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Modulatory Components of Kainate Receptors in Sensory Neurons and Heterologous Systems

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ABSTRACT

Modulatory Components of Kainate Receptors in Sensory Neurons and Heterologous Systems

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Kainate receptors (KARs) are expressed throughout the central and peripheral nervous systems. One of three subfamilies of ionotropic glutamate receptors, KARs are localized both pre- and post-synaptically and exert important modulatory control over neural circuits. This modulatory role in circuit function makes them a potential actor in wide variety of neurological disorders, and they play an established role in pathological but not acute pain. The basic constituents of KARs, the pore-forming subunits, were identified over 25 years ago; since then, a vast amount of research has described both the cellular regulation of KAR subunits and the functional contribution of these receptors to excitatory and inhibitory signaling. Differential combinations of auxiliary and pore-forming subunits, post-transcriptional and post-translational modifications, and dynamic protein–protein interactions comprise a complex collective of molecules that constitutes native receptors.

In this dissertation, I present work that furthers our understanding of two of these molecular KAR components: the auxiliary protein Neto2, and the *N*-glycan moieties attached to receptor subunits. In Chapter 2, I confirm Neto2 as a true KAR auxiliary protein, showing that it is a developmentally regulated constituent of KARs in peripheral sensory neurons that modulates neurite outgrowth. In Chapter 3, I show that changing the molecular content of recombinant KAR *N*-glycosylation alters functional properties of these receptors. Although auxiliary subunits and oligosaccharide chains are molecularly distinct, both are expected to be critical components

of native KARs. Understanding their contribution to receptor function is critical to improving our knowledge of KAR function within neural circuits, the role of these receptors in cognition and disease, and their potential as therapeutic targets.

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List of Abbreviations:

ACC: anterior cingulate cortex

AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolapropionic acid receptor

ATD: amino terminal domain

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

Cng-1: Congerin-1

CNS: central nervous system

ConA: ConcanavalinA

COPI: coat protein I

CUB: complement C1r/C1s, Uegf, Bmp1

DNA: deoxyribonucleic acid

DRG: dorsal root ganglia

EPSC: excitatory postsynaptic current

EPSP: excitatory postsynaptic potential

ER: endoplasmic reticulum

ERK: extracellular-signal related kinase

GABA: γ-aminobutyric acid

GlcAT-P: glucuronyltransferase-P

GlcNAc: *N*-acetylglucosamine

GPCR: G-protein coupled receptor

GRIP: glutamate receptor-interacting protein

HNK-1: human natural killer-1

HNK-1ST: HNK-1 sulfotransferase

iGluR: ionotropic glutamate receptor

IPSC: inhibitory postsynaptic current

IPSP: inhibitory postsynaptic potential

KAR: kainate receptor

KCC2: potassium–chloride co-transporter isoform 2

KRIP6: kainate receptor interacting protein for GluR6

LII: lamina II of the spinal cord

LBD: ligand binding domain

LTD: long-term depression

LTP: long-term potentiation

kDa: kilodalton

MAGUK: membrane-associated guanylate kinase

mAHP: medium afterhyperpolarization

Man: mannose

Neto: neuropilin- and tolloid-like

NMDAR: N-methyl-D-aspartate receptor

PAG: periaquaductal grey

PDZ domain: PSD-95–Dig1–zo-1 domain

PI3K: phosphatidylinositol-4,5-bisphosphate 3-kinase

PICK1: protein interacting with C-kinase 1

PKA: protein kinase A

PKC: protein kinase C

PNS: peripheral nervous system

PSA: polysialic acid

PSD-95: post-synaptic density protein 95

RNA: ribonucleic acid

sAHP: slow afterhyperpolarization

SAP102: synapse-associated protein 102

SNAP25: synaptosome-associated protein 25

ST: sialyltransferase

SUMO: small ubiquitin-like modifier

TARP: transmembrane AMPA receptor regulatory protein

TRP: transient receptor potential

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Chapter 1.

Introduction:

Molecular constituents and functional properties of kainate receptors.

The Oxford English Dictionary defines cognition as "the mental action or process of acquiring knowledge and understanding through thought, experience, and the senses." The nervous system processes sensations and experiences, transmitting this information in chemical and electrical signals through many circuits. Appropriate balance between excitatory and inhibitory signaling is critical for successful transmission between circuits and "normal" cognition. The majority of excitatory signals between cells are passed by the chemical signal glutamate, and two types of proteins detect glutamate signals and effect downstream signaling: ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors. Among iGluRs, three receptor subfamilies exist: AMPA receptors, NMDA receptors, and kainate receptors (KARs). This introduction is intended to provide a brief overview of the molecular components comprising KARs, from the genes encoding their individual subunits to post-translational modifications and protein-protein interactions that modify their function. KARs fulfill diverse signaling roles in neurons which lead them to modulate the balance between excitation and inhibition in neural circuits. I review what is known generally about KAR function in neurons before discussing their contributions to pain circuitry in more detail, as a portion of my thesis work investigates KAR composition in primary pain-sensing neurons. Consistent with their role in circuit modulation, KAR dysfunction is suggested in neuropathological conditions. I will review these, again with a focus on KAR contribution to pain, and will discuss the challenge of targeting these receptors therapeutically. A greater knowledge of KAR function is critical to advance our understanding of the contribution these receptors make to cognition and to disease, and my work on the protein and oligosaccharide components of KARs contributes to a more complete understanding of receptor function.

The pore-forming constituents of KARs

Prior to their cloning, ionotropic glutamate receptors (iGluRs) were categorized as NMDA, AMPA, and kainate receptors based on the pharmacological sensitivities of currents recorded from neurons. These studies predicted a molecularly diverse family of glutamate-sensitive channels that were located throughout the nervous system (Lodge, 2009). The genes encoding a protein provide valuable predictive information about its structure, function, and potential modifications, and the cloning of iGluRs represented an important step forward in detailing their molecular complexity.

Cloning of KARs

The first KAR subunit to be cloned was named GluR5, as it was discovered fifth among several structurally related channel proteins that were gated by glutamate and sensitive to AMPA or kainate (Bettler et al., 1990; Sommer et al., 1992). GluR5 can form homomeric receptors when expressed in heterologous systems and it has higher affinity for kainate than AMPA, defining it as a KAR subunit (Sommer et al., 1992). AMPA and kainate receptors have overlapping pharmacology, particularly with their namesake agonists, and the first AMPAR subunit cloned had been named GluR-K1 for the sensitivity of the receptor to kainate (Hollmann et al., 1989). It was renamed to GluR1 with the realization that it had greater affinity for quisqualate than kainate (Sakimura et al., 1990). GluR1 and three structurally similar subunits with greater affinity for AMPA than kainate named GluR2, GluR3, and GluR4, exhibit ~70% sequence identity and assemble to form homomeric and heteromeric AMPARs (Boulter et al., 1990; Keinanen et al., 1990; Nakanishi et al., 1990). GluR5 and the closely related GluR6 subunit have ~70% primary sequence identity with each other but only around 40% identity with AMPARs (Bettler et al., 1990; Egebjerg et al., 1991). Similar to GluR5, GluR6 forms homomeric receptors that have

high affinity for kainate but not AMPA (Egebjerg et al., 1991). A subunit named GluR7 was also identified that displayed a KAR agonist profile and sequence similarity to GluR5 and GluR6 rather than AMPARs, although it would be several years before GluR7-containing receptors were confirmed to gate currents (Bettler et al., 1992; Lomeli et al., 1992; Schiffer et al., 1997). The final two subunits in the KAR subfamily have even higher kainate affinity than GluR5–7. Initially called KA-1 and KA-2, these subunits have ~70% primary sequence identity with each other but only ~40% identity with the AMPAR and the lower affinity KAR subunits (Werner et al., 1991; Herb et al., 1992; Sakimura et al., 1992). They cannot form functional homomeric channels and are obligate heteromers with GluR5–7, conferring altered gating properties and pharmacological sensitivity upon these heteromeric assemblies (Herb et al., 1992; Sakimura et al., 1992; Swanson et al., 1996; Schiffer et al., 1997).

The discovery of iGluR subunits by multiple different groups led to several different naming conventions within each family of receptors, and different names were used for the homologous subunits in different species. Eventually, the International Union of Basic and Clinical Pharmacology (IUPHAR) proposed a standardized model for the naming of ligand-gated channels in which iGluR subunit protein names reflected the naming structure given to the human genes encoding the subunits (Collingridge et al., 2009). The KAR proteins are encoded by the genes *Grik1–5*, so GluR5–7 became known as GluK1–3, and KA1 and KA2 were renamed to GluK4 and GluK5. AMPAR subunits previously referred to as either GluR1–4 or GluR-A–D are now called GluA1–4 and I use this standardized naming convention exclusively throughout this dissertation.

In addition to AMPA and kainate receptors, there are two other subfamilies of iGluRs: NMDA receptors and ∂-receptors. NMDA receptors are composed of subunits GluN1, GluN2A–D, GluN3A&B (Moriyoshi et al., 1991; Monyer et al., 1992; Ciabarra et al., 1995; Sucher et al., 1995; Matsuda et al., 2002), require glycine as a co-agonist, and are critical to synaptic plasticity throughout the nervous system (Nicoll and Roche, 2013). The ∂-receptor subfamily consists of GluD1 and GluD2 (Yamazaki et al., 1992; Lomeli et al., 1993), and though they are structurally similar to the other iGluR families it remains unclear whether they gate currents. For AMPA, kainate, and NMDA receptors, the subunits described above are the minimal components necessary to form a channel pore gated by glutamate, and as such are referred to as "pore-forming subunits". The five genes that encode KAR subunits can assemble into a number of different tetrameric receptor combinations, and modifications of the subunit transcripts further increase the potential diversity of this family of receptors.

RNA Editing, Splice Variants, and Trafficking Motifs

Nine known splice variants occur between the GluK1, GluK2, and GluK3 subunits: one in the ATD of GluK1, called GluK1-1, that negatively influences gating of the homomeric receptor (Bettler et al., 1990), and 8 other variants at the intracellular C-terminal tails of their respective subunits (Sommer et al., 1992; Gregor et al., 1993; Schiffer et al., 1997; Barbon et al., 2001). An ER export motif promotes surface localization in the case of the GluK2a and GluK3a variants (Jaskolski et al., 2004; Yan et al., 2004; Jaskolski et al., 2005), and residues in GluK1-2b and GluK1-2c strongly retain these proteins intracellularly (Ren et al., 2003b; Jaskolski et al., 2004). GluK5 is also retained internally due to motifs in an intracellular loop and the C-terminus (Ren et al., 2003a; Nasu-Nishimura et al., 2006). In many cases heteromeric assembly of intracellularly retained subunits with forward trafficked subunits promotes the former's localization at the

membrane, perhaps representing a mechanism by which cells regulate the inclusion of certain subunits in surface-expressed receptors.

In addition to transcript splicing, GluK1 and GluK2 transcripts are subject to RNA editing, a post-transcriptional deamination that converts an adenosine to an inosine, causing it to be read as a guanosine by translational machinery and altering the amino acid coding at that site. RNA editing of a glutamine (Q) to an arginine (R) at a site in the pore domain of these subunits reduces calcium permeability, inward rectification, and single-channel conductance of receptors that contain edited subunits (Sommer et al., 1991; Egebjerg and Heinemann, 1993; Kohler et al., 1993; Swanson et al., 1996). GluK2 contains two other RNA editing sites in its first transmembrane domain (isoleucine to valine, tyrosine to cysteine), and the edited residues at these two sites also reduce receptor calcium permeability (Kohler et al., 1993). The so-called "Q/R site" is also present in the GluA2 subunit, which is nearly entirely edited in neurons, while GluK1 and GluK2 exhibit partial editing in the brain (Sommer et al., 1991). RNA editing at the Q/R site increases rapidly at the end of rodent embryonic development, with GluK1 becoming around half edited and GluK2 being predominantly edited in adult animals (Bernard and Khrestchatisky, 1994; Bernard et al., 1999). The precise time course and level of editing varies between regions; in DRG the GluK1 subunit is predominantly unedited until birth and becomes almost completely edited by post-natal day (P) 7 (Lee et al., 2001), a time frame that corresponds to spinal innervation by the KAR-expressing population of peripheral neurons (Fitzgerald and Gibson, 1984; Fitzgerald, 1987) and to the downregulation of the auxiliary subunit Neto2, which I present in Chapter 2. KAR transcripts represent the first layer of cellular regulation of receptor composition, and regional differences or developmental alterations of

KAR transcripts are likely critical to the signaling roles or regulation of the receptors they encode.

Modifications and KAR interaction partners

Trafficking chaperones and post-translational modifications

Motifs encoded within KARs mediate a number of protein-protein interactions that facilitate receptor transport to the cell membrane and affect their localization at the synapse. Domains in GluK2 and GluK5 mediate direct interactions with the PDZ-domain containing proteins PSD-95 and SAP102, postsynaptic organization proteins that cluster co-expressed KARs into a punctate pattern (Garcia et al., 1998). In addition to inducing clustered localization, PSD-95 modestly alters desensitization in recombinant KARs (Bowie et al., 2003). Several other PDZ-domain proteins like GRIP, PICK, and syntenin also interact with KAR C-termini, and PICK1 and GRIP regulate post-synaptic receptor localization (Hirbec et al., 2003). GluK2 associates with the postsynaptic ß-catenin–cadherin complexes and members of the MAGUK kinase family, an interaction that can redistribute receptors to cadherin-mediated cell-cell contacts (Coussen et al., 2002). KARs also interact with non-PDZ domain containing proteins such as KRIP6, which reduces desensitization in heterologous systems and competes with PICK1 binding to GluK2 (Laezza et al., 2007; Laezza et al., 2008). Besides promoting postysynaptic localization, protein interactions also regulate internalization of receptors and their removal from the synapse. A C-terminal motif in GluK5 mediates interactions with COPI complex proteins causing ER retention of this subunit, an effect that is overcome by heteromeric assembly with GluK2 (Vivithanaporn et al., 2006). The presynaptic release protein SNAP25 is also found postsynaptically where it regulates internalization of receptors containing GluK5 (Selak et al., 2009). Interactions between the cytoskeletal adaptor protein 4.1N and subunits GluK1 and GluK2 regulate activity dependent

internalization of KARs containing these subunits (Copits and Swanson, 2013). Actinfillin, a protein related to KRIP6, interacts with GluK2 linking it to an E3 ubiquitin ligase and potentially regulating receptor degradation (Salinas et al., 2006). Recent endeavors to describe the iGluR interactome have confirmed many of these interactions, and have identified other potential interaction partners whose impact on KAR function remains to be determined (Shanks et al., 2012). Tightly regulated trafficking and synaptic localization of KARs is a critical aspect of receptor function and much remains to be clarified about the dynamics of these interactions and cellular control of receptor localization.

KAR subunits are also substrates for several post-translational modifications that regulate receptor function: phosphorylation (Traynelis and Wahl, 1997), the addition of a phosphate; palmitoylation (Pickering et al., 1995), the addition of the fatty acid palmitate; and SUMOylation (Martin et al., 2007), the addition of small ubiquitin-like modifier (SUMO). Addition of these modifiers can alter functional properties of the receptor. Phosphorylation by PKA modifies GluK2 and increases open probability of the receptor, and similarly, phosphate removal by calcineurin reduces open probability (Traynelis and Wahl, 1997). Post-translational modification can also affect trafficking and localization of receptors by altering protein–protein interactions. Use-dependent PKC activation reduces KAR current amplitudes in DRG neurons, an effect that is opposed by calcineurin activity (Rivera et al., 2007). PKC also phosphorylates GluK2 via interactions with PICK1 and this phosphorylation is responsible for PICK1 stabilization of KARs in the synapse (Hirbec et al., 2003). Conversely, PKC-mediated endocytosis of GluK2-containing receptors is mediated by phosphorylation and subsequent SUMOylation (Martin and Henley, 2004; Martin et al., 2007; Nasu-Nishimura et al., 2010) and contributes to KAR LTD in the hippocampus (Chamberlain et al., 2012). PKC phosphorylation also reduces GluK2

interaction with the scaffolding protein 4.1N, while palmitoylation enhances 4.1N association and membrane localization of the receptor (Copits and Swanson, 2013). Post-translational modifications and protein–protein interactions are transient molecular components of KARs, particularly when contrasted with the pore-forming subunits, but they nevertheless contribute dynamically to receptor function.

N-linked glycosylation

A substantial post-translation modification occurring on KARs is the *N*-linked glycosylation that decorates the extracellular surface of these proteins (Figure 1.1). Oligosaccharide addition to at least some of the 8–11 consensus asparagines is critical for KAR biogenesis in mammalian cells and accounts for ~10% of the molecular weight of native subunits (Roche et al., 1994; Everts et al., 1999). These glycans have been heavily studied for their role as binding sites for lectins, carbohydrate-binding proteins that allosterically inhibit KAR desensitization. ConcanavalinA (ConA), a lectin isolated from the jack bean *Canavalia ensiformis*, potently inhibits desensitization of currents evoked from recombinant and native KARs (Huettner, 1990; Everts et al., 1997). Lectin potency on oocyte-expressed KARs correlates with carbohydrate binding preference, and their binding is thought to inhibit desensitization by stabilizing the open state of the receptor (Thalhammer et al., 2002; Bowie et al., 2003). That lectins can alter KAR gating properties suggests that their substrate glycans are located at places along the receptor that are also critical to gating. This interpretation is supported by the fact that removal of three sites between the amino-terminal and ligand-binding

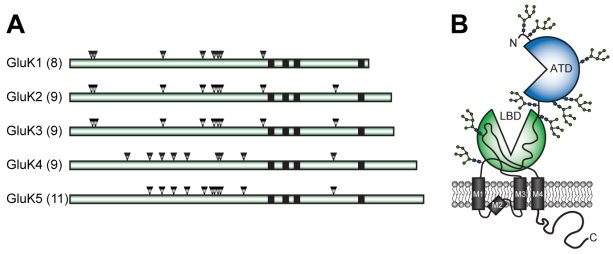

Figure 1.1. KAR subunits have between 8 and 11 predicted *N*-glycosylation sites. **A**) Putative glycosylation sites are indicated by black triangles along the polypeptide chains of KAR subunits. **B**) Cartoon depicting a fully-glycosylated, folded GluK2 subunit.

domains of GluK2, NG5, NG6, and NG7, significantly reduces the effect of ConA and completely occludes inhibition of desensitization by the vertebrate lectin Congerin-1 (Cng-1) (Fay and Bowie, 2006; Copits et al., 2014). Lectin–oligosaccharide binding has been a useful tool in investigating KAR gating properties, and evidence suggests that mammalian lectins could alter iGluR function in the nervous system (Copits et al., 2014). There is reason to ask, however, if *N*-linked glycans might contribute to KAR function beyond assisting receptor folding and mediating lectin interactions.

Beyond folding: glycans and channel gating

Whether or not *N*-glycans modulate iGluR functional properties has been investigated a handful of times, though no clear consensus has emerged (Everts et al., 1999; Pasternack et al., 2003; Vaithianathan et al., 2004; Sinitskiy et al., 2016). Tunicamycin-treated oocytes produce functional AMPA and kainate receptors, though agonist-evoked current amplitudes differ between treated and untreated cells suggesting that desensitization might be affected when glycosylation is precluded (Everts et al., 1997). Consistent with this interpretation, mutant GluK2-containing receptors that lack individual glycosylation sites showed potentially altered desensitization when expressed in oocytes, however this effect was less pronounced when desensitization kinetics were evaluated with higher resolution in mammalian cells (Everts et al., 1999). Mutation of two glycan attachment sites from GluA4 did not alter receptor properties or ligand-binding affinities (Pasternack et al., 2003), and the conclusion from these data has been that oligosaccharide content does not contribute to receptor function properties.

In contrast, native AMPARs show two distinct affinities for [^{3}H]-AMPA that are attributed to different maturity of oligosaccharide processing (Hall et al., 1992; Standley et al., 1998), and perhaps differing glycan structures modulate this important receptor property. Additionally, the open probability of purified native AMPARs is increased by polysialic acid (PSA) but only for receptors isolated from juvenile or neonatal tissue, not adult tissue (Vaithianathan et al., 2004). These studies support the idea that *N*-glycans modulate AMPAR function and indicate the possibility of developmental regulation of iGluRs by glycans. The NMDAR subunit GluN1 contains 12 consensus glycosylation sites, the most of all iGluR subunits, and is an obligate constituent of NMDARs. Glycosylation is required for NMDAR expression, even in oocytes (Everts et al., 1997), and native receptors contain both immature oligomannosidic and complex fucosylated glycans (Clark et al., 1998; Hanus et al., 2016; Kaniakova et al., 2016). Computational modeling indicates that sugar chains attached to the GluN1 LBD stabilize the closed conformation of this domain. Consistent with this conclusion, occluding glycan attachment at one of these sites increases the glycine EC_{50} of recombinant NMDARs, likely due to the loss of inter-lobe contacts mediated by the sugar chain (Sinitskiy et al., 2016). While glycan contribution to iGluR functional properties remains poorly defined, oligosaccharide chains clearly modulate voltage-gated channel properties (Scott and Panin, 2014). The sialylation level of voltage-gated sodium channels controls their gating properties in a developmental and region-specific manner (Tyrrell et al., 2001; Ufret-Vincenty et al., 2001b; Stocker and Bennett, 2006). Additionally, there is evidence that functional properties of TRP channels and $GABA_A$ receptors are influenced by their glycosylation state (Dietrich et al., 2003; Lo et al., 2010; Pertusa et al., 2012).

We have taken a different approach to this question by inhibiting or over-expressing glycan modifying enzymes to alter the structural composition of oligosaccharides attached to KARs and then characterizing functional properties of the receptors. Our work provides evidence that glycan content affects KAR functional properties, and shows that this depends on both the identity of the glycans and the subunits to which they are attached.

Auxiliary subunits: more than an interaction partner

I have described the molecular components of KARs that constitute their pore-forming subunits and provided an overview of the various modifications that occur in KAR transcripts and on subunits. These various modifications influence interactions these receptors have with proteins that variously modify their trafficking and localization, though these protein–protein interactions are generally transient components of the life-cycle of the receptor. A different class of interacting proteins has been described for receptor-channel proteins, that of auxiliary subunits. Auxiliary subunits constitute a permanent component of iGluRs and are considered to interact with a receptor for the lifetime of that protein, although they might functionally decouple during structural rearrangements such as desensitization (Morimoto-Tomita et al., 2009).

Discovery of iGluR auxiliary subunits

With the exception of the *∂* receptor subfamily, expression of cloned iGluR subunits in heterologous systems recapitulated the minimal two elements of these receptors previously identified in neurons: (1) cationic, depolarizing conductances that were (2) evoked by glutamate. Evidence that these receptors have protein constituents that neither directly bound ligands nor formed the channel pore came first for AMPA receptors. Genetic analysis determined that a

loss-of-function mutation in a protein called stargazin (also named γ-2) was the likely cause of ataxia and seizures that had developed spontaneously in a line of mice (Letts et al., 1998). Further characterization showed selective loss of AMPARs from cerebellar mossy fiber to granular cell synapses (Hashimoto et al., 1999). This and follow-up studies determined that stargazin was required for surface expression and postsynaptic localization of cerebellar AMPARs and that it modified recombinant receptor gating (Chen et al., 2000; Priel et al., 2005). Soon, related protein sequences were identified and it became clear that this new family of proteins altered AMPAR functional properties and contributed critically to native receptor function (Tomita et al., 2003; Yamazaki et al., 2004; Rouach et al., 2005; Tomita et al., 2005; Turetsky et al., 2005; Jackson and Nicoll, 2011); reflecting this, they were named transmembrane AMPA receptor regulatory proteins, or TARPs. This new development presented a major shift in the perception of the proteins that comprise a "complete" iGluR.

The broadened understanding of ligand-gated channel molecular constituents necessitated a framework for differentiating auxiliary subunits from interacting proteins. Towards this goal, Yan and Tomita (Yan and Tomita, 2012) proposed inclusion standards for ligand-gated channel auxiliary subunits that were based on previously established criteria from the voltage-gated channel field (Isom et al., 1994; Vacher et al., 2008). These standards require four criteria to be met for an interacting partner to be classified as an auxiliary subunit (Yan and Tomita, 2012). Logically, the protein must (1) not have any channel activity of itself, and it must (2) form a direct and stable interaction with a pore-forming subunit in its receptor-channel. An auxiliary subunit must (3) modulate channel properties and/or receptor trafficking in heterologous systems and must also be (4) necessary for *in vivo* channel properties, expression, and/or localization.

Neto proteins: the KAR auxiliary subunits

In 2009, the relatively uncharacterized protein neuropilin- and tolloid-like (Neto) 2 (Figure 1.2A) was identified as a KAR interaction partner that altered rates of deactivation, desensitization, and recovery from desensitization, and increased open probability of GluK2-containing receptors. Additionally, hippocampal cultures from Neto2-null (Neto2$^{-/-}$) mice showed altered glutamate:kainate current ratios, suggesting that the Neto2 modulation observed in recombinant receptors might translate to native KARs (Zhang et al., 2009). Concurrently, the highly similar Neto1 was shown to interact with NMDARs and to influence spatial memory, though no direct Neto1 modulation of NMDAR biophysical properties was shown (Ng et al., 2009). Neto1 and Neto2 are single-pass transmembrane domain proteins with 57% sequence identiy, containing two extracellular CUB domains similar to the neuropillin and tolloid proteins, for which are named (Stöhr et al., 2002). The Neto proteins substantially alter functional properties of several different KAR combinations, and Neto2 promotes post-synaptic localization of GluK1 when both are transfected into hippocampal cultures (Copits et al., 2011; Straub et al., 2011a). The discovery of significantly faster KAR-mediated excitatory post-synaptic currents (EPSCs) in hippocampal slices from Neto1$^{-/-}$ mice cemented the classification of these proteins as KAR auxiliary subunits, at least for Neto1 (Straub et al., 2011b; Tang et al., 2011).

