCaB1 when forming heteromeric receptors with GluK2, while GluK2 failed to immunoprecipitate NeCaB1 (Figure 13).

These experiments demonstrated that GluK5 containing kainate receptors are able to interact with NeCaB1, whereas GluK1 or GluK2 homomeric receptors are not.

3. Mapping NeCaB1 interaction on GluK5 C-terminal domain.

As we knew that NeCaB1 interacts in the C-terminal region of GluK5, we used the Bimolecular Fluorescence Complementation (BiFC) technique to visualize NeCaB1-GluK5 interaction in a living system, such as HEK cells. For that reason, we took advantage of several chimeric proteins where GluK5 with C-terminal deletions (Figure 14 A) were fused to the N-terminal half of Venus Yellow fluorescent protein (vYFP). On the other hand, C-terminal half of the vYFP was fused to NeCaB1 protein.

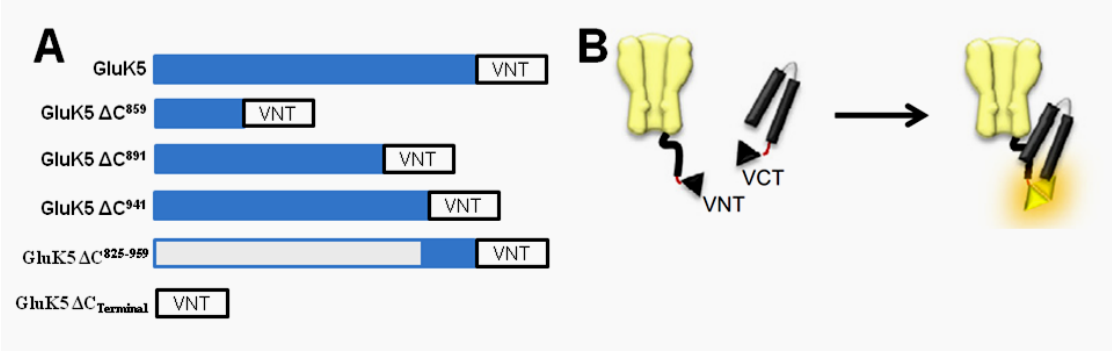

Figure 14. ***Chimeric GluK5 constructs used in Biomolecular fluorescence complementation technique*****.** A, schematic structure of different deletion constructs of the C-terminal domain of GluK5 subunit fused to Venus N-terminal (VNT) domain. B, Bimolecular Fluorescence complementation (BiFC) technique diagram.

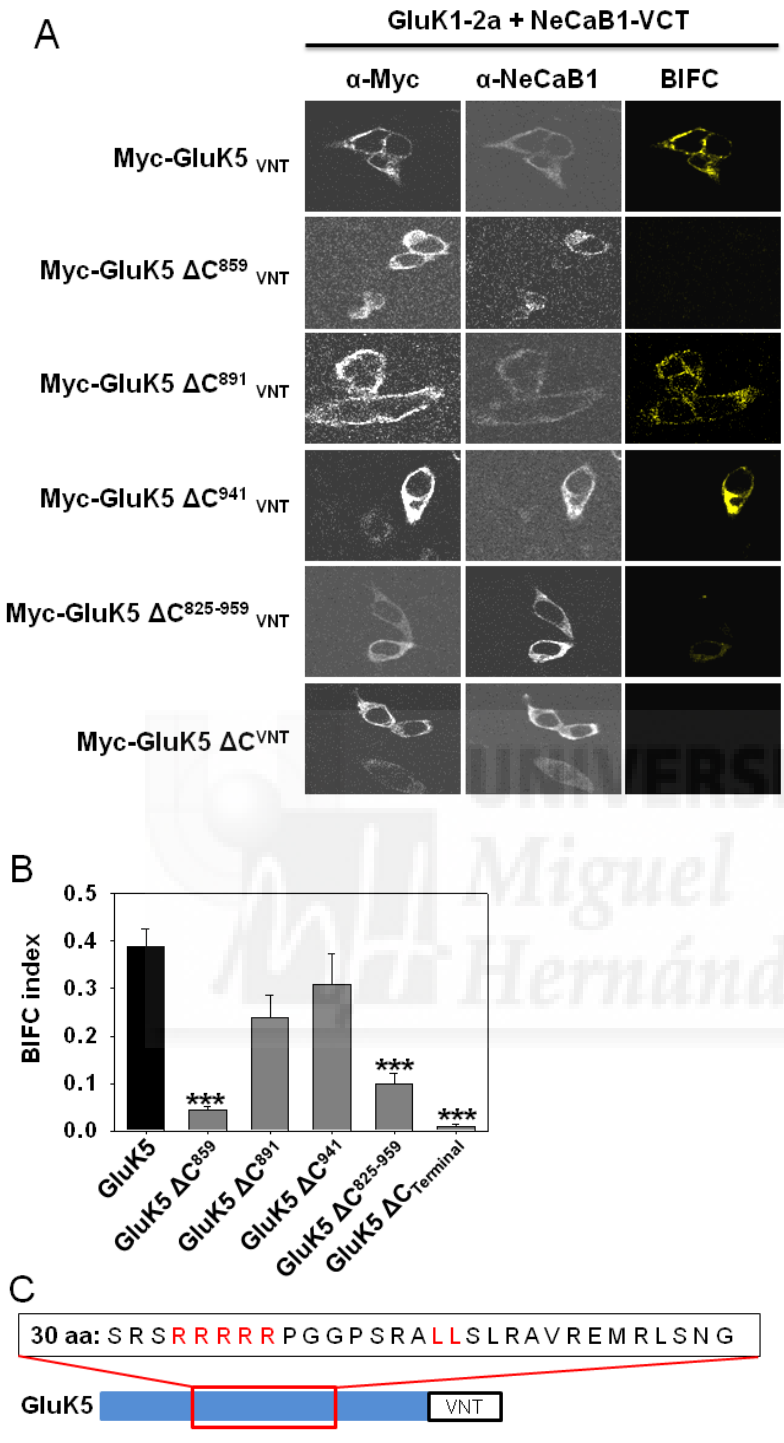

Figure 15. *"In vivo"* interaction between NeCaB1 and GluK5. A, representative illustrations of HEK cells transfected with GluK1 and different GluK5 constructs where the interaction with NeCaB1-VCT is revealed by the appearance of yellow fluorescence. B, quantification of BiFC index [BiFC signal/ (α-Myc + α-NeCaB1) fluorescence]. C, GluK5 C-terminal minimal region which is necessary for NeCaB1 interaction. Data are mean + SEM. ***p < 0.005.

Functional kainate receptors were reconstructed in HEK cells expressing GluK1, chimeric GluK5 with vYFP N-terminal (VNT) and chimeric NeCaB1 with vYFP C-terminal (VCT). To ensure that HEK cells contained at least GluK5 and NeCaB1, immunocytochemistry experiments were performed using NeCaB1 antibody and Myc immunoreactivity for labeling GluK5.

First, full length chimeric GluK5-VNT and NeCaB1-VCT were able to reconstruct vYFP fluorescence, obtaining a BiFC index (see material and methods, section 10) of 0.39 ± 0.04 (n=42). Then, different C-terminal region deletions were tested to determine the minimal region needed allowing GluK5 and NeCaB1 interaction. GluK5 859ΔC (which lacked amino acids 859 and on), showed a BiFC index of 0.04 ± 0.01 (n=44; $p<0.005$). GluK5 891ΔC (which lacked aminoacids from 891), had a BiFC value of 0.24 ± 0.05 (n=38; $p>0.05$). GluK5941ΔC (which lacked aminoacids from 941), had a BiFC value of 0.31 ± 0.06 (n=25; $p>0.05$). GluK5 825-966ΔC (which lacked aminoacids from 825 to 969), had a BiFC value of 0.1 ± 0.02 (n=22; $p<0.005$). GluK5ΔC (which lacked the hole C-terminal region), showed a BiFC value of 0.07 ± 0.01 (n=20; $p<0.005$) (Figure 15 B). Examples of cells from which previous values were obtained are shown in figure 15 A.

These experiments suggest that the minimal region allowing GluK5 and NeCaB1 interaction is a region included before the residue 891. As GluK5 859ΔC did not yield interaction, we could conclude that the region comprised between aminoacids 859 and 891 is the minimal region required for NeCaB1 and GluK5 interaction. This region is composed by 30 aminoacids which include two different endoplasmatic reticulum retention signals (red aminoacids in figure 15 C; Ren et al., 2003).

4. Ca^{2+} modulates NeCaB1 and GluK5 interaction.

NeCaB1 contains a calcium binding domain (Sugita et al., 2002). We wanted to determine whether this ion plays any role in the NeCaB1 and GluK5 interaction. We decided to study interaction between both proteins in an isolated environment to control free Ca^{2+} concentration. For that reason, pull-down experiments were performed under different conditions.

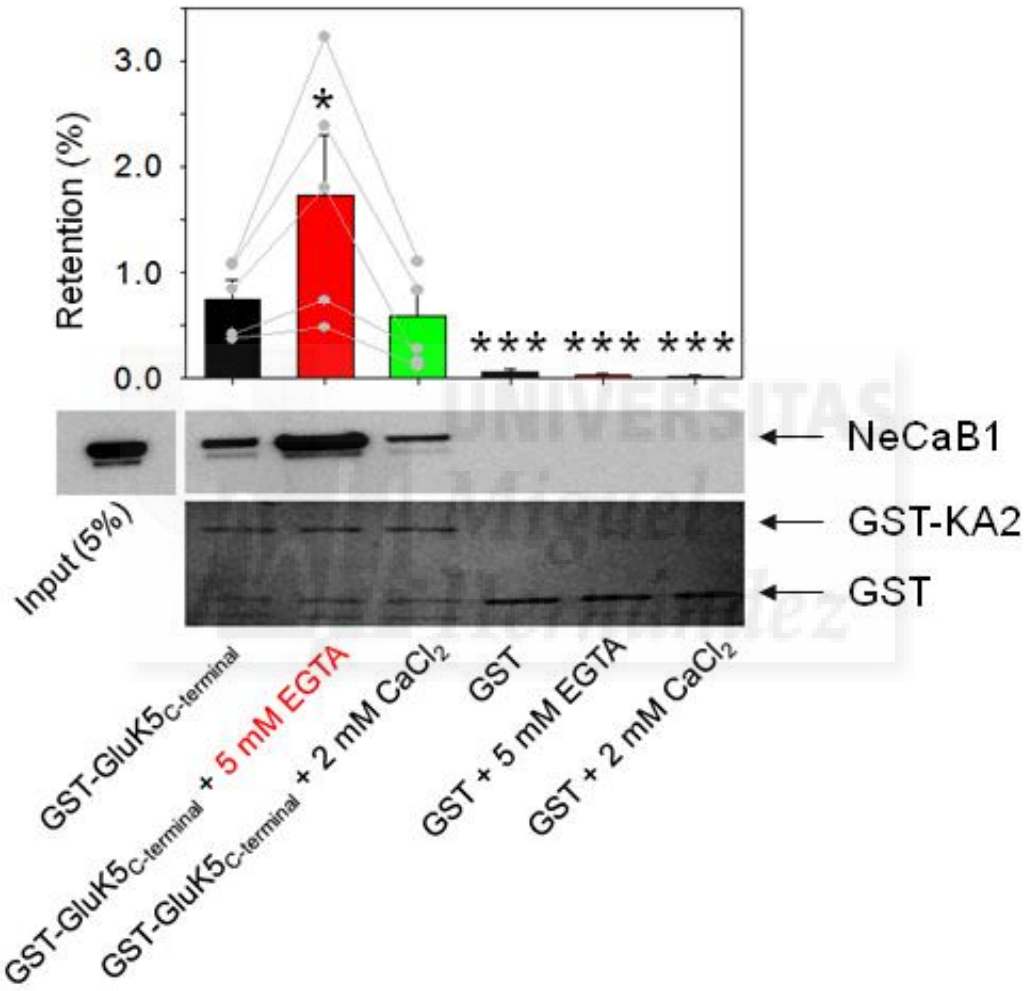

Figure 16. *"In vitro"* interaction between NeCaB1 and GluK5 C-terminal domain. Pull-down assays where HEK cells extracts expressing NeCaB1 are probed for interaction with GST fusion proteins containing the C-terminal domain of GluK5 subunit (bottom). The histogram shows the quantification of several pull-down assays, where paired experiments are linked with a line. The degree of NeCaB1 retention was referred to the input signal after normalization by the amount of GST fusion protein. Data are mean + SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

Purified GST or GST-GluK5$_{C\text{-terminal}}$ (GST fused to the C-terminal region of GluK5) were incubated with HEK cells lysates expressing NeCaB1. To test Ca^{2+} influence in NeCaB1 and GluK5$_{C\text{-terminal}}$ binding, a solution containing 5 mM EGTA (5 mM) was added to chelate any free Ca^{2+} was compared to others in which calcium was not added (control solution) or $CaCl_2$ was added to a concentration of 2 mM. As it can be seen in figure 16, GST-GluK5$_{C\text{-terminal}}$ was able to retain 0.75 ± 0.19 % (n=5) from total input when Ca^{2+} was not added or 0.58 ± 0.23 % (n=5) when the solution contained 2 mM Ca^{2+}, whereas in the absence of Ca^{2+} (5 mM EGTA added) NeCaB1 retention was enhanced to 1.73 ± 0.57 % (n=5; $p<0.05$) of total input. Experiments where GST alone was used, NeCaB1 was not retained, showing a specific binding with GluK5$_{C\text{-terminal}}$ region (Figure 16).

These experiments suggest that NeCaB1 binds to C-terminal region of GluK5 in a Ca^{2+} dependent manner, such that under normal Ca^{2+} levels, NeCaB1 binding to GluK5$_{C\text{-terminal}}$ is disfavored.

5. Functional impact of NeCaB1 on GluK1/5 KARs.

Kainate receptors were tested in HEK cells to control protein expression. Due to NeCaB1 and GluK5 interaction was isolated from dorsal root ganglia mRNA library and in that particular system the expressed kainate receptors are composed by GluK1 and GluK5 subunits (Petralia et al., 1994; Lafora et al., 2006), we studied the effect of NeCaB1 on GluK1/5 heteromeric receptors.

Heteromeric GluK1/5 kainate receptors where distinguished from homomeric GluK1 receptors due to their higher affinity for glutamate. To that end, we applied 10 mM as a saturating concentration, acting on both high and low affinity receptors, and 0.1 mM at which high affinity receptors are preferentially activated. The ratio between both concentrations served as readout of the density of high affinity receptors present at the plasma membrane.

To deal with modification of intracellular calcium concentration, we tested kainate receptors in HEK cells under normal conditions or transfected cells that

had previously been exposed to BAPTA-AM 5 µM (a Ca^{2+} chelator able to go through plasma membrane and accumulate inside the cells) for 1 hour.

In GluK1 homomeric receptors, the ratio of responses induced by 0.1 mM and 10 mM glutamate, was 0.16 ± 0.02 (n=9) and this was not different from when cells were treated with BAPTA-AM (0.15 ± 0.01; n=6; p>0.05, Student t-test). Heteromeric kainate receptors composed by GluK1/5 subunits, exhibited an increased affinity as this same ratio was 0.50 ± 0.04 (n=21) under control and 0.49 ± 0.03 (n=21) under low intracellular Ca^{2+} (BATA-AM treated). The coexpression of NeCaB1 significantly increased the ratio upon BAPTA-AM treatment (0.67 ± 0.04; n=23; p<0.01, Student t-test) (Figure 17).

These results may be interpreted as if high affinity kainate receptors (GluK1/5) increased at the cellular surface when NeCaB1 and GluK5 interaction is favored in low Ca^{2+} environment.

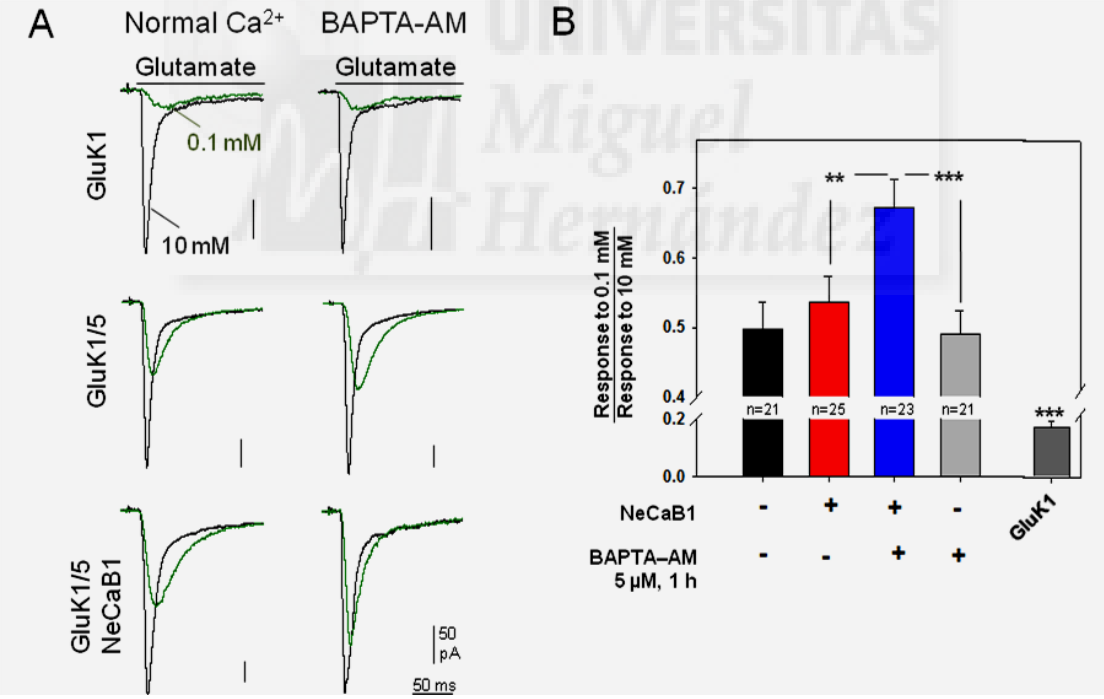

Figure 17. Effect of NeCaB1 on GluK5 containing KARs at low Calcium environment. A, current reponses evoked by 10 mM (black traces) and 0.1 mM (green traces) of glutamate in HEK cells transfected with different KARs in normal or low Ca^{2+} environment (BAPTA-AM treated). B, quantification of panel A showing the ratio between 0.1 mM and 10 mM glutamate, which served as readout of high affinity receptors at the surface. Data are mean + SEM. **p < 0.01; ***p<0.05; Student t-test.

6. NeCaB1 increases GluK5 surface expression.

To directly demonstrate a larger targeting of hetermoeric kainate receptors to the membrane when NeCaB1 and GluK5 interaction is favored, immuno-cytochemistry of GluK5 was performed under non permeabilization conditions to label exclusively membrane receptors. Intracellular Ca^{2+} concentration was modified as previously to modulate NeCaB1-GluK5 interaction. To differentiate between NeCaB1 positive and negative cells, NeCaB1 was tagged with GFP.

Myc-tagged GluK5 was detected and normalized to the cellular size. This was quantified as Surface Index (SI).

