UNIVERSITY OF CALIFORNIA, SAN DIEGO

Regulators of neuronal GIRK channels

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in

Biology

by

Rounak Nassirpour

Committee in charge:

Professor Paul A. Slesinger, Chair
Professor Darwin Berg
Professor Gentry Patrick
Professor Ronald Kuczenski
Professor Nicholas C. Spitzer
Professor Lisa Stowers

2008

The Dissertation of Rounak Nassirpour is approved, and it is acceptable in quality and form for publication on microfilm:

Chair

University of California, San Diego
2008

This thesis is dedicated to all the strong, independent, determined, ambitious, and intellectual women around the world who gave me the courage to believe in myself and follow my dreams.

.

Table of Contents

Signature Page iii

Dedication iv

Table of Contents v

List of Figures vii

Acknowledgments ix

Vita and Publications xii

Abstract of the dissertation xiii

I. Introduction 1

- Potassium channels 2
- G-protein gated potassium channels 3
- GIRK channel targeting 7
- The SAP family of proteins and synaptic localization 7
- SNX27 10
- Opioid receptors 13
- CaMKII and opioid receptors 18
- Neuronal plasticity and the GIRKs 21
- References 23

II. SNX27 regulates expression and intracellular trafficking of neuronal GIRK channels 42

- Abstract 42
- Introduction 42
- Methods 44
- Results 48
- Discussion 53
- Acknowledgments 58
- References 59

III. CaMKII dependent opioid modulation of GIRK channels selectively alters inhibitory input in hippocampal neurons 74

- Abstract 74

Introduction..... 75
Methods..... 77
Results..... 82
Discussion..... 88
Acknowledgments..... 92
References..... 93

IV. Conclusion..... 114

Synaptic plasticity and addiction……... 114
GIRKs and addiction……. 116
References..... 119

List of Figures

Chapter 1:

Fig. 1.1. A PDZ-containing sorting nexin, SNX27, associates with GIRK3 and GIRK2c channels. 41

Chapter 2:

Fig. 2.1. SNX27 contains a lipid binding motif (PX domain) that co-localizes GIRK3 in the early endosome..... 64

Fig. 2.2. SNX27 regulates cell surface expression of GIRK3 channel. 66

Fig. 2.3. Downregulation of GIRK channels by SNX27 requires lysosomal targeting and PDZ-binding motifs in GIRK3. 67

Fig. 2.4. Subunit-specific down regulation of GIRK channels by SNX27 68

Fig. 2.5. GIRK2c channels colocalize with SNX27 in the early endosomes, but show no change in cell surface expression. 69

Fig. 2.6. GIRK3 promotes downregulation of GIRK2c channels by SNX27. ……71

Fig. 2.7. Colocalization of SNX27b and GIRK channels in hippocampal neurons. 72

Fig. 2.8. Proposed pathway for subunit-specific down regulation of GIRK channels by SNX27..... 73

Chapter 3:

Fig. 3.1. GIRK2 is highly expressed in the dendritic shaft of dissociated hippocampal neurons and overlaps little with dendritic spine markers PSD95 and NMDA receptors..... 101

Fig. 3.2. Stimulation of opioid receptors with morphine alters GIRK2 localization in hippocampal neurons 102

Fig. 3.3. Morphine increases colocalization of GIRK2 with actin-labeled dendritic spines..... 103

Fig. 3.4. Morphine increases the expression of GIRK2 along the plasma membrane of hippocampal cells 105

Fig. 3.5. Morphine-dependent increase in GIRK2 and PSD95 colocalization does not require activity dependent release of presynaptic glutamate and GABA, or activation of PTX-sensitive G proteins 106

Fig. 3.6. Morphine-dependent increase in colocalization of GIRK2 and PSD95 requires activation of CaMKII 107

Fig. 3.7. Expression of activated CaMKII increases colocalization of GIRK2 and PSD95 in hippocampal neurons 108