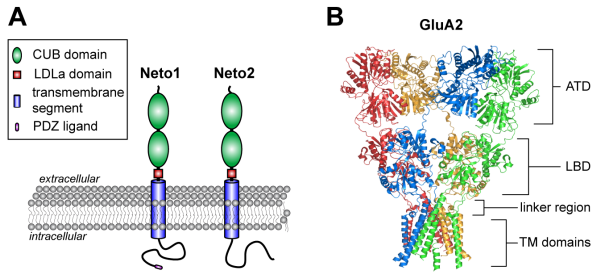

Figure 1.2. Neto and iGluR structure and domains. **A**) Cartoon depicting Neto protein structural domains. Modified from Copits & Swanon, 2012. **B**) Crystal structure of GluA2-containing AMPAR, reproduced from Sobolevsky, et al., 2009. Functional domains are noted: ATD, amino-terminal domain; LBD, ligand-binding domain; linker domain; TM, transmembrane domains and ion pore.

The question of whether Neto2 is also an auxiliary subunit rests primarily on whether it is a constituent of endogenous receptors. Genetic deletion of Neto1 and Neto2 does not reduce overall expression levels of primary KAR subunits but it does reduce the post-synaptic pool of KARs in the hippocampus and

cerebellum, respectively (Tang et al., 2011; Tang et al., 2012). Many groups observe Neto2-mediated slowing of KAR EPSCs in neurons where GluK1 and Neto2 are exogenously expressed (Zhang et al., 2009; Copits et al., 2011; Straub et al., 2011a; Sheng et al., 2015; Palacios-Filardo et al., 2016; Sheng et al., 2017), which confirms that Neto2 modulation of KARs is possible in neurons. This does not fulfill the auxiliary subunit criteria that Neto2 be a required component of native receptors, however, and it has remained unclear where Neto2-containing KARs might be expressed. Work presented in Chapter 2 confirms that Neto2 fulfills the fourth requirement for inclusion as a KAR auxiliary subunit, as it assembles with and alters KAR properties in DRG neurons.

Neto proteins have also been determined to interact with proteins other than KAR subunits. As mentioned above, Neto1 was first detected interacting with NMDARs and Neto1$^{-/-}$ mice have reduced post-synaptic localization of both NMDARs and KARs in the hippocampus (Wyeth et al., 2014). Direct Neto modulation of NMDAR channel properties has not been reported, but AMPAR:NMDAR ratio is increased and LTP is impaired in Neto1$^{-/-}$ hippocampal slices (Ng et al., 2009; Tang et al., 2011), perhaps due to altered receptor localization in the absence of Neto1. A non-KAR interaction partner has also been suggested for Neto2, the chloride co-transporter KCC2. Neto2 enhances KCC2 expression and function in hippocampal neurons (Ivakine et al., 2013; Mahadevan et al., 2014), and aberrantly depolarized chloride reversal potential in the hippocampus due to loss of KCC2 is proposed to underlie the increased seizure susceptibility observed in Neto2$^{-/-}$ mice (Mahadevan et al., 2015). Both KCC2 and KAR subunits interact with protein 4.1N (Li et al., 2007; Copits and Swanson, 2013), and it is possible that Neto2 modulation of KCC2 occurs within complexes of KAR interacting proteins. It is clear that Neto proteins are critical components of KARs, and the extent to which these "non-KAR" functions of

Neto proteins are truly segregated from their roles as KAR auxiliary proteins is yet to be determined.

Gating mechanisms and signaling pathways

Receptor structure and gating mechanisms

Studies elucidating iGluR structure provide three-dimensional perspective to our understanding of the molecular constituents of these receptors. The solved crystals of GluK1 and GluK2 ligand-binding domains (LBDs) showed a clamshell-like structure that forms the pocket for ligand docking, similar to other iGluRs (Nanao et al., 2005; Naur et al., 2005). These studies began the still-ongoing exercise of applying structural location information to our knowledge of functionally critical amino acid residues to better understand the complex physical relationship between agonist binding and channel gating (Mayer, 2005; Kumar and Mayer, 2013; Sobolevsky, 2015). Subsequent publication of a full-length GluA2 structure greatly advanced our understanding of iGluR composition ((Sobolevsky et al., 2009) reproduced in Figure 1.2B). The combination of this full AMPAR structure, solved crystals of isolated KAR domains, and full-length cryoEM structures of KARs have informed our "picture" of KARs, as a full-length KAR has not yet been crystallized (Nanao et al., 2005; Naur et al., 2005; Kumar et al., 2009; Meyerson et al., 2016). The GluA2 structure revealed an interesting shift in symmetry of tetrameric receptors: the ATD and LBD form dimers of dimers, in which the subunit pairs forming these dimers switch partners between the domains, and this two-fold symmetry shifts to four-fold symmetry at the level of the transmembrane domains and the channel pore (Sobolevsky et al., 2009). Linker domains, termed the S1-M1, M3-S2, and S2-M4 linkers, bridge this symmetry switch as they transduce the energy and conformational shifts of agonist binding to the ion pore within the transmembrane domain. In particular, the M3-S2 linker and a highly

conserved region at the extracellular edge of the M3 domain are critical determinants of both receptor gating and auxiliary modulation of receptor gating (Kohda et al., 2000; Schwarz et al., 2001; Yelshansky et al., 2004; Vivithanaporn et al., 2007; Griffith and Swanson, 2015).

These structures revealed three-dimensional location information of motifs and residues that had previously been discovered to be critical to gating. Receptors are proposed to occupy several different states throughout this process, that are each most highly associated with a particular level of agonist saturation at the ligand-binding site. KARs have a very low open probability in the absence of agonist and are primarily in the resting state. Resting state receptors are capable of activating upon agonist binding, and even partial agonist occupancy activates the channel, opening the pore to allow ion flux at several sub-conductance levels (Rosenmund et al., 1998); when agonist unbinds from an activated receptor, the receptor returns to resting, or deactivates (Swanson et al., 1998; Barberis et al., 2008). Complete agonist occupancy, such as that which occurs at saturating concentrations or prolonged application, destabilizes the LBD upper-lobe interface causing desensitization (Swanson et al., 2002; Robert and Howe, 2003; Weston et al., 2006). Desensitization, the conformation in which a ligand-gated channel is closed and unable to respond to agonist, results from drastic displacement of the LBDs and dissolution into quasi-four-fold symmetry at this level (Meyerson et al., 2014; Meyerson et al., 2016). KARs recover from this splayed, desensitized state over several seconds, which is quite slow compared with the hundreds of milliseconds in which AMPARs recover (Heckmann et al., 1996; Swanson and Heinemann, 1998; Robert and Howe, 2003). Movement between these conformational states is determined by the energetic stability of each state, a property that is obviously influenced by ligand binding but also depends on the composite of molecular interactions in and around functional domains. These interactions differ

between subunits and can be altered by extracellular ions (Bowie, 2002; Veran et al., 2012). Two sodium and one chloride ion bind in separate pockets at the LBD dimer interface and are a critical component of KAR but not AMPAR gating (Paternain et al., 2003; Wong et al., 2006; Plested and Mayer, 2007; Plested et al., 2008). *N*-glycans in KARs are attached at several sites in the LBD and near the LBD dimer interface, which positions them near functionally relevant domains within the receptor (Parker et al., 2013). Interactions between these sugars and the LBD might modulate NMDAR gating (Sinitskiy et al., 2016), however glycosylation is often removed or reduced to improve the success of protein crystallization and our understanding of these molecules as components of the KAR structure is limited.

Metabotropic signaling and G-protein interactions

KARs are clearly ionotropic receptor-channels and much of their signaling is thought to occur downstream of the currents they gate. Nonetheless, activation of KARs induces signaling through G-protein and PKC-sensitive pathways that can alter intrinsic excitability and GABA release. In hippocampal pyramidal neurons the sAHP and mAHP are reduced following KAR activation, and KAR activation in hippocampal interneurons and DRG neurons inhibits voltage-gated calcium currents (Rodriguez-Moreno and Lerma, 1998; Melyan et al., 2002; Rozas et al., 2003; Fisahn et al., 2005). The mechanism behind this phenomenon remains unclear. While generally evoked by exogenous activation of receptors, this signaling can be engaged by synaptically released glutamate (Melyan et al., 2004). These metabotropic pathways require intact GluK1 expression in DRG neurons or GluK2 expression in the hippocampus (Rozas et al., 2003; Fisahn et al., 2005). GluK5 subunits are also proposed to mediate this signaling (Ruiz et al., 2005) but their requirement has not been confirmed (Fernandes et al., 2009). Given the obvious ion-channel structure of KARs, an attractive explanation for their metabotropic action is

that G-protein activation is indirectly coupled to ionotropic KAR signaling, however recent experiments suggest a direct interaction between GluK1 and G_o (Rutkowska-Wlodarczyk et al., 2015). Additionally, ionotropic action of the receptors is not required to induce G-protein mediated signaling (Melyan et al., 2002; Rozas et al., 2003). How Neto proteins might contribute to the metabotropic action of KARs remains an open question, particularly as we find Neto2 to be a KAR constituent in DRG neurons where this signaling occurs.

Characterization of native KAR properties and function

These discoveries have deepened our understanding of the molecular elements that constitute KARs and contribute to their function. Many of these components have been reconstituted with KARs in heterologous cells, to test their contribution to receptor function in a reduced system. With one possible exception, however, these systems fail to reproduce the functional properties of native receptors. Recombinant KARs display large agonist-evoked currents with fast decays (Herb et al., 1992; Schiffer et al., 1997; Swanson et al., 1997; Paternain et al., 1998), which contrast with the small, slow KAR-mediated postsynaptic currents observed in neurons (discussed below). These slow kinetics of native receptors are observed at postsynaptic sites throughout the nervous system, and suggest that a critical component of native receptors has yet to be described.

Native KAR functional properties differ from recombinant receptor properties

Although KAR-mediated currents had been described and characterized in neurons using agonists (Agrawal and Evans, 1986; Huettner, 1990; Lerma et al., 1993), the discovery of mostly-selective AMPAR antagonists allowed KAR currents to be isolated and their function to

be probed (Paternain et al., 1995; Chittajallu et al., 1996). This revealed postsynaptic KAR responses at synapses in the hippocampus (Castillo et al., 1997; Vignes and Collingridge, 1997; Frerking et al., 1998), at thalamocortical synapses (Kidd and Isaac, 1999), and post-synaptic to C-fiber inputs in the spinal cord dorsal horn (Li et al., 1999), and it became clear that KARs fulfilled a role in neural circuits that was distinct from the closely related AMPARs. Initially surprising features of KAR-mediated excitatory postsynaptic currents (EPSCs) in neurons were their small amplitudes and slow decay kinetics, which contrasted with receptor properties measured in heterologous systems. Slow KAR EPSCs were determined to be intrinsic to these native receptors and not caused by slow exposure to diffusing glutamate, as might happen if the receptors were located perisynaptically (Petralia et al., 1994; Vignes and Collingridge, 1997; Bureau et al., 2000; Darstein et al., 2003). One component of the mismatch between recombinant and native KAR properties likely lies in the experimental set-up used to probe recombinant receptor function; heteromeric, though not homomeric, KARs deactivate with slow kinetics in response to sub-saturating glutamate (Barberis et al., 2008), which might more closely resemble synaptic glutamate exposure than does a long exposure to saturating glutamate. Additionally, it is clear that native KAR properties depend on receptor subunit composition, as KAR-mediated EPSCs are altered in mice genetically deficient for either pore-forming or auxiliary subunits (Mulle et al., 1998; Contractor et al., 2003; Fernandes et al., 2009; Straub et al., 2011b; Tang et al., 2011). With one possible exception, however (Barberis et al., 2008), matching expected subunit composition has not recapitulated the slow kinetics of native receptors and there are likely KAR components that are incompletely, incorrectly, or not at all recapitulated in recombinant systems that are nonetheless critical to native receptor function.

KARs function as modulators of circuit excitability

As mentioned above, AMPA and kainate receptors play drastically different roles within neural circuits. KARs are often localized presynaptically, from where they modulate both excitatory (Chittajallu et al., 1996; Contractor et al., 2001; Lauri et al., 2001; Schmitz et al., 2001; Kidd et al., 2002; Sun and Dobrunz, 2006; Pinheiro et al., 2007) and inhibitory signaling (Clarke et al., 1997; Rodriguez-Moreno et al., 1997; Min et al., 1999; Cossart et al., 2001; Jiang et al., 2001; Semyanov and Kullmann, 2001). Even when localized to post-synaptic sites, KARs do not mediate fast-excitatory transmission like AMPARs and their slow kinetics lead them to influence cellular excitability through EPSC summation and to increase EPSP-spike coupling (Frerking and Ohliger-Frerking, 2002; Miyata and Imoto, 2006; Goldin et al., 2007; Sachidhanandam et al., 2009). KAR activation can also increase interneuron firing rates, which increases sIPSC frequency but could also contribute to their negative regulation of evoked GABA release (Cossart et al., 1998; Frerking et al., 1999). These properties, considered with KAR modulation of cellular excitability via metabotropic signaling, suggest that KAR activation could diversely influence circuit function in a manner dependent on cell-specific subunit expression, subcellular receptor localization, and the precise downstream signaling the receptors engage.

KARs modulate pain circuitry

KARs are highly expressed and predicted to exert modulatory control in circuits throughout the pain neuraxis, a complex network that perceives and responds to danger in the environment. Intact pain sensation is critical for survival; it allows an organism to avoid tissue damage and to protect damaged tissue as it recovers. By contrast, pain that persists in the absence of tissue damage or pain sensed in response to innocuous environmental elements is deleterious, as it

can inhibit physical and cognitive capacities as well as disrupt emotional processing (Cohen and Mao, 2014; Baliki and Apkarian, 2015; Boakye et al., 2016).

KARs containing the GluK1 subunit are highly expressed in the unmyelinated, small-diameter C-fiber neurons with the DRG (Agrawal and Evans, 1986; Huettner, 1990). These neurons grow a pseudounipolar axon that bifurcates, sending one process out to peripheral tissues and one into the dorsal horn of the spinal cord, and they are the first step in transmitting sensory signals from peripheral tissues to the CNS. In the spinal cord they synapse onto projection neurons and interneurons within lamina II (LII). KARs are present at a number of sites in the dorsal horn: on the presynaptic terminals of incoming sensory neurons (Kerchner et al., 2001a), post-synaptic to these nociceptive inputs in LII (Li et al.), and as modulators of interneuron excitability and GABA release on LII interneurons (Kerchner et al., 2001b; Xu et al., 2006). In primary sensory neurons, the GluK1 subunit is an essential component of KARs, but within the spinal cord both the GluK1 and GluK2 subunits contribute to receptors. Given the many positions from which they could modulate excitability within this sensory circuit, it is not surprising that a GluK1-directed antagonist reduces spiking from the spinothalamic neurons in a primate neuropathy model (Palecek et al., 2004). Sensory signals proceed from the spinal cord to intermediate central processing centers on their way to cortical processing sites. A major recipient of spinal output signals is the thalamus, where GluK1-containing KARs modulate GABAergic tone in thalamic reticular neurons within the ventrobasal thalamus (Binns et al., 2003). The amygdala receives input from the thalamus among other brain regions, and GluK1-containing receptors in amydalar interneurons promote interneuron excitability and reduce anxiety-like behaviors (Wu et al., 2007b). GluK1-containing KARs contribute to presynaptic LTP at amydalar to ACC synapses (Koga et al., 2015) and layer 2/3 pyramidal cells in the anterior cingulate cortex (ACC)

express postsynaptic GluK1/GluK2-containing KARs, as do pyramidal cells in all layers of the insular cortex (Wu et al., 2005; Koga et al., 2012). In the ACC, GluK1-containing receptors also enhance GABAergic signaling (Wu et al., 2007a). Consistent with KAR expression at multiple locations in the ACC, intra-ACC infusion of a GluK1 antagonist reduces behavioral hypersensitivity caused by formalin injection (Descalzi et al., 2013). Descending sensory control from cortical regions passes through the amygdala and brainstem centers to modulate spinal excitability. Within descending circuits, the periaquaductal grey (PAG) expresses GluK1-containing receptors and their activation in PAG cultures increases mIPSC frequency (Nakamura et al., 2010). Notably, GluK1-directed antagonism reduces hypersensitive pain in animal models when compounds are injected systemically, intrathecally, and intra-ACC (Dominguez et al., 2005; Qiu et al., 2011; Descalzi et al., 2013). The multiplicity of potential influences KARs could have on sensory processing is great. The observations that KARs are poised to modulate pain signaling and that their antagonism modifies pain behaviors are particularly intriguing given that GluK1-targeted compounds do not affect acute pain (Sang et al., 1998; Qiu et al., 2011).

Animal studies of pain behavior strongly suggest a role for these receptors, specifically those containing the GluK1 subunit, in pathological pain (see (Bhangoo and Swanson, 2013) and Chapter 2). This is supported by experiments on healthy human volunteers showing that GluK1 antagonism blunted inflammatory hypersensitivity but did not alter basal pain thresholds (Sang et al., 1998). Administration of this GluK1 antagonist to migraine sufferers during an attack and was found to significantly reduce both migraine pain and secondary symptoms (Sang et al., 2004), in agreement with pre-clinical experiments (Filla et al., 2002; Weiss et al., 2006). KAR antagonism was also found to alleviate pain following dental surgery (Gilron et al.). Further

development of KAR-targeting therapeutics, however, is hampered by the relatively poor specificity of these agents and the predominance of compounds that target the glutamate binding site.

Pharmacology

Despite much effort, identification of pharmacological agents that are highly selective for KARs has proven a challenge that persists even today. An initial success on this front was the discovery that some 2,3-benzodiazepine compounds, notably GYKI52466 and GYKI53665, antagonize AMPARs at lower concentrations than KARs (Bleakman et al., 1996). This allowed segregation of KAR from AMPAR activation in neurons, and was an important tool for characterizing native KAR function (Paternain et al., 1995; Wilding and Huettner, 1995; Chittajallu et al., 1996; Castillo et al., 1997; Vignes and Collingridge, 1997). Among KAR subunits, GluK1 has proven the most amenable to pharmacological targeting but many of the compounds directed against this subunit show some level of activity at AMPAR or other KAR subunits (Alt et al., 2004; Jane et al., 2009; Perrais et al., 2009b). Synthesis and characterization of the AMPA derivative ATPA defined it as an agonist primarily at GluK1-containing receptors with minor activity at AMPARs (Clarke et al., 1997); natural products and their derivatives such as 5-iodowillardiine, UBP310, dysiherbaine, and MSVIII-19 have proven useful tools to dissect particulars of KAR function (Agrawal and Evans, 1986; Jane et al., 1997; Sakai et al., 2001; Swanson et al., 2002; Alt et al., 2004; Sanders et al., 2005; Dolman et al., 2007). The UBP compounds, derived from willardiine, are among the most selective antagonists for GluK1-containing receptors, though they might also antagonize receptors containing GluK3 (Perrais et al., 2009b). The Eli Lilly compound tested in humans, LY293558, is a competitive antagonists with moderate selectivity for GluK1 over AMPARs and its polar nature makes it a

poor therapeutic (Sang et al., 1998; Dominguez et al., 2005; Jones et al., 2006). Beyond the challenge of developing specific compounds, KARs have proven resistant to attempts at allosteric modulation; only a handful of allosteric modulators have been developed, and their activity and selectivity have not been thoroughly characterized (Valgeirsson et al., 2003; Christensen et al., 2004b; Valgeirsson et al., 2004; Kaczor et al., 2015).

The lack of specific and allosteric compounds directed against KARs has particularly hindered successful therapeutic targeting of these receptors, and limits our capability to test KAR contribution to human disease. To this point, GluK1-containing KARs are highly expressed in the retina, and a dose-limiting and likely on-target side effect of GluK1 antagonism in people is hazy vision (Sang et al., 1998; Puthussery et al., 2014). It is possible that the ability to pharmacologically discriminate between different receptor combinations that contain a common subunit could allow for directed targeting of KARs involved in pain rather than vision. Development of improved compounds is obviously important to remedying the pharmacological limitations in the KAR field, and critical to this goal is a more complete picture of the target KAR molecular constituents.

KAR role in neural function and disease

Broad expression of KARs throughout the nervous system and their modulation of the balance between excitation and inhibition suggest that their misregulation would prove deleterious in humans. In addition to their role in hypersensitive pain, studies in animals clearly show that excessive KAR signaling promotes seizures and epileptiform activity, that interneuronal KAR signaling might reduce seizure propagation, and that seizures induce aberrant KAR expression

in the hippocampus (Mulle et al., 1998; Khalilov et al., 2002; Smolders et al., 2002; Epsztein et al., 2005). Sprouting of recurrent hippocampal synapses occurs in human temporal lobe epilepsy, and in rodents these recurrent synapses express abnormal post-synaptic KARs that promote epileptiform activity (Epsztein et al., 2010). Direct implication of KAR function in human epilepsy has not been reported, though a recent case study linked a loss-of-function mutation in *Grik2* to neurodevelopmental delays and epilepsy in two siblings (Cordoba et al., 2015). Other studies have suggested links between mutations or deletions of KAR subunits and general neurodevelopmental disorders (Motazacker et al., 2007; Bonaglia et al., 2008; Takenouchi et al., 2014) as well as to the autism spectrum (Jamain et al., 2002; Shuang et al., 2004; Kim et al., 2007) (but see (Dutta et al., 2007)). Genetic linkage analyses have also implicated KAR variants in neuropsychiatric conditions such as schizophrenia and obsessive-compulsive disorder (Begni et al., 2002; Pickard et al., 2006; Wilson et al., 2006; Beneyto et al., 2007; Schiffer and Heinemann, 2007; Woo et al., 2007) (but see (Shibata et al., 2002)). Although much remains unknown about KAR function in these disorders, the inferred involvement of these receptors is consistent with dynamic KAR regulation during development and their modulatory role in circuit function.

Project goals

As discussed above, the molecular components that contribute to KAR signaling can be encoded in subunits, added post-translationally, or result from transient protein-protein interactions. My goal in this project is to further our understanding of two KAR constituents: the auxiliary protein Neto2 and the covalently attached *N*-glycans that decorate receptors. Although both are elements of KARs, the outstanding questions for these two receptor components are quite different. It is clear that Neto2 modulates important receptor properties and that it is

expressed in various brain regions where KAR pore-forming subunits are also present. Endogenous Neto2-containing KARs have not yet been identified, however, and Neto2 cannot be considered an auxiliary subunit if it is not a component of native receptors. Additionally, the location of Neto2-containing KARs must be determined before the contribution of Neto2 to native KAR function can be tested. I have made use of Neto2$^{-/-}$ mice to test the contribution of Neto2 to KAR properties and function in peripheral sensory neurons, a population of cells that highly express GluK1-containing KARs. My data implicate Neto2 as a developmentally-regulated KAR constituent in the peripheral nervous system, and the more general possibility that Neto2 is developmentally regulated in the central nervous system can now be tested.

On the other hand, a potential contribution of oligosaccharides to KAR function has not been clearly established. *N*-glycosylation is critical for receptor biogenesis but studies evaluating *N*-glycan modulation of functional properties have been equivocal. These previous studies have mutated consensus glycosylation sites or inhibited *N*-glycosylation altogether, which manipulate oligosaccharide chains in a way that is not likely to occur *in vivo*. We have taken a different approach in which we manipulate processing enzymes to shift the molecular content of oligosaccharides before measuring functional properties of glycan-altered recombinant receptors. Our study in a reduced system provides proof of concept that the glycan content on KARs can influence the complex structural rearrangements underlying receptor gating. Elucidating the oligosaccharide structures present on native receptors will be a critical next step to determining glycan contribution to KAR function in neurons. The work presented in this dissertation furthers our knowledge of two elements within many that contribute to KAR function. Molecular diversity of KARs is likely to be a cellular tool that regulates KAR signaling in neural

circuits, and a thorough characterization of this diversity is essential to deepen our understanding of the role that KARs play in cognition and disease.

Aim 1. To determine the contribution of Neto2 to KAR properties and to regulated process outgrowth in peripheral sensory neurons. This work is presented in Chapter 2.

Aim 2. To test how altered *N*-linked oligosaccharide content affects the functional properties of recombinant KARs. This work is presented in Chapter 3.

Chapter 2.

The auxiliary protein Neto2 modulates KAR functional properties in sensory neurons in a developmentally-regulated fashion.

Abstract

Peripheral sensory neurons in the dorsal root ganglia (DRG) are the initial transducers of sensory stimuli, including painful stimuli, from the periphery to central sensory and pain-processing centers. Small- to medium-diameter non-peptidergic neurons in the neonatal DRG express functional kainate receptors (KARs), one of three sub-families of ionotropic glutamate receptors, as well as the putative KAR auxiliary subunit Neto2. Neto2 markedly alters recombinant KAR function but has yet to be confirmed as an auxiliary subunit that assembles with and alters function of endogenous KARs. KARs in neonatal DRG require the GluK1 subunit as a necessary constituent, but it is unclear to what extent other KAR subunits contribute to the function and the proposed roles of KARs in sensory ganglia, which include promotion of neurite outgrowth and modulation of glutamate release at the DRG-dorsal horn synapse. Additionally, KARs containing the GluK1 subunit are implicated in modes of persistent but not acute pain signaling. We show here that Neto2 protein is highly expressed in neonatal DRG and modifies KAR gating in DRG neurons in a developmentally-regulated fashion in mice. While normally at very low levels in adult DRG neurons, Neto2 protein expression can be upregulated via MEK/ERK signaling and following sciatic nerve crush, and Neto2$^{-/-}$ neurons from adult mice have stunted neurite outgrowth. These data confirm that Neto2 is a *bona fide* KAR auxiliary subunit that is an important constituent of KARs early in sensory neuron development and suggest that Neto2 assembly is critical to KAR modulation of DRG neuron process outgrowth.