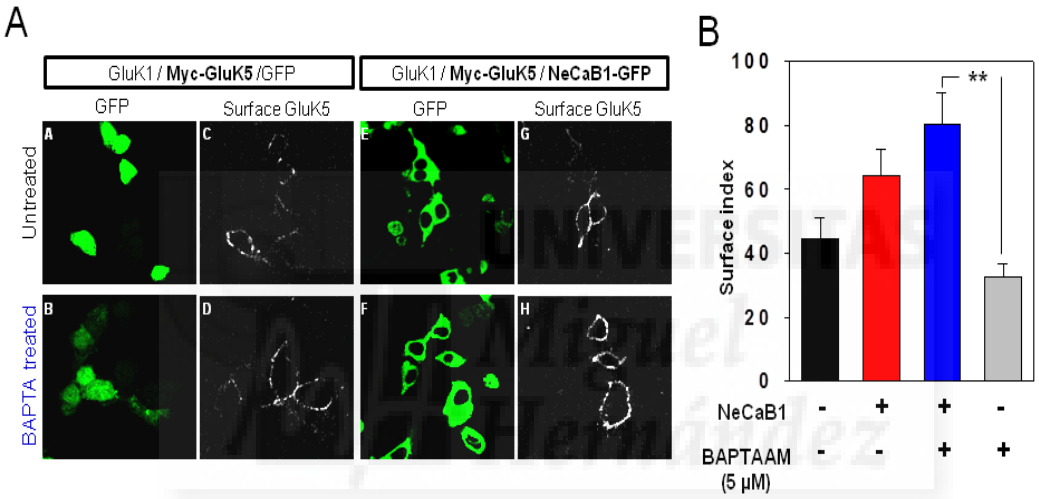

Figure 18. Increased GluK5 expression at the plasma membrane by NeCaB1 action. A, HEK cells expressing GluK1/5 KARs were tested for GluK5 membrane expression upon co-expression of GFP or NeCaB1-GFP under normal or low Ca^{2+} (5 µM BAPTA-AM treated for 1 hour). B, quantification of surface expression of GluK5 subunit reflects an increase of this protein at the membrane when NeCaB1 is expressed under low Ca^{2+}. Data are mean + SEM. p **< 0.01; Student t-test.

Control GFP expressing heteromeric GluK1/5 kainate receptor presented a surface index of 44.59 ± 6.62 (n=41) which was slightly, although not significantly, higher when NeCaB1 was coexpressed (64.38 ± 8.05, n=58). On the other hand, the surface index for GluK5 was increased when NeCaB1 was expressed and cells were kept under low Ca^{2+} environment (80.17 ± 9.84, n=58). This value was significantly different when compared with the same Ca^{2+} conditions in the absence of NeCaB1 expression (32.72 ± 4.19, n=45; $p<0.01$, Student t-test) (Figure 18).

To further substantiate this conclusion, we performed biotinylation experiments, where surface proteins may be differentiated from intracellular proteins.

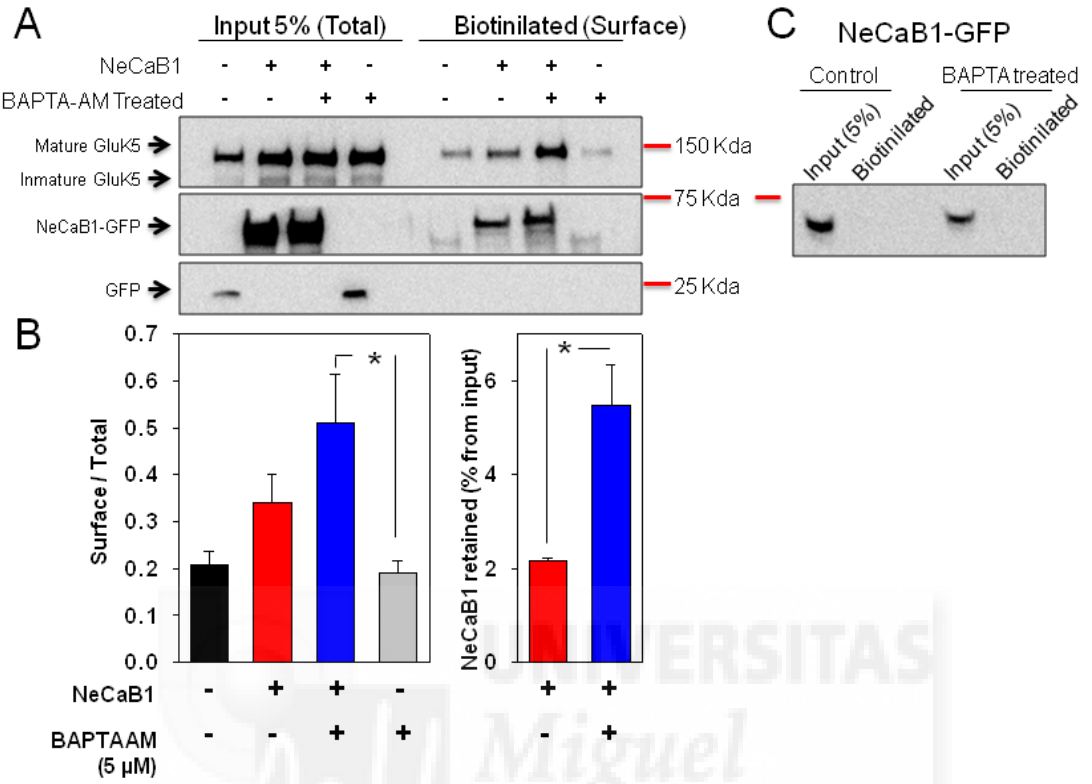

Figure 19. NeCaB1 enriched GluK5 containing KARs at the plasma membrane. A, Western blot analysis of total versus surface fraction (biotinylated) of GluK5 subunits, NeCaB1-GFP and GFP proteins. NeCaB1 trafficked GluK1/5 KARs whenever Ca^{2+} levels were reduced by BAPTA-AM treatment. Moreover, NeCaB1 remained present at membrane fraction, with a higher intensity in BAPTA treated cells. B, quantification of panel A was done measuring the ratio between surface and total fraction intensities (left) and NeCaB1 immunoreactivity was compared with input references (5 % of total sample). C, NeCaB1 is not localized to membrane fraction in the absence of GluK1/5 KARs either in control or BAPTA-AM (5 µM) treated cells. Data are mean + SEM. p *< 0.05; Student t-test.

GluK1 protein was co-expressed with GluK5 subunits to make functional heteromeric receptors. We were able to compare total GluK5 protein and surface expressed protein, obtaining a ratio of surface over total for control condition of 0.21 ± 0.03 (n=5) which was not significantly increased when NeCaB1 was co-expressed (0.34 ± 0.06, (n=5). On the other hand, GluK1/5 transfected cells untreated or treated with BAPTA-AM presented a similar ratio

(0.21 ± 0.03; n=5, and 0.19 ± 0.03, n=5, respectively), which was increased in the presence of NeCaB1 and BAPTA to 0.51 ± 0.1 (n=5, $p<0.05$; Student t-test; Figure 19 A, B).

Surprisingly, we observed NeCaB1 immunoreactivity in the surface labeled proteins, something not expected in principle for a soluble protein. The lack of immature GluK5 band and GFP band in surface protein fraction confirmed this conclusion. This surface immunoreactivity was larger in HEK cells when intracellular Ca^{2+} was decreased (2.17 ± 0.05 %, vs 5.48 ± 0.87 %, n=4, $p<0.05$; Student t-test). (Figure 19 A, B).

To clarify whether NeCaB1 could be inserted into surface fraction in the absence of kainate receptors, we transfected HEK cells with GFP tagged NeCaB1. In this case we did not observe NeCaB1 in the biotinylated fraction in normal or low Ca^{2+} conditions (Figure 19 C).

These results indicate that NeCaB1 clearly traffics GluK5 containing kainate receptors to the cellular surface under low Ca^{2+} environment. Nevertheless, in cells in which Ca^{2+} was not reduced to a minimum, NeCaB1 also exhibited some effect that not reached statistical significance. Furthermore, the appearance of NeCaB1 in the biotinylated fraction suggests that this protein is able to bind membrane anchored GluK5 containing kainate receptors

7. Trafficking and affinity modifications by NeCaB1.

The change in the ratio of response to 0.1 mM over 10 mM glutamate induced by the presence of NeCaB1 under low Ca^{2+} concentration was interpreted as indicative of the increased number of high affinity receptors (GluK1/5 heteromers) in the membrane. In keeping with this, the number of heteromeric receptors was demonstrated increased by both immunocytochemistry and biotinylation experiments under conditions favoring interaction between NeCaB1 and GluK5 subunits. If this is true, then it should be possible to demonstrate the existence of two populations of functional receptors, of low (i.e. GluK1) and high (GluK1/GluK5) affinity, which proportion

should be possible to alter upon interaction with NeCaB1. Therefore, we constructed dose-response curves for each of the conditions presented before.

To differentiate low affinity (homomeric GluK1) and high affinity (heteromeric GluK1/5) receptors, we fitted dose-response data as the sum of two logistic equations (Figure 20), where the first element of the equation revealed high affinity response (I_{max1}; K_{d1}) and the second one low affinity response (I_{max2}; K_{d2}). To facilitate convergence, we fixed Kd_2 to the affinity previously calculated from cells expressing exclusively homomeric GluK1 (i.e. 580 µM).

$$y = \frac{I_{max_1} * X}{K_{d_1} + X} + \frac{I_{max_2} * X}{K_{d_2} + X}$$

Figure 20. The sum of two logistic equations was used to fit data. Experimental data was fitted to the sum of two logistic equations, where I_{max1} and I_{max2} are the weighted components of each equation and K_{d1} and K_{d2} are the affinity values for each component.

In figure 21, experimentally obtained data are plotted for each condition together to the curves that better fitted the data. In control conditions, the magnitude of high and low affinity components presented similar values (45.8 ± 14.1 % and 54.9 ± 13.2 % of total response, respectively). These values did not change in BAPTA-AM treated cells (46.8 ± 9.6 % and 54.7 ± 10.1 % of total response). On the other hand, expression of NeCaB1 slightly changes the weight of each plot under normal conditions (54.6 ± 13.3 % and 46.6 ± 12.8 % of total response) but when NeCaB1 expressing cells were treated with BAPTA-AM the weight of the high affinity component remarkably augmented (67.8 ± 6.2 % vs 32.7 ± 6.3 % of total response).

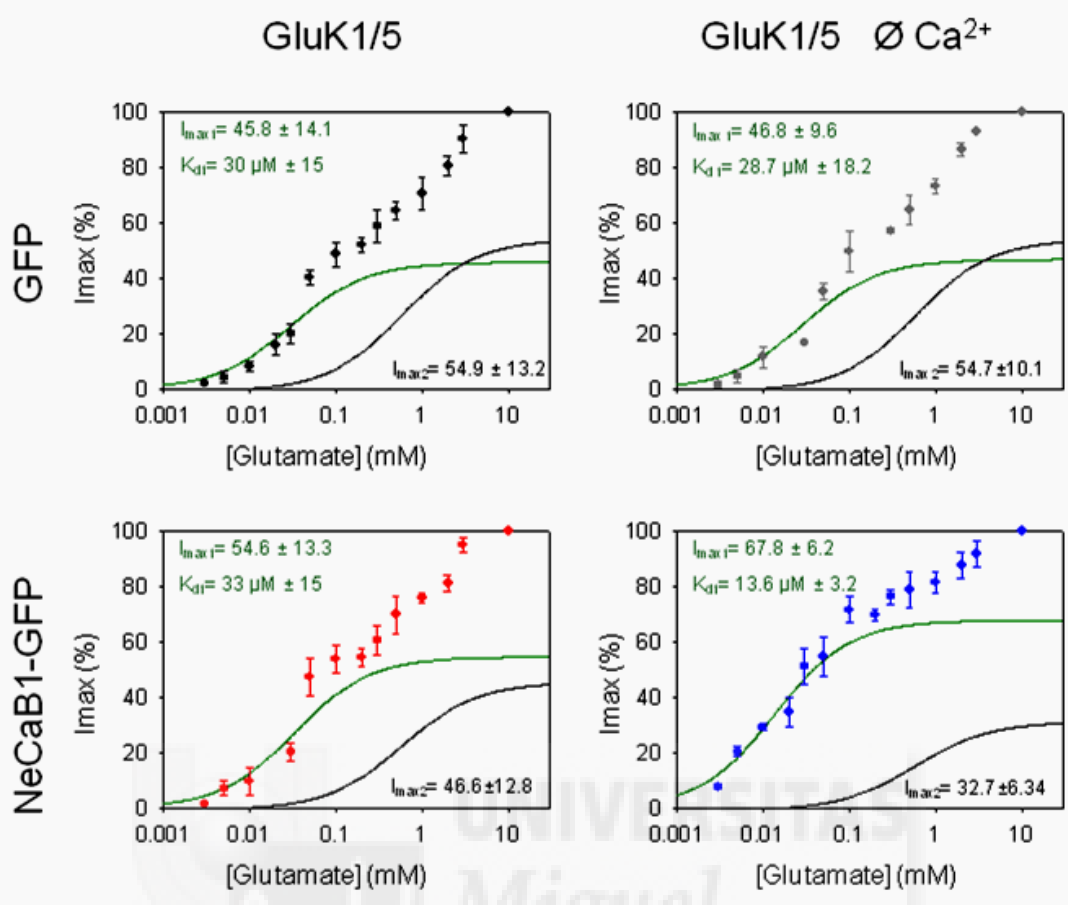

Figure 21. NeCaB1 increases GluK5 containing KARs glutamate affinity in low Calcium environment. Dose-response curves for GluK1/5 KARs in cells co-expressing GFP or NeCaB1-GFP in normal and low Ca^{2+} environment. Data were fitted to the sum of two logistic equation [$Y = I_{max} / (K_d + X)$], which are plotted independently (high affinity curve in green and low affinity in black). Parameters for both components of the logistic equation are plotted (I_{max}, which represent the weight of each component over the total equation and K_d, which represent the affinity of each component).

To our surprise, the affinity value of the high affinity component, which had a value of 33 ± 15 µM NeCaB1 expressing in normal Ca^{2+}, was further reduced when Ca^{2+} was chelated (13.6 ± 3.2 µM; Figure 21, 22).

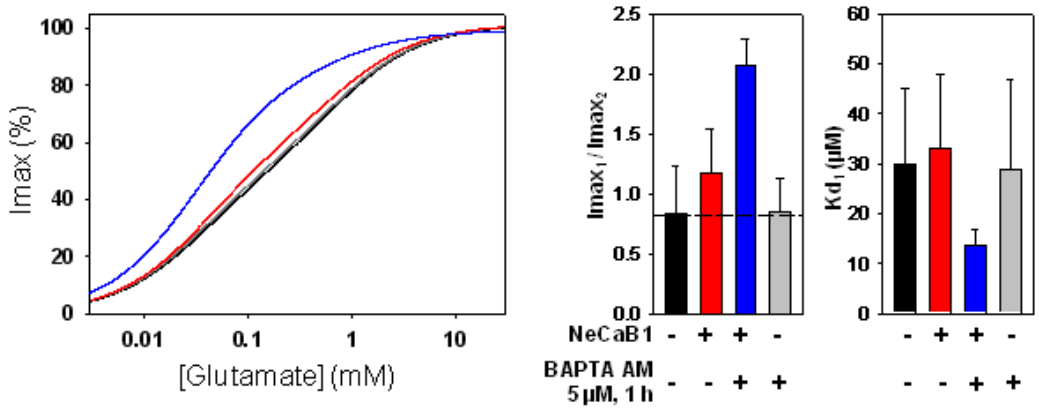

Figure 22. Quantification parameters show a dual effect in trafficking and affinity by NeCaB1. The fitted equations for each condition illustrated in Figure 21 are shown normalized by amplitude (left). On the right, the ratio between the amplitude values (I_{max}) of both components and the values for Kd for high affinity component are presented. Data are mean + SEM.

These data indicate that NeCaB1 is able to increase the number of high affinity receptors at the membrane when Ca^{2+} concentration is highly reduced, at the same time further increasing GluK1/5 receptor affinity.

8. NeCaB1 modulates trafficking and affinity of GluK2/5 KARs.

To determine the effect of NeCaB1 over a different combination of receptors, we looked at GluK2/5 heteromers in HEK cells. We generated two opposite scenarios to visualize the effect of Ca^{2+} and NeCaB1 on kainate receptors. First, we controlled the intracellular Ca^{2+} concentration to known values by including 0.3 mM EGTA in the pipette, which would set the intracellular concentration of free Ca^{2+} at 100 nM and second, we treated cells with BAPTA-AM as in previous experiments

In figure 23 A, saturating pulses of glutamate (10 mM) to activate the whole population of kainate receptors and of 1 mM ATPA, which activates just heteromeric receptors (homomeric GluK2 is not sensitive to ATPA), are shown. HEK cells expressing GluK2/5 heteromeric receptors presented similar ratios of

Glutamate to ATPA induced responses under control or when Ca^{2+} was reduced with BAPTA-AM (17.82 ± 4.32; n=9, vs 25.21 ± 5.48, n=11). When GluK2/5 receptors were co-expressed with NeCaB1, this ratio was slightly higher (32 ± 8.84, n=11) and further increased in cells treated with BAPTA-AM (66.02 ± 18.56, n=11; p<0.05; Student t-test) (Figure 23 B).

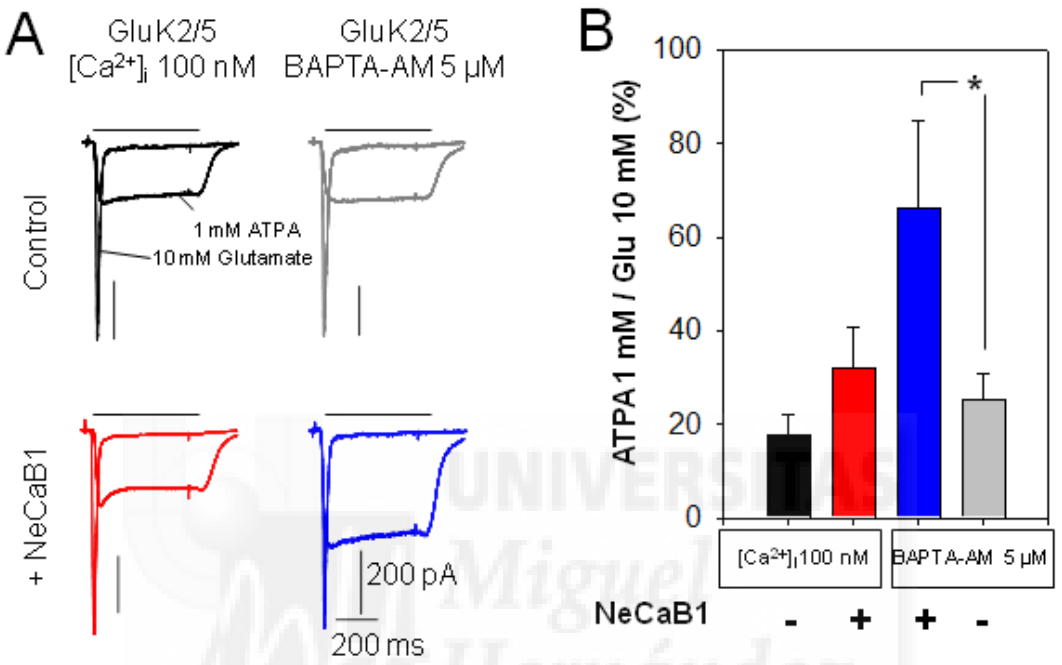

Figure 23. NeCaB1 increases GluK2/5 KARs number in a low Ca^{2+} environment. A, traces of HEK cells expressing GluK2/5 and GFP or NeCaB1-GFP were activated by either glutamate 10 mM or ATPA 1mM to measure abundance of heteromeric receptors, in normal (100 nM) or low (BAPTA-AM treated) Ca^{2+} environment. B, quantification of panel A. Data are mean + SEM. *p<0.05; Student T-test.

To further demostrate that the heteromeric population of kainate receptors bound to NeCaB1 undergoes an increase of agonist affinity, we used this same preparation expressing GluK2/GluK5 receptors and applied two different concentrations of ATPA to activate exclusively heteromeric receptors.