Fig. 3.8. Morphine-dependent increase in colocalization of GIRK2 and PSD95 in young neurons 109

Fig. 3.9. Morphine treatment and expression of activated CaMKII in hippocampal neurons reduce amplitude of $GABA_B$ receptor-activated GIRK currents 111

Fig. 3.10. Agonist-dependent changes in GIRK channel trafficking 113

Chapter 4:

Fig. 4.1. Mesolimbic dopamine system circuitry. 121

ACKNOWLWDGEMENTS

I was a direct admit graduate student into Eduardo Macagno's laboratory. I would like to thank him for this opportunity. I would also like to thank Iain Dykes for instructing me on many laboratory techniques such as in situ hybridization, cDNA library screening, and subcloning. I would also like to thank him for his endless support and encouragement during the hardest times in my academic life.

I consider myself a very fortunate grad student for having had the opportunity to have Paul Slesinger as my advisor. Paul took the challenge of training me at a low point in my career when I had lost confidence in myself. He patiently, caringly, and enthusiastically spent the time, almost every day, to teach me the skills and challenges of being a good scientist. He is one of the most critical thinkers that I have ever met in my life. His dedication to accuracy and reproducibility is admirable. Paul, as a biophysicist, has also taught me the importance of quantification in a scientific analysis. He is definitely one of my role models and I aspire to someday become as good a scientist as he is.

I also would like to thank both the past and the present members of the Slesinger lab. I would most like to thank my fellow grad students in the lab: Stephanie Boyer and Prafulla Aryal who were my pillars of support both at a scientific and a personal level. Especial thanks goes to Sinead Clancy who not only kindly trained me on many techniques such as western blots, q-RT-PCR, and immunocytochemistry, but she was also a great source of scientific knowledge. I also would like to thank members from the Vale's (especially Pina Ciarmela) and the, Sawchenko's (Carlos M. Arias and Charles Peto) labs for providing many "emergency" reagents and supplies. Special thanks goes to

Carlos who not only provided technical support whenever needed, but he also taught me perfusion, fixing, and slicing of rat brain. Throughout my time at the Salk, Debbie Johns was an instrumental help in organizing various affairs such as scientific meetings and other administrative issues.

I have met a lot of truly fantastic people during my time at UCSD and have formed friendships with many, too many to list. Friends like Serapio Baca, Eric Thompson, Lily Soni, John Bracht, Kate and Quentin Gaudry, Martha Bagnall and Ed Han, DJ Brasier and Aryn Gittis, Stephanie Boyer and Austin Nelson, and Krista Todd made the hard times easier and the good times some of the best of my life.

Thanks to all members of my family: my parents Feraidoon and Salveh, my sister Roya and brother-in-law Ken Mansouri, my brothers Babak and Siamak, and their wives Marjan and Sahar. My family has been most instrumental in giving me the love, support, and confidence needed to get me through graduate school. I am proud to be the first ever doctor in my family. Words can not describe how grateful I am to my children, Roozbeh and Dawna, for making it all worthwhile and keeping me focused. They are my inspiration, the loves of my life, and all that I ever wished for. Most of all, many thanks goes to my wonderful fiancé. Iain is not only an amazing scientist and has taught me a great deal, but he truly is my best friend, soul mate, and committed partner. By sharing my life, my laughs, my cries, my achievements, my failures, my good days and my bad, he has made all of this possible.

Figure 1.1 is a revised version of the figures that appeared in Lunn and Nassirpour et al. 2007. Nature Neuroscience. A unique sorting nexin regulates trafficking of

potassium channels via a PDZ domain interaction. Oct;10(10):1249-1259. It is included with the permission of all the authors on the final publication.

Chapter 2 is a revised version of Lunn and Nassirpour et al. 2007. A unique sorting nexin regulates trafficking of potassium channels via a PDZ domain interaction. Nature Neuroscience. Oct;10(10):1249-1259 and Nassirpour and Slesinger. 2007. Subunit-specific regulation of Kir3 channels by sorting nexin 27. 2007. Channels. Sept/Oct 1(5):331-333. The data included in Chapter 2 only comprise those for which the thesis author was primarily responsible.