Introduction

Kainate receptors (KARs), a subfamily of ionotropic glutamate receptors (iGluRs), modulate circuit activity and excitatory/inhibitory balance throughout the nervous system (Contractor et al., 2011). KARs assemble as a diverse array of heteromeric proteins found at both pre- and post-synaptic sites. Five pore-forming subunits (GluK1-GluK5) form the tetrameric core of these receptors, which differ in their biophysical properties and neuronal distribution dependent upon their subunit composition (Contractor et al., 2011). Additionally, the Neuropilin- and tolloid-like (Neto) proteins, Neto1 and Neto2, impact KAR biophysical properties and localization, providing an extra layer of functional variability dependent on the subunits expressed in a given cell type (Ng et al., 2009; Zhang et al., 2009; Copits et al., 2011; Straub et al., 2011b; Tang et al., 2011). Both Neto proteins were shown to alter recombinant KAR properties, but to date only Neto1 is known to incorporate into and alter native KAR function (Straub et al., 2011b; Tang et al., 2011). Modulation of native receptors represents a critical criterion that distinguishes between putative and validated auxiliary subunits (Yan and Tomita, 2012), and Neto2 therefore has yet to be confirmed as a *bone fide* KAR auxiliary subunit despite its robust impact on recombinant receptor properties (Zhang et al., 2009; Copits et al., 2011; Straub et al., 2011a; Tang et al., 2012).

KARs are expressed throughout the nervous system including in small-diameter non-peptidergic neurons located in the dorsal root ganglia (DRG) that are presumed to be nociceptors based on their molecular profile and their slow conduction velocity (Agrawal and Evans, 1986; Huettner, 1990; Lee et al., 2001; Usoskin et al., 2015). GluK1 mRNA is expressed in DRG at particularly high levels (Bettler et al., 1990; Partin et al., 1993), and KAR currents and calcium signals are dependent on expression of the GluK1 subunit (Mulle et al., 2000; Rozas et al., 2003). Glutamate-evoked currents in DRG neurons have properties similar to GluK1-containing

receptors (Swanson and Heinemann, 1998), though there is evidence for GluK5 mRNA (Herb et al., 1992; Partin et al., 1993) and Neto mRNA in DRG (ASCA, 2015).

The role of KARs in peripheral neurons is diverse. They act as autoreceptors at central terminals to modulate glutamate release onto dorsal horn neurons (Kerchner et al., 2001b; Kerchner et al., 2002), and their activation impacts neurite outgrowth in culture (Joseph et al., 2011; Marques et al., 2013). Peripheral KARs are thought to serve as glutamate sensors during inflammation or tissue damage (Du et al., 2006) and somatic KARs might sense glutamate released within the ganglia (Kung et al., 2013). GluK1-containing KARs also modulate pathologic pain, though this function does not necessarily arise from the KAR population in DRG neurons (Wu et al., 2005; Koga et al., 2012), and pharmacological inhibition or genetic ablation of GluK1 alleviates diverse models of hypersensitive pain in rodents (reviewed in (Bhangoo and Swanson, 2013) and in Chapter 1). While the obligatory role of the GluK1 subunit is clear, to what extent additional pore-forming or auxiliary subunits contribute to these receptors is not understood. Moreover, the physiological properties of KARs in adult DRG neurons, and how those might differ from neonatal receptors, have not been characterized despite their relevance to nociception and other activities. A better understanding of the molecular composition of KARs will be critical to successfully targeting the receptors in pathological pain in a therapeutically useful manner.

We confirm here that Neto2 is a *bona fide* KAR auxiliary subunit and a critical component of KARs in peripheral sensory neurons. Neto2 expression is highest in the first post-natal week, reducing to low levels around 2 weeks after birth. Adult neurons retain the capacity for high Neto2 expression, however, and their outgrowth in culture is altered in the absence of Neto2. Additionally, Neto2 is upregulated following crush injury to the sciatic nerve. These findings

show that Neto2 is a developmentally-regulated component of KARs in the peripheral nervous system and it is dynamically regulated in adult neurons during axon outgrowth.

Materials and Methods

Animals

All animals used in these studies were treated according to protocols approved by Northwestern University's Institutional Animal Care and Use Committee, which were consistent with standards of care established by the *Guide for the Care and Use of Animals*, edn 8, published by the US National Institutes of Health in 2011. Male and female mice were used for all experiments; no gender dependent differences were observed and so data from both genders was combined. For physiology and biochemical experiments, wildtype animals are C57Bl/6 from Charles River (Wilmington, MA) and The Jackson Laboratory (Bar Harbor, ME) or are wildtype littermates of the knockout animals used. For outgrowth and behavioral experiments, wildtype animals are littermates of the Neto1 knockout ($Neto1^{-/-}$) and $Neto2^{-/-}$ mice that were generously provided to us by Dr. Roderick McInnes (Ng et al., 2009; Tang et al., 2011). $GluK5^{-/-}$ mice were provided by Dr. Anis Contractor (Contractor et al., 2003). For all experiments, neonatal animals are between post-natal day 0 (P0) and P5, adult animals are P56 (8 weeks) and older, all other ages are noted.

Dissection and Neuron Culture

Dorsal root ganglia (DRG) neuron cultures were performed as described previously (Copits et al., 2014). Briefly, mice were anesthetized with isoflurane and rapidly decapitated. DRG were removed and cleaned of nerve processes and connective tissue. For electrophysiology, only lumbar ganglia were removed, for imaging and Western blotting, lumbar and thoracic ganglia were removed. Tissue was digested at 37^{o}C in collagenase A/D (1 mg/ml neonatal DRG, 3-5 mg/ml adult DRG) followed by 0.4 mg/ml activated papain (Roche, Indianapolis, IN). DRG were plated to poly-L-lysine/laminin coated glass coverslips in 50:50 DMEM:F12 media (Corning

Cellgro, Mannassas, VA) containing 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA) and 0.5% penicillin/streptomycin (Corning Cellgro, Mannassas, VA). Cells were plated in a minimal volume (100-200 ml) to each coverslip and wells were filled with media after 2 hours of culture. At this time, Nerve Growth Factor (10 ng/ml) (Promega, Madison, WI) was added to adult cultures, and U0126 (Abcam, Cambridge, MA), triciribine (Sigma, St. Louis, MO), and wortmannin (Sigma, St. Louis, MO) were added as noted in the results.

Western Blotting

Total solubilized protein was separated on a 10% denaturing polyacrylamide gel and transferred to a polyvinylidene fluoride membrane. Neto2 was detected using Abcam (Cambridge, MA) rabbit anti-Neto2 (1:2000, Abcam catalog number ab109288, RRID:AB_10863520). Actin was detected using Sigma-Alderich (St. Louis, MO) mouse anti-actin (1:2000, Sigma-Aldrich catalog number A4700, RRID:AB_476730). Goat anti-rabbit and Goat anti-mouse HRP-conjugated secondary antibodies were from Thermo Fisher Scientific (Waltham, MA; catalog number 31460, RRID:AB_228341, and catalog number 31430, RRID:AB_228307, respectively).

Imaging and Neurite Outgrowth Quantification

To visualize and quantify neuron axon outgrowth, dissociated neurons were electroporated with eGFP using the MaxCyte Electroporation System (Gaithersburg, MD) immediately prior to plating. Twenty-four hours after plating, neurons were fixed in 4% PFA for one hour by gently exchanging the growth media for PBS in four 50% volume exchanges, then exchanging the PBS for PFA in four 50% volume exchanges. PFA was again exchanged for PBS and coverslips were mounted in ProlongGold mounting media (Invitrogen, Carlsbad, CA) and dried overnight. DRG neurons were identified by their large, bright, round somas and were imaged on a Nikon

AR1 laser scanning microscope at the Northwestern University Center for Advanced Microscopy. Confocal image stacks were flattened and neurite arbors were traced using the Simple Neurite Tracer plugin in FIJI (Fiji Is Just ImageJ, RRID:SCR_002285) (Schindelin et al., 2012). Arbor information was exported to Excel and automated Sholl Analysis was performed at 5 um intervals in the Simple Neurite Tracer, exported to Excel, and statistics performed in Prism5 (GraphPad Software, La Jolla, CA). For statistical comparisons between equivalent maturation stages, cells were categorized as was done by Marques and colleagues. After analysis, defined parameters for each maturation stage were determined by evaluating cells in each stage from both genotypes combined. Cells with more than 60 branch points were Stage 2, regardless of the length of their longest axon. Cells with 60 or fewer branch points were categorized as Stage 1 if their longest axon was 150 mm long or less, and as Stage 3 if their longest axon was greater than 150 mm long. Experimenter was blind to genotype for the dissection, culture, imaging, tracing and analysis of all cells.

Electrophysiology

Whole-cell recordings from acutely isolated DRG neurons were performed as described previously (Copits et al., 2014). Currents were elicited by fast application of 10 mM glutamate (Sigma, St. Louis, MO) to lifted cells using a piezoceramic system, where rise times (10–90%) ranged from 0.5 to 3.0 ms. Weighted desensitization rates and relative proportions were calculated from bi-exponential fits of current decays during a 1 s application of glutamate using Clampfit10 (Molecular Devices, Sunnyvale, CA). Recordings were made and agonist was applied in our standard external solution containing (in mM): 150 NaCl, 2.8 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 10 glucose, and 10 HEPES, adjusted to pH 7.3. Intracellular solution contained (in mM): 95 CsF, 25 CsCl, 2 NaCl, 10 HEPES, 10 EGTA, 2 Mg-ATP, 10 QX-314, 5 TEA-Cl, and 5 4-

aminopyridine, adjusted to pH 7.3 with CsOH. To isolate KAR currents in adult neurons, AMPAR and NMDAR were blocked with 50 mM each of GYKI53655 (Tocris Bioscience, Bristol, UK) and D-APV (Abcam, Cambridge, MA). Acutely isolated neonatal neurons do not express detectible AMPAR or NMDAR currents prior to growing axons (Lovinger and Weight, 1988; Huettner, 1990). We confirmed this by recording glutamate-evoked currents from a subset of neonatal neurons in the presence of GYKI53655 and D-APV; no difference in current amplitudes or desensitization rates was observed compared to the absence of antagonists. Therefore, most neonatal KAR currents were evoked in the absence of antagonists.

Behavior

Experimenter was blind to genotype and injection (formalin or saline) for all behavioral experiments, which were carried out as described previously (Qiu et al., 2011). Briefly, thermal sensory thresholds were determined by Hargreaves test on a Plantar Test Hargreaves Apparatus from Ugo Basile (Varese, Italy); a cutoff time of 15 seconds was set to avoid tissue damage. Mechanical sensory thresholds were determined using increasing weights of von Frey sensory evaluator filaments (North Coast Medical, Inc., Gilroy, CA). Nocifensive reactions to 3 of 5 trials was considered a response, and that filament weight was recorded as threshold. For formalin-induced inflammation, 10 µl of saline or 5% formalin in saline was injected subcutaneously into the left hindpaw plantar. Immediately after formalin injection, mice were placed in an observation chamber. The amount of time spent biting, licking, or shaking the paw was measured and data were pooled into 5-minute bins. Total observation time was 60 minutes. Mice were habituated to the experimental chamber for 30 minutes prior to beginning any experiment.

Sciatic Nerve Crush

The sciatic nerve was crushed as previously described (Decosterd et al., 2002). Briefly, adult male and female C57Bl/6 mice were brought to a surgical plane of anesthesia with ketamine/xylazine and the sciatic nerve exposed at mid-thigh level. The nerve was crushed proximal to the trifurcation with a pair of hemostat forceps for 30 seconds. For sham animals, the nerve was exposed for 30 seconds without crush. Three, 7, and 10 days after surgery, the L3-L5 ganglia and the attached nerve to the trifurcation were recovered and homogenized for Western blotting.

Statistical Methods

Comparisons between two sets of data were performed with an unpaired *t*-test. One-sample *t*-tests evaluated the difference between samples and a theoretical mean of 1.0 and were used to determine significance of fold-change from baseline values. Data consisting of 3 or more groups were analyzed by one-way ANOVA, followed by Tukey's post-hoc comparison. Confidence intervals of proportions were calculated with the modified Wald method, and total numbers of KAR-positive and KAR-negative cells were compared using a Chi-square test. All other data is presented as mean ± S.E.M. Sholl curves were analyzed by two-way ANOVA, followed by Bonferroni post-hoc comparison. Spearman's or Pearson's correlation was used to evaluate correlations between time spent in culture and desensitization kinetics, based on results from Krustal-Wallis normality test performed in Origin (OriginLab Corp., Northampton, MA). All other statistical tests were performed in Prisim5 (GraphPad Software, La Jolla, CA).

Results

How the putative KAR auxiliary subunit Neto2 contributes to KAR signaling in the central and peripheral nervous systems is unknown. To address this question, we first identified neurons that expressed Neto2 mRNA and which were known to have detectable KAR currents. One such population of neurons was found in dorsal root ganglia (DRG) using the publicly available *in situ* hybridization data from the Allen Spinal Cord Atlas (ASCA; http://mousespinal.brain-map.org/), which showed Neto2 transcription in DRG tissue from P4 mice (Figure 2.1A and (ASCA, 2015)). Consistent with the ASCA data, Neto2 protein was detected in Western blots from wildtype P3 DRG but not from DRG isolated from Neto2$^{-/-}$ mice (Figure 2.1B). We also observed that Neto2 was most highly expressed at the earliest neonatal ages tested and was downregulated over development, plateauing at a barely detectable level of immunoreactivity by 2 weeks after birth and remaining low through adulthood (Figure 2.1C,

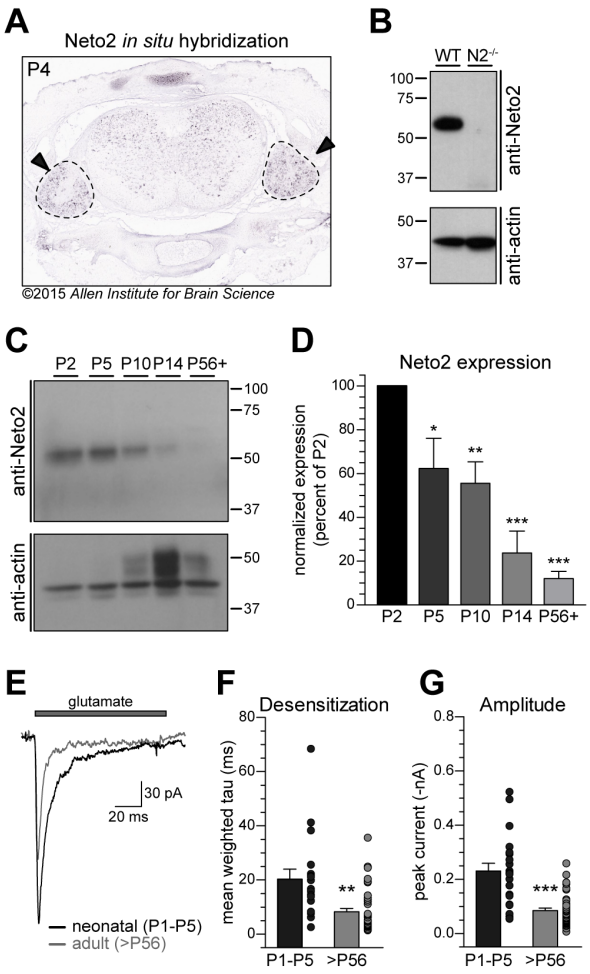

Figure 2.1. Neto2 is highly expressed in neonatal DRG and downregulated over development. **A**) In situ hybridization in a P4 spinal section with a probe directed against Neto2 transcripts. DRG are outlined with the dotted lines and denoted by arrowheads. **B**) Neto2 protein is detected by Western blot in wildtype but not Neto2$^{-/-}$ DRG homogenate. **C**) Representative Western blot showing Neto2 protein expression from acutely homogenized P2, P5, P10, P14, and adult DRG. **D**) Densitometry quantification of Neto2 expression normalized to actin. Represented as percent of Neto2 expression at P2. **E**) Representative current traces from neonatal and adult wildtype DRG neurons. Grey bar indicates glutamate (10 mM) application. Currents are reproduced from Figure 2A (neonatal) and Figure 3A (adult) for comparison. **F**) Quantification of mean weighted tau of glutamate-evoked desensitization for neonatal and adult DRG neurons. **G**) Quantification of peak current amplitude for neonatal and adult DRG neurons. Statistical significance is indicated as follows: * p<0.05, ** p<0.01, ***p<0.001.

2.1D; Percent of P2 expression: P5 = 62 ± 14%, P10 = 55 ± 10%, P14 = 24 ± 10%, Adult=12 ± 3%, n = 3, repeated measures ANOVA, $p<0.0001$).

We hypothesized that reduced Neto2 might alter KAR subunit composition and current properties in adult neurons compared to neonatal neurons. To test this possibility, glutamate (10 mM, 100 ms) was fast-applied to dissociated neurons of small to medium diameter to elicit KAR currents from acutely dissociated adult and neonatal wildtype neurons. Fitting of the current decay with either a one- or two-component exponential function yielded a mean weighted tau of 8.2 ± 1.2 ms (n = 42) in adult neurons, more than twice as fast as the 20.2 ± 3.7 ms (n = 18) glutamate-evoked desensitization rate from neonatal neurons ($p = 0.0063$) (Figure 2.1D, 2.1E). The adult neuron population also had smaller peak amplitudes (85 ± 9 pA, n = 43) than were seen in neonatal neurons (229 ± 29 pA, n = 22) ($p<0.0001$) (Figure 2.1D, 2.1F).

We next tested if Neto2 is indeed a component of KARs by comparing glutamate-evoked currents in DRG neurons acutely isolated from wildtype and Neto2$^{-/-}$ mice (Tang et al., 2011). Consistent with previous studies, glutamate elicited rapidly desensitizing currents in 71% of neonatal wildtype neurons recorded (95% CI = 53-85%) and 46% of neonatal Neto2$^{-/-}$ neurons (95% CI = 30-62%, Chi-square $p = 0.1358$, df = 3) (Figure 2.2A, 2.2B). KAR currents in neonatal wildtype neurons exhibit somewhat variable desensitization kinetics (Figure 2.1E, 2.2D), an observation supported by a high coefficient of variation (CV) of 0.78 for the fitted time course of desensitization. This inter-cell variability in neonatal neurons depended on genotype (Bartlett's test $p<0.0001$) and was much lower, 0.35, in neurons isolated from Neto2$^{-/-}$ mice, which showed a ~10-fold faster KAR desensitization rate of 2.1 ± 0.2 ms (n = 14, $p<0.0001$ with a Tukey's multiple comparison test) (Figure 2.2C). KAR currents in neurons from Neto2$^{-/-}$ mice also exhibited much lower peak amplitudes of their glutamate-evoked currents (28 ± 4 pA, n = 14)

compared to wildtype ($p<0.0001$; Figure 2.2E, 2.2F) and the CV for Neto2$^{-/-}$ current amplitudes (0.48) was lower than for wildtype neurons (0.60). These results support a role for Neto2 auxiliary proteins in shaping KARs in DRG neurons.

The very rapid desensitization of currents during glutamate application to Neto2$^{-/-}$ neurons appeared similar in time course to recombinant GluK1/GluK5-containing heteromeric KARs (Herb et al., 1992; Swanson et al., 1998). GluK5 is transcribed in both DRG and trigeminal ganglia and was suggested to assemble into KARs in trigeminal neurons (Sahara et al., 1997). We tested the inclusion of GluK5 in DRG KARs in additional comparative recordings of glutamate-evoked currents in neurons from GluK5$^{-/-}$ mice. KAR currents were evoked from 52% of neonatal GluK5$^{-/-}$ neurons (95% CI = 35-68%, Figure 2.2A, 2.2B) and exhibited significantly slower desensitization (39.5 ± 3.5 ms) and larger current amplitudes (449 ± 51 pA) relative to recordings from wildtype neurons (n = 15; $p<0.0001$ for both measures relative to wildtype) (Figure 2.2C & 2.2D, 2.2E & 2.2F). Additionally, the

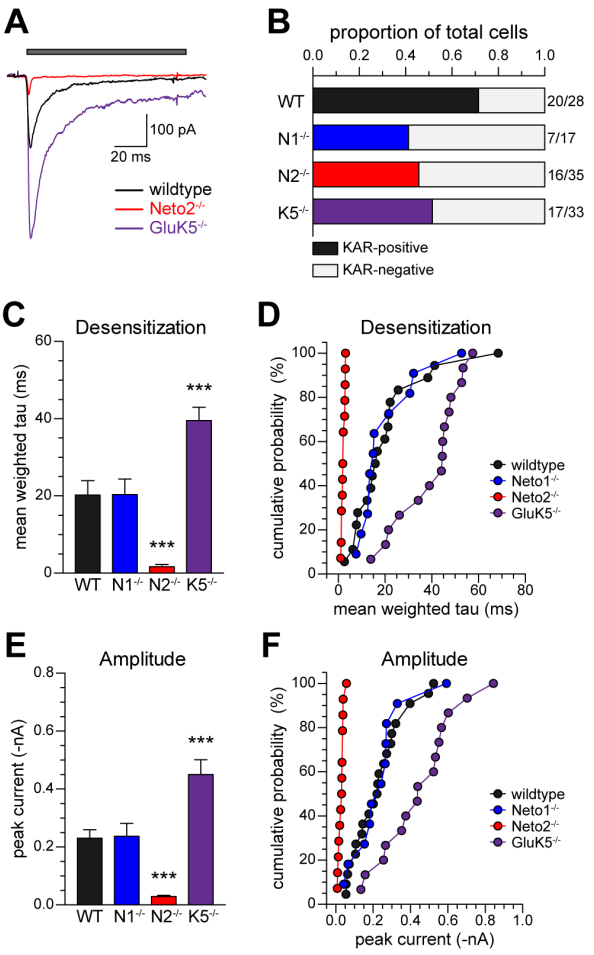

Figure 2.2. Neto2 assembles into functional GluK1/GluK5-containing KARs in neonatal DRG neurons. **A**) Representative current traces from wildtype (also presented in Figure 1E), Neto2$^{-/-}$, and GluK5$^{-/-}$ neonatal DRG neurons. Grey bar indicates glutamate (10 mM) application. **B**) The proportion of small- and medium-diameter cells patched that expressed KAR-mediated current is represented in the filled bars. Empty bars are the proportion of KAR-negative cells patched for each genotype. **C**) Quantification of mean weighted tau of glutamate-evoked desensitization for wildtype, Neto1$^{-/-}$, Neto2$^{-/-}$, and GluK5$^{-/-}$ neonatal DRG neurons. **D**) Cumulative probability histogram of individual cell desensitization rates for wildtype, Neto1$^{-/-}$, Neto2$^{-/-}$, and GluK5$^{-/-}$ neonatal DRG neurons. **E**) Quantification of peak glutamate-evoked current amplitude for wildtype, Neto1$^{-/-}$, Neto2$^{-/-}$, and GluK5$^{-/-}$ neonatal DRG neurons. **F**) Cumulative probability histogram of individual cell peak current amplitudes for wildtype, Neto1$^{-/-}$, Neto2$^{-/-}$, and GluK5$^{-/-}$ neonatal DRG neurons. Statistical significance is indicated as follows: **p<0.01, ***p<0.001. Error bars in column graphs represent s.e.m.

relative variability of currents evoked from GluK5$^{-/-}$ neurons was more similar to Neto2$^{-/-}$ neurons than to wildtype, in that the CV was 0.34 for desensitization rates and 0.44 for peak current amplitudes. Although the Allen Spinal Cord Atlas shows Neto1 mRNA in P4 DRG (ASCA, 2015), we found no evidence of Neto1 assembly into KARs in neonatal DRG neurons. KAR currents were elicited from 41% of Neto1$^{-/-}$ neurons (95% CI = 23-64%, Figure 2.2A, 2.2B) and similar to wildtype neurons, the mean weighted tau of desensitization was 20.4 ± 4.0 ms (n=11, p>0.05) (Figure 2.2C, 2.2D). Peak current amplitudes in Neto1$^{-/-}$ neurons were 236 ± 44 pA (n=11, p>0.05 compared to wildtype) (Figure 2.2C, 2.2E). Inter-cell variability in currents evoked from Neto1$^{-/-}$ neurons was similar to wildtype neurons with a CV of 0.66 for the mean weighted tau and 0.62 for peak current amplitudes. These results are consistent with the interpretation that neonatal wildtype neurons express KARs composed of the GluK1, GluK5, and Neto2 subunits.