We included 100 nM free Ca^{2+} in the recording pipette or treated cells with BAPTA-AM when including 0.3 mM EGTA inside the recording pipette. We compared responses to saturating and unsaturating concentrations of ATPA (0.05 mM ATPA vs 1 mM). Low ATPA induced a response which was 15.72 ± 1.28 % (n=13) of total current induced by saturating concentration in control conditions. This fractional response was similar upon NeCaB1 expression (17.05 ± 1.44 % of total response, n=13). However, in cells previously treated with BAPTA-AM and expressing NeCaB1, low ATPA induced a response which 25.87 ± 1.93 % of total current (n=15) which was significantly larger than when Ca^{2+} was removed without NeCaB1 being transfected (18.54 ± 2.03 %, ,n=12) (Figure 24 A, B).

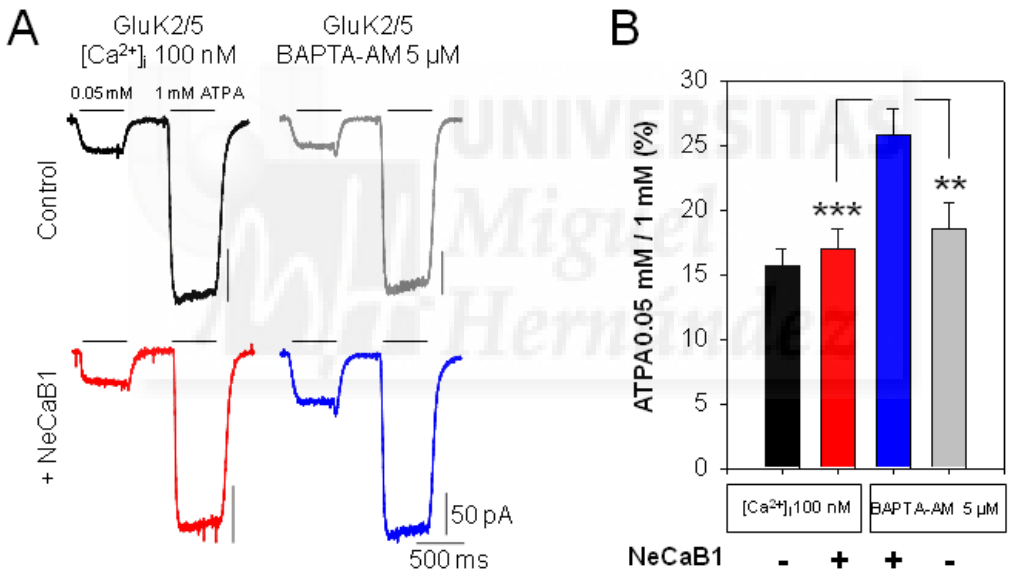

Figure 24. NeCaB1 increases GluK2/5 KARs agonist affinity in a low Ca^{2+} environment. A, traces of HEK cells transfected with GluK2/5 and co-expressing GFP or NeCaB1-GFP are activated by different concentration of ATPA to measure relative affinity between 0.05 mM and 1 mM, in normal (100 nM) or low (BAPTA-AM treated) Ca^{2+} environment. B, quantification of panel A Data are mean + SEM. **p< 0.01 and ***p< 0.005.

Altogether these data indicate that NeCaB1 increases the fraction of GluK5 containing kainate receptors expressed in the membrane and also increases the affinity of those receptors incorporating GluK5.

9. The modulation of GluK2/5 KARs affinity by NeCaB1 is rapid.

To assess whether fluctuations of Ca^{2+} concentration inside the cells could induce a rapid change GluK2/5 affinity by NeCaB1, we used a Ca^{2+} ionophore, Ionomycin, to rapidly increase intracellular Ca^{2+} (Beeler et al., 1979). After application of Ionomycin (1 µM) for 2 seconds, we calculate relative affinity of GluK2/GluK5 receptors for ATPA (i.e. comparing the ratio of responses to 0.05 mM and 1 mM ATPA) (figure 25 A). ATPA-induced responses maintained constant ratios before and 1 minute after Ionomycin application (14.92 ± 1.21 and 15.21 ± 1.24, n=16, respectively; figure 14 B, C).However, in cells expressing NeCaB1 this ratio was significantly affected by the ionomycin-induced increase of intracellular Ca^{2+}, undergoing a clear reduction of ATPA affinity, as judged by the significant reduction in the ratio of responses to ATPA (from 20.26 ± 1.25 to 17.27 ± 1.29, n=17, $p<0.05$; Student t-test). Such decreased value of ratio slowly returned to control values (figure 25 B, C).

These data indicate that Ca^{2+} activity controls the action of NeCaB1 on kainate receptors in a rapid manner, also reflecting the fine tuning that Ca^{2+} has over NeCaB1 action on GluK5 containing kainate receptors.

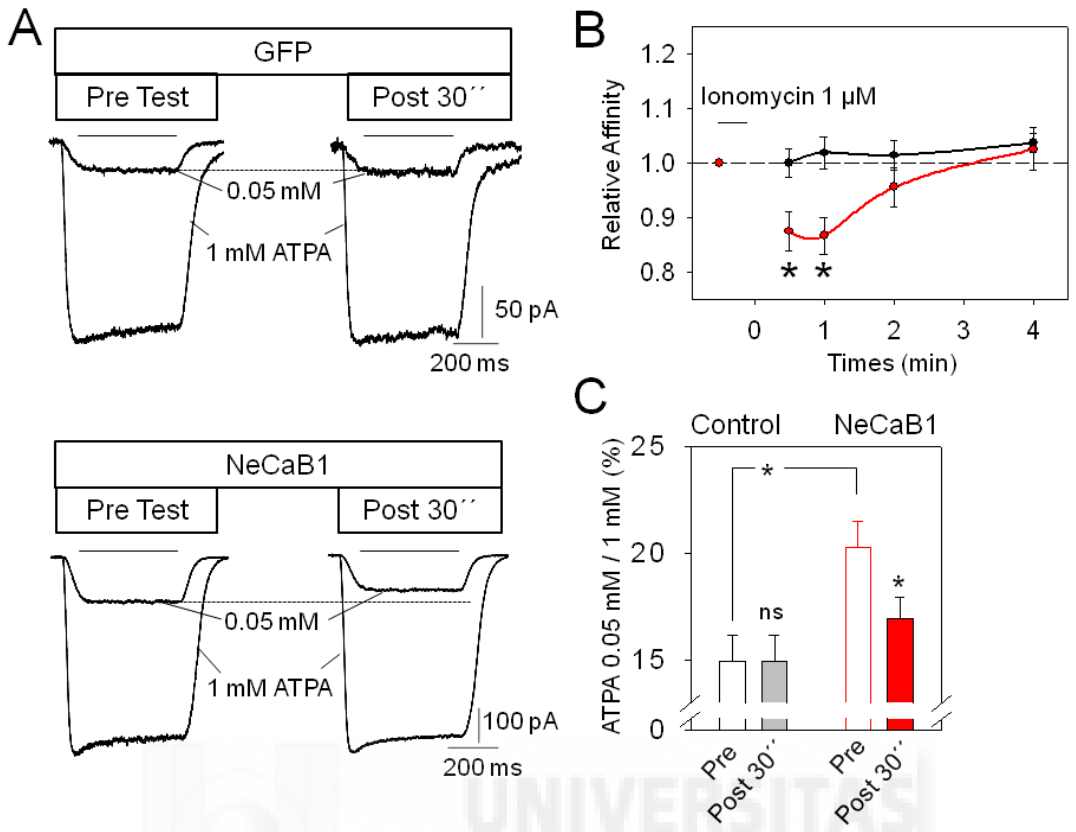

Figure 25. NeCaB1 changes GluK5 containing KARs affinity rapidly. HEK cells expressing GluK2/5 and GFP or NeCaB1-GFP were activated by ATPA 1mM and 0.05 mM to measure relative affinity. After a brief pulse (2s) of Ionomycin relative affinity to ATPA was checked at different time points. A, representative traces to both concentration of ATPA. B, Time course of the affinity for ATPA after ionomycin. C, a histogram showing average data from 3 experiments (n=17). Data are mean + SEM. *p < 0.05.

10. Synaptic localization of NeCaB1.

For NeCaB1 having an action on synaptic receptors, this protein should localize in the synaptic compartment. Therefore, we studied its localization in hippocampal cultures by using a postsynaptic marker, such as PSD-95 and a presynaptic marker, such as Bassoon.

We observed expression of NeCaB1 in a small proportion of cultured neurons. Figure 26 shows the native NeCaB1 localized at postsynaptic densities but not in the presynaptic compartment, as it co-localizes with PSD-95 in 64.23 ± 4.76 % of the puncta (figure 26 A, C) but not with Bassom (9.62 ± 1.67 % of the puncta; Figure 26 B, C).

These data reveal that NeCaB1 may be localized in active synapses.

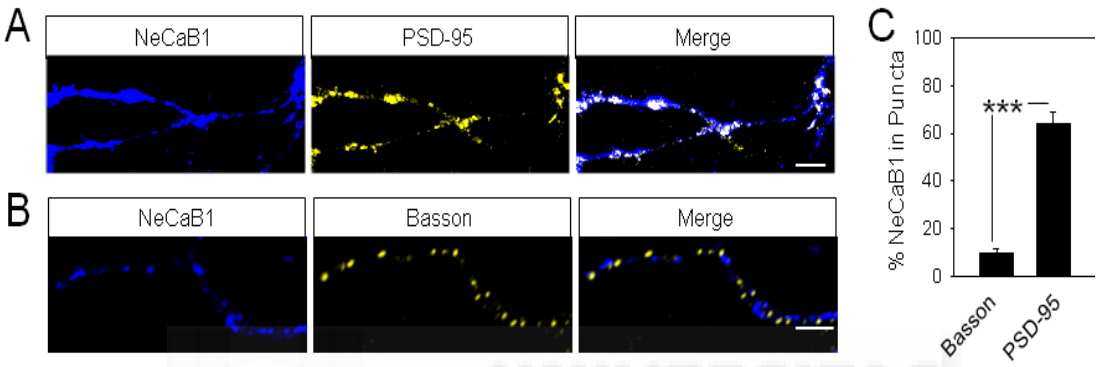

Figure 26. Subcellular localization of NeCaB1 at hippocampal cultures. Immunocytochemical images showing that NeCaB1 co-localized with PSD-95 puncta (functional postsynaptic sites) but not with Basson protein (as a marker of presynaptic sites; A, B). C, Colocalization of NeCaB1 with Basson or PSD-95 puncta is expressed as percent of the total puncta.

11. Discussion.

This work shows the interaction between NeCaB1 and GluK5 subunit of kainate receptors. NeCaB1 interaction with GluK5 takes place on the C-terminal domain and is regulated by Ca^{2+}, in that this ion reduces the interaction between both proteins. Our data highlight NeCaB1 as a trafficking and gating modulator, which augments the number of GluK5 containing kainate receptors at the plasma membrane and at the same time increases the receptor affinity. All these functional consequences are modulated by the intracellular Ca^{2+} concentration. Thus, NeCaB1 may dynamically determine the kind of kainate receptors at synapses according to synaptic activity.

NeCaB1 binds GluK5 C-terminal domain.

Several maneuvers, including co-immunoprecipitation, pull-down assays and Bimolecular Fluorescence Complementation, confirmed that GluK5 C-terminal domain interacts with NeCaB1. In addition, NeCaB1 interaction is independent of the subunits partnering GluK5 subunits, since GluK5 heteromeric kainate receptors interacted with NeCaB1 protein independently of the partner subunit (GluK1 or GluK2). This interaction is modulated by intrinsic factors in GluK5 C-terminal, such as amino acidic sequence. More in detail, a stretch of 30 aminoacids, which contains two different endoplasmic reticulum retention signals consistent in five repetitions of arginine (R) and two consecutive leucines (L) (Ren et al., 2004), is involved in this interaction. NeCaB1 contains one functional Ca^{2+}-binding domain (Sugita et al., 2002). The binding of Ca^{2+} to NeCaB1 seems to disturb its interaction with GluK5. GST pulldown experiments indicated that at nominally free Ca^{+2} concentration (presumably Ca^{2+} free buffer contains endogenous calcium at micromolar concentration), NeCaB1 seemed to have the Ca^{2+} binding site fully occupied, since under this situation interaction with GluK5 was disfavored and no further inhibition was obtained by including Ca^{2+} at 2 mM. On the other hand, efficient removal of free Ca^{2+} by 5 mM EGTA (a soft Ca^{2+} chelator) drastically enhanced the interaction between GluK5 and NeCaB1.

Other NeCaB family member (NeCaB2) has been shown to interact with target proteins in a Ca^{2+} dependent manner; Ca^{2+} disfavoring the interaction. NeCaB2 has been shown to interact with adenosine A_{2A} receptors (Canela et al., 2007) and with mGluR5 receptors (Canela et al., 2009). Hence, one might hypothesize that NeCaB family is capable to bind target proteins in low Ca^{2+} environments. This might imply that after intracellular Ca^{2+} rise, NeCaB1 would dissociate from its target but might bind to others targets, since NeCaB1 also binds C_2A domain of synaptotagmin, requiring some amount of Ca^{2+} (Sugita et al., 2002).

NeCaB1 promotes GluK5 containing KARs to the cellular surface.

We investigated NeCaB1 function on GluK1/5 kainate receptors by co-expressing these subunits in HEK cells. Our strategy to reduce intracellular Ca^{2+} made use of the chelator BAPTA-AM (5 µM) applied one hour before recording. BAPTA-AM is supposed to accumulate inside the cell removing all available free Ca^{2+}. We used the increase of kainate receptor affinity imposed by GluK5 subunit incorporation to the receptor (Alt et al., 2004; Baberis et al., 2008) to discriminate GluK1 homomeric from GluK1/5 heteromeric receptor population. Hence, the ratio between saturating (10 mM) and low (0.1 mM) concentrations of glutamate served as readout of functional heteromeric receptors at the membrane. We observed that NeCaB1 under low intracellular Ca^{2+} concentration increased that ratio. This effect was Ca^{2+} dependent since the expression of NeCaB1 did not have any action in normal Ca^{2+}. Interestingly, including a soft Ca^{2+} chelator in the recording pipette (0.3 mM EGTA) was not enough to reproduce the effect of BAPTA-AM, indicating that 0.3 mM of EGTA is not sufficient to displace Ca^{2+} from NeCaB1 protein.

Structural homology between NeCaB1 and NeCaB2 might suggest a similar role for both proteins. Indeed, NeCaB2 traffics adenosine A_{2A} receptors (Canela et al., 2007) and mGluR5 receptors (Canela et al., 2009) to the plasma membrane. Our immunocytochemical experiments further showed that NeCaB1 increases GluK5 surface expression under low Ca^{2+} conditions. Moreover, biotinylation experiments also indicated an increment of GluK5 protein at the surface triggered by NeCaB1 under low Ca^{2+} concentration but not significantly different under normal Ca^{2+}. NeCaB1 did not have any effect on trafficking under normal Ca^{2+} concentration, arguing that only when Ca^{2+} is reduced NeCaB1 exerts its control over GluK5 containing kainate receptors.

Interestingly, the fact that NeCaB1 remained present in the surface fraction denoted an interaction in the plasma membrane of NeCaB1 and GluK5 functional receptors. This was not seen in the absence of GluK1/5 kainate receptors, discarding an intrinsic effect of NeCaB1, such as other Ca^{2+} binding proteins (e.g. Neuronal Calcium Sensor 1, NCS-1), which can be translocated to the inner surface of the membrane by its myristoyl group (Amici et al., 2009).

NeCaB1 increases affinity of GluK5 containing KARs.

Together with a role in trafficking GluK1/5 kainate receptors, NeCaB1 affects affinity of these heteromeric receptors. We tested this hypothesis constructing an accurate dose-response curve, where both affinity components, one due to the presence of GluK1 homomeric receptors; the other due to the presence of heteromeric, higher affinity, GluK1/GluK5 receptors could be detected. We separated both affinity components by fitting experimental data to two component logistic equation. We defined the low affinity K_d for homomeric GluK1 receptors to a fix value (580 µM, obtained in parallel experiments) and allowed fitting each logistic equation according to their weight (I_{max1} –for low affinity and I_{max2} –for high affinity). An increase of I_{max1} over I_{max2} indicated a larger presence of high affinity receptors at the plasma membrane. Again, this pointed to a role for NeCaB1 in trafficking GluK1/5 kainate receptors under low Ca^{2+} environment. However, at the same time, we observed an evident reduction on the high affinity equation K_d in NeCaB1 expressing cells under low Ca^{2+} concentration. These results led us to conclude that binding to NeCaB1 further increases the affinity of GluK5 containing receptors.

Trafficking and affinity modulation by NeCaB1 are reproduced on GluK2/5 heteromeric KARs.

NeCaB1 interaction with GluK5 did not depend on the partner which makes functional this subunit. Therefore, we made use of GluK2/5 heteromers because the presence of GluK2 made possible the pharmacological differentiation between GluK2 homomers and GluK5/GluK2 heteromers by using ATPA. Using this strategy, we confirmed the trafficking effect of NecaB1 on GluK2/5 kainate receptors under low Ca^{2+} concentration. In this case, BAPTA-AM treated cells (5 µM for 1 hour) were compared with cells that were recorded with a modified recording solution which included 100 nM of free Ca^{2+}. This trafficking assay was performed visualizing the whole surface population of kainate receptors (GluK2 plus GluK2/5 receptors) with 10 mM glutamate and compared with heteromeric receptors activation, activated by 1 mM ATPA.

With the same strategy, we further confirmed the effect of NeCaB1 on the affinity of heteromeric receptors in isolation. Two different ATPA concentrations were used to test GluK2/5 receptor affinity: a saturating concentration of ATPA (1 mM) and a low ATPA concentration (0.05 mM) to show that NeCaB1 increased GluK2/5 receptor affinity in a Ca^{2+} dependent manner.

Rapid modulation of GluK2/5 KARs affinity by NeCaB1.

Bath applied (for 2 seconds) Ionomycin 1 µM was used to allow rapid and specific entry of Ca^{2+} inside the cell (Perney et al., 1984). We compared the effect of Ionomycin for modulation of ATPA affinity over time (30 s, 60s, 2 min and 4 min after Ionomycin). The rapid increase of Ca^{2+} did not have any action on GluK2/5 receptors when NeCaB1 was not expressed, indicating that GluK2/5 receptors lack an intrinsic regulation by Ca^{2+}. On the other hand, when GluK2/5 receptors and NeCaB1 were co-expressed, the rapid increase in intracellular Ca^{2+} significantly reduced the affinity for ATPA at the earliest time point measured (30 s), returning to normal values after 4 minutes. These data demonstrate a rapid modulation of affinity by NeCaB1 that may play a role in modulating kainate receptor affinity by endogenous fluctuations of Ca^{2+}.

Working model for NeCaB1 action on GluK5 containing KARs.

Even though we are not working at physiological Ca^{2+} concentration, we demonstrate that NeCaB1 modulates trafficking and affinity in a Ca^{2+} dependent manner. Trafficking modulation by NeCaB1 seems to occur as a long lasting effect. In contrast, the modulation of receptor affinity seems to occur rapidly and reversibly (figure 25).

We propose that trafficking and affinity of heteromeric GluK5 containing receptors are favored under a situation where intracellular Ca^{2+} is low, a scenario that could happen under scarce synaptic activity. Therefore, GluK5 containing kainate receptors in the intracellular stores could be trafficked to the surface by the effect of NeCaB1, which by remaining bound to GluK5 increases the affinity of the complex for the ligand. These two effects would work towards the same direction: to increase the synaptic sensitivity to neurotransmitter. Upon an increase in intracellular Ca^{2+} signal (e.g. as a consequence of increased synaptic activity), NeCaB1 will unbind from GluK5 containing receptor, decreasing the affinity of them for the agonist and at the same time disfavoring the insertion in the membrane of GluK5-containing receptors, further decreasing the sensitivity to the neurotransmitter (Figure 27). This may represent a homeostatic mechanism for regulation of synaptic sensitivity (See figure 28).