Chapter 3 is a modified version of the material as it appears in Nassirpour, Lujan and Slesinger. 2008 (in preparation). It also includes additional data not included in the manuscript. The dissertation author was the primary author of this paper.

Vita and Publications

1998 B.Sc, Biology, University of Toronto, Canada/ CSUSM, summa cum laude

2001 M.Sc., Biology, CSUSM, summa cum laude

2008 Ph.D., Biology, University of California, San Diego

Publications

Lunn ML*, Nassirpour R*, Arrabit C, Tan J, McLeod I, Arias CM, Sawchenko PE, Yates JR 3rd, Slesinger PA. 2007. A unique sorting nexin regulates trafficking of potassium channels via a PDZ domain interaction. Nature Neuroscience. Oct;10(10):1249-1259
* = equal contribution

Nassirpour R and Slesinger PA. 2007. Subunit-specific regulation of Kir3 channels by sorting nexin 27. Channels. Sept/Oct 1(5):331-333.

Nassirpour R and Slesinger PA. CaMKII dependent opioid modulation of GIRK channels in hippocampal neurons. In preparation

Nassirpour R, Norris BJ. 2007. A Vasopressin-like peptide in the CNS of the medicinal leech, Hirudo medicinalis. In revision.

Bouvet M, Wang J, Nardin SR, Nassirpour R, Yang M, Baranov E, Jiang P, Moossa AR, Hoffman RM. 2002. Real-time optical imaging of primary tumor growth and multiple metastatic events in a pancreatic cancer orthotopic model. Cancer Research. 62(5): 1534-40.

Pirocanac EC, Nassirpour R, Yang M, Wang J, Nardin SR, Gu J, Fang B, Moossa AR, Hoffman RM, Bouvet M. 2002. Bax-induction gene therapy of pancreatic cancer. Journal of Surgical Research. 106(2): 346-51.

Sun FX, Tohgo A, Bouvet M, Yagi S, Nassirpour R, Moossa AR, Hoffman RM. 2003. Efficacy of Camptothecin analog DX-8951f (Exatecan Mesylate) on human pancreatic cancer in an orthotopic metastatic model. Cancer Research. 63(1): 80-85.

Awards

2001	Summa Cum Laude
1998	Summa Cum Laude
1997-1998	Keith and Jane Kellogg academic scholarship
1994-1998	Dean's list

ABSTRACT OF THE DISSERTATION

Regulators of neuronal GIRK channels

by

Rounak Nassirpour

Doctor of Philosophy in Biology

University of California, San Diego 2008

Professor Paul A Slesinger, chair

Balancing inhibitory and excitatory inputs is essential for proper signaling in the brain. A significant component of inhibition involves the G protein-gated inwardly rectifying potassium (GIRK/Kir3) channels. GIRK channels have been shown to play a role in learning, pain sensation and drug addiction. Little is known about mechanisms for regulating the trafficking of GIRK channels. Using a proteomics approach, we have identified a unique rodent intracellular protein, sorting nexin 27 (SNX27), which regulates the trafficking of GIRK channels. SNX27 promotes the endosomal movement of GIRK channels, leading to reduced surface expression, increased degradation and smaller GIRK potassium currents. The regulation of endosomal trafficking via sorting nexins reveals a previously unknown mechanism for controlling potassium channel surface expression. We further describe the first agonist dependent regulation of these channels. Using immunohistochemistry at both light and electron microscopic levels, we discovered that chronic (24 hr) morphine treatment increases the colocalization of GIRK2 and PSD95 within dendritic spines. This change in expression requires activation of

CaMKII and is mimicked by constitutively active form of this enzyme. The net effect of morphine treatment and CaMKII activation is to reduce the amplitude of $GABA_B$ receptor-activated GIRK currents. Our results demonstrate that stimulation of opioid receptors produces a form of CaMKII-dependent plasticity for the $GABA_B$ receptor–GIRK channel inhibitory pathway in the hippocampus. Both of these studies demonstrate changes in GIRK expression and may establish an important link between trafficking of potassium channels and drug actions in the brain, possibly opening up new avenues for treating drug addictions.