Our data suggest that Neto2 could be the principle component that differentiates properties of neonatal and adult DRG KARs. To test this, we evoked KAR-mediated currents in adult neurons from wildtype and knockout mice. Because adult neurons grew processes more slowly than neonatal neurons, we were able to lift and record from healthy adult neurons over a longer time span (3-22 hours) than for neonatal neurons (3-12 hours), while still achieving the solution exchange required to resolved rapid glutamate-evoked currents (example traces are shown in Figure 2.3A). Adult wildtype neurons had KAR currents in 54% of small- and medium-diameter neurons (95% CI = 43-64%), which was similar in all the genotypes examined (Figure 2.3B). Notably, we observed a time in culture-dependent slowing of KAR current desensitization in adult neurons. That is, in the first 12 hours after plating, currents from wildtype cells desensitized with a mean weighted tau of 3.1 ± 0.3 ms (n = 16), similar to the decay rate we observed in adult Neto2$^{-/-}$ neurons in this same time frame (2.2 ± 0.2 ms, n = 15, p>0.05). At

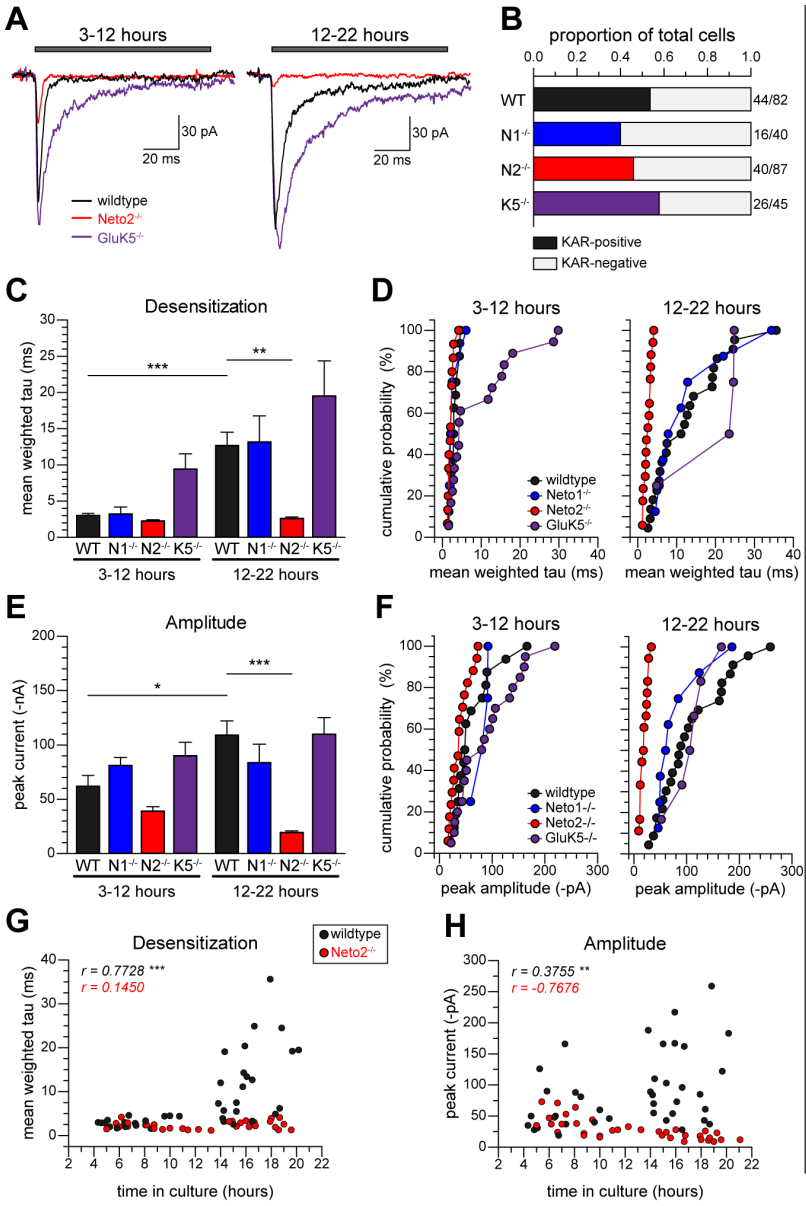

Figure 2.3. KARs in acutely plated adult DRG do not contain Neto2 but increase Neto2 incorporation over time in culture. **A**) Representative current traces from wildtype, Neto2$^{-/-}$, and GluK5$^{-/-}$ adult DRG neurons are shown for both early and late recording time points: 3-12 hours or 12-22 hours in culture, respectively. **B**) The proportion of small- and medium-diameter cells patched that expressed KAR-mediated current is represented in the filled bars. Empty bars are the proportion of KAR-negative cells patched for each genotype. **C**) Quantification of mean weighted tau of glutamate-evoked desensitization for wildtype, Neto1$^{-/-}$, Neto2$^{-/-}$, and GluK5$^{-/-}$ adult DRG neurons. Data is grouped by whether the cell was recorded between 3-12 hours or 12-22 hours after the cultures were plated. **D**) Cumulative probability histogram of individual cell desensitization rates for wildtype, Neto1$^{-/-}$, Neto2$^{-/-}$, and GluK5$^{-/-}$ adult DRG neurons. **E**) Quantification of peak glutamate-evoked current amplitudes for wildtype, Neto1$^{-/-}$, Neto2$^{-/-}$, and GluK5$^{-/-}$ adult DRG neurons. Data is grouped by whether the cell was recorded 3-12 hours or 12- 22 hours after the cultures were plated. **F**) Cumulative probability histogram of individual cell peak current amplitudes for wildtype, Neto1$^{-/-}$, Neto2$^{-/-}$, and GluK5$^{-/-}$ adult DRG neurons. **G**) Wildtype and Neto2$^{-/-}$ data from Figure 3D is represented as desensitization rate versus time-in-culture to visually demonstrate the correlations reported in the text. **H**) Wildtype and Neto2$^{-/-}$ data from Figure 3E is represented as peak current amplitude versus time-in-culture to visually demonstrate the correlations reported in the text. Statistical significance is indicated as follows: ***p<0.001. Error bars in column graphs represent s.e.m.

later time points, however, neurons with more slowly desensitizing kinetics emerged in cultures from adult wildtype mice, shifting the mean desensitization rate to 12.6 ± 1.9 ms (n = 22, p<0.001 compared to adult wildtype neurons recorded 3-12 hours after dissociation) (Figure 2.3C, 2.3D). The slower desensitization of currents in wildtype neurons was accompanied by greater variability (CV = 0.70) compared to those recorded at less than 12 h in culture (CV = 0.33). In contrast, currents evoked from Neto2$^{-/-}$ neurons desensitized rapidly at later time points (2.5 ± 0.2 ms, n = 17; p<0.0001 versus wildtype 12-22 hours, p>0.05 versus wildtype 3-12

hours) and the variability of desensitization kinetics remained relatively low (Figure 2.3C, 2.3D). There was a positive correlation between the mean weighted tau of desensitization and the time wildtype neurons spent in culture prior to the recordings ($n = 38$, $r = 0.7728$, $p<0.0001$ Spearman's correlation) that was not seen for adult Neto2$^{-/-}$ neurons ($n = 32$, $r = 0.1450$, $p = 0.2143$ Pearson's correlation) (Figure 2.3G). These data underscore the difference in KAR current properties between neonatal and adult sensory neurons, and demonstrates that Neto2 incorporation critically distinguishes KARs at different ages. Additionally, the data suggest that Neto2 incorporation increases over time in adult sensory neuron cultures.

Consistent with this interpretation, current amplitudes in wildtype adult neurons increased from 62 ± 10 pA at 3-12 hours post-plating to 109 ± 13 pA at 12-22 hours ($p<0.05$). Current amplitudes in Neto2$^{-/-}$ neurons were 38 ± 4 pA from 3-12 hours and 19 ± 2 pA after 12-22 hours in culture, which were similar to currents from wildtype neurons at early time points ($p>0.05$) but significantly different from recordings made more than 12 h after plating the cells ($p<0.001$) (Figure 2.3E, 2.3F). We detect a modest but significant positive correlation of wildtype peak current amplitudes with time in culture ($r = 0.3755$ and $p = 0.0092$) that was not observed in Neto2$^{-/-}$ neurons ($r = -0.7660$, $p>0.05$) (Figure 2.3H), supporting our interpretation that Neto2 incorporation into KARs increases in adult neurons over time in culture.

As in neonatal neurons, fast desensitization of currents evoked from Neto2$^{-/-}$ and early wildtype neurons was suggestive of GluK5 subunit incorporation. KAR currents evoked in GluK5$^{-/-}$ neurons desensitized with a mean weighted tau of 9.3 ± 2.1 ms ($n = 18$) (Figure 2.3C). The presence of two somewhat distinct groups of desensitization rates, as reflected in the cumulative probability plot (Figure 2.3D), produced a very high CV of 0.97; accordingly, the mean weighted tau of GluK5$^{-/-}$ currents in the 3-12 hours was not different from wildtype decay

either at early or at late time points ($p>0.05$ for both comparisons). From 12-22 hours GluK5$^{-/-}$ neurons had a mean weighted tau of 19.4 ± 4.9 ms, similar to wild type neurons at late, rather than early, time points ($p>0.05$ and $p<0.001$, respectively) (Figure 2.3C, 2.3D). Additionally, desensitization rate was not positively correlated with time in culture in GluK5$^{-/-}$ neurons ($r = 0.3120$, $p = 0.0787$, Spearman's correlation, data not shown), unlike currents in wildtype neurons. KAR current amplitudes from adult GluK5$^{-/-}$neurons were similar to those in wildtype neurons (89 ± 13 pA from 3-12 hours and 109 ± 15 pA from 12-22 hours, $p>0.05$ compared to wildtype) (Figure 2.3E, 2.3F). These data support the interpretation that GluK5 incorporates into adult DRG KARs, perhaps with some degree of heterogeneity.

Similar to neonatal neurons, we did not find evidence for Neto1 incorporation into adult KARs. The 40% of Neto1$^{-/-}$ neurons with KAR-mediated currents (95% CI = 26-55%, Figure 2.3B) exhibited fast desensitization kinetics of 3.1 ± 1.0 ms in the first 12 hours after dissociation ($n = 4$) and cells with slower desensitization kinetics emerged from 12-22 hours, shifting the mean desensitization rate to 13.1 ± 3.6 ms ($n = 8$) (Figure 2.3C, 2.3D). Like adult wildtype neurons, Neto1$^{-/-}$ desensitization rates correlated positively with the time neurons spent in culture ($r = 0.8601$, $p = 0.0002$, Spearman's correlation, data not shown) and peak current amplitudes of 81 ± 8 pA at early and 83 ± 17 pA at late time points were not different from wildtype ($p>0.05$) (Figure 2.3E, 2.3F). These data show that DRG KAR composition is more complex than homomeric GluK1-containing receptors (Swanson and Heinemann, 1998). We propose that most DRG KARs are heteromeric GluK1/GluK5-containing receptors, that a variable proportion of KARs incorporate Neto2, and that Neto2 incorporation is a critical distinguishing factor between receptors in neonatal and adult neurons.

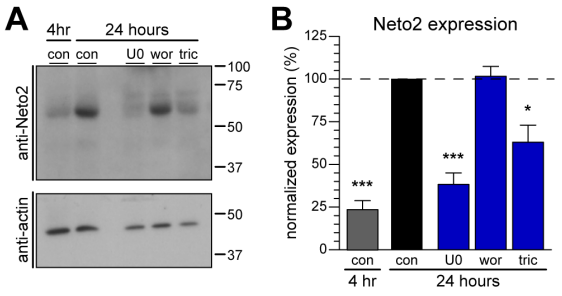

Figure 2.4. ERK activation is required for Neto2 upregulation in adult DRG neurons. **A**) Representative Western blot of Neto2 expression in adult wildtype DRG cultures at 4 hours and 24 hours in untreated cultures and at 24 hours in cultures treated with ERK1/2 inhibitor U0126 (50 μM), PI3K inhibitor wortmannin (10 nM), and Akt inhibitor triciribine (20 μM). **B**) Densitometry quantification of Neto2 expression, normalized to actin. Represented as percent of control culture at 24 hours. Error bars represent s.e.m. Statistical significance is indicated as follows: *p<0.05, ***p<0.001. Abbreviations indicate untreated control cultures (con) and cultures treated with U0126 (U0), wortmannin (wor), and triciribine (tric).

To test the hypothesis that Neto2 protein is upregulated with time in culture, we cultured wildtype DRG neurons for either 4 or 24 hours before lysing the cells and probing for Neto2 expression by Western blot. In agreement with our physiology data, we found that Neto2 immunoreactivity from cultures lysed at 4 hours was only 28 ± 5% of the Neto2 signal from cultures lysed after 24 hours ($p<0.001$) (Figure 2.4A, 2.4B). Furthermore, this upregulation of Neto2 in culture depended on intact ERK1/2 signaling, as inhibition of ERK phosphorylation by 50 μM U0126 maintained Neto2 expression at 38 ± 7% of control cultures at 24 hours ($p<0.001$ compared to untreated cultures at 24 hours) (Figure 2.4A, 2.4B). Phosphorylated ERK1/2 is transported from the site of axonal injury to the nucleus in DRG neurons and is a critical early component of the cellular program mobilized for process regeneration in sensory neurons (Perlson et al., 2005). Acutely isolated DRG neurons undergo exuberant axon regrowth in the 24 hours during which we see Neto2 upregulation. We therefore tested the contribution of other signaling molecules implicated in axon outgrowth, PI3K (Saijilafu et al., 2013) and its downstream effector Akt, to Neto2 upregulation in the adult DRG cultures. Neto2 upregulation was unaffected by the PI3K inhibitor wortmannin (10 nM, 102 ± 6% of control immunoreactivity; $p>0.05$ compared to untreated cultures at 24 hours) but was significantly reduced by the Akt inhibitor triciribine (20 μM), which reduced Neto2 immunoreactivity to 63 ± 10% of control ($p<0.05$ compared to untreated cultures at 24 hours) (Figure 2.4A, 2.4B). It seems, therefore, that Neto2 upregulation is supported by multiple kinases but is not a general component of growth permissive signaling pathways.

KARs in DRG neurons have been implicated in multiple functional roles as peripheral chemosensors (Carlton et al., 1995), presynaptic autoreceptors in the spinal cord (Kerchner et al., 2001a; Kerchner et al., 2002), and as trophic stimulators of axon outgrowth (Joseph et al., 2011; Marques et al., 2013). Pharmacological inhibition or genetic ablation of GluK1-containing KARs also reduces some forms of inflammatory or neuropathic pain (Simmons et al., 1998; Qiu et al., 2011). We tested the role of Neto2 in mediating persistent pain modalities and neurite outgrowth in cultured adult DRG neurons. First, we evaluated baseline and short-term pain behaviors in Neto1$^{-/-}$ and Neto2$^{-/-}$ mice, an experiment which had the additional use of evaluating a known GluK1-dependent behavioral phenotype in KAR auxiliary subunit knockout mice whose behavior has been minimally characterized to date (Ng et al., 2009; Mahadevan et al., 2015). Given that GluK1-containing KARs are not involved in acute pain signaling (Sang et al., 1998; Qiu et al., 2011) we expected basal pain thresholds to be intact in the absence of Neto proteins. Neto1$^{-/-}$ mice showed withdrawal latencies from a calibrated heat source of 6.7 ± 0.4 s (n = 33), which were indistinguishable from the 7.0 ± 0.4 s latencies (n = 31) of their wildtype littermates (p=0.5726); Neto2$^{-/-}$ mice also withdrew from the heat source similar to their wildtype littermates with latencies of 5.2 ± 0.3 s (n=36) and 5.8 ± 0.2 s (n=42), respectively (p = 0.0825) (data not shown). Like thermal sensitivity, mechanical sensitivity was unchanged by genetic ablation of Neto proteins. Wildtype and Neto1$^{-/-}$ littermates responded at 0.72 ± 0.06 g (n = 30) and 0.80 ± 0.07 g (n = 27), respectively (p = 0.3962), and Neto2$^{-/-}$ animals (n = 25) and their wildtype littermates (n=21) had indistinguishable mechanical thresholds of 0.61 ± 0.05 g and 0.67 ± 0.06 g (p = 0.429).

While our data (Figures 2.1 and 2.3) suggest that Neto2 in adult peripheral neurons would not contribute to immediate inflammatory pain behaviors, it was possible that KARs elsewhere in

the pain neuraxis would be compromised by loss of an auxiliary subunit and that this would result in a deficit in spontaneous formalin behaviors like that seen in the GluK1$^{-/-}$ mouse (Ko et al., 2005). We found, however, that Neto1$^{-/-}$ (n = 20) and Neto2$^{-/-}$ mice (n = 22) exhibited the same two-phase spontaneous pain behaviors as their respective wildtype littermates (n = 18 Neto1$^{+/+}$ and n = 25 Neto2$^{+/+}$ animals) with genotype not altering the time course of either curve (two-way repeated measures ANOVA, Neto1 comparison p = 0.0554, Neto2 comparison p = 0.5974) (Figure 2.5A). Additionally, it was possible that inflammation would upregulate Neto2 in DRG neurons similar to what we saw in cultures and that we would observe a Neto2-dependent deficit in inflammatory hypersensitivity. To test this idea, we re-tested the latency to withdrawal from heat 3 hours after either formalin or saline injection. Saline-injected mice showed unchanged heat sensitivity in both the Neto1 (0.98 ± 0.09 and 0.92 ± 0.06 fold-change from baseline for Neto1$^{+/+}$ and Neto1$^{-/-}$, respectively; n = 13 for both groups, one-sample *t*-test p = 0.8388 and 0.1733) and Neto2 strains (0.94 ± 0.06 (n=18) and 1.04 ± 0.09 (n=14) fold-change was observed for Neto2$^{+/+}$ and Neto2$^{-/-}$ mice, p = 0.3198 and 0.6354) (Figure 2.5B). Formalin-inflamed Neto1$^{-/-}$ mice (n=20) developed hypersensitivity that reduced their heat tolerance to

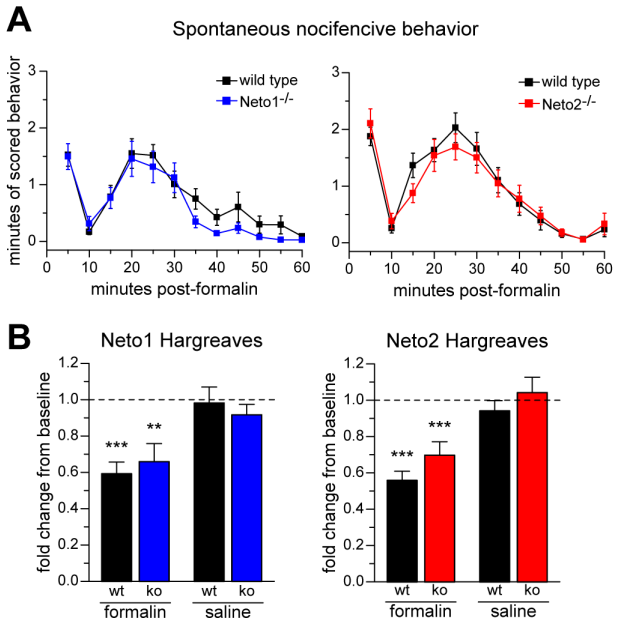

Figure 2.5. Neto$^{-/-}$ mice show normal formalin pain behaviors and normal formalin-induced inflammatory heat hypersensitivity. **A**) Neto1$^{-/-}$ and Neto2$^{-/-}$ mouse spontaneous pain behavior following formalin injection, compared to their wildtype littermates. Measured time spent on nocifensive behaviors was pooled into 5 minute bins. Saline-injected animals did not show nocifensive behaviors over the 60 minutes following injection, and this data is omitted from graphs for the purpose of clarity. **B**) Neto1$^{-/-}$ and Neto2$^{-/-}$ mouse thermal hypersensitivity, compared to their wildtype littermates. Hypersensitivity was measured by Hargreaves test 3 hours after formalin injection and is represented as the fold-change from the withdrawal latency measured prior to formalin injection. Error bars represent s.e.m. Statistical significance is indicated as follows: *p<0.05, ***p<0.001.

0.66 ± 0.10 fold of their baseline withdrawal latency ($p = 0.0029$), similar to 0.59 ± 0.07 fold heat tolerance seen in their formalin-injected wildtype littermates ($n=18$, $p<0.0001$) (Figure 2.5B). The same normal hypersensitivity developed in both genotypes of Neto2 animals, where heat tolerance was reduced to 0.56 ± 0.05 fold of baseline in wildtype littermates ($n=25$, $p<0.0001$) and to 0.70 ± 0.07 fold of baseline thresholds in Neto2$^{-/-}$ mice ($n = 22$, $p = 0.0006$) (Figure 2.5B). Thus, these knockout mice have intact short-term hypersensitivity.

KARs are known to modulate axon outgrowth in cultured DRG neurons from early developmental stages, and the Neto2 upregulation we observed in adult neuron cultures coincides with a period of rapid axon regeneration that, like Neto2 upregulation, is stunted by ERK1/2 inhibition. Additionally, our recordings from neurons that had not yet grown neurites (Figure 2.3) suggest that Neto2 upregulation begins prior to axonal outgrowth. To determine if upregulation of Neto2-containing KARs modulated neurite outgrowth in our adult cultures, we electroporated neurons from wildtype and Neto2$^{-/-}$ mice with eGFP and fixed them 24 hours after plating. At this time in culture, neurons are still maturing and show multiple stages of outgrowth (Smith and Skene, 1997; Marques et al., 2013). Neuronal axon arbors were imaged and traced to quantify outgrowth; experiments and analysis was performed blinded to genotype. To compare neurons at similar growth stages, we divided neurons into three groups based on the size and complexity of their axon arbors: immature (Stage 1), intermediate (Stage 2), or mature/elongating (Stage 3) (see Materials and Methods for details, Figure 2.6A for example images). Twenty-four hours after plating 51% of wildtype neurons had reached the intermediate growth stage with highly ramified arbors, 16% had fully matured and reached elongating growth, and only 33% remained in early stages of growth. By contrast, 42% of Neto2$^{-/-}$ neurons remained in the early growth stage, and smaller proportions of 46% and 12% reached intermediate and mature/elongating growth, respectively (Figure 2.6B). In wildtype neurons the

length of the longest neurite on each cell increased significantly from 30 ± 8 µm in Stage 1 neurons (n=22) to 266 ± 14 µm in Stage 2 (n=34) and 462 ± 60 µm in Stage 3 neurons (n=11) (ANOVA p<0.0001, p<0.001 for all comparisons) (Figure 2.6C). We found that Neto2$^{-/-}$ neurons also showed an increase in longest neurite length between Stage 1 and Stage 2 (37 ± 10 µm (n=17) and 232 ± 16 µm (n=19), respectively; p<0.001). However, neurites on Stage 3 neurons from Neto2$^{-/-}$ mice elongated to only 241 ± 25 µm (n=5), a length that was not longer than the Stage 2 Neto2$^{-/-}$ neurites (p>0.05) and significantly shorter than the longest axons measured on Stage 3 wildtype neurons (p<0.001) (Figure 2.6C). At each stage we observed no difference

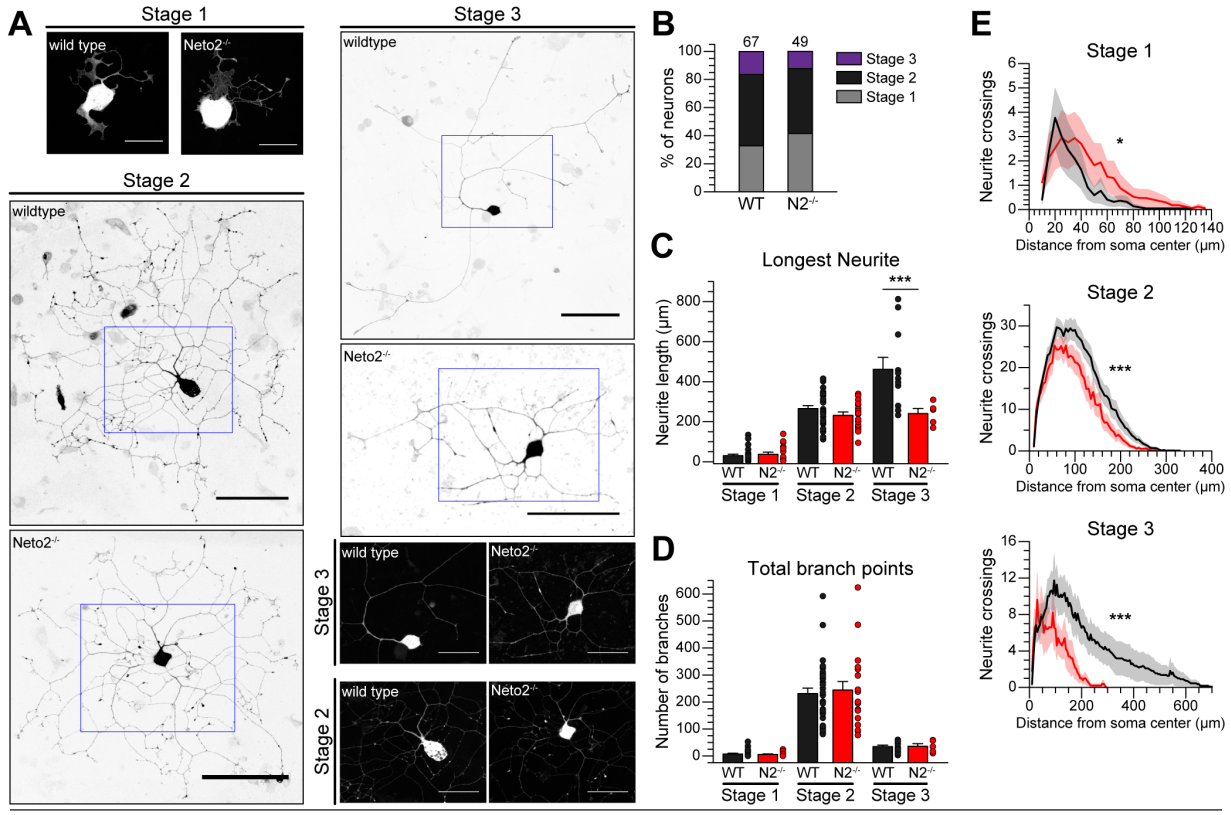

Figure 2.6. Neto2$^{-/-}$ DRG neurons from adult mice show stunted neurite outgrowth and maturation in culture compared to wildtype neurons. **A**) Representative images of wildtype and Neto2$^{-/-}$ neurons from each maturation stage. Stage 2 and 3 inverted images show the full axon arbor for each cell, and the area within the blue boxes is magnified to demonstrate branching. Black scale bars are 100 µm long, white scale bars are 50 µm. **B**) The percentage of wildtype and Neto2$^{-/-}$ cells at each maturation stage (see Materials and Methods for details). **C**) Length of the longest neurite measured from each cell in wildtype and Neto2$^{-/-}$ adult neurons. Cells were electroporated with eGFP and fixed after 24 hours in culture. Data are grouped by the cell's maturation stage. **D**) Number of branch points measured on each cell in wildtype and Neto2$^{-/-}$ adult neurons. Data are grouped by the cell's maturation stage. **E**) Sholl analysis was performed on axon arbor tracings of wildtype and Neto2$^{-/-}$ adult neurons at 5 µm intervals from the soma center. Sholl data is grouped by maturation stage for presentation and analysis. Error bars on column graphs represent s.e.m. Statistical significance is indicated as follows: *p<0.05, **p<0.01, ***p<0.001.

between wildtype and Neto2$^{-/-}$ neuron branch number (Stage 1: 7 ± 3 and 6 ± 2; Stage 2: 231 ± 20 and 244 ± 32; Stage 3: 34 ± 6 and 36 ± 10; $p>0.05$ for all within-stage comparisons) (Figure 2.6D).