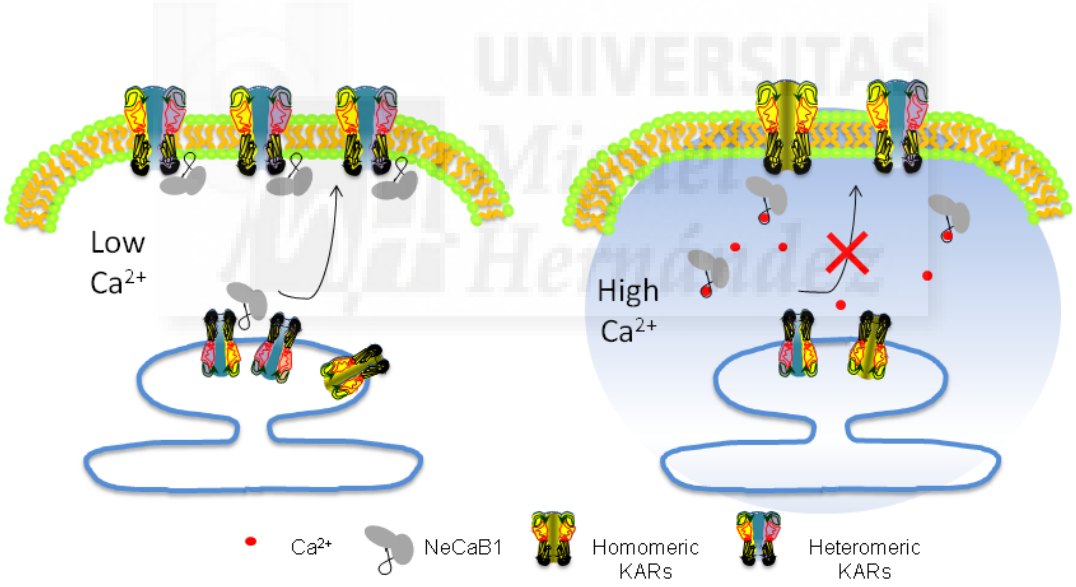

Figure 27. Working model of NeCaB1 over GluK5 containing KARs at low and high Ca^{2+}. NeCaB1 traffics GluK5 containing KARs from the endoplasmatic reticulum to the membrane and increase glutamate affinity of surface receptors. These modulations occur at low Ca^{2+} environment, where NeCaB1 does preferentially interact with GluK5. Cytoplasmatic Ca^{2+} increase, avoid NeCaB1 binding to GluK5 and thus avoid trafficking and affinity modulation over KARs. This mechanism allows increasing heteromeric high affinity GluK5 containing KARs depending on intracellular Ca^{2+} levels.

Whereas, it is easy to propose that through the interaction with retention signals in the C-terminal of GluK5, NeCaB1 could regulate receptor trafficking, it is difficult to figure out how an interaction in the C-terminal region is able to modulate receptor affinity. Interestingly, it has been shown that Calmodulin, a well known Ca^{2+} binding protein, is able to modulate NMDA receptors gating interacting with the C-terminal region of GluN1 subunits (Ehlers et al., 1996). Similarly, Calmodulin has been shown to interact with $Ca_v1.4$ carboxy terminal domain, depressing inactivation of Cav1.4 chanels. How NeCaB1 could regulate GluK5 containing kainate receptor gating remains to be determined.

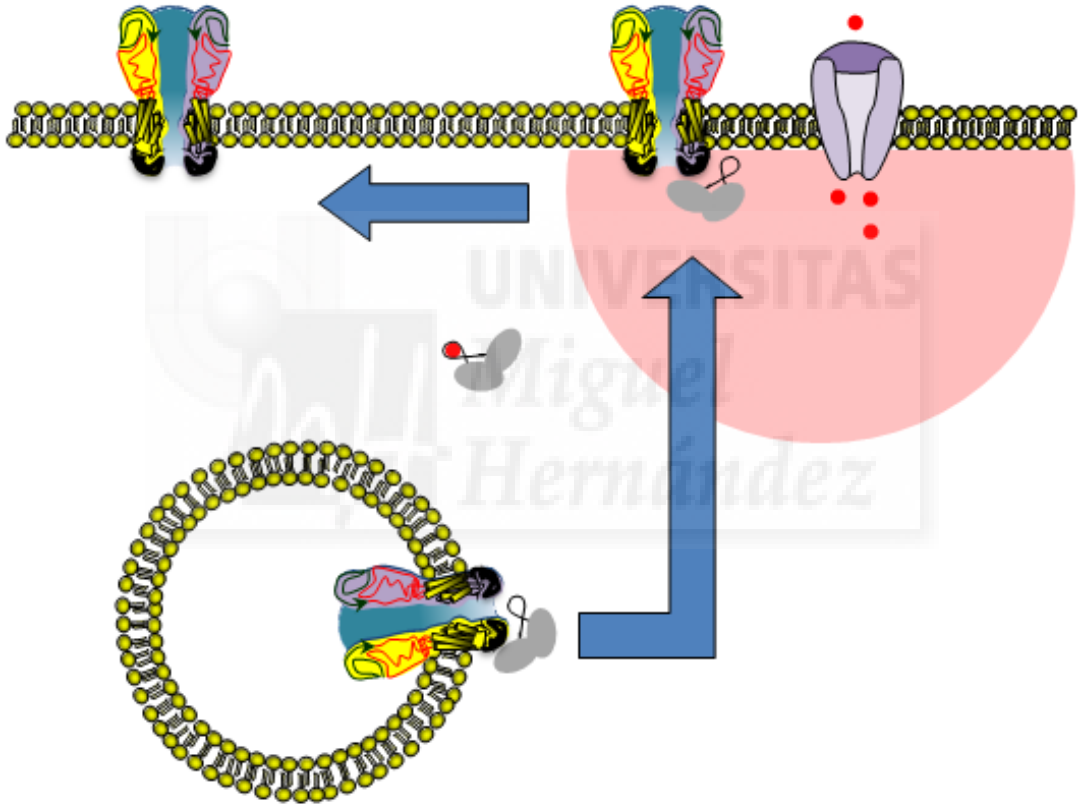

Figure 28. Activity dependent trafficking of KARs may form part of a homeostatic mechanism. Ca^{2+} depletion favors NeCaB1 interaction with the C-terminal domain of GluK5 containing kainate receptors. This interaction traffics receptors to the plasma membrane and increases agonist affinity, favoring activation of kainate receptors. Upon activation of Ca^{2+} permeable channels, NeCaB1 unbinds from kainate receptors in the membrane, decreasing affinity of receptors. Thus initial sensitivity for glutamate is restored.

Other Ca^{2+} binding proteins have been described to modulate kainate receptor trafficking, such as, neurocalcin δ (Coussen et al., 2006), Visinin-like proteins (Coussen and Mulle, 2006) and CAMKinase II (Carta et al., 2013). Interestingly, CAMKinase II is expressed by principal cells within the hippocampus and phosphorylates GluK5 subunit in a Ca^{2+} dependent manner to uncouple these receptors from PSD-95 proteins, enhancing receptor mobility. This phosphorylation promotes the movement of GluK5 containing receptors out of the synaptic active zone, reducing the abundance of kainate receptors at the synapse (Carta et al., 2013).

IV. Results II. Similarities and dissimilarities of KARs tethered to Neto proteins.

Recently, the existence of two membrane integral proteins have been described and it seems that they correspond to true ancillary proteins of kainate receptors (Zhang et al., 2009; Straub et al., 2011; Tang et al., 2011). Neurophilin Tolloid-like 1 and 2 (Neto1 and Neto2) are auxiliary proteins of native kainate receptors that exert an important influence on their function and that impart differential properties on these receptors, which could account for a number of previously unexplained characteristics of these receptors (see Copits and Swanson, 2012; Lerma, 2011; Tomita and Castillo, 2012 for recent reviews). Neto1 was first identified as a protein that interacts with the NMDA receptor (Ng et al, 2009), although a number of studies then illustrated that it has a more striking influence on the function of kainate receptors.

1. Neto proteins differentially express during development.

Neto1 and Neto2 share an identical and unique domain structure, representing a novel subfamily of transmembrane proteins containing CUB (complement C1r/C1s, Uegf, Bmp1) and LDLα- (Low-density Lipoprotein) domains. It has been shown that CUB domain containing proteins are developmentally regulated (Bork and Beckmann, 1993). Therefore, we first examined the expression of Neto 1 and Neto2 in the brain during mouse embryogenesis (E12 and E18) and in postnatal development (P0 to P29; figure 29) by real time PCR. Values were corrected by the expression of ciclophylin (a house keeping gene). Neto1 showed a delayed expression profile with a peak of expression at P14,

which declined subsequently to steady levels (ca. 25%). On the other hand, Neto2 was expressed as early as E12, with a peak of expression at P0 that was maintained until P14. Both proteins showed a reduced expression in adult brain that may be ascribed to the restricted expression to some brain areas (these experiments were done by Dra. Isabel Aller in the laboratory).

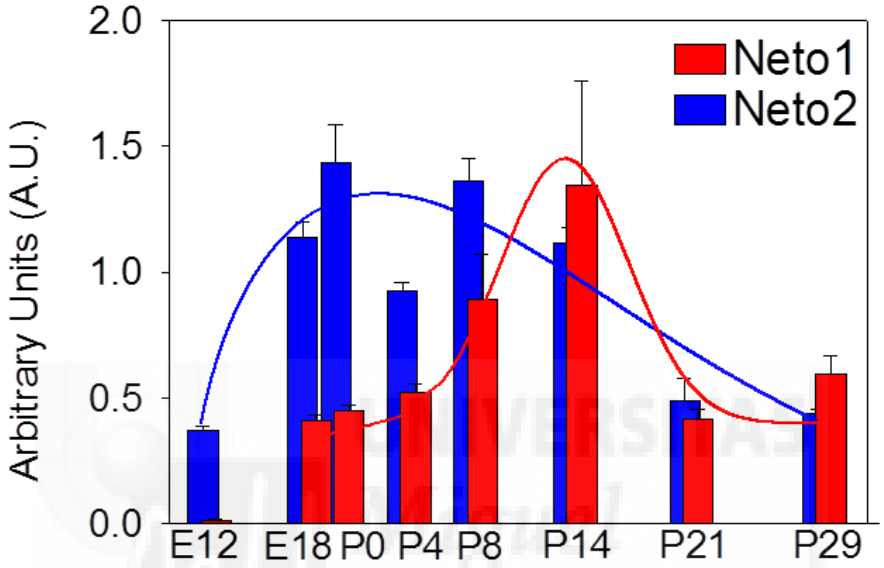

Figure 29. Normalized Neto1 and Neto2 gene expression level during development in mice. Quantitative PCR analysis of Neto1 and Neto2 mRNA at different key developmental stages: Embryonic states E12-E18 and P0, P4, P8, P14, P21 and P29 postnatal mouse brain. Values in bars were fitted (lines plot) using a log normal peak equation.

2. Neto1 and Neto2 differentially modulate KAR gating.

It has been shown that Neto1 and Neto2 are able to bind and modify GluK1 and GluK2 receptors gating. We transfected HEK293 cells (with and without Neto1 or Neto2) to check if these auxiliary subunits influence GluK1-3 kainate receptor subunits. Neto1 coexpression induced the presence of larger amplitudes mediated by kainate receptors in GluK1 expressing cells (280.35% average increase; from 28.5 ± 5.2 pA/pF, n=17 without to 79.9 ± 18.5 pA/pF, n=14, with Neto1) and speeded up desensitization rate. Neto2 also increased current responses (624.84%; to a value of 178.08 ± 19.75 pA/pF, n=22) but slowed down desensitization rate (figure 30 A, B). Desensitization was fitted by

two exponentials and the weigthened time constant (τ) was calculated. This was 67.8 ± 12.9 ms (n=17) in GluK1 expressed alone, while the combination with Neto1 accelerated desensitization to 35.4 ± 7.5 ms (n=14; p<0.05). In contrast, Neto2 dramatically slowed desensitization to 439.1 ± 71.4 ms of tau (n=22; p<0.005). The change in gating properties could account for the observed change in amplitude. To examine this possibility, we made a plot (figure 30 C) to look at the relation between amount of current and amplitude. As can be seen, whilst Neto1does not fit to the diagonal line, Neto2 does.

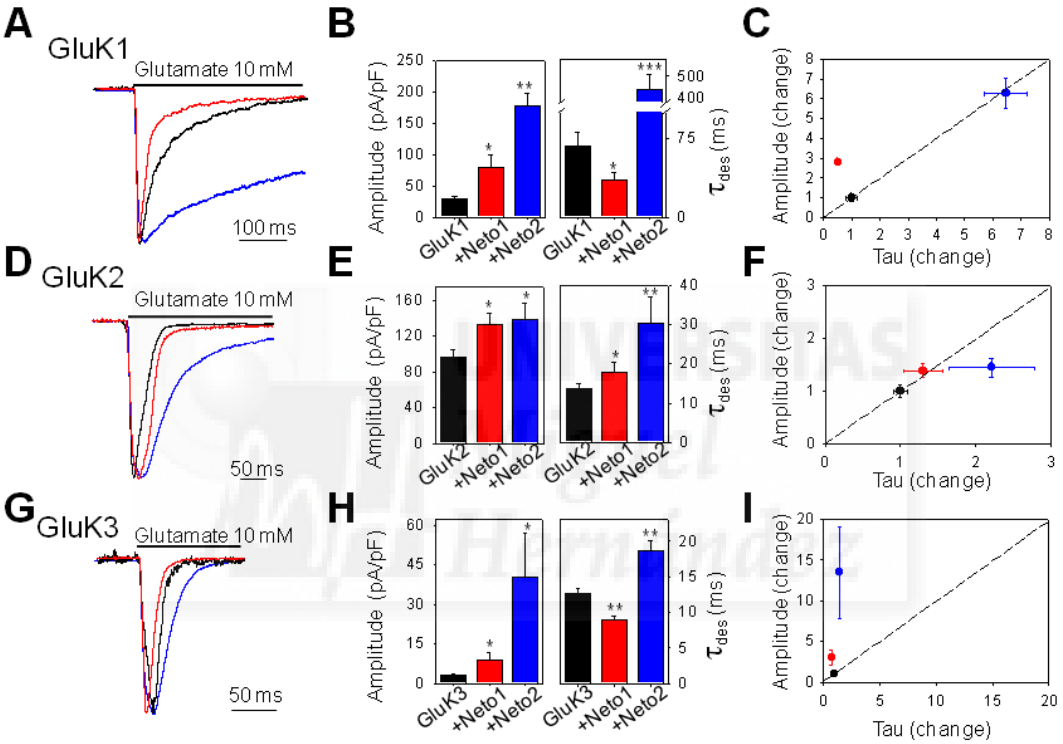

Figure 30. KARs amplitude and desensitization rate modification by Neto auxiliary subunits. Responses elicited by glutamate (10 mM, 500 ms) in HEK 293 cells transfected with different KARs subunit plus Neto1 or Neto2. Traces in A, D and G correspond to GluK1-3 with and without Neto proteins after normalization. Current amplitude is measured as pA/pF, the normalized units of current to cell size (pF). B, E and H represent quantification of current amplitude and desensitization rate of GluK1 (n≥8), GluK2 (n≥5) and GluK3 (n≥7) respectively. C, F, and I, show the relation between desensitization and current amplitude change, normalize to KARs without Neto proteins. In black, KARs subunit GluK1-3; in red, GluK1-3 & Neto1 and in blue, GluK1-3 & Neto2. Data are shown as mean + SEM. *p<0.05; **p<0.01; ***p<0.005.

Kainate receptors made of GluK2 subunits showed a milder modification in channel gating when coexpressed with Neto proteins. With either Neto1 or Neto2, GluK2 kainate receptors displayed bigger current amplitude and slower desensitization rate (figure 30 D, E). In these responses, desensitization was well fitted by a single exponential process (τ=13.7 ms ± 1.2 ms in control, n=9), 17.9 ± 2.5 ms with Neto1 (n=11; $p<0.05$), and 30.2 ± 7.8 ms with Neto2 (n=12; $p<0.01$). The presence of Neto1 enhanced amplitude according to the change in desensitization kinetics, while Neto2 did not (figure 30 F).

Homomeric kainate receptors composed of GluK3 subunit, were also modulated by Neto1 and Neto2. While Neto1 notably increased current amplitude from 3 ± 0.6 pA/pF (n=8) to 9.1 ± 2.7 pA/pF, (n=5: $p<0.05$), it slightly accelerated desensitization rate (from 12.5 ± 0.7 ms to 9.1 ± 0.5 ms, n=5: $p<0.01$). On the other hand Neto2 dramatically increased the response amplitude (to 40.3 ± 16.7 pA/pF .n=8; $p<0.05$) and decreased desensitization rate (τ=18.7 ± 1.4 ms, n=8; $p<0.01$) (figure 30 G, H). The increase in current amplitude could not be explained by the change desensitization kinetics (figure 30 I).

Taken together, these data indicate that Neto1 and Neto2 functionally interact with the three major kainate receptor subunits (GluK1-3), altering degree of response and desensitization properties, although, these modifications in biophysical properties are dissimilar in the three receptor subunits studied.

3. Neto proteins increase membrane KARs

Increased amplitude of kainate receptors tethered with Neto proteins could be explained not only by the change in desensitization kinetics, but also by the increase in receptor number at the cell surface. For that purpose, biotinylation experiments were performed to determine the insertion of kainate receptors in the cell surface, with and without Neto proteins.

All three kainate receptors (GluK1-3) exhibited increased membrane insertion when Neto proteins were co-expressed. Non-biotinylated kainate receptors were taken as control and the change in the ratio (surface/total) was plotted. The specificity of biotinylation for the membrane fraction was evaluated by the

absence of GAPDH in this fraction. For GluK1, Neto1 increases the ratio by 2.28 ± 0.68 fold (n=4; p<0.05; Student T-test) and Neto2 by 2.54 ± 0.79 fold (figure 20A, B; n=4; p<0.05; Student T-test). GluK2 containing kainate receptors increased the ratio by 2.15 ± 0.96 fold (n=5; p<0.05; Student T-test) when co-expressed with Neto1 and 1.77 ± 0.36 fold with Neto2 (figure 20 C, D; n=5; p<0.05; Student T-test). In the case of GluK3, Neto1 also increased the ratio by 1.67 ± 0.23 fold (n=5; p<0.05; Student T-test) and Neto2 by 2.68 ± 0.56 fold (figure 31 E, F; n=5; p<0.05; Student T-test).