I. Introduction

The cellular processes that direct the assembly, transport, targeting, localization and surface expression of receptors and ion channels are of fundamental importance for neuronal function. For example, in the hippocampus, which has served as a cellular model of learning and memory, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR) have recently been demonstrated to constitutively recycle in the postsynaptic membrane with a surface expression half life of about 10 minutes [1]. It is evident that other ion channels, most notably γ-amino-butyric acid (GABA) A and glycine receptors, are also cycling between the cell surface and intracellular compartments [2, 3]. A series of recent studies revealed that N-methyl-D-aspartate receptors (NMDAR) cycle rapidly into and out of synapses, and cumulatively over long timescales this trafficking can modify the number and composition of NMDARs [4]. Together these results suggest that rapid cycling of receptors in and out of synaptic membranes may be a general means of controlling neuronal excitability and may underlie certain forms of synaptic plasticity.

Aside from the requisite voltage-gated ion channels, neurons express an array of other ion channels that influence excitability. Inwardly rectifying potassium (Kir) channels are one class of channels that subserve this role – they contribute little to the formation of the action potential but instead have large effects on the resting membrane potential. Thus, regulating the activity of Kir channels would be expected to change the firing properties of neurons.

While there is still much to learn about the subcellular localization and synaptic targeting of receptors and ion channels, a large number of proteins are already implicated

in this process. We now know that the dynamically regulated trafficking of synaptic proteins is activity dependent and plays an important role in different kinds of synaptic plasticity. In particular, PDZ binding proteins have been implicated in the activity dependent trafficking of various synaptic receptors.

Potassium channels

Potassium currents play an array of roles in cellular function. In excitable tissues they shape the action potential by determining repolarization and play a major part in shaping repetitive firing. In addition, they influence transmitter and hormone release and are involved in an array of cellular function such as cell volume regulation, extracellular K^+ buffering and apoptosis [5].

Potassium channels are one of the largest ion channel gene families and they are present in all living organisms from bacteria to humans [6, 7]. In very broad terms, potassium currents can be categorized into voltage gated, inwardly rectifying and calcium activated currents. All three families share a highly conserved motif, a hairpin H5 loop that briefly enters and exits the membrane. A large body of mutagenesis work [8] and the relatively recent crystal structure of the bacterial potassium channel [9] show this region to be an important component of the channel pore and to contain residues critical for potassium selectivity. Generally this potassium signature sequence contains the GYG motif [10].

Aside from the requisite voltage-gated ion channels, neurons express an array of other ion channels that influence excitability. Inwardly rectifying potassium (Kir) channels are one class of channels that subserve this role – they contribute little to the

formation of the action potential but instead have large effects on the resting membrane potential. Thus, regulating the activity of Kir channels would be expected to change the firing properties of neurons.

Kir channel proteins are characterized by a common molecular motif of two membrane-spanning domains, one pore-forming domain, and cytoplasmic N- and C-terminal domains [11]. These channels have the distinctive property of allowing more inward than outward current to flow. However, it is the small amount of outward current these channels carry that is important for physiological function. These currents do not shape the propagating action potential but can influence repetitive firing by modulating resting membrane potential and pace maker activity [12]. The G-protein gated inwardly rectifying potassium (GIRK) channels form one branch of the larger Kir family.

G-protein Gated Potassium Channels (GIRKs)

The first GIRK protein was identified in atrial myocytes and was found to be responsible for acetylcholine induced slowing of the heart [13]. Analogous GIRK currents are present in neurons and neuroendocrine cells [14]. Neuronal channels regulate firing rates, membrane potentials, and neurotransmitter release in the brain [12].