To further characterize neurite outgrowth in these cultures we performed Sholl analysis on the traced neurite arbors, quantifying the total number of neurite crossings at 5 µm increasing intervals from the soma. Both Stage 2 and Stage 3 neurons from Neto2$^{-/-}$ cultures showed significantly reduced arbor complexity when compared to wildtype neurons of the same stage (two-way ANOVA, $p<0.0001$ for genotype as a source of variation at both Stage 2 and Stage 3) (Figure 2.6E). Interestingly, Sholl analysis of Stage 1 neurons showed increased complexity in Neto2$^{-/-}$ neurons compared to their wildtype counterparts ($p = 0.0123$ for genotype as a source of variation) (Figure 2.6E), suggesting that the contribution of Neto2-containing receptors early in neurite outgrowth might differ from their role in later stages of growth.

The peripheral branch of DRG axons retains the capacity for regeneration in the adult. Crush injury to the sciatic nerve induces regrowth of axons through the site of injury and recovery of motor control and sensation in the distal toes within about 10 weeks in mouse (Decosterd et al., 2002). To determine whether Neto2 might be upregulated in this *in vivo* regeneration, as would be predicted from our

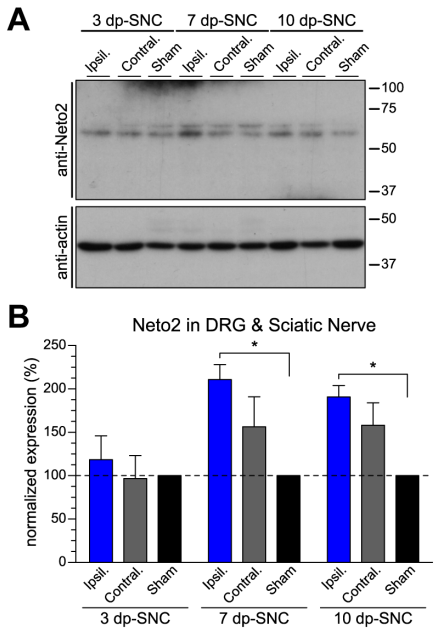

Figure 2.7. Sciatic nerve crush upregulates Neto2 *in vivo*. **A**) Representative Western blot showing Neto2 and actin expression in homogenized DRG and sciatic nerve tissue taken 3, 7, or 10 days post sciatic nerve crush (dp-SNC). Tissue was taken ipsilateral to the crushed nerve (Ipsil.), contralateral to the crushed nerve (Contral.), or ipsilateral to an exposed surgical sham nerve (Sham). **B**) Densitometry quantification of Neto2, normalized to actin. Within each time-point, Neto2 expression in Sham tissue was set to 100%. Error bars represent s.e.m. Statistical significance is indicated as follows: *p<0.05.

results in cultured neurons, we performed nerve crush injury on wildtype mice and then tested Neto2 protein levels in DRG and sciatic nerve by Western blot 3, 7, and 10 days following surgery (Figure 2.7A). We found that Neto2 protein expression ipsilateral to the crush injury increased to twice that of sham after 7 days ($p = 0.0467$, Repeated measures ANOVA, $n = 3$, $p<0.05$ day 7 ipsilateral crush versus sham) and 10 days ($p = 0.0538$, $p<0.05$ day 10 ipsilateral crush versus sham) (Figure 2.7B). Neto2 expression was not different between crushed and sham tissue 3 days following nerve crush injury ($p = 0.6626$) (Figure 2.7B). Thus, Neto2 protein is upregulated in an injury model that induces axon outgrowth from sensory ganglia neurons.

Discussion

We report here that Neto2 is a *bone fide* KAR auxiliary subunit because it assembles with KARs and impacts their functional properties in peripheral sensory neurons. Moreover, we find a substantial difference in the composition of KARs in neonatal and adult DRG neurons that primarily results from developmental down-regulation of Neto2. Finally, our data suggest that Neto2-containing KARs contribute to regulated process outgrowth from sensory neurons rather than mediating an inflammatory pain state previously shown to involve KAR signaling.

KARs in DRG neurons contain the GluK1 subunit as an essential constituent (Mulle et al., 2000; Kerchner et al., 2002; Rozas et al., 2003), but the extent to which other subunits contribute to KAR signaling is less clear. GluK1, GluK5, and Neto2 mRNAs are observed in DRG (Bettler et al., 1990; Herb et al., 1992; Partin et al., 1993; ASCA, 2015). The biophysical properties of KARs in sensory neurons are markedly similar to those of homomeric recombinant GluK1 receptors (Swanson and Heinemann, 1998). It is clear that recombinant Neto2 modulates recombinant KARs in both heterologous systems and in neurons, altering KAR biophysical properties and promoting KAR post-synaptic localization (Zhang et al., 2009; Tang et al., 2011; Tang et al., 2012; Copits et al., 2014; Wyeth et al., 2014). The only evidence to date that Neto2 plays a role in native KAR function, however, is the observation that silencing Neto2 alters KAR agonist sensitivity in cultured hippocampal neurons (Zhang et al., 2009). Here we show that Neto2 shapes KAR currents evoked from DRG neurons. KAR currents desensitize at different rates depending on the subunit composition of the receptors, with GluK5 incorporation speeding the desensitization rate of recombinant GluK1-containing receptors (Swanson et al., 1998) and Neto2 incorporation slowing both GluK1-containing and GluK1/GluK5-containing receptor desensitization (Copits et al., 2011; Straub et al., 2011a). We found that gene-targeting of Neto2 makes KAR currents in neonatal neurons desensitize more rapidly, whereas elimination of

GluK5 has the opposite effect, suggesting these neurons express predominantly GluK1/GluK5/Neto2-containing KARs. DRG KAR desensitization is slower than for recombinant GluK1/GluK5/Neto2-containing receptors, however, which could reflect mixed composition of receptors on the cell surface or might be due to an additional component of native receptors that has yet to be recapitulated in recombinant systems. Regardless, our data confirm that Neto2 is a component of endogenous KARs in sensory neurons.

Despite the obvious relevance of adult nociceptor function to pain, KAR composition and function have not been studied in adult DRG. We found that high neonatal Neto2 expression was down-regulated by P14, and adult KAR currents differ strikingly from neonatal currents in a Neto2-dependent manner. Distinct KAR subunit composition between adult and neonatal neurons is intriguing given that GluK1-containing KARs in DRG are expressed specifically in nociceptors, known to be involved in persistent pain but not acute pain in adult animals, and known to modulate neonatal DRG neurite outgrowth. It is possible that KAR function in sensory neurons is altered over the rodent lifetime as the subunit composition of these receptors changes.

The selective expression of KARs in peripheral nociceptors is of particular interest in the search to better understand KAR contribution to pathological pain. GluK1$^{-/-}$ mice have reduced formalin-induced pain behaviors (Ko et al., 2005), and functional antagonists directed against the GluK1 subunit alleviate hypersensitive pain states in rodents (Simmons et al., 1998; Dominguez et al., 2005; Qiu et al., 2011). Additionally, a GluK1 functional antagonist was shown to alleviate inflammatory thermal hypersensitivity and migraine pain in human volunteers (Sang et al., 1998; Sang et al., 2004). It remains unclear precisely where in the pain neuraxis GluK1-containing KAR signaling supports these pathologic pain states, however, because in addition to

nociceptors GluK1 is expressed in many central pain centers where receptors containing this subunit modulate various functions related to circuit excitability and pain behaviors (Kerchner et al., 2001a; Kerchner et al., 2002; Binns et al., 2003; Palecek et al., 2004; Wu et al., 2007b; Nakamura et al., 2010; Descalzi et al., 2013; Koga et al., 2015). It also remains unclear what additional subunits compose these pain-supportive KARs, as other pore-forming subunits and Neto proteins are expressed throughout the pain neuraxis. We find that Neto1$^{-/-}$ and Neto2$^{-/-}$ mice have normal acute thermal and mechanical pain thresholds, a finding we expected given that KARs do not affect acute pain signaling (Sang et al., 1998; Qiu et al., 2011). Our behavioral data also show that Neto proteins do not contribute to short-term inflammatory pain, consistent with the predominantly Neto-lacking KARs expressed in adult sensory neurons and suggestive that these GluK1/GluK5-containing receptors could be a candidate target for alleviating short-term inflammatory pain. It will be important to determine whether KAR contribution to long-term persistent pain is also independent of Neto proteins or if Neto assembly differentiates KARs that modulate long- and short-term pathologic pain.

In contrast to their less defined contribution to pain, the peripheral sensory neuron population of KARs does modulate neurite outgrowth. In embryonic cultures, the agonist kainate inhibited elongating outgrowth, and KAR antagonists promoted elongation (Joseph et al., 2011). Further, KARs bidirectionally modulated early and elongating outgrowth and the rate at which neonatal neurons progressed through outgrowth stages (Marques et al., 2013). This bidirectional modulation of outgrowth was mediated by G_O- and PKC-dependent phosphorylation of different sites on the growth-associated, GluK5-interacting CRMP2 protein. How Neto2 contributes to these processes is unknown, but the high Neto2 expression we observe in neonatal DRG neurons suggests it is a component of this neonatal KAR signaling complex modulating outgrowth. KAR composition and function in adult DRG neurons have not previously been

described, yet the molecular constituents of adult nociceptors are more relevant to the mechanisms of pathological pain than are neonatal nociceptors. Our finding that Neto2 expression is low in adult DRG is consistent with the static growth state of these neurons, and up-regulation of Neto2 in culture is consistent with rapid neurite outgrowth to which Neto2 contributes, presumably through its incorporation into KARs. We observe aberrant Neto2$^{-/-}$ arbor complexity at both early and later growth stages. Most of these alterations are similar to the previously reported effect of high kainate concentrations on young wildtype cultures: reduced elongating outgrowth, and reduced intermediate and elongating arbor complexity (Joseph et al., 2011; Marques et al., 2013). Neto2 loss does not exactly recapitulate kainate-induced signaling however, as low but not high concentrations of kainate increased arbor complexity at early growth stages (Marques et al., 2013). Genetic removal of a modulatory subunit does not necessarily alter signaling in the same way as exogenous agonists, and differences in signaling downstream of KARs in adult and neonatal neurons could alter the impact of receptors on outgrowth. Regrowth of adult neurons in culture likely depends on regenerative signaling pathways engaged by axotomy during dissection, and KAR modulation of axon regeneration might differ somewhat from developmental axon growth.

An alteration in KAR signaling is the most straightforward interpretation of the altered outgrowth we see in Neto2$^{-/-}$ neurons, given the previous evidence that these receptors play a role in this process (Joseph et al., 2011; Marques et al., 2013). However, other mechanisms that we cannot rule out include compensatory responses to genetic deletion of Neto2 or interactions between Neto2 and non-KAR signaling systems that change the process of axon regeneration. With respect to compensation, Neto2$^{-/-}$ mice show normal expression of kainate, AMPA, and NMDA receptor subunits in the hippocampus (Tang et al., 2011; Tang et al., 2012), and no evidence exists for functional replacement of the auxiliary protein in KAR currents. Neto2 does

interact with and modulate the function of the chloride transporter KCC2, and Neto2$^{-/-}$ hippocampal lysates show reduced KCC2 protein (Ivakine et al., 2013). This interaction is unlikely to be relevant to our outgrowth data, however, because intracellular chloride levels in DRG neurons are controlled primarily by NKCC1 and KCC3 rather than the KCC2 isoform (Sung et al., 2000; Lucas et al., 2012; Mao et al., 2012). Thus, the weight of evidence supports our working hypothesis that Neto2 has its role in axon outgrowth as a result of assembly into KARs and consequent alteration of their function.

In addition to modulating outgrowth, KARs function as autoreceptors at the DRG to dorsal horn synapse in young mice (Kerchner et al., 2001a; Kerchner et al., 2002). Whether Neto2 is a critical component of neonatal KAR autoreceptors and how Neto2 downregulation might alter KAR autoreceptors in adult spinal circuits remain open questions. Neto2 increases postsynaptic localization of KARs in hippocampal and cerebellar neurons (Copits et al., 2011; Tang et al., 2012), and it will be important to address how Neto2 might impact receptor localization to spinal DRG terminals. Our observation that Neto2 deletion alters neurite outgrowth suggests that Neto2-containing KARs are found in or near growth cones, but it is unclear if Neto2 loss in these neurons alters receptor signaling, receptor localization, or both.

Altered neurite growth in adult Neto2$^{-/-}$ DRG cultures is consistent with our finding that Neto2 up-regulation in adult neurons depended on activation of the positive regenerative injury signal pERK. Transportation of pERK1/2 from the axonal site of injury to the nucleus is critical to neurite regrowth and the outgrowth-priming effect of peripheral nerve injury (Perlson et al., 2005). PI3K-mediated signaling also promotes neurite regrowth; however, the regeneration-permissive pathways downstream of PI3K differ between peripheral and central neurons with GSK3 inhibition mediating PNS regeneration, and Akt and mTOR activation being growth

permissive in CNS neurons but dispensable in the PNS (Park et al., 2008; Christie et al., 2010; Saijilafu et al., 2013). We found that inhibition of MEK/ERK signaling blocked Neto2 up-regulation in adult DRG cultures. Akt is the major effector of PI3K (Saijilafu et al., 2013), yet Akt-mediated signaling partially contributes to Neto2 up-regulation while PI3K-mediated signaling does not. It is becoming evident that a number of kinases can activate Akt independently of PI3K (Mahajan and Mahajan, 2012), among which the Src family of kinases has several known functions in DRG including transducing growth-supportive signaling (Tucker et al., 2008). Critical points that remain to be resolved include determining if the same signaling cascades control Neto2 expression in neonatal and adult neurons and testing if Neto2-containing KARs promote the rate of non-peptidergic fiber innervation during embryonic and early post-natal spinal cord development.

That Neto2 modulates axon regrowth in neurons isolated from adult animals suggests KARs could critically contribute to peripheral regeneration after injury. The observation that Neto2 increased following sciatic nerve crush confirms that expression of this auxiliary protein in adult neurons is malleable under pathological conditions. Nerve crush injury induces a broad array of changes in peripheral neurons as distal axons degenerate and neurons switch from a signal-conducting expression profile to a regenerative expression profile (Navarro et al., 2007). Neto2 expression peaks approximately one week after injury, following a slower time course than was observed in our cultures. While peripheral axons can sprout growth cones and begin regenerating within the first day after injury, the majority of axons initiate growth more slowly, with fewer than half reaching 3 mm past the proximal site of injury after 5 days (Pan et al., 2003). A regenerative lag period exists during the first 3 days following nerve crush before axons reach their maximal growth rate of 1-3 mm/day (Danielsen et al., 1986; Pan et al., 2003; Sta et al., 2014); the lack of Neto2 upregulation at 3 days post-crush suggests that Neto2 is not

involved in initial growth cone sprouting but rather in established regrowth, an interpretation that is consistent with our culture outgrowth data. It will be important to determine what effect loss of Neto2 has on regenerating peripheral axons; our culture experiments suggest that the initial growth sprouting could be more robust but that elongating regrowth would be ultimately stunted in Neto2$^{-/-}$ mice, potentially impacting the rate or extent of functional recovery.

In conclusion, we demonstrate that Neto2 is a *bone fide* KAR auxiliary subunit that is a developmentally downregulated but dynamic component of KARs in nociceptors and a critical modulator of adult sensory axon regrowth. These findings raise the possibility of differential KAR functions in neonatal and adult nociceptors, and they suggest that KAR composition and therefore KAR function might not be static in the adult state. This information is critical to improving our mechanistic understanding of neural circuit modulation by KARs and the contributions of KAR-mediated signaling to normal development and disease states.

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Chapter 3.

N-glycan content modulates kainate receptor functional properties.

Abstract

Ionotropic glutamate receptors (iGluRs) are tetrameric proteins with between 4 and 12 consensus sites for *N*-glycosylation on each subunit, which potentially allows for an incredible amount of structural diversity conferred by this post-translational modification. The functional properties of iGluRs are central to excitatory synaptic function. *N*-glycosylation is required for proper folding of iGluRs in mammalian cells, but the impact of oligosaccharides on the function of successfully folded receptors is less clear. Glycan moieties are large, polar, occasionally charged, and mediate many protein-protein interactions throughout the nervous system. Additionally, they are attached at sites along iGluR subunits that position them for involvement in the structural changes underlying gating. We show here that altering glycan content on KARs changes functional properties of the receptors in a manner dependent on the identity of both the modified sugars and the subunit composition of the receptor to which they are attached. Glycosylation patterns likely differ between cell types, across development, or with pathologies, and thus our findings reveal a new mechanism for context-specific fine-tuning of iGluR function through diversity in glycan structure.

Introduction

Post-translational modifications of ionotropic glutamate receptor (iGluR) subunits have the potential to diversify channel function and impact intracellular trafficking (Traynelis et al., 2010). Phosphorylation of the AMPA receptor GluA1 subunit at discrete sites in the cytoplasmic domain, for example, is thought to be a key step in regulated targeting of synaptic receptors underlying plasticity of excitatory transmission (Boehm et al., 2006). Fundamental steps in the biogenesis of iGluRs, such as protein folding and egress from the endoplasmic reticulum, require a different form of post-translational modification, *N*-glycosylation, which consists of the conjugation and processing of oligosaccharides attached to asparagines in the extracellular domains of subunit proteins (Everts et al., 1999). For tetrameric iGluRs, the potential structural diversity conferred by variable oligosaccharide content far exceeds any other form of post-translational modification. iGluR subunit proteins have between 4 and 12 *N*-glycosylation consensus motifs, and oligosaccharides can constitute ~10% of the mass of the mature subunit proteins (e.g., Rogers et al., 1991; Roche et al., 1994). Discrete sites of *N*-glycosylation in neuronal AMPA, kainate, and NMDA receptors were identified in a proteomic analysis of glycans and glycopeptides (Parker et al., 2013). These sites of oligosaccharide conjugation included asparagines within the first extracellular linker domain connecting the amino terminal and ligand binding domains in the receptor subunits, which positions oligosaccharide chains on native receptors adjacent to key structural components involved in channel gating.

The proximity of substantial, highly polar, and potentially charged sugar chains to a critical functional domain in iGluRs suggested to us that oligosaccharides might directly affect receptor gating. This hypothesis has been tested in a number of ways over the last two decades but no clear consensus has emerged. For example, tunicamycin inhibition of *N*-glycosylation in

Xenopus oocytes diversely impacted, but did not preclude, AMPA and kainate receptor currents and had little effect on agonist EC_{50} values; however, a possible effect of glycans on desensitization properties was inferred from changes in relative glutamate and kainate current amplitudes (Everts et al., 1997). Consistent with this inference, GluK2 kainate receptors (KARs) lacking single *N*-glycosylation sites exhibited variable desensitization rates when expressed in mammalian cells and examined with an agonist exchange system able to resolve the rapid kinetics of entry into desensitization (Everts et al., 1999). On the other hand, elimination by mutagenesis of two *N*-glycosylation sites in the initial S1 segment of the ligand-binding domain (LBD) of GluA4 AMPA receptors had no qualitatively measurable effect on receptor function or ligand binding affinity (Pasternack et al., 2003). Radioligand binding assays have been similarly equivocal; AMPA receptors exhibit two discrete [^{3}H]AMPA binding affinities (Hall et al., 1992), which was ascribed to differentially glycosylated subunit isoforms (Standley et al., 1998). Finally, the sulfated trisaccharide human natural killer-1 (HNK-1) epitope is added to GluA2 in the hippocampus, where it mediates subunit interactions with N-cadherin that are critical for the induction of plasticity in CA1 (Yamamoto et al., 2002; Morita et al., 2009).

In this study, we took a different tack to test the hypothesis that glycan chemical content impacts iGluR function. KAR currents were recorded after either pharmacologically inhibiting key oligosaccharide processing enzymes in the Golgi or after over-expressing enzymes responsible for transferring capping sugars to complex oligosaccharides. Our results suggest that alterations in *N*-glycan identity produce subtle differences in recombinant KAR functional properties in a manner that depends on both the receptor subunit composition and the identity of the sugars attached to the protein.

Materials and Methods

Materials

DNAs used in these studies were provided to us by Dr. Derek Bowie (McGill University, rat GluK2(ΔNG5,6,7) cDNA), Dr. Sakari Kellokumpu (University of Oulu, eGFP-ST3 and eGFP-ST6 cDNAs), Dr. Shogo Oka (Kyoto University, pIRES-GlcAT-P-HNK-1ST) and Dr. Susumu Tomita (Yale University School of Medicine, Neto2 cDNA). The GluA2, GluK1, and GluK2 cDNAs used in these experiments expressed unedited (glutamine-containing) receptors. Swainsonine and kifunensine were purchased from Sigma-Alderich (St. Louis, MO).

Cell Culture and Transfection

Human embryonic kidney expressing T-antigen, clone 17 (HEK293T/17) cells from American Type Culture Collections (Manassas, VA) were cultured in Dulbecco's modified essential medium (Corning Cellgro, Manassas, VA) supplemented with 10% heat-inactivated fetal bovine serum (Gemini Bio-Products, West Sacramento, CA), 100 μg ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin (Corning Cellgro, Manassas, VA), at 37^{o}C with 5% CO_2. Transfections were performed according to the manufacturer's protocol using a ratio of 1 μg cDNA to 3 μl Mirius Bio *Trans*-IT reagent (Mirius Bio Corporation, Madison, WI). An enhanced green fluorescent protein (eGFP) was co-transfected in order to identify receptor-expressing cells. Swainsonine (20 μM) and kifunensine (5 μM) (Sigma-Alderich, St. Louis, MO) treatments were added to the culture medium at least 4 hours prior to transfection.

Electrophysiology

Whole-cell or outside-out patch recordings were made from transfected HEK293T/17 cells held at –70 mV as described previously (Vivithanaporn et al., 2007). Currents were elicited by rapid application of 10 mM glutamate, to receptor-expressing cells using a piezoceramic system. Rise times (10-90%) were less than 2.0 ms. Weighted desensitization rates were calculated from bi-exponential fits of 1 second glutamate applications in Clampfit10 (Molecular Devices, Sunnyvale, CA). Recovery rates were calculated with single exponential association fits in Prism5 (GraphPad Software, La Jolla, CA). External solution contained (in mM): 150 NaCl, 2.8 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 10 glucose, and 10 Hepes, adjusted to pH 7.3. Intracellular solution contained (in mM): 110 CsF, 30 CsCl, 4 NaCl, 0.5 $CaCl_2$, 10 Hepes, and 5 EGTA, adjusted to pH 7.3.

Western Blotting

Recombinant proteins were expressed in HEK293T/17 for 72 hours prior to washing with ice cold DPBS and lysing in lysis buffer. Samples were solubilized for one hour at 4^{o}C and cleared of cell debris by centrifugation. Proteins were separated on a denaturing 8% polyacrylamide gel and transferred to a PVDF membrane. Proteins were detected using a rabbit anti-myc antibody (product number 06-549, EMD Millipore, Darmstadt, Germany), a rabbit anti-GluR6/7 antibody (product number 04-921, EMD Millipore, Darmstadt, Germany). Antibody binding was imaged on an Odyssey CLx Imager (LI-COR Biosciences, Lincoln, NE). Western blot images were analyzed using Image Studio Software (LI-COR Biosciences, Lincoln, NE).

Statistical Methods

Comparisons between two conditions were made by *t*-test. Comparisons between 3 or more groups were made with a one-way ANOVA followed by Dunnett's multiple comparison. Equivalent results were obtained using either parametric or non-parametric tests, and we report the statistical results from parametric tests here. The time courses of recovery from desensitization were fit with a one-phase association exponential function. Statistical tests were performed in Prism5 (GraphPad Software, La Jolla, CA).