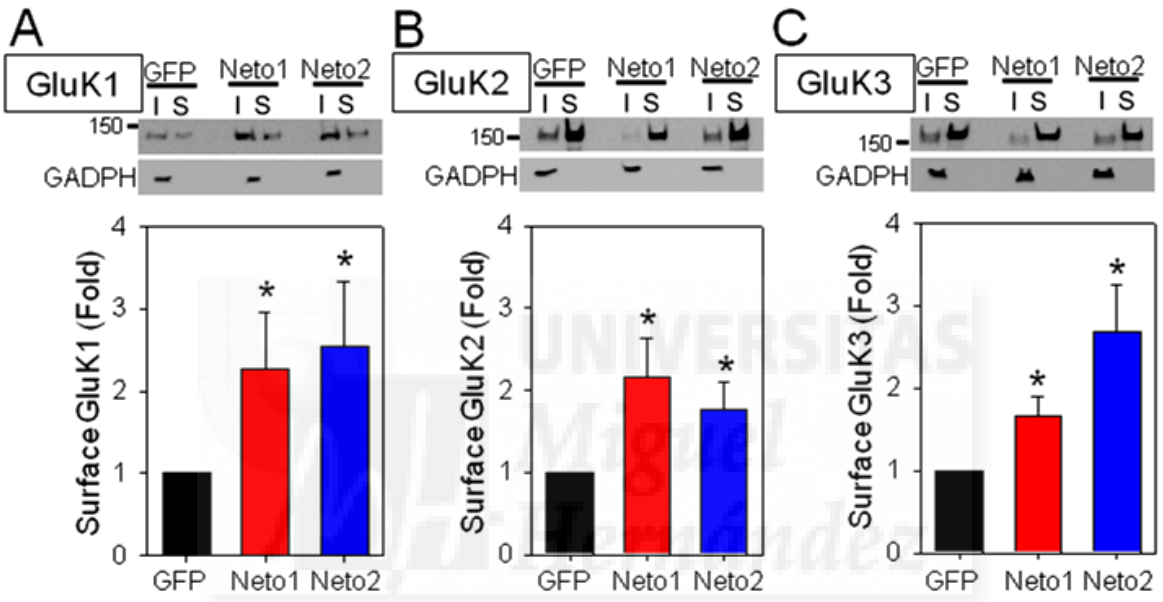

Figure 31. Neto1 and Neto2 increase the surface expression of KARs. HEK cells where transfected with GluK1-3 subunits KARs and probed the surface expression (S) level when coexpress with Neto1 or Neto2. A, C, and E show western blots of biotinylated GluK1-3 homomeric KARs, where 5% of the input (I) was loaded as quantification control. The density of the biotinylated band was compared with the input fraction to calculate amount of receptors at the cell surface. Experimental data (n≥4) show the action of Neto1 and Neto2 related to receptor without the auxiliary subunits. In black, KARs subunit GluK1-3; in red, GluK1-3 & Neto1 and in blue, GluK1-3 & Neto2. Data are shown as mean + SEM. *p<0.05; Student T-test.

These experiments indicate a role for Neto proteins in increasing the membrane abundance of kainate receptors in the plasma membrane.

4. Neto proteins increase affinity of KARs.

It is well known that reconstituted kainate receptors in HEK cells do not yield the same agonist affinity than native receptors (Lerma, 1997). To assess whether glutamate affinity of kainate receptors was altered in the presence of Neto proteins, we constructed dose response curves for the three types of receptor when expressed alone or in combination with either Neto1 or Neto2. We observed that there was a general action of Neto proteins increasing kainate receptors affinity, although no to the same degree among the different types (figure 32 B, D and F). GluK1 homomeric receptors revealed an EC_{50} of 580 ± 74 µM (data from 8 cells) while in combination with Neto1 it was 14 ± 28 µM (5 cells) and 56 ± 38 µM (9 cells) with Neto2 (figure 32 A, B). Neto proteins also produced an affinity increase of GluK2 subunits. While the homomeric form presented an EC_{50} of 603 ± 82 µM (7 cells), Neto1 reduced it to 208 ± 91 µM (4 cells) and Neto2 to 139 ± 63 µM (9 cells) (Fig.32 C, D). Furthermore, GluK3 subunit showed a very low glutamate affinity and even at 30 mM glutamate, it could not reach saturation. Therefore, it was not possible to calculate an accurate EC_{50}. However, Neto1 and Neto2 were able to significantly shift to the left the dose response curve, indicating an increase in apparent affinity (figure 32 E, F).

This data indicate that Neto proteins increase the affinity of kainate receptors for their endogenous agonist, glutamate.

5. Neto proteins alter desensitization recovery rate of KARs.

The recovery rate from desensitization may play a crucial role in receptor function at the plasma membrane. To evaluate the effect of Neto proteins on this aspect, we studied the recovery of kainate receptors desensitization from a single 500 ms, 10 mM glutamate pulse.

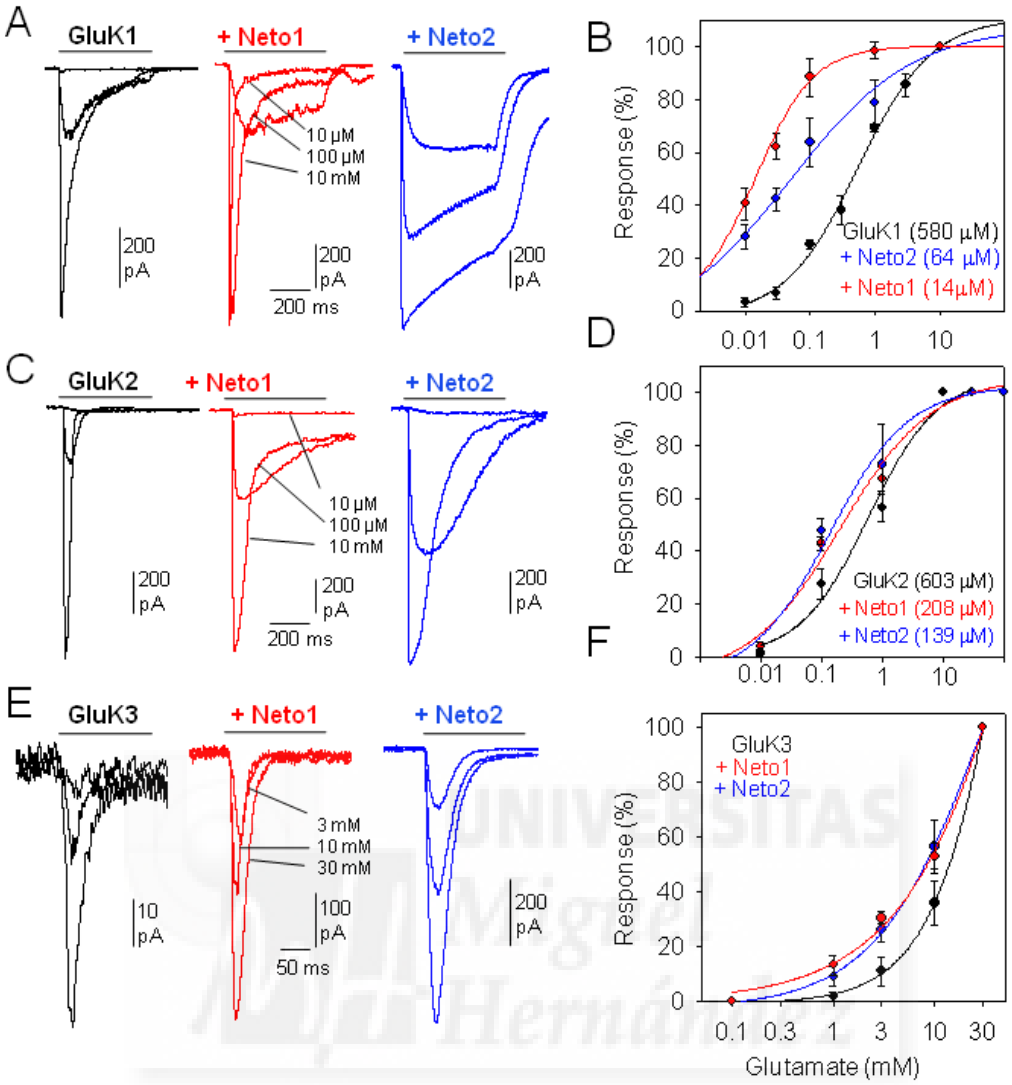

Figure 32. KARs affinity is modified by co-expression of Neto proteins. HEK cells were transfected with GluK1-3 and Neto1 or Neto2. Dose response curves were calculated in each case fitting it to a logistic equation. A, C and E show traces for GluK1-3 KARs subunit alone or in combination with Neto1 or Neto2 of different glutamate concentration (10 mM, 100µM and 10 µM for GluK1 & GluK2; 30 mM, 10 mM and 3 mM for GluK3 containing KARs complexes). B, D and F show dose response curves for GluK1, GluK2 ad GluK3, respectively, where Neto1 and Neto2 increase affinity of KAR complexes. In the case of GluK3 Ec50 was not calculated due to 30 mM of glutamate was not saturating concentration of this low affinity receptor. In black color GluK1-3 receptor without auxiliary proteins. Red and blue correspond to the action of Neto1 and Neto2, respectively.

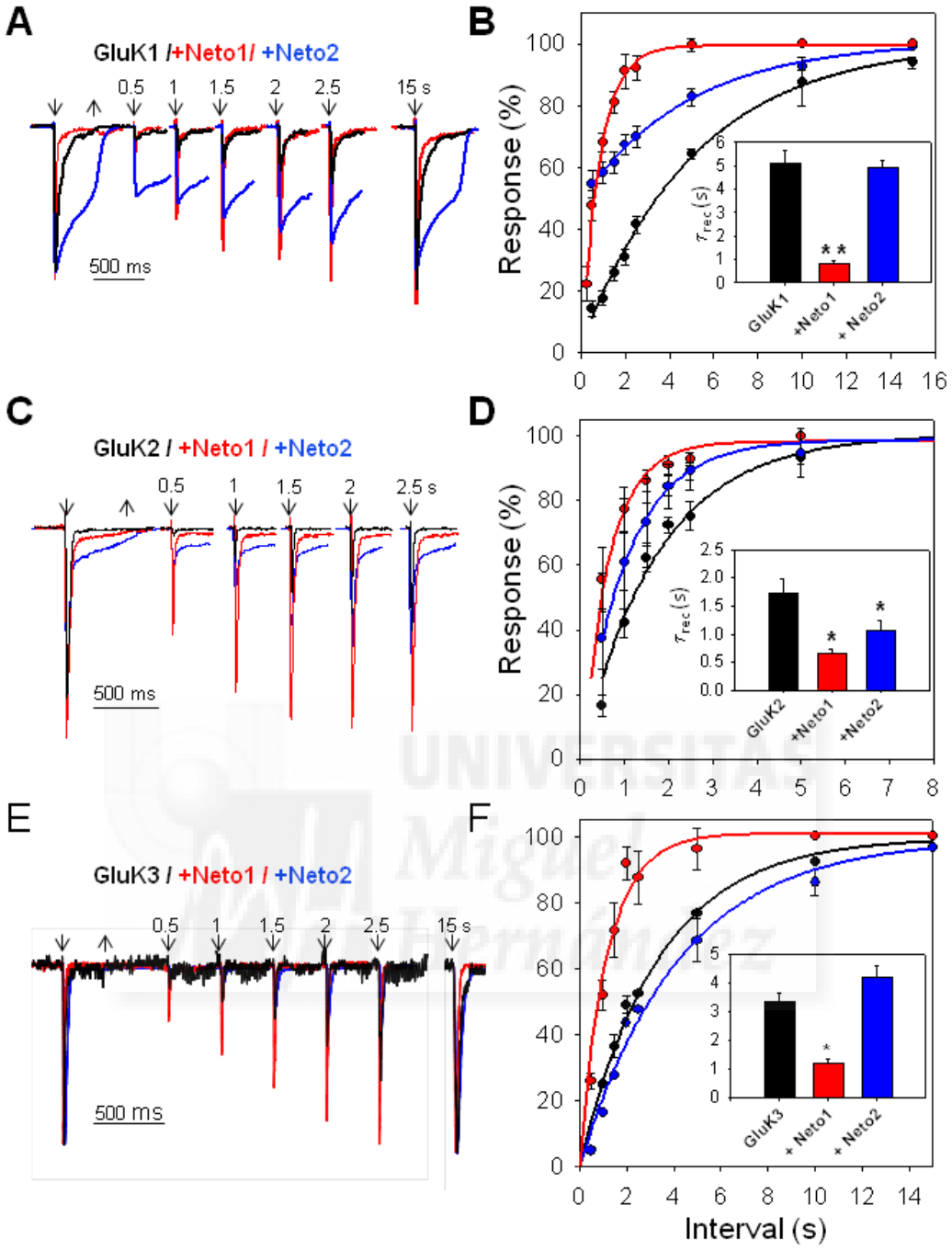

Figure 33 . Variable effect of Neto proteins in KARs recovery rate. Recovery from desensitization was measured in HEK cells transfected with KAR plus Neto proteins. KARs complexes were activated by 10 mM and 500 ms pulse of glutamate at different intervals. A, C and E traces from GluK1-3 KAR (black) superimposed with Reponses obtained when co-expressing Neto1 or Neto2 (red and blue, respectively). Responses to glutamate were evoked with a 500 ms intervals and range from 500 ms to 5 s or longer. B, D and F plots measuring kinetics of recovery from desensitization, fitted to exponentials to measure the time constant (τ). Data are shown as mean + SEM. *p<0.05; **p<0.001; Student T-test.

GluK1 recovery from desensitization was strongly modulated by Neto1, but not altered by Neto2 (figure 33 A, B). Desensitization recovery was fitted to single exponentials with time constants of 5.1 ± 0.5 s (n=9), 1.7 ± 0.2 s (n=6), and 3.4 ± 0.3 s (n=3) for GluK1, GluK2 and GluK3, respectively (figure 33 B, D, F). Neto1 accelerated recovery of desensitization for all these receptors (0.8 ± 0.1 s, n=7, $p<0.01$; Student T-test; 0.7 ± 0.1 s, n=7, $p<0.05$; Student T-test and 1.2 ± 0.1 s, n=3, $p<0.05$; Student T-test, respectively for Neto1 plus GluK1, GluK2 and GluK3). In contrast, Neto2 did not alter GluK1 (4.9 ± 1.3 s, n=14), or GluK3 (4.2 ± 0.4 s, n=3) desensitization recovery rate, but accelerated GluK2 recovery (1.1 ± 0.2 s, n=6, $p<0.05$; Student T-test), as Neto1 did.

These results indicate a conserved action for Neto1 accelerating recovery from desensitization of all kainate receptors, an effect that is similar for Neto2 on GluK2, whereas Neto2 did not alter this parameter in GluK1 or GluK3 receptors.

6. Neto proteins reduce sodium dependence gating.

Kainate receptors channel gating exhibit sensitivity to different ions (Paternain et al., 2003). This is particularly remarkable for sodium ions (Na^+) in that in the absence of Na^+, channel does not gate. Crystallographic studies have related such dependence with agonist stability at the binding domain and dimmer interface stabilization (Plested and Mayer, 2007). Indeed, GluK2/5 heteromeric receptors exhibit less sodium dependence due to the biophysical properties added to the heteromeric complex by GluK5 subunit (Paternain et al., 2003). Since Neto proteins seem to introduce drastic conformational changes on the receptor structure, we wanted to assess whether these proteins, which are likely a part of the in vivo receptor complex, would alter the sodium dependence of kainate receptors gating. For that reason, extracellular Na^+ was totally replaced by Cs^+ and response amplitudes compared in both conditions.

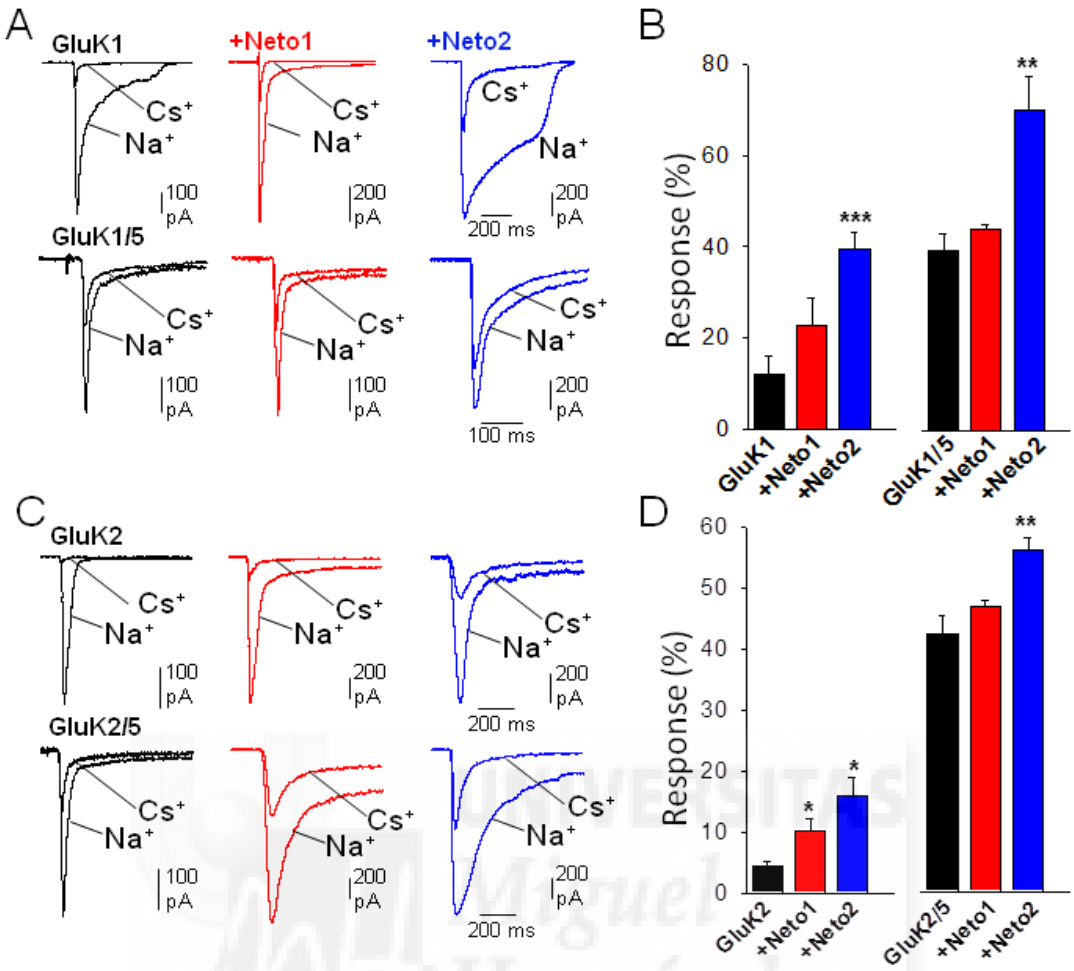

Figure 34. Neto proteins modify Na^+ dependence for channel gating of different KARs when evoked with glutamate. HEK cells transfected with KARs were evaluated by Cs^+ permeability in a Na^+ free external solution in the presence or absence of Neto1 or Neto2. Glutamate (10 mM) elicited responses were plotted over Na^+ containing external solution. A and C show examples of traces of GluK1 and GluK2, in homomeric or GluK5 heteromeric complexes. B and D show the quantification of Cs^+ permeability (%) over a Na^+ containing external solution for GluK1 or GluK2 containing KARs respectively. KARs without Neto proteins black; in combination with Neto1 red and with Neto2 in blue. Data are shown as mean + SEM, where n≥4. *p<0.05; **p<0.001.;***p<0.0005; Student T-test.

In the absence of Na^+, GluK1 homomeric receptors exhibited a small response to glutamate (12.2 ± 4 % of the current in Na^+, n=6). This remaining current was increased when GluK1 was co-expressed with GluK5 subunit (39.5 ± 3.5 %, n=8: p<0.005; Student T-test) (figure 34 A, B) and similar reductions were observed by activating the receptor with kainate (figure 35 A, B). Neto1 did not

modify sodium dependence of gating of GluK1 homomeric (22.7% ± 6.1% n=7) or GluK1/5 heteromeric receptors (44.2 ± 0.7 %) (figure 34 A, B). Similar results were obtained with kainate as an agonist (figure 35A, B). In contrast, Neto2 alleviated the sodium dependence (39.6 ± 3.6 %, n=8: $p<0.005$; Student T-test) of GluK1 homomeric receptors and heteromeric GluK1/GluK5 receptor (70.1 ± 7.2 %, n=6, $p<0.01$) (figure 34 A, B). Similar results were observed with kainate (figure 35 A, B).