Activation of these channels decreases membrane excitability by hyperpolarizing the membrane potential and slowing membrane depolarization [12] thus loss of neuronal GIRK channels leads to hyperexcitability and seizures [15].

GIRK channel stoichiometry

The mammalian GIRK family includes four subunits to date, GIRKs 1-4 [16-19]. Functional channels are believed to be homotetramers of GIRK 2 and 4 [20, 21], or heterotetramers of GIRK 1/2, GIRK 1/3, and GIRK1/4. These hetrotetrameric channels have indistinguishable single channel conductances, open times, and $G_{\beta\gamma}$ sensitivities [22]. By studying the biophysical properties of concatenated subunits, Silverman et al [23] suggested that various subunit arrangements are possible for GIRK1/4 and GIRK 1/2 channels [23, 24]. Subsequently, utilizing tandemly linked tetramers of GIRK 1 and 4, Tucker et al. [24] found that the most efficient channel comprises two subunits of each type in an alternating fashion. Although GIRK3, like GIRK1, is consistently detected using RT PCR [25] coexpression of GIRK3 with GIRK1 leads to reduced expression of GIRK1 at the cell surface. It has been suggested that GIRK3 plays a regulatory role in the expression and function of GIRK1 containing heteromers [26-28]. A most intriguing finding by Ma and colleagues is that despite its high similarity to GIRK2 and GIRK4, GIRK3 not only lacks an ER export signal, but also contains a lysosomal targeting signal [28]. GIRK tetramers without GIRK1 may be physiologically relevant. In fact, Jelacic et al [29] demonstrated that GIRK2/3 form functional channels in the native brain tissue. However these channels are 5 fold less sensitive to activation by $G_{\beta\gamma}$ and have a slightly smaller conductance than GIRK1/GIRK X channels.

GIRK 2 or 4 subunits alone produce spiky, irregular amplitude channels with brief open times [17, 30-34]. There are four different splice variants of GIRK2 that differ in their sequences at the extreme N- and C-termini of the channel. GIRK2b, which is truncated at the C terminus and lacks the acidic cluster, shows reduced surface

expression. Like GIRK3, GIRK2C, has a Postsynaptic Density-95 (PSD-95)/Discs Large/Zona Occludens-1- (PDZ)-binding domain. GIRK2D lacks several residues in the N terminus including the essential VL motif, leading to increased levels of this splice variant on the surface. GIRK 1 subunits alone do not make functional channels [22]. It seems unlikely that neuronal channels are homomeric GIRK1 or GIRK3, which are believed to reside in the endoplasmic reticulum (ER).

GIRK channel localization

In contrast to GIRK1, which is present in both the heart and the brain, GIRK4 is expressed mainly in the heart [17], while both GIRK 2 and 3 are specifically localized to the brain [19]. GIRK4 mRNA can be detected in only a few regions of the mouse brain, including the deep cortical pyramidal neurons, the endopiriform nucleus and claustrum of the insular cortex, the globus pallidus, the ventromedial hypothalamic nucleus, parafascicular and paraventricular thalamic nuclei, and a few brainstem nuclei (e.g., the inferior olive and vestibular nuclei). Karschin et al. [35] reported that, with the exception of GIRK4 which is expressed at a low level in the above regions, most GIRK proteins show a similar distribution in the brain with high expression levels in the olfactory bulb, hippocampus, cortex, thalamus and cerebellum. At the cellular level GIRK2 was localized in the neuronal cell body and dendrites, but distinct differences were seen in the degree of dendritic staining among neuronal groups. For some the staining of the distal dendrites and spines was strong, while others showed preferential somatodendritic labeling. Furthermore, some results suggest that GIRK2 might be localized in distal axonal terminal fields [36]. In addition, Immuno- elctromicroscopic studies of rat brain

revealed that GIRK1 is postsynaptically expressed in dendrites of hippocampal neurons [37]and can associate with microtubules [38]. Hippocampal GIRK channels are primarily composed of GIRK1 and GIRK2A subunits. GIRK1/2C heteromeric channels are also abundantly present but they appear to be trafficked to specific neuronal compartments and thus exhibit different expression patterns [25].