Results

Biochemical manipulation of glycan content

We tested the hypothesis that restricting glycan processing can alter iGluR functional properties by expressing recombinant receptors in the presence of enzyme inhibitors of α-mannosidases, the *cis*-Golgi enzymes responsible for trimming branches from immature, mannose-containing oligosaccharide branches. Receptor-transfected HEK293T/17 cells were treated with either kifunensine, an α-mannosidase I inhibitor, or swainsonine, an α-mannosidase II inhibitor (Figure 3.1A). These pharmacological tools restrict processing to immature or hybrid glycan structures, respectively (Figure 3.1A). Treatment with the two inhibitors incrementally reduced the

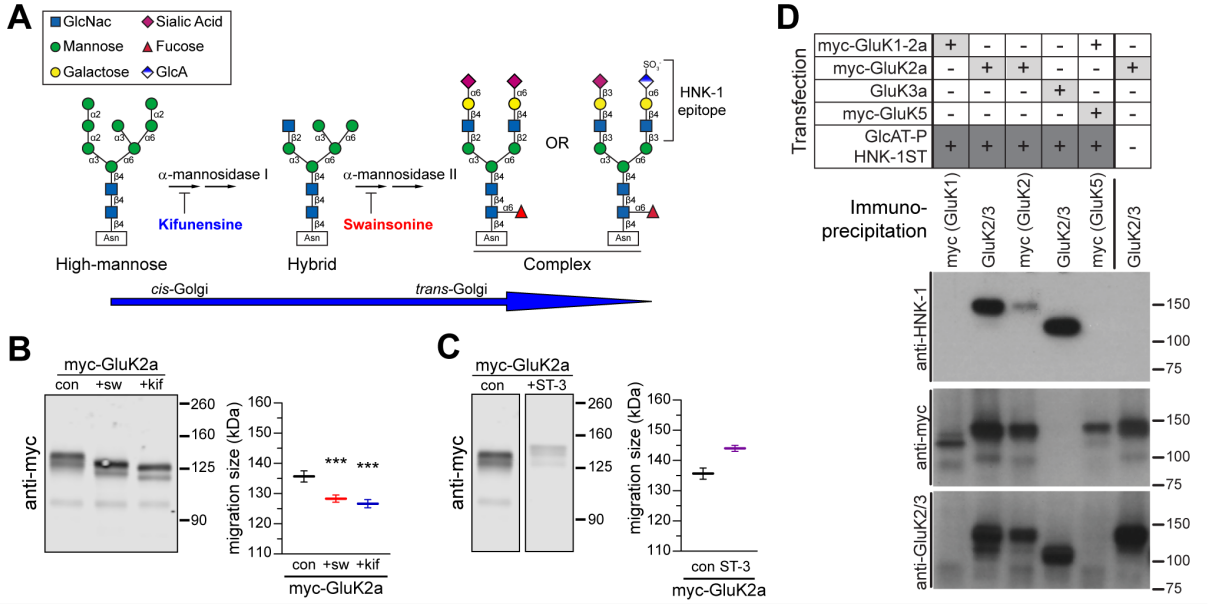

Figure 3.1. α-mannosidase inhibition and transferase over-expression change the glycan content on GluK2a-containing receptors. **A**) Swainsonine inhibits α-mannosidase II and kifunensine inhibits α-mannosidase I, respectively blocking hybrid and complex oligosaccharide formation. Diagram modified from Copits, et al., 2014. **B**) myc-GluK2a was expressed in untreated control, swainsonine-treated, or kifunensine-treated HEK293T/17 cells, detected by immunoblotting with an anti-myc antibody, and myc-GluK2a MW was measured from gel migration. **C**) myc-GluK2a was expressed alone or with α-2,3-sialyltransferase in HEK293T/17 cells, myc-GluK2a was detected by immunoblotting with an anti-myc antibody, and myc-GluK2a MW is measured from gel migration. **D**) Recombinant KAR subunits were co-expressed in HEK293T/17 cells with or without GlcAT-P and HNK-1ST, the two enzymes responsible for conjugating the HNK-1 epitope to complex glycan branches, as denoted by the table of transfection combinations. KAR subunits were immunoprecipitated from cell lysates with an antibody against myc or the GluK2/3 subunits. Immunoprecipitation of subunit-conjugated HNK-1 was detected by immunoblotting for the HNK-1 epitope; enrichment of KAR subunits was confirmed by immunoblotting for myc or GluK2/3. Abbreviations are untreated control (con), swainsonine treatment (sw), kifunensine treatment (kif), and α-2,3-sialyltransferase co-expression (ST-3). Error bars represent s.e.m. Statistical significance is indicated as follows: ***p<0.001.

molecular weight (MW) of recombinant myc-tagged GluK2a protein, as detected with anti-myc antibody (Figure 3.1B). Swainsonine reduced GluK2a monomers from 136 ± 2 kDa to 128 ± 1 kDa, and kifunensine further decreased the MW of GluK2a subunits to 127 ± 1 kDa (repeated measures ANOVA, $p = 0.0002$; $n = 3$) (Figure 3.1B).

We also drove processing of oligosaccharides to more complex structures by transfecting transferase enzymes that either capped branches with sialic acids or that catalyzed generation of an unusual sulfated trisaccharide, the HNK-1 epitope, which is known to be attached to GluA2 subunits in the mouse brain (Morita et al., 2009). Co-expression of GluK2a with α-2,3-sialyltransferase (ST-3) increased the MW of the protein to ~144 kDa ($n = 2$) while clearly reducing the total protein expression (Figure 3.1C). The basis for the observed reduction in equilibrium protein expression is unclear but could include enhanced degradation or slowed biogenesis induced by the addition of sialic acid to GluK2a-containing receptors.

The HNK-1 epitope was previously shown to be a constituent of neuronal GluA2 subunits that affected receptor stability in the plasma membrane (Morita et al., 2009), but it was unknown if KARs were similarly modified. We found that a subset of receptor subunits act as substrates for HNK-1 conjugation. We expressed plasmid DNA encoding glucuronyltransferase-P (GlcAT-P) and the HNK-1 sulfotransferase (HNK-1ST) together with myc-GluK1-2a, myc-GluK2a, GluK3, or myc-GluK5, immunoprecipitated with either anti-myc or anti-GluK2/3 antibody, and immunoblotted with an anti-HNK-1 antibody (Figure 3.1D). Myc-GluK2a and GluK3a subunits contained the HNK-1 epitope, whereas myc-GluK1-2a and myc-GluK5 subunit proteins did not.

The blots were stripped and re-probed with either anti-myc or anti-GluK2/3 to verify that each of the subunit proteins were expressed in the HEK293T/17 cells.

Functional consequences of restricting glycan processing

The biochemical data show that we are able to manipulate the oligosaccharide moieties attached to iGluR subunits with variable efficacy dependent on the identity of the receptor subunit. We next determined if these manipulations altered the functional properties of GluK2a-containing KARs in patch-clamp recordings from transfected HEK293T/17 cells that had been treated with normal media, swainsonine, or kifunensine. Glutamate (10 mM) was applied to the cells for 100 ms to evoke whole-cell currents; representative traces are shown in Figure 3.2A.

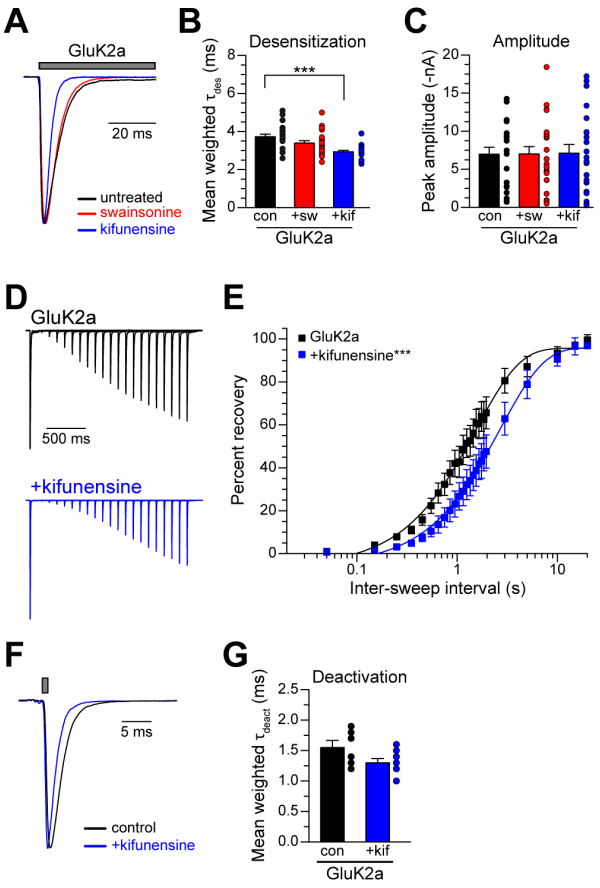

Figure 3.2. α-mannosidase inhibition alters GluK2a-containing receptor desensitization. **A**) Representative current traces from untreated control, swainsonine-treated, and kifunensine-treated myc-GluK2a-expressing HEK293T/17 cells. Grey bar indicates glutamate (10 mM) application. Amplitudes are scaled. **B**) Quantification of glutamate-evoked desensitization from untreated and treated myc-GluK2a-expressing HEK293T/17 cells. **C**) Quantification of glutamate-evoked current amplitudes from untreated and treated myc-GluK2a-expressing HEK293T/17 cells. **D**) Representative current traces in two-pulse glutamate (10 mM) recovery experiments recorded from untreated and kifunensine-treated myc-GluK2a-expressing HEK293T/17 cells. Intervals between glutamate exposures range from 50 ms to 2 s in the traces shown. Amplitudes of the first glutamate application are scaled. **E**) Quantification of mean glutamate recovery for all myc-GluK2a-expressing cells with and without kifunensine. Amplitude of the second glutamate application in a two-pulse experiment is reported as a normalized percentage of the first glutamate application. Results were fitted with a single component exponential equation. **F**) Representative current traces from outside-out patches pulled from untreated and kifunensine-treated myc-GluK2a-expressing HEK293T/17 cells. Grey bar indicates glutamate (10 mM) application. Amplitudes are scaled. **G**) Quantification of glutamate-evoked deactivation from myc-GluK2a-containing patches pulled from untreated and kifunensine-treated HEK293T/17 cells. Abbreviations are untreated control (con), swainsonine treatment (sw), and kifunensine treatment (kif). Error bars represent s.e.m. Statistical significance is indicated as follows: ***p<0.001.

We found that currents elicited from cells incubated with α-mannosidase I inhibitor exhibited more rapid desensitization. Recordings from kifunensine-treated cells desensitized faster than glutamate currents in untreated cells (control: τ_{des} = 3.7 ± 0.1 ms, n = 22; kif: 2.9 ± 0.1 ms, n = 22; p<0.0001, Dunnett's multiple comparison to control), whereas currents from swainsonine-treated GluK2a KARs were not different than control (3.4 ± 0.1 ms; n = 23) (Figure 3.2B). Whole-cell current amplitudes were not altered by either α-mannosidase inhibitor, with peak amplitudes of 6.9 ± 0.9 nA in control cells, 7.0 ± 1.0 nA in swainsonine-treated cells, and 7.1 ± 1.1 nA in kifunensine-treated cells (p = 0.9947) (Figure 3.2C). The rate of recovery from desensitization also was altered by kifunensine treatment. Recovery curves were generated by applying glutamate twice at varying intervals (Figure 3.2D). Kifunensine-treated GluK2a-containing receptors recovered from glutamate-evoked desensitization with a tau of 2.7 s, which was ~2-fold slower than the control recovery of 1.5 s (one-phase association exponential equation, parameters of best fit lines differ with p<0.0001) (Figure 3.2E). Finally, we tested whether restricting glycan processing altered deactivation of GluK2a-containing receptors (Figure 3.2F). Brief (1 ms) application of glutamate to outside-out patches from kifunensine-treated GluK2a KARs evoked currents that deactivated with a τ of 1.3 ± 0.1 ms (n = 8), which was not different from the 1.6 ± 0.1 ms (n = 6) deactivation time course in untreated patches (unpaired *t*-test, p = 0.0747) (Figure 3.2G). These data show that restricting glycan processing on GluK2a-containing KARs alter receptor desensitization kinetics, suggesting that sugar composition can influence the complex structural rearrangements underlying this functional property.

We next attempted to determine the gating properties of GluK2a KARs lacking all *N*-glycans by treating live cells with the glycosidase PNGase F. PNGase F cleaves glycans at the linkage

between asparagine side chain and the inner-most GlcNAc, removing entire glycan chains from the protein structure. However, we found that the MW of GluK2a protein was not reduced following treatment of live GluK2a-transfected cells with PNGase F for 2 hours prior to lysing (Figure 3.3A). Longer incubations of up to 24 hours also were ineffective at catalyzing cleavage (data not shown). These data suggest that the native receptor conformation presents a steric

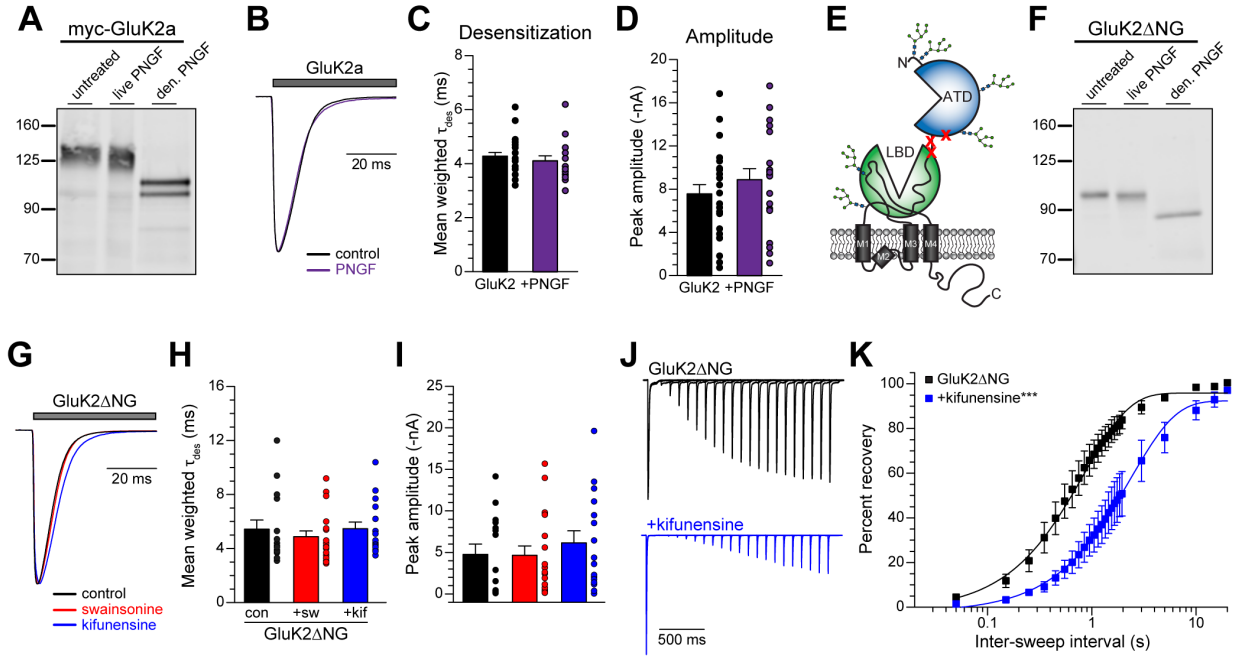

Figure 3.3. Glycan chains attached between the ATD and LBD are critical modulators of KAR desensitization. **A**) myc-GluK2a-expressing cells were incubated in buffer (untreated) or buffer containing PNGase F (live PNGF) for two hours prior to lysing. 5 ug of total protein from untreated cell lysates was denatured, digested with PNGF (den. PNGF), and run as a positive control for PNGase F-mediated glycan removal. **B**) Representative traces of currents evoked from untreated (control) or live PNGase F-incubated (PNGF) HEK293T/17 cells expressing myc-GluK2a. Grey bar indicates glutamate (10 mM) application. **C**) Quantification of whole-cell glutamate-evoked desensitization from untreated and PNGF-incubated cells expressing myc-GluK2a. **D**) Quantification of peak current amplitudes from untreated and PNGF-incubated cells expressing myc-GluK2a. **E**) Cartoon depiction of the mutated glycosylation sites in a GluK2ΔNG5,6,7 subunit. **F**) GluK2ΔNG5,6,7-expressing cells were incubated in buffer (untreated) or buffer containing PNGase F (live PNGF) for two hours prior to lysing. 5 ug of total protein from untreated cell lysates was denatured, digested with PNGF (den. PNGF), and run as a positive control for PNGase F-mediated glycan removal. **G**) Representative current traces from untreated control, swainsonine-treated, and kifunensine-treated GluK2Δ NG5,6,7-expressing HEK293T/17 cells. Grey bar indicates glutamate (10 mM) application. Amplitudes are scaled. **H**) Quantification of glutamate-evoked desensitization from untreated and treated GluK2ΔNG5,6,7-expressing HEK293T/17 cells. **I**) Quantification of glutamate-evoked current amplitudes from untreated and treated GluK2ΔNG5,6,7-expressing HEK293T/17 cells. **J**) Representative current traces in two-pulse glutamate (10 mM) recovery experiments recorded from untreated and kifunesine-treated GluK2ΔNG5,6,7-expressing HEK293T/17 cells. Intervals between glutamate exposures range from 50 ms to 2 s in the traces shown. Amplitudes from the first glutamate application are scaled. **K**) Quantification of mean glutamate recovery for all GluK2ΔNG5,6,7-expressing cells with and without kifunensine treatment. Amplitude of the second glutamate application in a two-pulse experiment is reported as a normalized percentage of the first glutamate application. Results were fitted with a single component exponential equation. Treatment abbreviations are untreated control (con), swainsonine treatment (sw), and kifunensine treatment (kif). Error bars represent s.e.m. Statistical significance is indicated as follows: ***p<0.001.

hindrance that precludes PNGase F from reaching its substrate site along the polypeptide chain. PNGase F also did not alter glutamate-evoked current amplitudes or desensitization measured in recordings from GluK2a-expressing cells (Figure 3.3B, 3.3C) (untreated: 7.5 ± 0.9 nA, τ_{des} = 4.3 ± 0.1 ms, n = 23; PNGase F: 8.9 ± 1.0 nA, τ_{des} = 4.1 ± 0.2 ms, n = 20). Thus, were unable to test the effect of complete enzymatic removal of glycans on KAR functional properties.

In the GluK2a subunit, plant and vertebrate lectins that allosterically modulate receptor function bind to oligosaccharides conjugated to one or more of three asparagines at the interface between the amino-terminal domain (ATD) and ligand-binding domain (LBD) (glycan sites 5, 6, and 7; Figure 3.3E) (Fay and Bowie, 2006; Copits et al., 2014). We hypothesized that these oligosaccharide chains also acted as the key mediators of the glycan-dependent functional changes observed in the preceding experiments. To test that idea, we analyzed the biochemical and functional properties of a receptor, GluK2ΔNG5,6,7, in which each consensus site for glycosylation had been mutated within the consensus sequence. The mutant protein carried approximately 10 kDa of glycosylation as measured from the difference in MW between the untreated and PNGase F digested subunits (Figure 3.3F), as compared to 30-40 kDa of glycosylation that was added to wildtype GluK2 (Figure 3.3A). This means that more than 66% of the sugar on GluK2 is attached at only 3 of 9 glycosylation sites, and oligosaccharides attached to the remaining sites also exhibited resistance to PNGase F-mediated cleavage. Homomeric GluK2ΔNG5,6,7 receptors desensitized with the same time course regardless of enzyme inhibitor treatment (Figure 3.3G), in contrast to wildtype GluK2a receptors (untreated: τ_{des} = 5.4 ± 0.7 ms, n = 15; swainsonine: τ_{des} = 4.9 ± 0.4 ms, n = 19; kifunensine τ_{des} = 5.5 ± 0.5 ms, n = 16; p = 0.6598) (Figure 3.3H). Restricting glycan processing did not alter peak current

amplitudes from GluK2ΔNG5,6,7 receptors (Figure 3.3I). This suggests that glycans conjugated to one or more of these discrete sites influence GluK2 KAR receptor gating.

Surprisingly, kifunensine treatment significantly slowed recovery from glutamate-evoked desensitization of GluK2ΔNG5,6,7 receptors despite its lack of effect on the rate of entry into desensitization (Figure 3.3J). Untreated GluK2ΔNG5,6,7 receptor currents recovered from desensitization with a τ of 0.85 seconds (n = 3), whereas treatment with kifunensine slowed recovery of these mutant receptors to a τ of 2.36 seconds (n = 6) (parameters of best fit lines differ $p<0.0001$) (Figure 3.3K), which was similar to the rate at which wildtype kifunensine-treated receptors recovered from desensitization. These data suggest that the glycans with the greatest impact on macroscopic desensitization and those that most strongly affect recovery from desensitization are located at non-overlapping or only partially overlapping sites along the GluK2a receptor subunit.

Given the subunit-specific effects that our manipulations had on both size-shift and functional properties, we tested whether oligosaccharide composition influenced desensitization rates of other iGluRs. Homomeric GluK1-2a KARs expressed in untreated cells desensitized with a mean weighted τ_{des} of 13.4 ± 1.9 ms (n = 19) (Figure 3.4A), which was significantly slower than the 8.3 ± 0.9 ms τ_{des} measured from swainsonine-treated cells (n = 15, $p<0.05$) and 8.2 ± 1.1 ms from kifunensine-treated cells (n = 18, $p<0.05$) ($p = 0.0176$; Figure 3.4B), showing that restricted glycan processing similarly affects homomeric GluK1-2a and GluK2a-containing receptors. Neither swainsonine nor kifunensine treatment altered mean peak current amplitudes ($p = 0.7202$) (Figure 3.4C). In contrast, heteromeric GluK2a/GluK5 KARs were not affected by

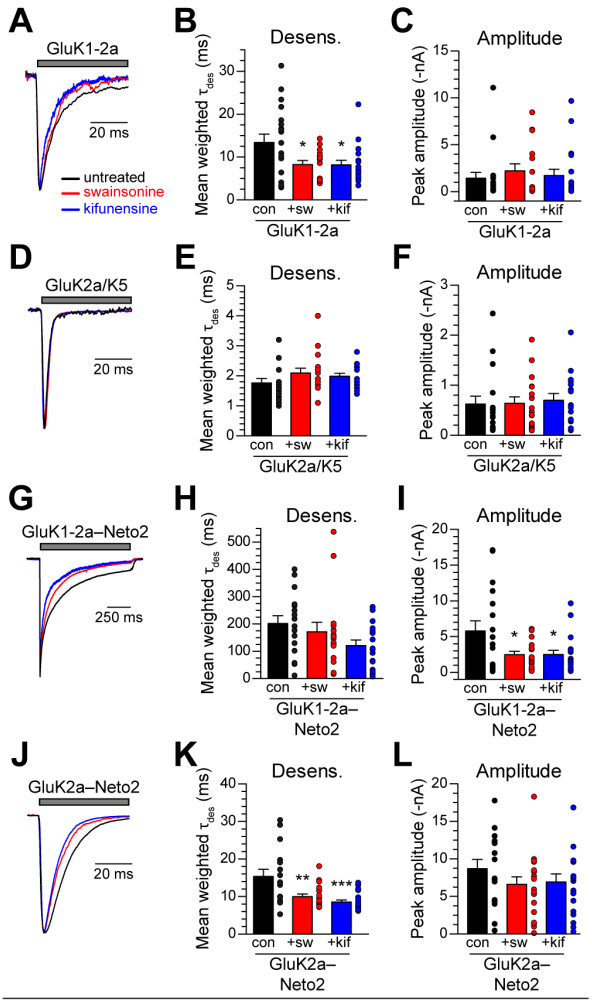

Figure 3.4. α-mannosidase inhibition speeds the rate of desensitization of other KAR subunit combinations. **A, D, G, J**) Representative current traces from untreated control, swainsonine-treated, and kifunensine-treated HEK293T/17 cells expressing myc-GluK1-2a (A), myc-GluK2a with myc-GluK5 (D), myc-GluK1-2a with Neto2 (G), or myc-GluK2a with Neto2 (J). Grey bar indicates glutamate (10 mM) application. Amplitudes are scaled. **B, E, H, K**) Quantification of glutamate-evoked desensitization from untreated and treated HEK293T/17 cells expressing myc-GluK1-2a (B), myc-GluK2a with myc-GluK5 (E), myc-GluK1-2a with Neto2 (H), or myc-GluK2a with Neto2 (K). **C, F, I, L**) Quantification of glutamate-evoked current amplitudes from untreated and treated HEK293T/17 cells expressing myc-GluK1-2a (C), myc-GluK2a with myc-GluK5 (F), myc-GluK1-2a with Neto2 (I), or myc-GluK2a with Neto2 (L). Error bars represent s.e.m. Abbreviations are untreated control (con), swainsonine treatment (sw), and kifunensine treatment (kif). Statistical significance is indicated as follows: *p<0.05, **p<0.01, ***p<0.001.

restricted glycan processing (Figure 3.4D). Glutamate-evoked currents from control cells desensitized at a rate of 1.8 ± 0.1 ms (n = 17), and this was not significantly altered by either swainsonine (2.1 ± 0.2 ms, n = 17) or by kifunensine (2.4 ± 0.4 ms, n = 17) (p = 0.2636; Figure 3.4E). Peak current amplitudes for GluK2a/K5-containing receptors were unaltered by restricted glycan processing (p = 0.9242) (Figure 3.4F).

We next tested whether glycan composition affected functional properties of KARs containing auxiliary subunits. We found that assembly of GluK1-2a with its auxiliary subunit Neto2 occluded a significant effect of restricting oligosaccharide processing (Figure 3.4G). GluK1-2a/Neto2 desensitization was highly variable, as expected, and currents decayed with a τ_{des} of 201.6 ± 28.8 ms (n = 16) in control cells. Swainsonine and kifunensine treatment resulted in τ_{des} of 171.1 ± 34.6 ms (n = 16) and 120.8 ± 20.6 ms (n = 18), respectively, which were not different from untreated cells (p = 0.1259) (Figure 3.4H). Current amplitudes were slightly smaller following α-mannosidase

inhibition, however (control: 5.8 ± 1.4 nA; swainsonine: 2.5 ± 0.5 nA; kifunensine: 2.5 ± 0.6 nA; p = 0.0200) (Figure 3.4I). In contrast, glycan processing did affect the desensitization rate of GluK2a-containing receptors that were co-assembled with Neto2 (Figure 3.4J). Currents mediated by GluK2a/Neto2 KARs desensitized with a τ_{des} of 15.3 ± 1.9 ms in untreated cells (n = 16). Desensitization was faster for currents evoked from cells treated with both swainsonine (10.0 ± 0.7 ms n = 19) and kifunensine (8.5 ± 0.6 ms, n = 19; p = 0.0003) (Figure 3.4K). The amplitude of currents from GluK2a/Neto2 KARs were not different in untreated cells compared to α-mannosidase inhibition (p = 0.3791; Figure 3.4L).

Functional consequences of incorporating negatively charged glycans

Sialic acids are common capping sugars that terminate extension of *N*-glycans (Zamze et al., 1998), whereas the sulfated trisaccharide HNK-1 is more restricted in its incorporation into on CNS glycoproteins (Schwarting et al., 1987). Both contribute negative charge to the distal end of oligosaccharide antennae and could, in principle, interact with determinants in receptor proteins to modulate function. To test this possibility, we next carried out experiments in which the functional properties of homomeric GluK2a KARs were analyzed in cells co-expressing either enzymes necessary for generation of the HNK-1 epitope or two sialyltransferases found in the brain.