For GluK2 subunits, both Neto1 and Neto2 attenuated sodium dependency when receptors were activated by glutamate (figure 34 C, D) (4.4 ± 0.8 %, n=6, for GluK2 without Neto proteins and 10.2 ± 1.9, n=6, $p<0.05$ and 15.2 ± 3.2 %, n=6, $p<0.05$; Student T-test, for Neto1 and Neto2, respectively). GluK2 heteromerization with GluK5 significantly increased responses in the absence of Na^+ (Paternain et al, 2003) (42.2 ± 2.8%, n=3) and co-expression with Neto1 did not alter remaining current (46.7± 0.9 %, n=3) whilst co-expression with Neto2 slightly increased it (55.01 ± 3.7 %, n=3: $p<0.01$) (figure 34 C, D). Interestingly, when GluK2 containing kainate receptors were activated by kainate (50 µM), homomeric receptors displayed less sodium dependency when combined with Neto proteins (figure 35 C, D) (3.8 ± 1.1%, n=4) for GluK2 alone; 13.4 ± 2.1 %, n=4, $p<0.01$, for Neto1 and 20.9 ± 3.1 %, n=4, $p<0.01$; Student T-test, for Neto2). Similarly, heteromerization with GluK5 produced a similar effect (20.4 ± 1 %, n=4, for GluK2-5 without Neto and 26.6 ± 5.3 %, n=4, $p<0.05$ with Neto1 and 46.3 ± 16.1 %, n=4, $p<0.05$; Student T-test, with Neto2 (figure 35 C, D).

These results indicate a similar action of Neto proteins on different types of homomeric kainate receptors, in that association with Neto 1 and Neto2 reduce Na^+ requirements for channel gating in GluK1 and GluK2 subunits, although to different magnitudes. In most cases, some effect of Na^+ removal was also observed in kainate receptors containing GluK5 subunit, although the heteromerization totally occluded the action of Neto1 on both GluK1/GluK5 and GluK2/GluK5 receptors.

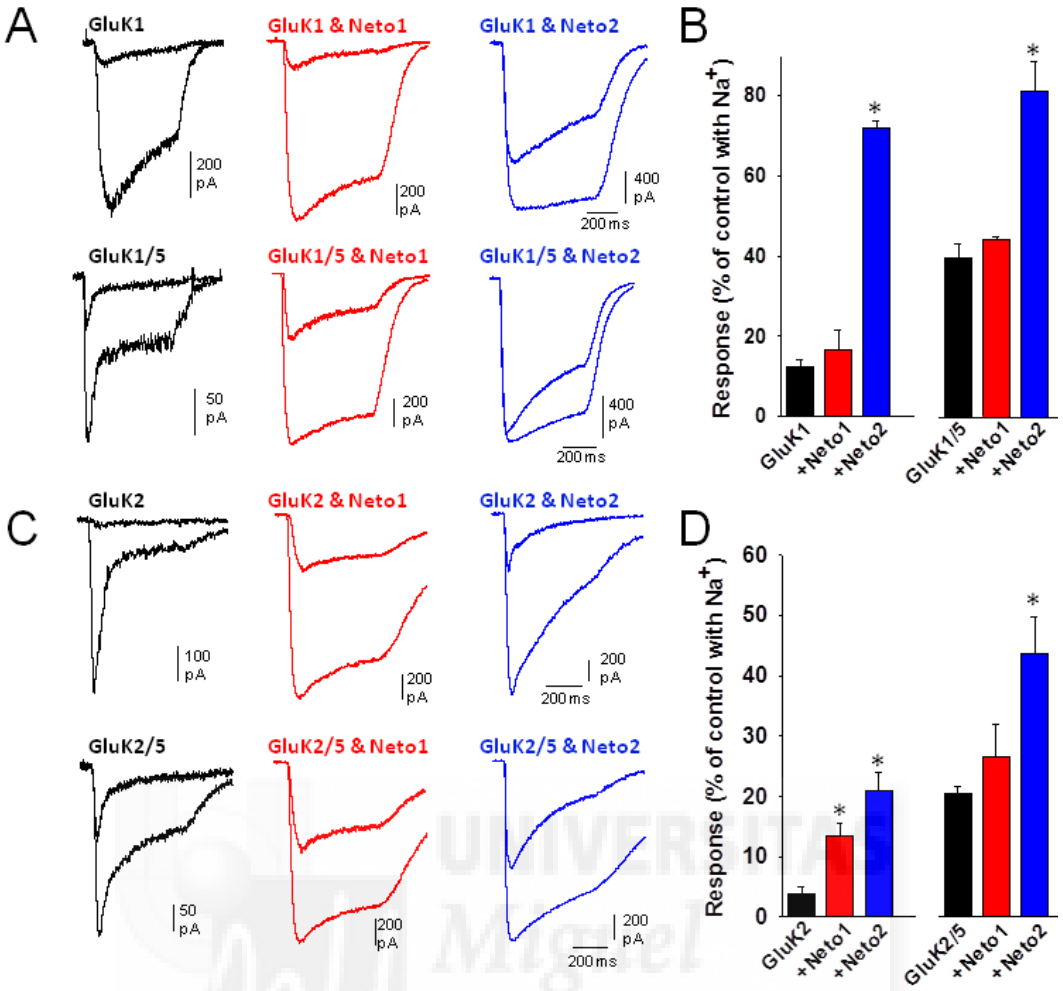

Figure 35. Neto proteins modify Na^+ dependence for channel gating of different KARs when evoked with Kainate. HEK cells transfected with KARs were evaluated by Cs^+ permeability in a Na^+ free external solution in the presence or absence of Neto1 or Neto2. Kainate (50 µM) elicited responses were plotted over Na^+ containing external solution. A and C show examples of traces of GluK1 and GluK2, in homomeric or GluK5 heteromeric complexes. B and D show the quantification of Cs^+ permeability (%) over a Na^+ containing external solution for GluK1 or GluK2 containing KARs respectively. KARs without Neto proteins black; in combination with Neto1 red and with Neto2 in blue. Data are shown as mean + SEM, where n≥4. *p<0.05; **p<0.001.;***p<0.0005; Student T-test.

7. Neto proteins drastically alter steady state desensitization of KARs when activated by kainate.

To further analyze the effect of kainate agonist on Neto protein containing kainate receptors, we probed kainate 50 µM as an agonist. We realized that the desensitization was variable depending on the presence of Neto1 or Neto2. We measured the ratio of the steady-state to the peak current under different conditions (figure 36 A, B). Thus, GluK1 homomeric kainate receptors exhibited a ratio of 0.58 ± 0.03. The presence of Neto1 increased the steady-state current, yielding a ratio of 0.75 ± 0.04 (n=4; p<0.005; Student T-test) and a ratio of 0.9 ± 0.02 for Neto2 (n=4; p<0.005; Student T-test). The presence of the GluK5 subunits also altered the steady-state, reducing it to a ratio of 0.36 ± 0.037 in the case of GluK1. Neto proteins had a similar effect on heteromeric receptors, increasing the steady-state to peak ratios to 0.79 ± 0.07 (n=4; p<0.005; Student T-test) for Neto1 and 0.79 ± 0.027 (n=4; p<0.005; Student T-test) for Neto2.

GluK2 homomeric kainate receptors presented a small steady-state to peak ratio (figure 36 C, D), 0.12 ± 0.02 and this was increased by both Neto1 to 0.4 ± 0.07 (n=4; p<0.01; Student T-test) and Neto2 to 0.39 ± 0.03 (n=4; p<0.005; Student T-test). GluK2/5 heteromeric kainate receptors, the major type of postsynaptic kainate receptors in the hippocampus, also presented modulation of the steady-state by Neto proteins from 0.21 ± 0.05 for naïve receptors to 0.76 ± 0.06 (n=4; p<0.005; Student T-test) for Neto1 and to 0.42 ± 0.05 (n=4; p<0.005; Student T-test) for Neto2.

This data indicate that kainate-induce steady-state currents are dramatically increased by Neto proteins, being this effect more evident for heteromeric kainate receptors.

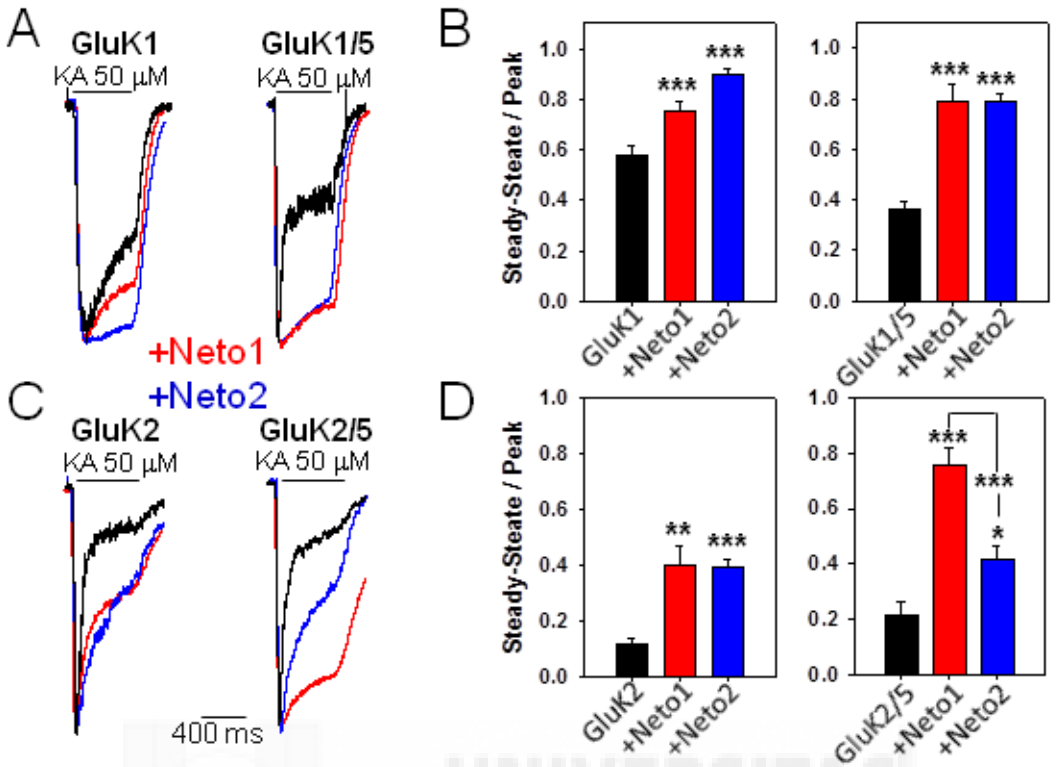

Figure 36. Kainate mediated action on Neto protein containing KARs. HEK cells transfected with GluK1 or GluK2 and the heteromeric forms with GluK5 subunits were probed with 50 µM of Kainate. Steady-state currents were analyzed after 0.5 second sustained pulse. A and B, show current elicited by 50 µM Kainate and the quantification for the steady-state ratio over the peak current for GluK1 subunits, Neto1, Neto2 and the heteromeric forms with GluK5 subunits. C and D, for GluK2 and the effect of Neto1-2 and the heteromeric KARs with GluK5 subunits. Gluk1 is presented in black, Neto1 in red and Neto2 in blue. Data are shown as mean + SEM, where n≥4. *p<0.05; **p<0.01.;***p<0.005.

8. Discussion.

We have studied the biophysical properties of kainate receptors and their modification induced by the interaction of these receptors with Neto proteins. We also determined a difference in expression time between Neto1 and Neto2 in that Neto2 mRNA had an earlier expression (with a peak in P0 and sustained levels until P14; Figure 18) while Neto1 mRNA peaked at P14 (Figure 29). These differences may indicate a possible role during developmental

processes, such as, growth cone motility or path finding and control of neuronal activity during development (Ibarretxe et al., 2007; Tashiro et al., 2003; Marques et al., 2013) for Neto 2, while Neto1 might participate in synaptogenesis and circuit maturation (Segerstrale et al., 2010), activities in which kainate receptors has been already implicated.

We demonstrated that Neto1 and Neto2 combine with the three major kainate receptors subunit (GluK1-3) and we assessed the functional alteration of biophysical properties of these receptors when they associate to Neto proteins (in table 4 is a summary of Neto1 and Neto2 actions on GluK1-3 kainate receptors). In agreement with previous data, we found that Neto1 accelerates desensitization rate of GluK1 while slowed-down GluK2 desensitization and that Neto2 reduces desensitization rate of GluK1, GluK2 (Zhang et al., 2009; Copits et al., 2011; Straub et al., 2011). Similarly to GluK1, Neto proteins have a dual modulation of GluK3, accelerating desensitization in the case of Neto1 and decreasing its rate when associated with Neto2 (figure 30). In all cases Neto1 and Neto2 showed conserved action incrementing current amplitude of kainate receptors. When amplitude and desensitization are compared, we saw a variety of correlations, suggesting that the amplitude increase is not due to the change in desensitization rate (figure 30 C, F and I). Rather, we propose that Neto1 and Neto2 increase the current responses because they promote the membrane insertion of kainate receptors (figure 31). The increased density of kainate receptors in the cell membrane might be due to either larger trafficking to the membrane or increased stabilization of membrane receptors. The PDZ binding domain located on the C-terminal region of Neto1 might account for receptor stabilization at the membrane. Accordingly Neto2, which lacks a PDZ binding domain, has been shown to increase receptor stability in the membrane through its interaction with Glutamate Receptor Interacting Protein 1 (GRIP1) in the cerebellum (Tang et al., 2012).

Table 4. Effects of Neto1 and Neto2 on recombinant kainate Receptors

	Current Amplitude (% from naïve)		Desensitization						Agonist Affinity (Kd, µM)		
			Onset time constant (ms)			Recovery time constant (s)					
	Neto1	Neto2	Naïve	Neto1	Neto2	Naïve	Neto1	Neto2	Naïve	Neto1	Neto2
GluK1	282 ±65 n=14	573 ±63 n=21	67.8 ±12.9 n=17	35.4 ±7.4 n=14	439.1 ±71.5 n=21	5.1 ±0.5 n=9	0.9 ±0.1 n=7	4.9 ±0.3 n=14	485 ±74 n=8	14 ±7 n=5	64 ±28 n=9
GluK2	143 ±13 n=9	146 ±18 n=11	13.7 ±1.2 n=9	17.9 ±2.6 n=11	30.4 ±6.8 n=12	1.7 ±0.2 n=6	0.6 ±0.1 n=7	1.1 ±0.2 n=6	603 ±29 n=7	208 ±81 n=4	139 ±62 n=9
GluK3	303 ±92 n=5	1348 ±535 n=8	12.6 ±0.7 n=8	9.1 ±0.5 n=5	18.7 ±1.4 n=8	3.4 ±0.3 n=4	1.2 ±0.1 n=4	4.2 ±0.4 n=3	>1000 n=4	< Naïve n=4	< Naïve n=4

Data are Mean±SEM (n, number of cells studied). Red: significant increase ($p<0.05$, Student t-test); blue:

The reason why kainate receptors exhibit a lower affinity for glutamate in heterologous systems than in *"in vivo"* situation has been elusive for years (Wisden and Seeburg, 1993, Paternain et al., 1998). We showed that combination with Neto proteins, drastically increases receptor affinity (figure 32). Therefore, EC_{50} calculated for kainate receptors in combination with Neto proteins is much less than glutamate concentration in synaptic cleft at the hippocampus (~1 mM) (Clements et al., 1992) and exhibit a major effect to ambient glutamate in physiological systems. Nevertheless, it has been shown recently that in the cerebellar granule cells, the estimated glutamate concentration in the synaptic cleft is close to 300 µM (Yan et al., 2013). In our experiments, there was a considerable increase in affinity that yielded 1 mM glutamate as a saturating concentration for GluK1 in combination with Neto1 or 80% in combination with Neto2. In the case of GluK2, 1 mM glutamate increased responses to 75% of the maximum in combination with Neto1 or Neto2. In addition, we have observed that the increase in affinity carried a decrease in desensitization when the agonist concentration was reduced in all kainate receptors subunits (figure 32), in agreement with previous data (Paternain et al., 1998). Interestingly, combination of GluK1 and Neto2 made non desensitizing currents to a 10 µM glutamate concentration and slightly desensitizing for Neto1 combination at this concentration. Extracellular glutamate concentration, which stills a debate, range from 20 nM to 20 µM depending on the technology used to measured it (Moussawi et al, 2011). This could give a significant argument for the importance of Neto in kainate receptor physiology at basal agonist concentration. For example, at the synaptic cleft modulated by the tripartite synapse (Araque, 2006), glial cells might modulate synaptic responses acting through extrasynaptic kainate receptors sensing glutamate from synaptic spill over (Rodriguez-Moreno et al., 1997; Liu et al., 2004). At the pathological level, it has been shown that at ischemic episodes glutamate is increased until 100 µM (Benveniste et al., 1984). While non tethered kainate receptors showed a variable activation to 100 µM (heteromeric receptors including GluK4 or GluK5 display higher affinity than homomeric receptors), association with Neto did not only change activation efficacy but also desensitization. Thus, 100 µM did not desensitize complete kainate receptors

allowing a tonic current, which one might hypothesize to be responsible of excitotoxicity.

Due to relatively high frequency of synaptic transmission, the kinetics of receptor desensitization may be a relevant aspect to consider. Neto1 protein showed a conserved action along three major kainate receptors subunits strongly speeding the rate of recovery from desensitization. Neto2 slows down onset of desensitization, although slightly increases recovery rate to GluK2 containing kainate receptors but does not change GluK1 or GluK3 rates (figure 33). Therefore, the association to either Neto1 or Neto2 of different types of kainate receptors would have a significant impact in synaptic transmission, since the accumulated desensitization during high frequency activation of synaptic receptors will vary in each case.

Among several ligand-gated ion channels, kainate receptors are unique to require external ions (such as Na^+) for gating activity (Bowie, 2002; Paternain et al., 2003). In our experiments, we demonstrated that Neto2 is much more efficient than Neto1 in relieving kainate receptors from Na^+ dependency (figure 34, 35). Heteromerization with GluK5 itself significantly relieved Na^+ dependence (Paternain et al., 2003). Since Neto2 further relieved Na^+ dependency of GluK5 containing heteromeric receptors. We think of a different mechanism for each protein to attenuate Na^+ dependency. A feasible explanation could be that Na^+ requirement is related with dimer interface stability, based on models from crystallized structures of kainate receptors (Plested et al., 2008). The stability of dimer interface has been also related to desensitization. Therefore, the action of Neto proteins on kainate receptors desensitization and Na^+ dependence seems to have in common the improvement of dimmer stability. Thus, Neto2 reduces desensitization of GluK1 kainate receptors to the same degree of modulation observed for Na^+ dependence. Similarly, Neto1/2 reduce both desensitization and Na^+ dependence of GluK2 homomeric kainate receptors. The fact that GluK5 occluded Neto1 effect in GluK2/5 heteromeric receptors is in agreement with this conclusion: GluK5 has a similar effect than Neto1 and slightly weaker than Neto2 in GluK2 receptors. Indeed, in the absence of Na^+, desensitization gets faster (Paternain et al., 2003; Dawe et al., 2013). We therefore could

hypothesize that modulation of gating by Neto proteins are mediated by dimer interface stabilization. In this way, GluK1 dimer interface stabilization might be increased by Neto2 but not by Neto1, whereas both auxiliary proteins increase stabilization of dimer interface of GluK2 containing kainate receptors.

It is known that kainate evoke a non-desensitizing or pronounced steady-state current in native tissue (Lerma et al., 2001), while this property has not been reproduced with recombinant receptors (Paternain et al., 1998). We explained how both Neto1 and Neto2 increase steady-state current either to homomeric (GluK1 and GluK2) or heteromeric kainate receptors with GluK5 subunits (figure 36). These recapitulates an observation showed in native kainate receptors and might explain the importance of GluK2 containing receptors for kainate induced seizures (Mulle et al., 1998).

In summary, we have shown that main characteristics observed in native kainate receptors, which are lacking when expressed in heterologous systems could be rescued upon Neto1 or Neto2 coupling. In this way, receptor amplitude, desensitization (onset and recovery), affinity and sodium dependence are all altered towards increasing sensitivity to agonist. These demonstrated that Neto1 or Neto2 are part of native kainate receptors with profound structural and functional implications.