GIRK channel physiological significance

GIRK channels play pivotal roles in generation of slow inhibitory postsynaptic potentials in the brain as they are coupled to various inhibitory receptors including M2-muscarinic cholinergic, μ, κ, and δ opioid, A1 purinergic, $\acute{\alpha}_2$ adrenergic, D2 dopaminergic, 5HT, somatostatin, substance P, and $GABA_B$ receptors [28, 39, 40]. GIRK channels are activated by a direct, membrane delimited interaction with the PTX-sensitive G-proteins [41-43]. A recent study performed in the Slesinger lab indicates that GIRK channels are part of a membrane signaling complex that contains G-proteins, GPCRs, and the channels [43, 44]. They are activated by most combinations of $G_{\beta\gamma}$ subunits, yet only stimulation of $G_{\alpha i/o}$ activates GIRKs in pyramidal hippocampal neurons but are also regulated by G_{α} subunits [45]. A number of studies have sought to explain why these channels, when expressed in Xenopus laevis oocytes, deactivate more slowly than the native atrial current upon termination of the receptor stimulus. Identification of the RGS family has resolved this discrepancy. Recently, Benians et al. [46] found that these regulators of G-proteins form a quarternary complex with the agonist, receptor, and G-protein. Taken together with results from Clancy et al. [47], one can assume the

trafficking complex to be composed of the G proteins, their regulators, the GPCRs, and the GIRK channels.

GIRK channel Targeting

ER export is the rate-limiting step for surface delivery of many integral membrane proteins [48]. An ER export signal, FCYENE, was identified from the Kir 2.1 channel [49-51]. A related but distance ER export signal was also found in Kir 1.1 by Stockklausner et al. [51]. ER export signals are also present in GIRK2 and 4 [49, 50]. Ma and colleagues show that a region in the N-terminus of GIRK4 is involved in the ER export of channels because a deletion in this region leads to channel accumulation in the ER. Once GIRK4 channels exit the ER, the majority of them localize to the plasma membrane, although some channel proteins can also be found in vesicles and juxtanuclear regions. A VL motif was shown to be critical for targeting the channel to the endosomes. Deletions in the GIRK4 C-terminus that remove a cluster of acidic residues lead to the accumulation of a large number of channels in vesicles, resulting in a reduction in surface expression. In GIRK2, a similar cluster of acidic residues in the C terminus contains a consensus Ser-Thr phosphorylation site. Mutation of Thr to Ala at this site leads to reduced surface expression of these channels; however, mutation of Thr to Asp, mimicking the phosphorylated state of the channel, leads to enhanced surface expression [49, 50].

The SAP (synapse associated proteins) family of proteins and synaptic localization

SAPs have recently been shown to be involved in targeting, clustering, and scaffolding of proteins post- and presynaptically [52-55]. They are characterized by a

number of repeating protein-protein interaction modules known as PDZ domains. PDZ is an acronym for three proteins that include this domain namely PSD-95 (postsynaptic density protein, 95KD, also known as SAP90) Dlg (discs large, a Drosophila protein found at septate junctions) and zona occludens 1 (ZO-1, a protein found at vertebrate tight junctions). PDZ domains bind via a T/SXV motif present in the C-terminus of the interacting protein. The 4 different NR2 subunits (A-D), for example, contain a long cytoplasmic C-terminus with conserved ESDV or ESEV sequence, which mediates binding to PSD95.

A large number of SAP family members also include an inactive guanylate kinase-like domain and lead to further abbreviations, namely membrane associated guanylate kinase (MAGUK) and Chapsyns (channel associated proteins of the synapse). They also often contain a SH3 domain. In mammals this family includes 4 members, PSD95, Chapsyn 110, SAP102, and SAP97. Generally, SAP97 is axonal and presynaptic while PSD95, Chapsyn110 and SAP102 are largely postsynaptic.