HNK-1 conjugation altered the functional properties of GluK2a KARs (Figure 3.5A). Glutamate-evoked desensitization was slowed over two-fold from a mean τ_{des} of 3.5 ± 0.3 ms (n = 9) in control recordings to 7.7 ± 0.6 ms (n = 19) with HNK-1 (p = 0.0001; Figure 3.5B), whereas mean peak amplitudes were unaffected (Figure 3.5C). GluK2a-containing receptors expressed with

HNK-1 enzymes also recover from desensitization more rapidly (control: 4.0 s, 95% CI of 3.60 – 4.55, n = 4; HNK-1: 0.95 s, 95% CI of 0.87 – 1.06, n = 7; p<0.0001) (Figure 3.5D, 3.5E). Finally, HNK-1 conjugation slowed deactivation of the receptors in outside-out patch recordings from a control τ of 1.6 ± 0.0 ms (n = 4) to 2.7 ± 0.3 ms (p = 0.0094) (Figure 3.5G).

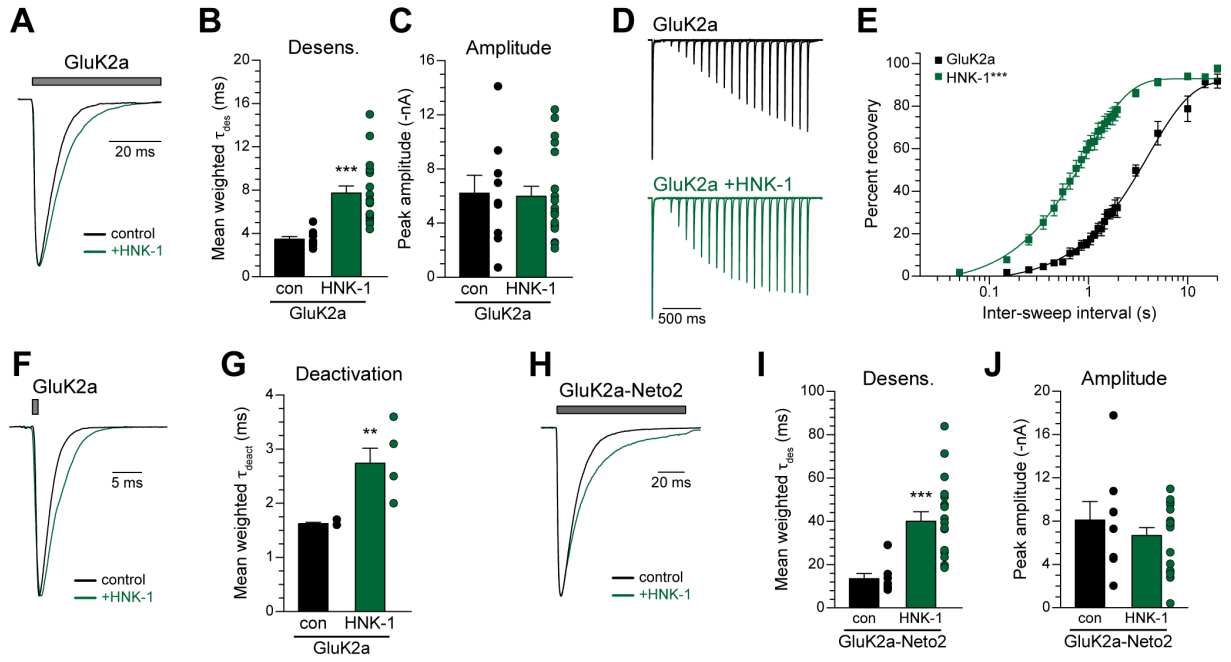

Figure 3.5. Conjugation of the HNK-1 epitope alters desensitization and deactivation of GluK2a-containing receptors. **A**) Representative current traces from control HEK293T/17 cells expressing only myc-GluK2a and from cells where myc-GluK2a was co-expressed with the HNK-1-conjugating transferases GlcAT-P and HNK-1ST. Grey bar indicates glutamate (10 mM) application. Amplitudes are scaled. **B**) Quantification of glutamate-evoked desensitization from myc-GluK2a alone and HNK-1 co-expressing HEK293T/17 cells. **C**) Quantification of glutamate-evoked current amplitudes from myc-GluK2a alone and HNK-1 co-expressing HEK293T/17 cells. **D**) Representative current traces in two-pulse glutamate (10 mM) recovery experiments recorded from myc-GluK2a expressing HEK293T/17 cells transfected either alone or with HNK-1-conjugating transferases. Intervals between glutamate exposures range from 50 ms to 2 s in the traces shown. Amplitudes from the first glutamate application are scaled. **E**) Quantification of mean glutamate recovery for all myc-GluK2a-expressing cells with and without HNK-1 conjugation. Amplitude of the second glutamate application in a two-pulse experiment is reported as a normalized percentage of the first glutamate application. Results were fit with a single component exponential. **F**) Representative current traces from outside-out patches pulled from myc-GluK2a-expressing HEK293T/17 cells transfected either with receptor subunit plasmid alone or with HNK-1-conjugating transferases. Grey bar indicates glutamate (10 mM) application. Amplitudes are scaled. **G**) Quantification of glutamate-evoked deactivation from myc-GluK2a-containing patches pulled from control and HNK-1-conjugating HEK293T/17 cells. **H**) Representative current traces from control HEK293T/17 cells expressing myc-GluK2a and Neto2 and from cells where the subunit plasmids were co-expressed with HNK-1-conjugating transferases. Grey bar indicates glutamate (10 mM) application. Amplitudes are scaled. **I**) Quantification of glutamate-evoked desensitization from myc-GluK2a–Neto2 alone and HNK-1 co-expressing HEK293T/17 cells. **J**) Quantification of glutamate-evoked current amplitudes from myc-GluK2a–Neto2 alone and HNK-1 co-expressing HEK293T/17 cells. Abbreviations are control transfection of receptor alone (con) and co-transfection with GlucAT-P and HNK-1ST (HNK-1). Error bars represent s.e.m. Statistical significance is indicated as follows: **p<0.01, ***p<0.001.

Properties of GluK2a-containing KARs were affected by glycan restriction even when assembled with Neto2. Similarly, HNK-1 conjugation to Neto-containing receptors slowed the desensitization of currents (Figure 3.5H). The desensitization rate of GluK2a/Neto2 KARs was 3-fold slower in cells co-transfected with the HNK-1 transferases, increasing the τ_{des} from 13.5 ± 2.4 ms (n = 8) to 40.0 ± 4.5 ms (n = 18, p = 0.0009) in the presence of HNK-1 (Figure 3.5I). As for GluK2a without Neto2, peak current amplitudes were not affected in HNK-1-expressing cells (p = 0.3745; Figure 3.5J). These data therefore demonstrate that this sulfated oligosaccharide modifies a number of receptor functional parameters, including deactivation.

We tested if HNK-1 modulation of receptor desensitization was dependent on the trio of glycan conjugation sites, between the ATD and LBD, that are eliminated in the GluK2ΔNG5,6,7 receptor mutant (Figure 3.6A). The mean weighted tau of desensitization measured from currents evoked from cells expressing GluK2ΔNG5,6,7 alone was 6.9 ± 1.1 ms (n = 7). Surprisingly, HNK-1 conjugation caused glutamate-evoked currents to desensitize faster (4.0 ± 0.2 ms, n = 12; p = 0.0034, Figure 3.6B) and to recover from desensitization slower (control τ_{rec} of 1.22 s, 95% CI 1.02 – 1.51, n = 5; HNK-1 τ_{rec} of 2.33 s, 95% CI 2.00 – 2.79, n = 6; p<0.0001, Figure 3.6D, 3.6E), which is opposite the effect that HNK-1 addition had on wildtype receptors. We conclude from this data that HNK-1 modification of GluK2a subunits can occur at two or more sites of glycosylation to effect opposing changes on receptor kinetics.

To determine if another negatively charged glycan, sialic acid, had similar effects on GluK2a receptor function, we co-expressed GFP-tagged sialyltransferases with GluK2a receptors. Neither ST3 nor α-2,6-sialyltransferase (ST6) altered desensitization of glutamate-evoked

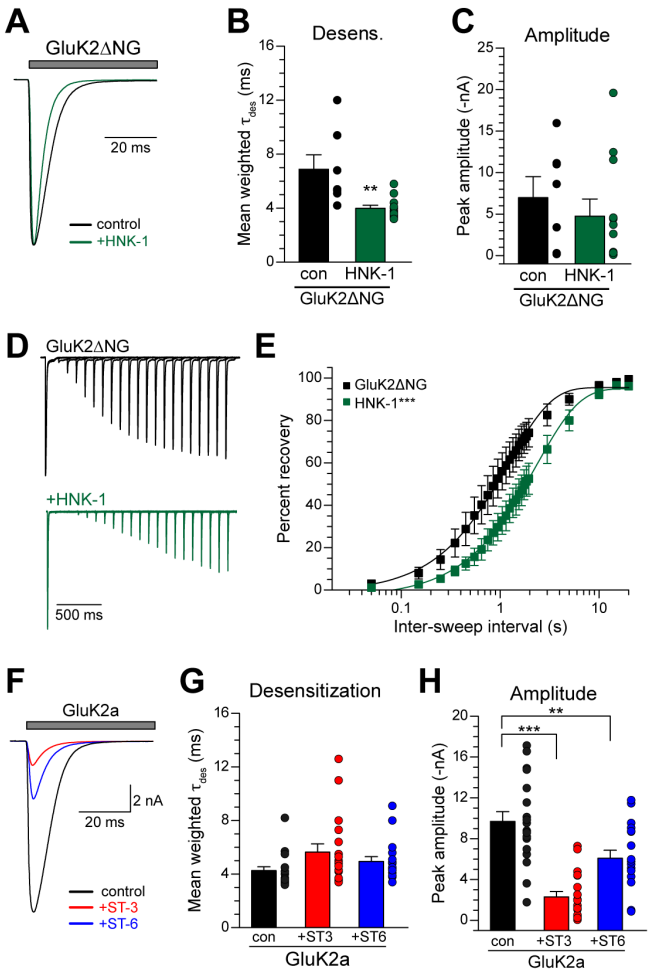

Figure 3.6. HNK-1 conjugation differentially modulates desensitization of KARs lacking glycan attachment sites, and sialic acid conjugation does not mimic the effect of HNK-1 conjugation. **A**) Representative current traces from control HEK293T/17 cells expressing only GluK2ΔNG5,6,7 and from cells where GluK2ΔNG5,6,7 was co-expressed with the HNK-1-conjugating transferases GlcAT-P and HNK-1ST. Grey bar indicates glutamate (10 mM) application. Amplitudes are scaled. **B**) Quantification of glutamate-evoked desensitization from GluK2ΔNG5,6,7 alone and HNK-1 co-expressing HEK293T/17 cells. **C**) Quantification of glutamate-evoked current amplitudes from GluK2ΔNG5,6,7 alone and HNK-1 co-expressing HEK293T/17 cells. **D**) Representative current traces in two-pulse glutamate (10 mM) recovery experiments recorded from GluK2ΔNG5,6,7 expressing HEK293T/17 cells transfected either alone or with HNK-1-conjugating transferases. Intervals between glutamate exposures range from 50 ms to 2 s in the traces shown. Amplitudes from the first glutamate application are scaled. **E**) Quantification of mean glutamate recovery for all GluK2ΔNG5,6,7-expressing cells with and without HNK-1 conjugation. Amplitude of the second glutamate application in a two-pulse experiment is reported as a normalized percentage of the first glutamate application. Results were fitted with a single component exponential equation. **F**) Representative current traces from control HEK293T/17 cells expressing only myc-GluK2a and from cells where myc-GluK2a was co-expressed with either α-2,3-sialyltransferase (ST-3) or α-2,6-sialyltransferase (ST-6). Grey bar indicates glutamate (10 mM) application. Amplitudes are scaled. **G**) Quantification of glutamate-evoked desensitization from myc-GluK2a alone and ST-3 or ST-6 co-expressing HEK293T/17 cells. **H**) Quantification of glutamate-evoked current amplitudes from myc-GluK2a alone and ST-3 or ST-6 co-expressing HEK293T/17 cells. Abbreviations are control transfection of receptor alone (con), co-transfection with GlucAT-P and HNK-1ST (HNK-1), and co-transfection with sialyltransferases (ST-3 or ST-6). Error bars represent s.e.m. Statistical significance is indicated as follows: **p<0.01, ***p<0.001.

currents (Figure 3.6F and 3.6G, p = 0.0960). In contrast, peak current amplitudes were lower from ST6 expressing cells 2.3 ± 0.5 nA (n = 18) compared to control recordings (9.7 ± 0.97 nA, n = 18) and ST3 expressing cells (6.1 ± 0.8 nA; n = 18) (p<0.0001 for ST6 vs. control, Figure 3.6H). These data show that the effect of HNK-1 on slowing GluK2a KAR currents is not replicated by adding a different charged capping sugar, suggesting a specificity of the contribution that HNK-1 makes to receptor function.

The marked effect that HNK-1 conjugation had on GluK2a currents prompted us to test if HNK-1 had equivalent modulatory actions on receptors composed of another KAR subunit, GluK3a. These receptors exhibit a very low sensitivity to glutamate (Schiffer et al., 1997), which arises in part because partially occupied receptors desensitize rapidly (Perrais et al., 2009a). We hypothesized that if HNK-1 addition to GluK3a slowed desensitization of currents evoked from KARs containing this subunit, as is the case for homomeric GluK2a KARs, we should observe

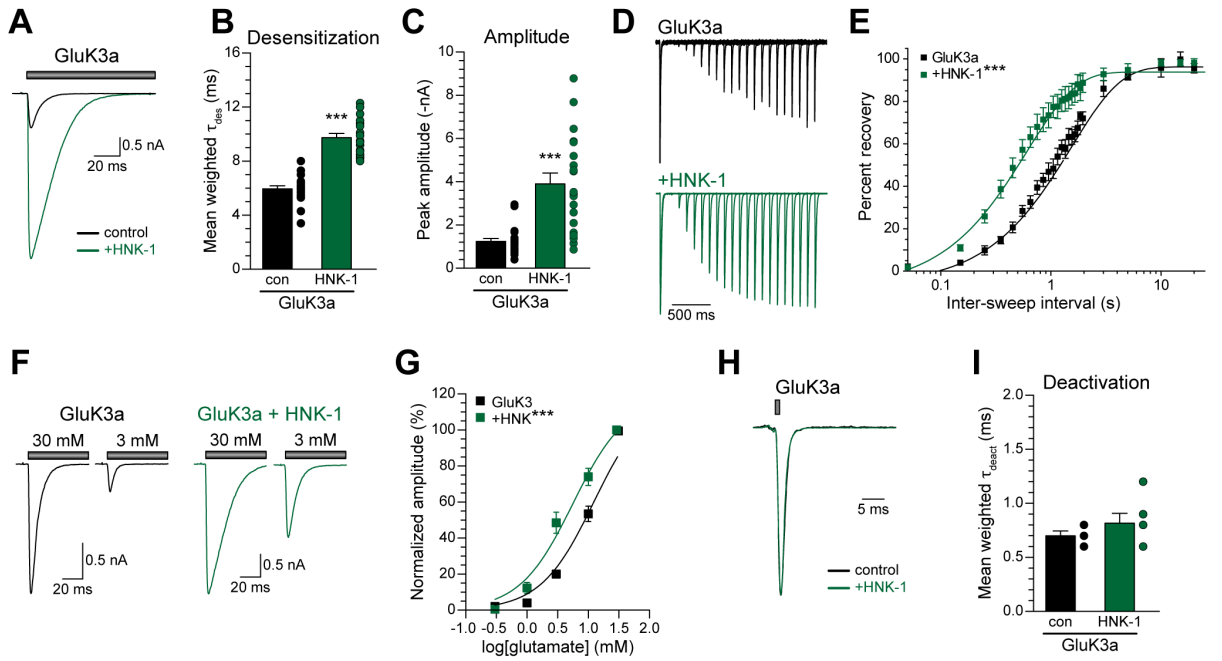

Figure 3.7. HNK-1 conjugation alters desensitization and apparent glutamate affinity of GluK3a-expressing KARs. **A**) Representative current traces from control HEK293T/17 cells expressing only GluK3a and from cells where GluK3a was co-expressed with the HNK-1-conjugating transferases GlcAT-P and HNK-1ST. Grey bar indicates glutamate (30 mM) application. Amplitudes are scaled. **B**) Quantification of glutamate-evoked desensitization from GluK3a alone and HNK-1 co-expressing HEK293T/17 cells. **C**) Quantification of glutamate-evoked current amplitudes from GluK3a alone and HNK-1 co-expressing HEK293T/17 cells. **D**) Representative current traces in two-pulse glutamate (30 mM) recovery experiments recorded from GluK3a expressing HEK293T/17 cells transfected either alone or with HNK-1-conjugating transferases. Intervals between glutamate exposures range from 50 ms to 2 s in the traces shown. Amplitudes from the first glutamate application are scaled. **E**) Quantification of mean glutamate recovery for all GluK3a-expressing cells with and without HNK-1 conjugation. Amplitude of the second glutamate application in a two-pulse experiment is reported as a normalized percentage of the first glutamate application. Results were fitted with a single component exponential equation. **F**) Representative traces of currents evoked with 3 mM or 30 mM glutamate application from HEK293T/17 cells expressing GluK3a, either alone or with HNK-1-conjugating transferases. **G**) Current were evoked from GluK3a-expressing HEK293T/17 cells with 0.3 mM, 1 mM, 3 mM, and 10 mM glutamate, and were normalized to the amplitude of currents evoked with 30 mM glutamate. These measurements were log-transformed and fit with a single component exponential. **H**) Representative current traces from outside-out patches pulled from GluK3a-expressing HEK293T/17 cells transfected either with receptor subunit plasmid alone or with HNK-1-conjugating transferases. Grey bar indicates glutamate (30 mM) application. Amplitudes are scaled. **I**) Quantification of glutamate-evoked deactivation from GluK3a-containing patches pulled from control and HNK-1-conjugating HEK293T/17 cells. Abbreviations are control transfection of receptor alone (con) and co-transfection with GlucAT-P and HNK-1ST (HNK-1). Error bars represent s.e.m. Statistical significance is indicated as follows: ***p<0.001.

an increase in peak current amplitudes in addition to the change in desensitization kinetics. To test this, we evoked whole-cell currents with 30 mM glutamate from cells expressing GluK3a with and without HNK-1-generating transferases (Figure 3.7A). Similar to GluK2a KARs, the desensitization rate of GluK3a-containing receptors was slowed, from a τ_{des} of 6.0 ± 0.2 ms in control cells (n = 23) to 9.7 ± 0.3 ms with the addition of HNK-1 (n = 20, p<0.0001) (Figure 3.7B). Moreover, mean peak current amplitudes increased by 3-fold at this concentration of glutamate, from a control of 1.2 ± 0.1 nA to 3.9 ± 0.5 nA in cells expressing GluK3a with HNK-1 (p<0.0001) (Figure 3.7C). HNK-1 made the rate of recovery of GluK3 KARs ~3-fold faster (control: τ_{rec} of 1.36 s, 95% CI 0.86 – 1.04, n = 4; HNK-1: τ_{rec} of 0.55 s, 95% CI 0.47 – 0.67, n = 4; Figure 3.7C). Concentration-response relationships revealed that the HNK-1-dependent slowing of desensitization shifted the macroscopic EC_{50} of GluK3a KARs from a control of 12.3 mM (95% CI 7.1 – 21.4, df = 21) to 5.6 mM (95% CI 3.5 – 9.0, df = 17) when HNK-1 was conjugated to receptors (p = 0.0001) (Figure 3.7G). Finally, we tested whether HNK-1 addition slowed deactivation of homomeric GluK3a KARs (Figure 3.7H). A 1 ms application of 30 mM glutamate evoked currents with mean weighted τ_{des} of 0.7 ± 0.0 ms (n = 5), which did not detectably change with HNK-1 conjugation (τ_{des} 0.8 ± 0.1 ms, n = 6; p = 0.3097) (Figure 3.7I). These data show that HNK-1 also modifies GluK3a KARs in similar ways as GluK2a receptors, although the extremely rapid deactivation of GluK3a receptors might have precluded detection of a modest slowing of this aspect of channel function. Overall, these data provide a proof a concept that the functional properties of AMPA and kainate receptors depend not only on the subunit composition of the receptor but also on the identity and complexity of the *N*-glycosylation moieties attached to these subunits, and they suggest a nuanced relationship between glycan identity, receptor composition, and receptor function.

Discussion

We find that manipulation of the oligosaccharide composition on iGluRs affects functional properties of these receptors in a manner dependent on both the identity of the glycan structures and the subunit composition of the receptors to which they are attached. This finding suggests that differential expression of Golgi oligosaccharide processing machinery in distinct cell types, brain regions, or over development could impact the fine-tuning of iGluR function, and represents another layer of complexity in the molecular control of these receptors.

Perhaps the most thoroughly investigated role of glycans on iGluRs is as lectin substrate binding sites. Plant lectins impact both AMPA and KA receptor desensitization (Everts et al., 1997), an effect that depends on the sugar affinity of the lectin for KARs (Thalhammer et al., 2002). Mammalian lectins alter AMPA and KA receptor desensitization in a manner that depends on both receptor subunit identity and their affinity for the sugars attached to receptors, and we showed previously that galectin-1 slows desensitization of KAR currents evoked from neonatal DRG neurons (Copits et al., 2014). A different approach to investigate glycosylation of native iGluRs found that polysialic acid (PSA) increased the P_{open} of AMPA receptors purified from rat brain and reconstituted in lipid bilayers. Furthermore, PSA increased the current density through AMPA receptors expressed by cultured hippocampal neurons from young animals but not from adult animals (Vaithianathan et al., 2004), emphasizing the likelihood that developmental differences exist in iGluR modulation by glycans.

Ligand affinity is also altered by Golgi processing, and therefore thought to depend somewhat on oligosaccharide maturity. As iGluRs move through the secretory system [^{3}H]AMPA binding

affinity in hippocampal sections is reduced, suggesting that increased oligosaccharide complexity reduces AMPA affinity of the receptor (Standley et al., 1998). It remains unclear what relevance this might have for receptor function at the synapse, as presumably glycan-dependent affinity is lowered before receptors exit the secretory system. It suggests, however, that changes in oligosaccharide content could alter this important receptor property. The GluN1 subunit has 12 consensus glycosylation sites, more than any other iGluR subunit, and it depends heavily on N-linked glycosylation for successful processing and exit from the secretory system (Everts et al., 1997). Computational modeling suggests that glycans attached to the NMDAR LBD increase the stability of the closed clamshell conformation (Sinitskiy et al., 2016). Eliminating the GluN1 *N*-glycan attachment site identified in this model reduced NMDAR glycine, which mirrors our finding that increased glycan complexity on GluK3-containing receptors increases glutamate sensitivity of these receptors. Differential glycosylation of iGluRs might also impact their protein-protein interactions. GluA2 is a substrate for the complex sugar HNK-1 in the hippocampus, a modification that stabilizes GluA2-containing receptors at the cell surface via HNK-1-mediated interactions with N-cadherin and supports hippocampal LTP (Morita et al., 2009). Further supporting this, hybrid/complex glycans are critical to surface retention of GluA2-containing receptors and increasing oligomannosidic content promotes receptor internalization (Hanus et al., 2016). Our system did not allow us to investigate the potential contribution of HNK-1 to GluA2-containing receptor function, but our data suggest that HNK-1 might alter more than the surface stability of these receptors. Additionally, our observation that KAR subunits can be substrates for HNK-1 in recombinant systems invites the question of whether HNK-1 might be attached to these receptors in neurons. The structural pattern of oligosaccharides on native iGluRs and the functional implication of changes to glycan content are outstanding questions with critical importance for understanding receptor function in neuronal circuits.

Determining the glycan content of native AMPA and KA receptors is no small task, considering that each subunit has between 4 and 11 *N*-glycosylation consensus sequences and each site could be differentially modified by the oligosaccharide processing enzymes of a given cell. AMPA and NMDA receptors purified from rat forebrain synaptosomes with the lectin ConA contain a majority of neutral sugars, about half of which are oligomannosidic (Clark et al., 1998). More detailed investigation confirms that these receptors have high oligomannosidic content but they also are conjugated to hybrid and complex sugars, which could contribute important elements to receptor function (Hanus et al., 2016; Kaniakova et al., 2016). Additionally, a glycoproteomics scan in rat whole brain confirmed AMPARs to be *N*-glycosylated at all or nearly all of their consensus sites and found GluK2, GluK3, and GluK5 to be glycosylated at multiple sites, including the *N*-glycosylation sites 5, 6, and 7 on GluK2 which we found to be critical determinants of oligosaccharide modulation of receptor properties (Parker et al., 2013). These studies confirm that multiple types of oligosaccharides are present on native iGluRs and at functionally relevant locations, although the question of which sugars occupy what space along the receptor remains open.

Numerous factors regulate the modification of oligosaccharide chains. Protein structure dictates the accessibility of *N*-glycan attachment sites for subsequent modifications, which affects the degree of core fucosylation and branching found on oligosaccharide chains (Thaysen-Andersen and Packer, 2012). Genes involved in glycometabolism show tissue-specific and developmentally-regulated differences in expression, which often correlates with alterations in the glycome detected in a particular tissue (Ishii et al., 2007; Nairn et al., 2008; Neelamegham

and Mahal, 2016). Notably, the sulfotransferase that catalyzes HNK-1 epitope formation is more highly transcribed in the brain than in peripheral tissues like kidney and liver (Nairn et al., 2008). This carbohydrate is a developmentally regulated component of glycolipids in the brain, and is found through adulthood on a select number of high molecular weight glycoproteins such as cell-adhesion molecules (Schwarting et al., 1987). Nearly all HNK-1 immunoreactivity in the brain is lost in mice that have the glucuronyltransferase isoform GlcAT-P knocked out, and the remaining GlcAT-S isoform catalyzes spatially restricted HNK-1 formation only in perineuronal nets (Yamamoto et al., 2002). Characterization of iGluR glycan content in different brain regions and over development will be a critical step in understanding the contribution these macromolecules make to receptor function.