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IV. Results III. Trafficking KARs to the synapses.

One of the emergences from the existence of multiprotein complexes formed by neurotransmitter receptors and interacting proteins is that biophysical and pharmacological properties of these receptors may be drastically altered (as it has been shown before in this thesis, *Results II*). Also, the association with auxiliary proteins may guide their synaptic targeting in the CNS. Whilst this is true for most of the synaptic receptors, it is becoming particularly apparent for ionotropic glutamate receptors. Auxiliary subunits tethered to AMPA receptors, such as stargazin and others TARPS subunits (Jacson & Nicoll, 2011) and Cysteine-Knot AMPA receptor modulating protein of 44 KDa (CKAMP44) and cornichon (von Engelhardt et al., 2010; Schwenk et al., 2009), have helped to understand differences in trafficking and biophysical properties of AMPA receptors among cell types. Kainate receptors have been long term elusive to this analysis but now that a number of interacting proteins have being identified, some of them may account for the mismatch observed when properties displayed in recombinant systems are compared to native receptors.

It is known that hippocampal culture do not express synaptic kainate receptors (Lerma et al., 1997) even though there is a set of functional extrasynaptic kainate receptors. As native kainate receptors in hippocampal cultures are similar to heterologously expressed homomeric GluK2 (Paternain et al., 1998), we wondered which proteins are expressed in hippocampal cultures, with the purpose of expressing those lacking proteins to determine any role of those proteins in kainate receptor trafficking to the synapses.

1. Hippocampal primary cultures do not express Neto auxiliary proteins or GluK5 containing KARs.

Semiquantitative PCR analysis from hippocampal primary cultures demonstrates that these cultured cells do not express Neto1 or Neto2 mRNA (figure 37 A, B). We compared mRNA expression of mice brain at P0 (0.53 ± 0.24, for Neto1 and 0.37 ± 0.05, for Neto2; n=3) with hippocampal primary cultures with 18 days in vitro (DIV) (0.01 ± 0.01, $p<0.01$ and 0.02 ± 0.03, $p<0.05$; Student T-test; n=3 for Neto1 and Neto2 respectively) to test that cultures do not express Neto1 and Neto2 genes. To prove the specificity of our primers and the validation of transfection, we quantified Neto1 and Neto2 mRNA levels in transfected cultures, obtaining high specificity for both primers (figure 37 B).

Previous data from single cell PCR experiments carried out in our lab (Ruano et al., 1994) indicated that cultured hippocampal neurons presenting functional kainate receptors consistently expressed GluK2. In these experiments, however, we were unable to amplify GluK4 or GluK5. Here, we have further checked the lack of GluK4/5 expression pharmacologically. Under kainate receptor isolation (i.e. in the presence of APV, GYKI and Picrotoxin for preventing activation of NMDAR, AMPAR and $GABA_A$ receptors, respectively), kainate was able to elicit kainate receptors-mediated currents (155.0 ± 44.5 pA n=6). However, ATPA (50 µM), a kainate receptor agonist active on GluK2 receptors whenever this heteromerizes with GluK1, GluK4 or GluK5 (see Paternain et al., 2000) was unable to elicit any response, further indicating the lack of heteromeric receptors containing either GluK4 or GluK5 subunits. In contrast, ATPA consistently induced non desensitizing responses in neurons transfected with GluK5 (15.07 ± 2.47 pA, n=6; $p< 0.01$; Student T-test) (figure 37 C, D). Kainate induced responses with similar amplitudes in these same GluK5 expressing cells and untransfected neurons (205.4 ± 61.0 pA, n=6) but the kinetic properties were more consistent with the activation of heteromeric GluK2/GluK5 receptors in the former case (figure 37 C, D).

These demonstrate that in control conditions kainate receptors are formed by homomeric GluK2 receptors while after GluK5 transfection, heteromeric receptors are functionally expressed.

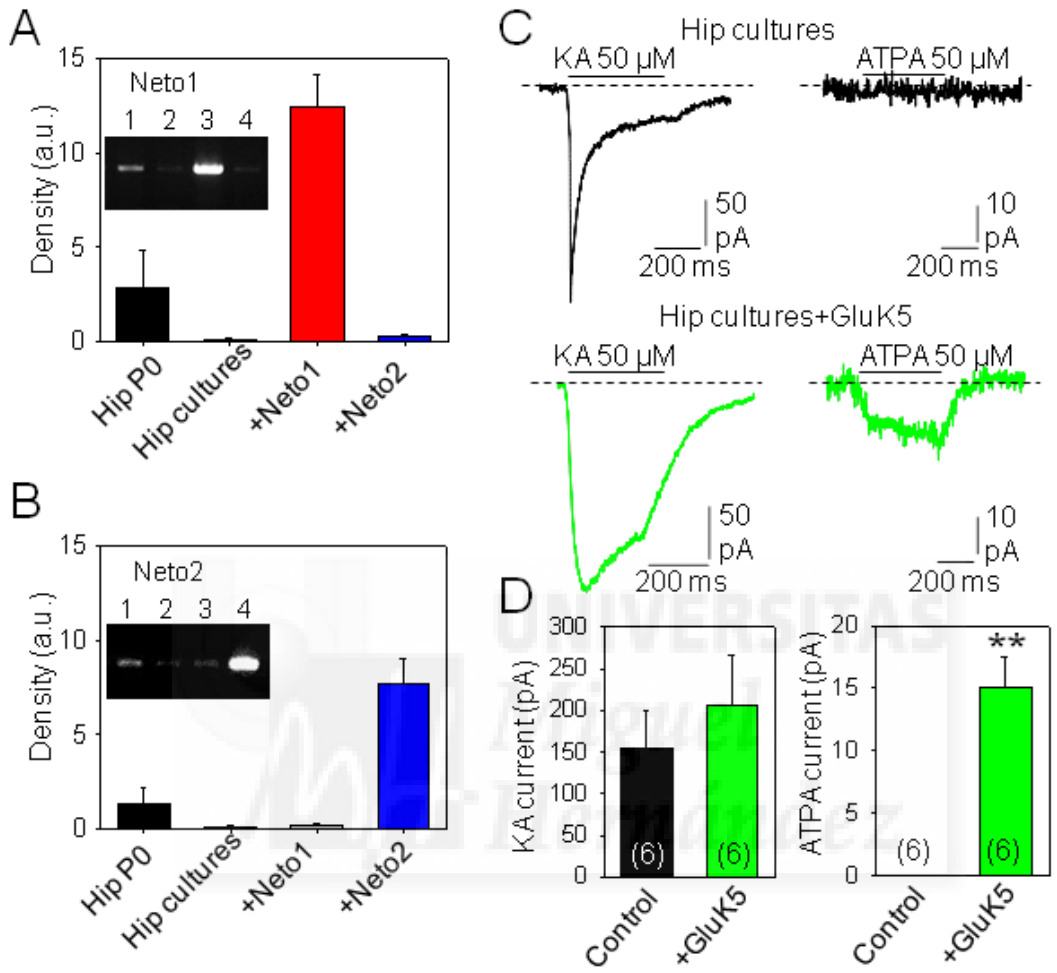

Figure 37. Hippocampal cultures did not expressed Neto proteins or GluK5 subunits. Neto1 and Neto2 mRNA was amplified by RT-PCR from either the hippocampus of P0 mice or 17-20 DIV hippocampal neuronal cultures that had been transfected with Neto1 (A) or Neto2 (B), or not. In each case, 4 different animals and cultures were analyzed. (C), control (black) and GluK5 transfected (green) cultures were exposed to Kainate (KA) to activate the whole population of KARs, or to ATPA to test the presence of heteromeric GluK5-containing receptors. Only cells in cultures transfected with GluK5 responded to ATPA (lower row). (D), quantification of these data shown as the mean ± SEM, where the numbers in parenthesis indicate the number of neurons studied: **p<0.001, Student t-test.

2. Neto1 and Neto2 KAR favor insertion of KARs at synaptic sites.

We decided to introduce exogenous Neto1 and Neto2 to check whether these proteins are necessary and sufficient to obtain kainate receptors mediated synaptic responses. The association of endogenous kainate receptors with exogenous Neto proteins was demonstrated by looking at gating differences in control or after Neto1 or Neto2 transfection (300 µM; figure 38 A). Current amplitude and steady state to peak ratio of kainate-induced responses increased significantly in Neto transfected cells (figure 38), and this was used as a functional readout of Neto association with endogenous kainate receptors.

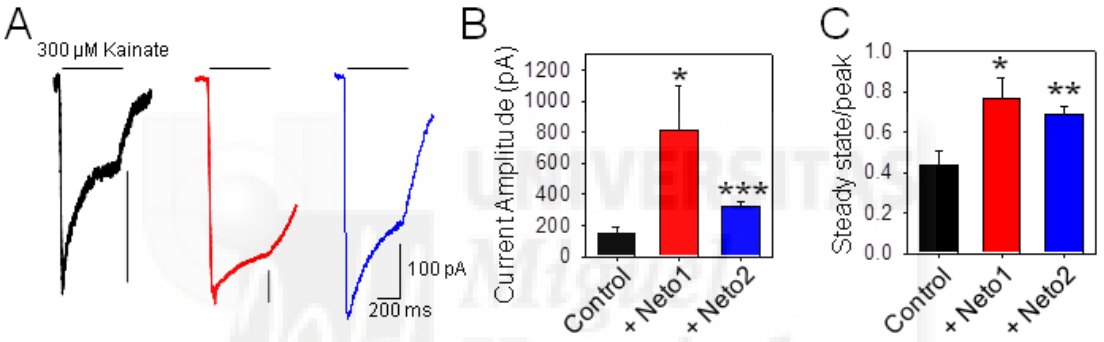

Figure 38. Effect of Neto proteins on endogenous KARs in hippocampal cultures. Hippocampal neuronal cultures were transfected with Neto1 or Neto2 to probe association with endogenous KARs after 16-20 day in vitro (DIV). A, neuronal responses to 300 µM Kainate of control (black), Neto1 (in red) or Neto2 (inblue). B, quantification of current amplitude: 153.6 ± 33.61 pA, n=8 for control, 813.29 ± 282.73 pA, n=4, $p<0.05$ for Neto1 and 324.71 ± 30.98 pA, n=4, $p<0.005$ for Neto2. C, quantification of steady-state to peak ratio in control (0.43 ± 0.07, n=8), Neto1 (0.77 ± 0.1, n=4, $p<0.05$) or Neto2 (0.69 ± 0.03, n=4, $p<0.01$) conditions showed an increase upon Neto proteins transfection. Data shown as the mean ± SEM, where the numbers in parenthesis indicate the number of neurons studied: *$p<0.05$; **$p<0.001$; ***$p<0.005$, Student t-test.

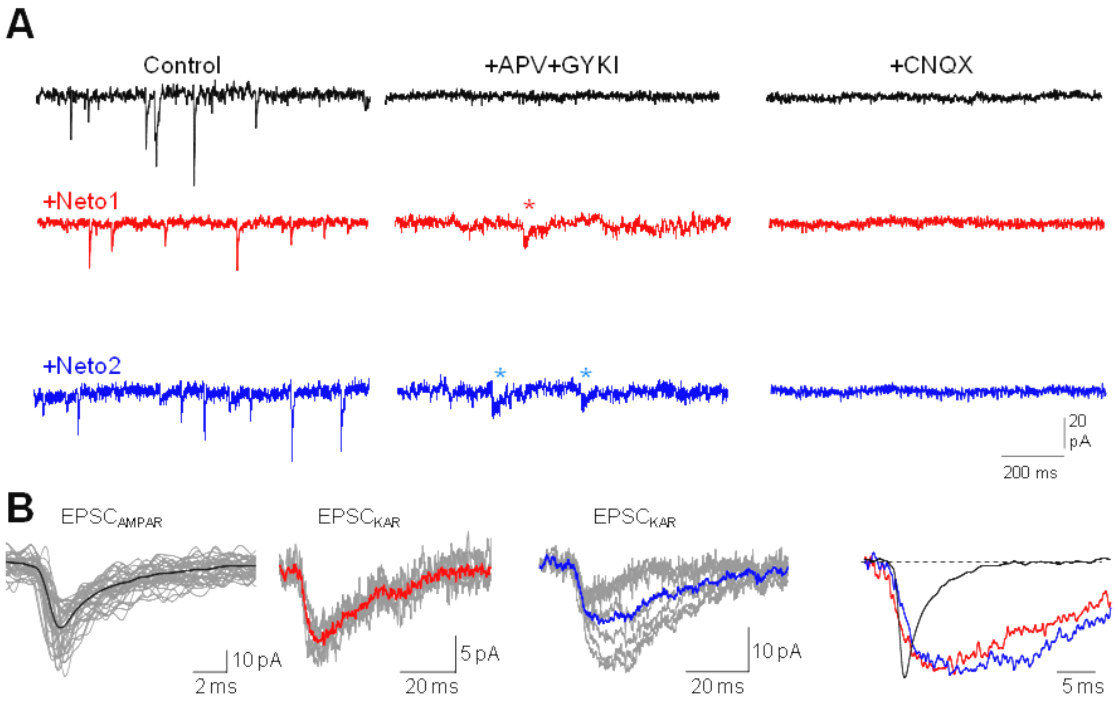

Figure 39. Auxiliary Neto proteins promote KARs synaptic responses. Hippocampal cultured neurons were recorded after 16-20 DIV. A, Excitatory synaptic transmission was recorded after blocking inhibitory transmission (Picrotoxin 50 µM: Control traces) and after additional blockade of NMDA and AMPA receptors (APV 50 µM and GYKI 50 µM). Only in Neto1 (red traces) or Neto2 (blue traces) transfected neurons could some synaptic activity be detected (asterisks) after AMPA and NMDA blockade. B, Amplification of these small synaptic responses from Neto1-2 transfected cells.

Despite the presence of native kainate receptors in the membrane of cultured hippocampal neurons, synaptic responses are not readily observed (Lerma et al., 1997). This was confirmed by the total absence of synaptic activity after blockade of NMDA and AMPA receptors, in cultures with high degree of spontaneous EPSCs before blocking glutamate receptors (figure 39 A, top). However, in cells transfected with Neto1 or Neto2, application of APV and GYKI unmasked the presence of small and slow EPSC (figure 39 A, red and blue traces), which were further abolished by introducing CNQX, an AMPA and kainate receptors mixed antagonist. Examination of excitatory postsynaptic currents mediated by kainate receptors ($EPSC_{KAR}$) revealed slow activation and deactivation kinetics with similar values to those $EPSC_{KAR}$ found in hippocampal slices (Castillo et al., 1997). However these were rare in that they were found in small proportion of neurons (23-26%) and the frequency of events was

extremely low (0.58±0.1 and 0.75±0.2 events/min for Neto1 and Neto2, respectively).

These data indicate that Neto1 and Neto2 favor to some extent trafficking of kainate receptors to the synapse.

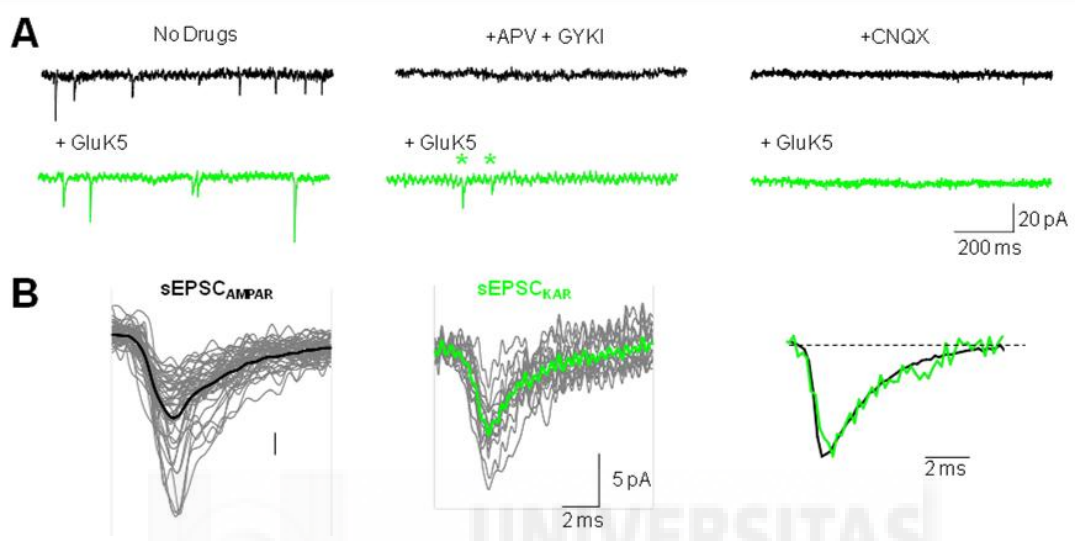

Figure 40. The GluK5 subunit targets KARs to synapses. Spontaneous excitatory synaptic transmission was recorded in 16-20 DIV hippocampal neurons before (A) and after (B) blocking AMPA and NMDA receptors (GYKI 50 µM and APV 50 µM, respectively). KAR-mediated synaptic EPSCs (asterisks) only appeared in GluK5 transfected neurons (green traces). C, ESPCs mediated by AMPARs (black) and KARs (green) are superimposed after amplitude normalization.

3. GluK5 traffics KARs to the synapse.

Since all the endogenously expressed kainate receptors at hippocampal cultures are homomeric receptors not trafficked to the synapse, we wondered whether the assembly into heteromeric kainate receptors would be sufficient for targeting these receptors to synapses, forming functional receptors. Data from GluK5 GluK4 double KO mice, revealed no kainate receptors mediated ionotropic synaptic responses in CA3 neurons (Fernandes et al., 2009). Therefore, we transfected GluK5 in hippocampal cultured neurons and looked for the appearance of $EPSC_{KAR}$. Spontaneous synaptic activity recorded from control or GluK5 transfected neurons showed no differences while NMDAR and AMPAR were not blocked (figure 40 A). However, in GluK5 transfected

neurons, $EPSC_{KAR}$ readily appeared in a proportion higher than with Neto proteins (42%), figure 40 A). $EPSC_{KAR}$ did not show slow activation-deactivation kinetics, but presented nearly identical shape as AMPA receptors mediated EPSCs (Figure 40 B).

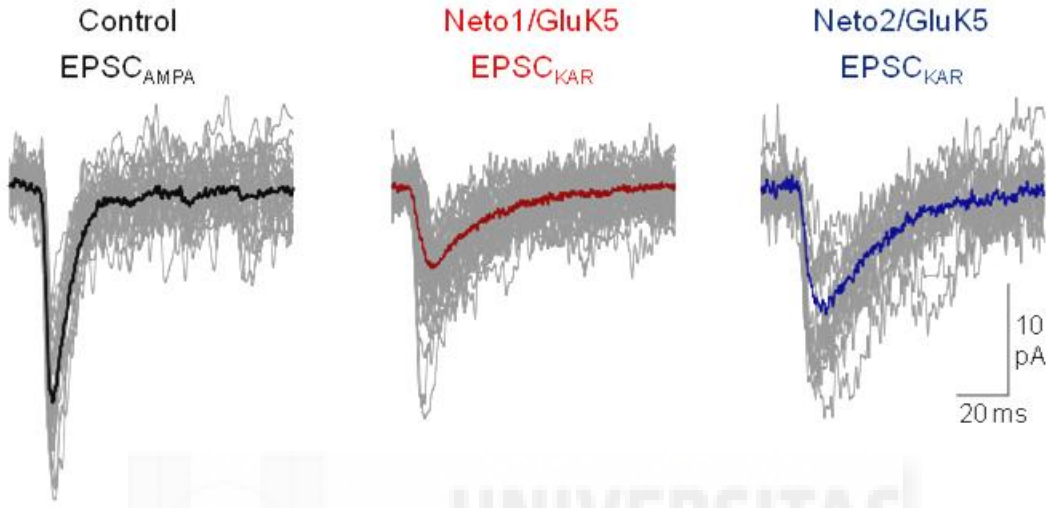

Figure 41. Comparison of $sEPSC_{AMPA}$ and $sEPSC_{KAR}$ after neto1 or Neto2 and GluK5 transfection. For control conditions, AMPA receptors mediated synaptic transmission were superimposed and the media was represented in black. For Neto1 or Neto2 both in combination with GluK5 (dark red or dark blue, respectively) transfection similar approach was taken.