The various domains of the SAP family have been shown to organize a complex signaling matrix. Such a molecular scaffold plays an important role not only in synaptogenesis, but also in synaptic plasticity. For example, PSD-95 is involved in the clustering of certain ion channels and receptors through its different domains [56]. SH2 domain of Fyn, a non-receptor Src tyrosine kinase, associates with NMDAR via binding to the PDZ3 of PSD95 [57]. This PSD95 mediated ternary complex facilitates the phosphorylation of NR2A by Fyn which may play a role in regulating the subcellular distribution of NMDARs [58].

The GK domain of PSD95 family proteins acts as another modular site for protein-protein interaction. The GK domain binds to abundant family of proteins in the PSD, termed GKAP, SAPAP, DAP. These proteins in turn link NMDARs to other proteins in the PSD. For instance, GKAP binds to Shank family of scaffolding proteins, which in turn bind to Homer and contactin [59-61]. This links the NMDARs to intracellular calcium stores, since Homer interacts with inositol 1,4,5-triphosphate receptors (IP3R) [61].

SAPs may also interact with signaling molecules such as nNOS, synGAP to form signaling complexes. Neuronal nitric oxide synthase (nNOS) binds to PSD95 via a PDZ-PDZ interaction [62]. nNOS is a calcium/calmodulin regulated enzyme that is preferentially activated by calcium influx through NMDA receptors. SynGAP, a GTPase activating protein for Ras, has a C-terminus that interacts with all three PDZ domains of PSD95 [63, 64]. SynGAP is an abundant PSD protein. These interactions however have not been established for potassium channels.

A number of potassium channels from various families contain the PDZ binding motif and potentially can interact with PDZ containing proteins. These include Kv 1.1-1.6, KV3.2b, 3.3b, Kv 4.1-4.3, Kir 2.1-2.3, Kir 4.1 and Task-1 as well as GIRK2c and GIRK3 [55]. SAPs may modulate the function of potassium channels. For example, Kir 4.1 current is increased when coexpressed with SAP97 [65]. Additionally, the GK domain of SAP97 was shown to be necessary for the function of Kir 2.3c [66]. Conversely, coexpression of PSD95 with Kir 2.3 led to current suppression because of a reduction in single channel conductance [67]. Although GIRK2c and GIRK3 also contain a PDZ binding motif, Nehring et al. reported that in contrast to Kir2.1 and Kir2.3, all

GIRK fragments failed to bind PSD-95, which was supported by the lack of co-immunoprecipitation and colocalization in mammalian cells. Nehring et al. found no colocalization between GIRKs and PSD-95. Another study by Ianobe et al. [68] showed that postsynaptically on dendrites of dopaminergic substantia nigra neurons neither PSD95 nor SAP97 coaggregated with the abundant GIRK2. Thus, although interactions with PSD95 and even SAP97 are unlikely, GIRK channels might bind to other PDZ containing proteins. Chapter 2 describes a unique PDZ protein that not only directly and specifically binds GIRK2c and GIRK3 but it also regulates the GIRK channel intracellular trafficking and function.

Sorting Nexin 27(SNX27)

The endosomal system functions as a sorting compartment to regulate the intracellular trafficking of plasma membrane proteins. The mammalian endosomal system consists of a variety of compartments, including early endosomes, recycling endosomes, late endosomes and lysosomes [69]. Internalized membrane proteins are delivered to early endosomes following endocytosis and are sorted for recycling back to the membrane, or to degradation pathways.

Sorting Nexins (SNX) form a large family of diverse cytoplasmic and membrane associated proteins, which are involved in intracellular sorting of various proteins. 29 human SNXs have been identified to date [70]. SNXs are characterized by the presence of a subclass of the phosphoinositide-binding phox (PX) homology domain. The *PX* domain derives its name from the protein complex from which it was originally identified—the PHagocyte NADPH OXidase (phox). *PX* domains typically interact

preferentially with PtdIns(3)P, and thus have a tendency to be localized to early endosomal compartments [71]. Due to their PX domain, SNXs have been suggested to have various endosomal sorting functions including pro-degradative sorting, internalization, endosomal recycling, or simply endosomal trafficking [72].