Glycans are added to and processed on members of several other channel and receptor-channel families, where the identity of the conjugated sugars modulates variable aspects of protein function. TRPC3 constitutive activation can be reduced by the addition of an extra glycosylation site found in TRPC6, and removal of this site from TRPC6 confers TRPC3-like constitutive activity (Dietrich et al., 2003). Temperature sensitivity of another TRP channel isoform, TRPM8, is altered when normal glycosylation of the receptor is precluded both in DRG neurons and recombinant systems (Pertusa et al., 2012). Protein function can also be impacted by receptor trafficking, and TRPV5 is stabilized on the membrane of kidney cells following glycan cleavage by the lectin Klotho and subsequent binding of Galectin-1 to the newly-exposed sugars (Chang et al., 2005; Cha et al., 2008). A different family of ligand-gated channel, $GABA_A$ receptor ß2-subunits require their two most C-terminal glycosylation sites for proper assembly and successful exit from the Golgi, and removal of any of their three consensus sequences impacts open-channel probability by reducing the amount of time receptors spend in the long

open state (Lo et al., 2010). How these findings inform native TRPC, TRPM, and $GABA_A$ receptor function remains to be elucidated, but it is clear that attached oligosaccharides can modulate diverse functional properties of ligand-gated ion channels.

Glycans also contribute to biophysical properties of voltage-gated channels, a phenomenon that has been extensively studied in cardiomyocytes where changes in sialic acid content shift the gating thresholds of voltage-gated potassium and sodium channels. Charged sialic acid moieties set the voltage dependence of the rapidly-inactivating transient outward potassium current in cardiomyocytes, likely due to their impact on $K_V4.3$ gating (Ufret-Vincenty et al., 2001a). Mice with genetic ablation of muscle LIM protein are prone to arrhythmia-induced heart failure, and myocytes from these mice exhibit depolarized sodium channel current-voltage relationships and slowed tau of inactivation, properties that can be reproduced in wild type myocytes by desialidation with neuraminidase (Ufret-Vincenty et al., 2001b). Additionally, developmental control of sialidation allows for cell-specific control of sodium current properties. The level of sodium channel sialylation is initially low in ventricular myocytes and increases over development, but sialylation is high at all ages in atrial myocytes. As a result, sodium channel gating threshold differs between ventricular and atrial myocytes in neonatal mice but not in adult mice (Stocker and Bennett, 2006). In dorsal root ganglion neurons, $Na_v1.9$ is more heavily sialylated at neonatal ages than at embryonic, weanling, or adult ages and the post-natal increase in sialidation is responsible for a concurrent hyperpolarized shift in sodium current voltage of inactivation (Tyrrell et al., 2001). These experiments highlight that cell-type and developmental stage are critical components of glycosylation-mediated differences in channel function, and this is almost certain to be true for functional properties of native iGluRs that might be modulated by oligosaccharide composition. As discussed above, there is evidence that

association or attachment to PSA differentially impacts AMPARs from neonatal and adult animals. The possibility that *N*-linked glycans could be a cellular tool for developmental regulation of iGluR function is exciting; much work remains to be done both to reveal regional and developmental patterns of glycosylation on iGluRs and to determine what precise functional properties are altered by different oligosaccharide fingerprints.

Here we provide evidence that the oligosaccharides attached to iGluRs impact receptor functional properties beyond protein folding, forward trafficking, and lectin binding. Our data suggest that oligosaccharides on both AMPARs and KARs are important components of the structural changes that occur upon agonist binding. This study of AMPA and kainate receptor glycosylation in a reduced, recombinant system is an important first step towards better understanding the full spectrum of elements contributing to iGluR function in neurons. How these findings inform the function of endogenous receptors is an important and exciting open question.

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Chapter 4.

Discussion and Concluding Remarks

Discussion

I have presented an overview of the molecular elements that modulate KAR function and signaling, considering both interacting partners and covalently attached molecules. Data presented in Chapter 2 confirm that the proposed KAR auxiliary subunit Neto2 is an endogenous component of these receptors in DRG neurons, and they suggest that Neto2-containing KARs regulate process outgrowth in these neurons. It is tempting to speculate that Neto2 assembly could tune KAR function to the needs of developing neurons and that Neto2-containing KARs might be developmentally regulated in other regions of the nervous system. In Chapter 3, we used a reduced system to show that *N*-glycans contribute critically to KAR functional properties. Tissue-specific and developmentally regulated activity of glycan-processing enzymes importantly determines the general composition of the glycome, and it is reasonable to suspect that oligosaccharide structures on KARs could be affected in a regional or developmental manner. In these concluding remarks, I will focus on outstanding questions around both glycan and auxiliary protein modulation of KAR function and will speculate on the potential for region-specific, developmentally-regulated, and pathological control of receptor function.

KAR diversity through regulated subunit expression

KARs are found broadly throughout the nervous system, however the individual subunits exhibit cell-specific expression and subcellular localization of receptors can be tightly regulated. Subunit knockout mice have revealed critical roles for GluK2 and GluK3 in presynaptic KARs (Contractor et al., 2001; Pinheiro et al., 2007) and for GluK2 in post-synaptic KARs (Mulle et al., 1998; Kerchner et al., 2001b). Immunohistochemical experiments suggest that the high affinity subunit GluK4 is predominantly presynaptic while GluK5 is mostly postsynaptic (Petralia et al.,

1994; Darstein et al., 2003). Currents recorded from GluK5$^{-/-}$ mice confirm this subunit to be a component of post-synaptic KARs in CA3 neurons, although GluK4 subunits might contribute as well (Contractor et al., 2003; Fernandes et al., 2009). Pharmacological manipulation of receptors and experiments using knockout mice indicate that GluK1 is primarily a constituent of interneuronal KARs, which function both presynaptically and somato-dendritically (Cossart et al., 1998; Mulle et al., 2000; Ali et al., 2001; Kerchner et al., 2001b; Binns et al., 2003; Christensen et al., 2004a; Lauri et al., 2005; Wu et al., 2007b). Retinal bipolar interneurons express GluK1-containing KARs that are a critical postsynaptic component of OFF-cell signaling (DeVries and Schwartz, 1999; Lindstrom et al., 2014) and interestingly, a soluble splice variant of Neto1 is expressed exclusively in the retina though the relevance of this soluble auxiliary protein to KAR function and retinal processing is yet unclear (Stöhr et al., 2002; Puthussery et al., 2014). Differential expression and localization of KAR subunits presumably fulfills different cellular requirements for KAR-mediated signaling, though much remains to be discovered about how this is regulated and what distinct physiological purposes these receptors serve.

Perhaps the most intriguing example of KAR compartmentalization is in CA3 pyramidal neurons in the hippocampus, where KARs are localized postsynaptic to mossy fiber inputs from the dentate gyrus but are excluded from the associational-commisural synapses located slightly more distal along CA3 dendrites (Castillo et al., 1997; Vignes and Collingridge, 1997). The mechanism behind regulated subcellular compartmentalization is not fully understood, but it depends somewhat on subunit expression levels and the various protein–protein interactions KARs engage (Fievre et al., 2016). The identity of presynaptic fibers plays a critical role in the postsynaptic segregation of KARs at hippocampal synapses, where mossy fibers secrete C1ql proteins that bind to the ATD of select KAR subunits and link them to presynaptic neurexins,

securing them specifically at this synapse (Matsuda et al., 2016; Straub et al., 2016). The recent elucidation of this trans-synaptic modulation of KAR localization underscores that much continues to be discovered about the molecular elements that regulate KAR function in neural circuits.

The contribution that Neto proteins make to KAR localization remains to be fully explored. Exogenous expression of Neto2 with KAR subunits strongly promotes post-synaptic localization of these receptors (Copits et al., 2011; Tang et al., 2012; Sheng et al., 2015). How Neto2 might regulate KAR localization in DRG neurons is not clear, as these neurons do not have dendrites but rather project a bifurcating axon to central and peripheral contact sites. Does Neto2 also promote presynaptic localization of KARs, and could presynaptic versus postsynaptic localization of Neto2-containing receptors be cell-type dependent? Are Neto2-containing KARs trafficked equally to peripheral terminals and to central spinal synapses in DRG neurons? DRG neurons might also exhibit somatic KARs that detect ambient glutamate in the ganglia (Kung et al., 2013), but it remains to be determined what physiological purpose this serves and the extent to which Neto2 might contribute. Compartmentalization and cell-specific expression of KARs with varied molecular constituents, in peripheral neurons and throughout the CNS, suggests that specific receptor combinations could be selectively targeted for therapeutic gains. Further characterization of the subunit composition of different KAR populations and the functional relevance of these differences will be necessary for this possibility to become a reality.

Developmental regulation of KARs

Developmental changes exist in KAR RNA editing and expression, and much remains to be understood about the dynamic perinatal regulation of these receptors. Thalamocortical synapses switch from kainate to AMPA receptor-mediated EPSCs over the first postnatal week, and KAR-mediated inhibition of presynaptic release from thalamaocortical projections also decreases during this developmental window (Kidd and Isaac, 1999; Kidd et al., 2002). In the hippocampus, by contrast, postsynaptic KARs are absent from mossy fiber-CA3 synapses neonatally but increase after the first week of life, a change that contributes to the maturation of postsynaptic AMPAR-mediated events (Marchal and Mulle, 2004). In the case of KAR maturation at CA3-CA1 synapses in the hippocampus, KAR activation inhibits evoked glutamate release at both neonatal and juvenile synapses but only exerts tonic downregulation of glutamate release at neonatal synapses (Lauri et al., 2006). Tonic activation of KARs at immature synapses is likely due to higher glutamate affinity of these receptors than that of KARs at mature synapses, and this could depend on differential incorporation of GluK1 splice variants in these receptors (Vesikansa et al., 2012). Neto2 modulates the agonist sensitivity of KARs (Zhang et al., 2009); could Neto2 be a developmentally downregulated KAR constituent in the CNS, or it its developmental regulation particular to the PNS? Presynaptic KARs at the DRG to dorsal horn synapse contain GluK1 and their activation with exogenous agonists suppresses glutamatergic transmission onto dorsal horn neurons (Kerchner et al., 2001a; Kerchner et al., 2002). It is not clear how this KAR-mediated signaling is engaged by synaptically-released glutamate, however, and whether their function as auto-receptors depends on the expression of Neto2. An obvious extension of the work in Chapter 2 is to test the ability of GluK1-directed agonists to alter evoked and spontaneous DRG–LII excitatory transmission in Neto2$^{-/-}$ mice. If Neto2 proves to be a component of these autoreceptors, comparing KAR regulation of

excitatory transmission between neonatal and juvenile mice will be important. Perhaps spinal DRG KARs are tonically activated at neonatal synapses as they are in CA1, and application of GluK1-directed antagonists could test KAR modulation DRG–LII transmission in response to synaptically released glutamate and how this might differ between neonatal and juvenile animals.

It is tempting to speculate that KAR (Joseph et al., 2011; Marques et al., 2013) and Neto2 (Chapter 2) modulation of neurite regrowth in culture might reflect an underlying Neto2-containing KAR contribution to sensory circuit development. Non-peptidergic C-fibers, approximately 60% of which express KARs (Lee et al., 2001; Usoskin et al., 2015), enter the spinal cord at late embryonic ages but do not achieve the innervation density seen in adult LII until P5 (Fitzgerald and Gibson, 1984). C-fiber activity in LII is critical for the eventual regression of A-fibers to the deeper laminae around 4-weeks of age (Fitzgerald et al., 1994; Beggs et al., 2002), meaning that both innervation and important developmental plasticity occur in dorsal horn circuits over the same age window that we detect maturation of DRG KAR subunit composition. Recent work suggests that unedited KARs are critical to axonal development and synapse formation, and that this role depends on metabotropic signaling (Sakha et al., 2016). Consistent with this, KARs in DRG neurons are unedited until these axons have innervated the spinal cord and they regulate neurite outgrowth, potentially through calcium-dependent or G-protein mediated signaling (Lee et al., 2001; Marques et al., 2013). There is a wealth of circumstantial evidence that DRG KARs contribute to sensory neuron development and that Neto2 is a critical component of these receptors, however we observe intact basic pain processing in adult mice (Chapter 2) and grossly normal non-peptidergic fiber innervation in adult spinal cord (data not shown, B. McClarty and Y.F. Guzmán). It is possible that the timing

of circuit development is affected by loss of Neto2 but that this is normalized by the time mice reach adulthood, or that these mice have deficits in spinal circuit function and pain processing that we have not tested. Characterization of the time course of MRGPRD-positive fiber innervation from late embryonic to ~one week postnatal in wildtype and Neto2$^{-/-}$ mice could test the hypothesis that Neto2-containing KARs regulate DRG axon development. Using MRGPRD as a fiber marker might provide a more clear description of KAR-positive fibers than our preliminary experiment using the broad non-peptidergic marker IB4, as MRGPRD and GluK1 mRNA overlap within non-peptidergic neurons more completely than other DRG subtype markers that have been used (Lee et al., 2001; Usoskin et al., 2015). Moreover, MRGPRD and GluK1 are transcribed in nearly all of the cells in this proposed sub-class of non-peptidergic C-fibers (Usoskin et al., 2015). Successfully determining the contribution that KARs make to DRG neuron outgrowth and development depends on identifying the KAR-expressing subpopulation of fibers, a difficult task given the poor specificity of GluK1-directed antibodies and the mediocre overlap of KARs with previously-tested immunohistochemical markers. In this age of big data, perhaps a better marker for KAR-expressing neurons can be gleaned from the comprehensive transcriptional profiling of these neurons (Chiu et al., 2014; Usoskin et al., 2015; Hu et al., 2016) and the contribution of these receptors to peripheral circuit development can be probed.

Regional and developmental patterns of glycosylation

Enzymes involved in oligosaccharide processing are regionally and developmentally regulated (Ishii et al., 2007; Nairn et al., 2008). As discussed in Chapter 3, cell-type specific and developmentally regulated sialylation modulates the gating properties of voltage-gated channels, and could be a cellular tool for regulating receptor-channel properties. Knowing the basal glycosylation pattern on iGluRs is key to understanding how alterations in glycan structure

influence receptor function, yet predicting the structural content of sugars on individual proteins is challenging. Differences in transferase and glycosidase transcription correlate generally with differences in the glycome between tissues and across development (Ishii et al., 2007; Nairn et al., 2008), but post-transcriptional factors like miRNAs regulate translation of these enzymes as well (Agrawal et al., 2014; Neelamegham and Mahal, 2016). The pattern of oligosaccharides on glycoproteins depends on expression of glycan processing enzymes, but also on enzyme affinity for the substrate protein and on tertiary and quaternary protein structure surrounding the consensus asparagine (Zielinska et al., 2010; Moremen et al., 2012).

iGluRs share a conserved two-lobe, dimer-of-dimers extracellular structure and several glycosylation sites are conserved between and within AMPAR and KARs, and it is possible that these related proteins present similar substrates to glycan processing enzymes. For some sites, this could be true. All kainate and AMPA receptor subunits have consensus glycosylation sites located between the ATD and LTD. With the potential exceptions of GluK1 and GluK4, every subunit carries *N*-glycosylation at one or more of these sites in rat brain (Parker et al., 2013), perhaps reflecting a conserved availability of these sites to glycan processing machinery. Computational modeling of the GluN1 subunit indicates a critical role for the GluN1-N440 glycan chain in the upper lobe of the LBD interacting with a hydrophilic region of the lower LBD lobe and stabilizing the closed conformation of the LBD (Sinitskiy et al., 2016). This site is analogous to the GluK2-N430 glycan site NG7 that was eliminated in our studies with the mutant subunit GluK2ΔNG. Sinitskiy and colleagues determined that polar interactions between the mannose constituents of immature glycans and the lower LBD lobe are possible only when the LBD is in the closed conformation, and the addition of these interactions in their model increased the likelihood of this domain assuming the closed state. Similar interactions likely occur in KARS.

We found that the presence of glycan chains at the interface between the ATD and LBD were required for kifunensine treatment to affect receptor entry into desensitization. Kifunensine treatment restricts oligosaccharide structures to an immature high mannose composition, structures that are likely smaller than their hybrid and complex counterparts. Perhaps this shorter chain structure restricts conformational flexibility of the LBD and promotes receptor desensitization, or perhaps the mannose chain has a higher affinity for interaction sites on the lower LBD lobe. The mechanism by which a charged oligosaccharide like HNK-1 at this site alters LBD stability and receptor gating is not clear, though one would predict that the negative charge on HNK-1 would substantially affect the glycan chain interactions. In the GluN1 model, oxygens from the oligomannosidic chain at GluN1-N440 primarily interact with glutamates, a glutamine, and an aspartate, and a strong negative charge on the glycan would be predicted to interfere with this interaction. On the other hand, the HNK-1 epitope is added to complex glycan chains that are expected to be much longer than the $Man_5GlcNAc_2$ used in the GluN1 model, and this epitope might interact with a different, positively-charged target on the LBD. It is interesting that we observed opposite effects of HNK-1 conjugation to wildtype and GluK2ΔNG KARs. This suggests that the HNK-1–receptor interactions that promote recovery from desensitization and slow entry into desensitization are specific to oligosaccharides attached at the three critical sites between the ATD and LBD, though clearly HNK-1 attachment at other locations alters mutant receptor gating. Modeling studies would be a useful approach to understand how glycans attached at different sites interact with KAR functional domains, although limits to computing power restrict the size of molecules that one can practically model (Sinitskiy et al., 2016). In theory, such models could suggest amino acid residues and glycan structures that would interact in a functionally relevant manner, and these interactions could then be tested *in vitro*, similar to what Sinitskiy and colleagues have done.

The structural make-up of oligosaccharides along native iGluRs is only beginning to be understood. Investigating iGluR oligosaccharide content from the perspective of receptor co-expression with enzymes is not likely to be useful, as the wealth of possibilities is somewhat overwhelming. Approximately 700 genes contribute to sugar transport, glycan processing, and lectin interactions, and these can conceivably arrange several thousand different structural combinations (Nairn et al., 2008; Cummings, 2009). Direct investigation of sugar content on iGluRs has revealed that NMDA and AMPA receptors in forebrain and cerebellum contain a large proportion of oligomannosidic sugars, though both receptor types also contain complex glycans (Clark et al., 1998; Hanus et al., 2016; Kaniakova et al., 2016). This composition is in line with the general prevalence of these structures in the cortex, with high-mannose glycans accounting for approximately 45% of the total cortical glycome (Ishii et al., 2007). Broad characterization of oligosaccharide content is useful, but can overlook important contributions of individual structures. For instance, conjugation of the complex glycoepitope HNK-1 to GluA2 is a critical component of hippocampal plasticity (Morita et al., 2009), however the presence of this particular structure is not appreciated in broad descriptions of AMPAR glycosylation (Clark et al., 1998; Hanus et al., 2016). Consistent with the fact that HNK-1-conjugation retains AMPARs at the synapse, the surface stability of GluA2 is higher for receptors containing complex glycans than for immature glycosylated receptors and this is rapidly regulated by network activity (Hanus et al., 2016). Might HNK-1 conjugation be a component of KAR-mediated presynaptic LTP in the hippocampus? Presynaptic GluK2 and GluK3 contribute critically to mossy fiber–CA3 LTP (Contractor et al., 2001; Pinheiro et al., 2007) and we find that HNK-1 conjugation substantially alters the properties of receptors composed of these subunits. The possibility that HNK-1 conjugation alters GluK3-containing receptor properties is particularly intriguing, given that the

rapid desensitization and low glutamate sensitivity of recombinant receptors are somewhat at odds with our expectation that GluK3 plays a critical role responding to synaptically-released glutamate. Preliminary work in our laboratory suggests that HNK-1 might be conjugated to GluK2 or GluK3 in mouse brain, though this may be more ubiquitous in the cortex than in the hippocampus (data not shown, Y.F. Guzmán). Additionally, we find that KAR EPSCs in CA3 are unaltered in HNK-1-deficient mice (data not shown, T. Ishii). Since our immunoprecipitations suggest greater conjugation of the HNK-1 epitope to cortical KARs, are the kinetics of postsynaptic KARs altered when this epitope is absent in cortical regions like the ACC?

The potential for direct contribution of glycans to the structural rearrangements underlying iGluR functional properties is certainly exciting and is likely under-appreciated. Still, the relevance of glycan content to receptor function extends beyond those sugars that are directly attached to the polypeptide chain. It is possible that lectin–receptor interactions at the cell surface could alter KAR functional properties or localization (Copits et al., 2014), as is the case for TRPV5 in the kidney (Chang et al., 2005). It is worth considering that lectin binding could interfere with interactions between the distal ends of glycan chains and functionally important domains of the receptor, effectively inhibiting that glycan chain from stabilizing the receptor. It is also possible that interactions with glycan content on other synaptic proteins or glycolipids could affect iGluR function. Exogenous application of PSA to purified native AMPARs increased receptor open probability of neonatal but not adult AMPARs (Vaithianathan et al., 2004). The *in vivo* relevance of this has not been explored, but it is possible that AMPAR proximity to heavily sialylated proteins such as voltage-gated sodium channels or PSA-NCAM might be sufficient to alter receptor properties at certain developmental stages.

Alterations of iGluRs in pathological states

As mentioned in Chapter 1, KARs have been implicated in a number of neuropathologies but their mechanistic contribution to these disorders remains unclear. We did not observe differences in acute pain thresholds or short-term inflammatory pain in either Neto1$^{-/-}$ or Neto2$^{-/-}$ mice (Chapter 2). We also describe a critical role for Neto2 in DRG neurite re-growth and detect Neto2 upregulation in DRG following a regeneration-inducing injury to the sciatic nerve (Chapter 2). In contrast to central neurons where axons do not regenerate, peripheral nerves regenerate following injury. Despite this, functional recovery after peripheral nerve injury varies widely in humans and outcomes are often poor (Wood et al., 2011). Peripheral nerve injuries also cause stimulus-evoked hypersensitivities due to aberrant signaling from both regenerating nerves and sprouting neighboring nerves (Bester et al., 2000; Decosterd and Woolf, 2000; Decosterd et al., 2002). Neto2 regulates process regrowth in culture and is particularly important for elongating outgrowth (Figure 2.6). This suggests that Neto2-containing KARs could promote growth of the peripheral branch of DRG neurons, but what physiological purpose does Neto2 upregulation serve during peripheral nerve regeneration *in vivo*? Nerve regeneration has several similarities to axon growth during development, but there are obvious differences in the environment of developing and regenerating axons. Immediately following injury, the distal nerve stump degenerates and axon regrowth from the proximal stump varies between neuron subtypes. Of particular interest is the observation that small-diameter, unmyelinated sensory neurons are more prone to delayed death following peripheral nerve injury than their larger, myelinated counterparts (Coggeshall et al., 1997; Tandrup et al., 2000); might Neto2-containing KARs promote regeneration and reduce apoptosis in this particular population of sensory neurons? This possibility could be tested in the Neto2$^{-/-}$ mice by quantifying sensory nerve regeneration following crush, and focusing particularly on small-diameter non-peptidergic neurons.

Understanding the mechanism of KAR contribution to regenerative growth is key to asking further questions about how Neto2 expression might influence regeneration, functional recovery, or post-injury hypersensitivity.

Additionally, KARs are implicated in many aspects of seizure activity. Neto2$^{-/-}$ mice are more susceptible to seizures that their wildtype counterparts, however this is attributed to reduced KCC2 expression in hippocampus rather than Neto2 modulation of KAR function (Mahadevan et al., 2015). Both KCC2 and KARs interact with protein 4.1N (Li et al., 2007; Copits and Swanson, 2013), and it is possible that Neto2 regulates KCC2 function through common KAR-containing complexes (Mahadevan et al., 2014). Interneurons in the hippocampus express KARs that contribute critically to seizures in animal models (Khalilov et al., 2002; Smolders et al., 2002), and these interneuronal KARs have yet to be tested for Neto2 incorporation. The increased seizure susceptibility of Neto2$^{-/-}$ mice strongly suggests that the auxiliary protein regulates KAR function in the hippocampus.

Congential disorders of glycosylation generally result in severe multi-system pathologies with notable neurological components (Freeze and Ng, 2011; Barone et al., 2012; Freeze et al., 2012). The most severe pathologies of glycosylation occur with deficits at early stages of glycan processing, and disease modeling with genetically altered mice additionally suggests that deficits late in glycan processing result in more mild phenotypes (Ohtsubo and Marth, 2006). Both glycan structural content and lectin expression change with the onset of inflammation and diseases such as cancer (Dube and Bertozzi, 2005; Rabinovich and Toscano, 2009; D'Haene et al., 2014), and as aging progresses (Sato and Endo, 2010). It is interesting to speculate that iGluR function might be affected by aging- or disease-related changes to glycan metabolism.

Given that network activity can rapidly alter surface GluA2 glycosylation patterns that contribute to dynamic cellular processes such as LTP (Morita et al., 2009; Hanus et al., 2016), it is possible that changes in cellular metabolism could induce aberrant iGluR function or impair normally dynamic regulation of these receptors. Differences in iGluR glycosylation state are observed in the post-mortem prefrontal cortex from patients with schizophrenia, with GluA2 and GluA4 exhibiting increased and GluK2 exhibiting reduced complex glycan content compared to subunits isolated from healthy tissue (Tucholski et al., 2013b; Tucholski et al., 2013a). Whether the shift in iGluR glycan processing is caused by schizophrenia or whether it might be a causative element of dysregulated circuit function in this disorder is unknown.

Conclusion

In this dissertation, I present work that establishes Neto2 as a true KAR auxiliary subunit and suggests that *N*-linked glycans contribute to structural changes underlying KAR gating. Auxiliary proteins and oligosaccharides are quite different, yet both contribute important molecular content to receptors. They represent two of the myriad of molecular elements that combine to influence KAR trafficking, localization, and functional properties in neurons. These distinct projects show perhaps little overlap at first appearance, but each represents a step towards a more complete picture of native KAR function. The goal of my thesis work has been to elucidate a better understanding of those molecular components that contribute to and regulate KAR function. This is critical to improving our mechanistic understanding of the receptors' modulation of circuit excitability, their roles in cognition and disease, and their potential therapeutic utility.

Chapter 5.

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