Interestingly, transfection of Neto1 or Neto2 and GluK5 in hippocampal neurons yielded $EPSC_{KAR}$ with slow activation and deactivation kinetics (Figure 41) after blocking AMPA and NMDA receptors. In this case, the proportion of neurons where $EPSC_{KAR}$ were considerably increased (c.a. 67%).

These results evidence that expression of GluK5 targets kainate receptors to synaptic sites much more efficiently than Neto1 or Neto2 does.

4. Different properties of Neto1/2 and GluK5 driven synaptic KARs.

According to the kinetics of synaptic events, we showed that GluK5 transfection induced fast $EPSC_{KARs}$ similar to AMPA receptor synaptic responses. On the other hand, Neto1 and Neto2 slowed down kinetics of synaptic kainate receptors. These observations are highlighted in a plot relating rise time and decay time, the main characteristics that shape synaptic kinetics (figure 42 B, filled circles). A combination of Neto1 or Neto2 and GluK5 in hippocampal cultures, displayed slow kinetics events, even though some cells present fast or mixed events (figure 42 B, empty circles).

Focusing at the frequency of $EPSC_{KAR}$ events, we found differences in the efficiency to find kainate receptor synaptic events at different conditions (as mentioned before, 0 % for control, 26 % for Neto1 and Neto2, 42 % for GluK5 and 66 % for dual combination of Neto and GluK5 proteins). We decided to analyze the frequency of cells where $EPSC_{KAR}$ were found (figure 42 C). All recorded neurons presented high level of connectivity when excitatory synaptic transmission was quantified ($sEPSC_{AMPA}$ 210.87 ± 35.43 events per minute). GluK5 transfection made synaptic kainate receptors to appear at a frequency of 4.88 ± 0.8 events per minute, which suppose a significant increase when compared with Neto1 or Neto2 (0.58 ± 0.14 events per minute, n=5 cells, $p<0.01$ and 0.75 ± 0.17 events per minute, n=7 cells, $p<0.01$; Student T-test, respectively). Neto1 plus GluK5 seemed to have a synergistic effect since the frequency obtained was higher than the sum of both separately (8.75 ± 2.9, n=6). On the other hand, Neto2 plus GluK5 did not yield such a synergistic effect (5.43 ± 1.97, n=6). In both cases, it is clear that Neto proteins plus GluK5 increase considerably the frequency when compared with Neto1 or Neto2 unaccompanied by GluK5 ($p<0.01$; Student T-test).

Concerning the amplitude of $EPSC_{KAR}$ we did not observe a significant difference between the five types of $sEPSC_{KAR}$ (8.13 ± 2.01 pA, n=5 cells for GluK5, 8.08 ± 1.72 pA, n=5 cells for Neto1, 14.2 ± 2.13 pA, n=7 cells for Neto2, 11.75 ± 1.06 pA for Neto1 GluK5 and 8.07 ± 1.91 pA for Neto2 GluK5 ; Figure 42 D).

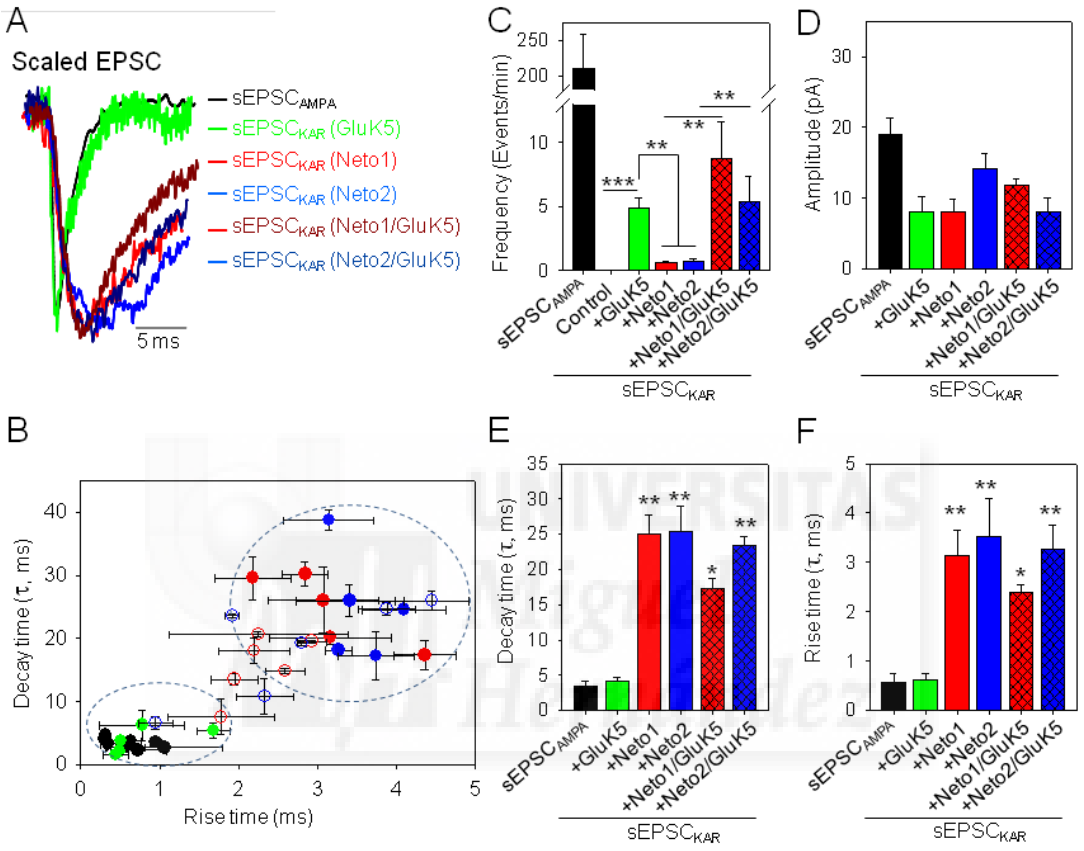

Figure 42. Properties of KAR-mediated EPSCs. A, KAR-mediated EPSCs were averaged for each condition and superimposed to AMPAR-mediated EPSC (black) after amplitude normalization to show the different time course presented in one or another experimental situation. B, Averaged rise time was plotted vs averaged decay time for all EPSCs recorded from each cell. The color code is as in A. KAR-mediated EPSC incorporating GluK5 present similar kinetics than AMPARs, whilst those incorporating Neto1 or Neto2 have considerable slower kinetics. Comparison of frequency and amplitude (C) and decay and rise times (D) of synaptic events from different experimental conditions. Data are mean ± SEM, **p<0.001;***p<0.0005, Student t-test.

Native kainate receptors in the brain are characterized by slow activation and deactivation kinetics (Castillo et al., 1997; Cosart et al., 2002). We analyze the kinetics of $EPSC_{KAR}$ obtained after transfecting Neto auxiliary proteins or GluK5

subunit of kainate receptors. We observed no differences in rise or decay time between control and GluK5 transfected neurons (0.58 ± 0.17 ms, 3.39 ± 0.8 ms; n=21 cells for controls and 0.63 ± 0.11 ms, 4.11 ± 0.54 ms; n=5 cells for GluK5, rise time and decay time respectively). Oppositely, Neto1 and Neto2 both increased rise and decay times significantly, being different from GluK5 transfection, but not between them (3.71 ± 0.5, 25.11 ± 2.69; n=5 for Neto1 and 4.03 ± 0.77, 25.36 ± 3.57; n=5 for Neto2, rise time $p<0.01$ and decay time $p<0.005$ respectively; Figure 42 E, F). Interestingly, receptors containing GluK5 and Neto1 or Neto2 had the same slow kinetics (2.38 ± 0.17 ms; n=6 and 3.26 ± 0.5 ms; n=6 raise time for Neto1 and Neto2 respectively and 17.35 ± 1.36 ms; n=6 and 23.39 ± 1.3 ms; n=6 decay time for Neto1 and Neto2 respectively).

Altogether, our data reveal a fundamental role of GluK5 subunits to target synaptic kainate receptors and Neto1 or Neto2 for modulating the kinetics of synaptic kainate receptors.

5. Discussion.

Our experiments have been done in cultured hippocampal neurons, a situation in which, by unknown reasons, the expression of Neto proteins is suppressed or is not activated during in vitro cellular differentiation and maturation (figure 37 A, B). The lack of Neto proteins at hippocampal culture neurons was suspected due to biophysical properties of native kainate receptors, which could be reproduced in heterologous systems by expressing GluK2 kainate receptors (Paternain et al., 1998). In the same way, previous work reported no expression for GluK4 or GluK5 subunits at all and GluK1 in a few cells (Ruano et al., 1994). We tested the presence of GluK5 subunit containing kainate receptors by pharmacological activation with ATPA (figure 37 C, D). In control conditions ATPA failed to elicit any response, indicating that pyramidal neurons from hippocampal cultures do not express GluK1 or GluK4-5 containing kainate receptors. Moreover, when GluK5 was exogenously introduced, ATPA elicited depolarizing responses, indicating the presence of GluK5 into functional responses. Altogether, indicates that under control conditions hippocampal pyramidal neurons express homomeric GluK2

receptors and introduction of Neto proteins (figure 38) or GluK5 (figure 37 C, D) yield incorporation of these proteins into functional receptors.

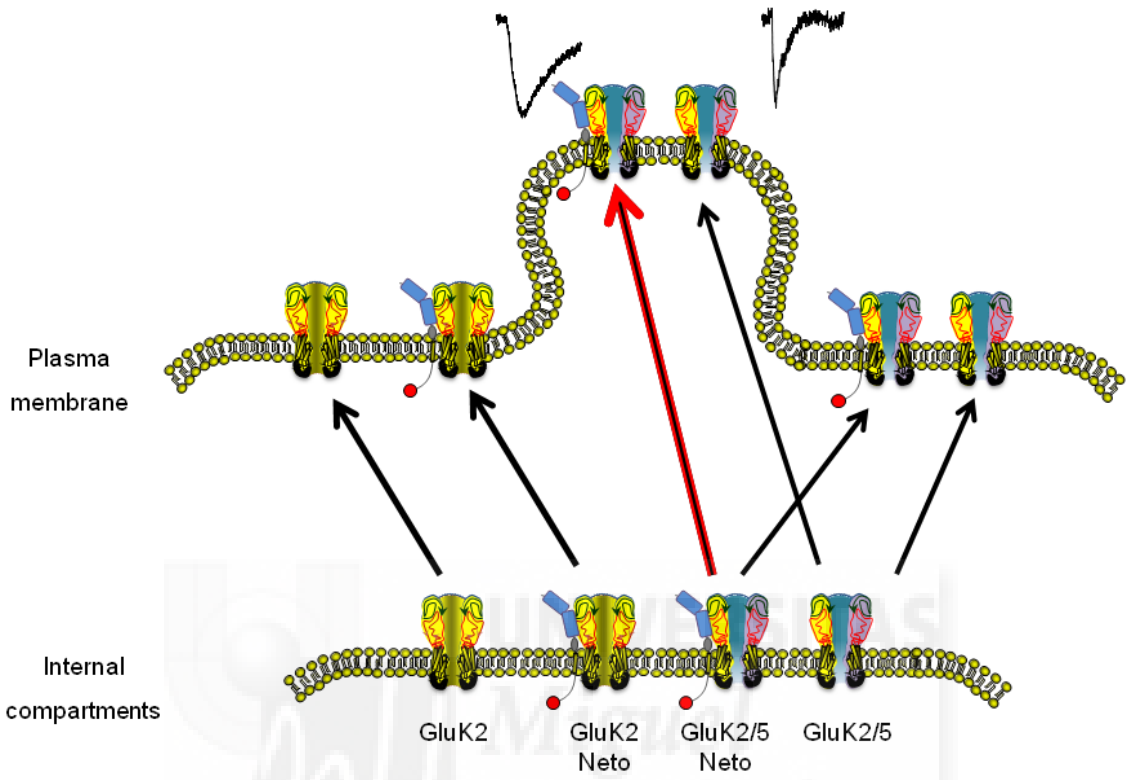

Figure 43. Working model for synaptic targeting of Kainate receptors. Homomeric GluK2 Kainate receptors do not reach synaptic structures but stay at extrasynaptic sites. In the same way, Neto1 or Neto2 bound to homomeric GluK2 receptors very rarely are found in synaptic sites. In the other hand, both GluK2/5 heteromeric Kainate receptors and those tethered to Neto proteins (with a higher efficacy of Neto1-GluK2/5 receptors) are placed at synapses but are differentiated by their rise and decay kinetics, which are fast for non Neto bound receptors and slow for Neto coupled (a hallmark of native Kainate receptors).

We have dissected kainate receptor requirements for synapse targeting. Thus, Neto1, Neto2 and GluK5 trafficked kainate receptors to the synapses but with different efficiency. We evaluated that efficiency by quantifying frequency of $EPSC_{KAR}$ events. Incorporation of Neto1 or Neto2 induced appearance of $EPSC_{KAR}$ with a very low frequency. Introduction of GluK5 increased almost 5 times that frequency (figure 42). The observed low efficiency of Neto proteins for targeting kainate receptors to the synapse is in agreement with Copits et al.

(2009). These authors observed a negligible appearance of $EPSC_{KAR}$ in cultured hippocampal neurons, activity that was induced upon Neto2. Our data show a synergistic effect when Neto1 and GluK5 were co-expressed. Co-transfection of Neto2 with GluK5 did not produce, however, such a synergism, although we have to admit that in these experiments we could not be sure of the amount of Neto and GluK5 that was incorporated in transfected neurons.

Altogether, our data indicate (figure 43) that synaptic kainate receptors are obliged to include GluK5 subunit. However, it should be taken into account that GluK4 subunits might also do this job, since compensatory mechanisms are seen between both subunits (Fernandes et al., 2009). Interestingly, heteromeric receptors without Neto proteins displayed fast kinetics, while slow kinetics (a hallmark for most native synaptic kainate receptors) are only found were Neto proteins form part of kainate receptor complex.

V. General discussion.

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Assembly of receptors with other proteins into higher order complexes is believed to be the mechanism by which cellular signaling occurs. Kainate receptors bind a vast number of proteins to fulfill all the different roles that had been ascribed to them in the central nervous system. To unravel the identification of those interacting proteins might help to dissect kainate receptor function. Different attempts have been made to clarify this issue. In this work, we analyze NeCaB1 interaction with GluK5, discovered by yeast two hybrid screening. NeCaB1 was identified using a DRG neuron mRNA library screening, where kainate receptors have been described to control neurotransmitter release (Rozas et al., 2003) and neuronal maturation (Marques et al., 2014). Present data indicate that this novel interactor favors the presence of GluK5 containing receptors at the same time that enhances their affinity for the agonist and that this both actions could be switched on and off by Ca^{2+}.

Moreover, we found that Neto1 and Neto2 bind without specificity for kainate receptor subunits. Neto interaction with different subunits of kainate receptors could reproduce "*in vitro*" several of the properties found in native receptors, such as affinity, desensitization rate and Na^{+} dependence channel gating. In the same line of evidence, Neto proteins are key proteins at synaptic kainate receptors imposing slow kinetics but not playing a key role in the trafficking of these receptors. Rather, GluK5 subunits seem to play this key function.

Notably GluK5 subunit, which is sufficient to traffic kainate receptors to the synapses, is also responsible for NeCaB1 interaction. GluK5 emerges then as

hub subunit within kainate receptor complexes, since it also influences gating mechanisms and pharmacological properties of the channel. GluK5 subunits do not form functional receptors by themselves nor are trafficked to the membrane alone, but they need the collaboration of at least one type of the other subunits, GluK1-3. GluK5 appears as a fundamental source of regulation for mature kainate receptors.

From a molecular point of view, GluK5 subunit stabilizes dimmer interface in the amino terminal domain (Kumar and Mayer, 2010). It has been proposed that for that reason, heteromeric GluK5 receptors exhibit higher glutamate sensitivity and slower deactivation rate (Barberis et al., 2008).

Moreover, the cytoplasmatic tail contains several regulatory regions that make GluK5 complexes an exquisite regulatory entity. First, several endoplasmatic reticulum retention signals avoid trafficking to the surface unless combined with GluK1-3 subunit (Ren et al., 2000). As a part of functional receptor provides with a number of phosphorylation sites, targeted by PKC and CAMKinase II that may modulate the density of functional receptors in the cell surface. Examples of these include phosphorylation at serine 833, 836 and 840 triggered by activation of mGluR1 that increase surface expression of GluK5 containing kainate receptors (Rojas et al., 2012). On the other hand, phosphorylation at serine 859, 892 and 976 by CaMKinaseII uncouples GluK5 from PSD-95 scaffolding proteins at the postsynaptic density favoring lateral diffusion and thus reducing the synaptic content in kainate receptors (Carta et al., 2013). Last, its molecular determinant for protein binding does also influence receptor function. SNAP-25 interacts with the C-terminal region of GluK5 at leads to receptor internalization in the postsynaptic membrane in an activity dependent manner (Selak et al., 2009). Interestingly, genetic removal of GluK5 abolishes this type of plasticity, presumably due to SNAP-25 interaction does not occur with other kainate receptors lacking GluK5.

Visualizing kainate receptor complexes from a global point of view, we could predict receptor function by analyzing each punctual interacting protein inside the complex. Supporting this idea, we provide new mechanisms for the synaptic targeting and explanations on how the slow kinetics of native kainate receptors

arise. Thus, we might hypothesize that interacting proteins with overlapping binding sequences compete each other and the resulting complex could display different roles depending on its partners. We have shown different developmental expression pattern for Neto1 and Neto2, which influences the incorporation of kainate receptor at complexes. In addition, there exist also different cellular expression pattern for these proteins (Straub et al., 2011; Tang et al., 2012). Interestingly, we provide evidence of the existence of transient interactions between proteins in that they could bind and unbind rapidly depending on environmental cues, such as Ca^{2+} levels. This is interesting, since indicates that functional properties of kainate receptor complexes due to protein-protein interactions may be more dynamic than expected, and therefore susceptible to be modulated by synaptic activity

Specific targeting of one or other subunit might provide insights onto kainate receptor function at a systems level. Similarly, disruption of transient interactions might alter specific behaviors of kainate receptors. Thus, as more interactors are found, larger possibilities of finding a doable target for kainate receptor function to be used in specific pathophysiological conditions.

VI. Conclusiones.

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Conclusiones

Siendo nuestro objetivo revelar la función de las proteínas que interactúan con los receptores de kainato para explicar el funcionamiento de estos receptores, podemos concluir:

1. La interacción de NeCaB1 con los receptores de kainato que incluyen subunidades GluK5, está regulada con la presencia de Calcio.

2. NeCaB1 incrementa el número de receptores que incluyen la subunidad GluK5 y aumenta la afinidad de estos receptores.

3. Las proteínas Neto1 y Neto2 modulan los receptores de kainato a dos niveles. Por una parte, aumentan la expresión en superficie de los receptores de kainato. Por otra, alteran las propiedades de apertura de los receptores, aumentando la amplitud de su respuesta, afinidad y ralentizando su desensibilización en la mayoría de los casos.

4. La subunidad GluK5 es la principal promotora de la localización de los receptores de kainato en las sinapsis, mientras que las proteínas Neto1 y Neto2 sustentan una cinética lenta para estos receptores.

VII. References.

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