Several SNX members localize to the endosomal structures, and some directly interact with the transmembrane receptors to direct their sorting in the endosomal pathway. For instance, SNX1 enhances the degradation of the epidermal growth factor receptor, probably by a facilitation of the endosome-to-lysosome targeting [73]. Later studies indicated that SNX1 participates in the sorting of the protease activated receptor1 from early endosomes to lysosomes [74]. Another sorting nexin, SNX3, drastically enhanced surface receptor transport from early to recycling endosomes [75]. SNX9 has been shown to assemble with AP-2 [49] and dynamin-2 at the plasma membrane and thus play a role in clathrin dependent endocytic machinery [76].

Sorting nexin 27 is a unique member of the SNX family in that it is the only SNX that contains a PDZ domain (Figure 1.1c). Cell surface expression of many ion channels and receptors are regulated by PDZ proteins (see above). SNX 27 was initially cloned from the human genome. Two different forms of the protein were further identified in rat and are identical to the human SNX27a and b (Figure 1.1c). Encoded by a single gene these transcripts are only different in their last few C-terminus amino acids. Both proteins are predominantly expressed in the brain, including the hippocampus [77, 78]. SNX27b is up-regulated in the neocortex of adult rats after chronic methamphetamine treatment, hence the name methamphetamine regulated transcript 1 (MRT1). In contrast,

the level of SNX27a (Mrt1a) mRNA, which is constitutively expressed, is not affected by this treatment [78].

Very few reports exist on SNX27 and its functional significance. SNX27 was first described as a binding partner for the 5-hydroxytryptamine (5-HT) type 4 receptor [79]. The authors showed that over expression of SNX27a redirects the 5-HT receptor through a PDZ dependent mechanism from the plasma membrane to early endosomes. However, using an overlay in vitro binding assay, the Slesinger lab did not observe any significant binding of the C-terminal domain of GIRK3 to the classical PDZ proteins (Figure 1.1b). Utilizing a proteomics approach using the C-terminal end of GIRK3, the Slesinger lab identified the first regulatory protein for these ion channels. The PDZ domain of SNX27 associates strongly and specifically with GIRK3 and not other PDZ binding motif containing potassium channels (Figure 1.1d). These results indicate that the PDZ domain of SNX27 discriminates among similar PDZ-binding motifs (–ESEI versus –ESKV). Consistent with the idea the PDZ-binding motif of GIRK3 binds the PDZ domain of SNX27, the C-terminal domain of GIRK2c, but not that of other GIRK subunits, associated with the PDZ domain of SNX27 (Fig. 1.1e). Thus, the two GIRK subunits that contain the PDZ-binding motif, –ESKV, interacted directly with the PDZ domain of SNX27. The binding of these channels to SNX27 modulates expression of GIRK channels through a PDZ domain interaction (Figure 1.1a and chapter 2).

SNX27 also contains a Ras Associating (RA) domain (Figure 1.1c). In other proteins, the RA domain stimulates the dissociation of GDP from Ras-related proteins, allowing the binding of GTP and activation of the GTPase [70]. The role of this domain in SNX27 has not been tested.

Opioid Receptors

The endogenous opioid system consists of three distinct neuronal pathways that are widely distributed throughout the CNS. The three opioid precursors are proopiomelanocortin [80], proenkephalin [81], and prodynorphin [82]each of which is released by specific opioidergic neurons. These peptides exert their physiological actions by interacting with various classes of opioid receptors [83-90] present on both pre- and postsynaptic membranes of opioid and opioid target neurons [91, 92]. The most important opioid peptide seems to be the pentapeptides enkephalins [93, 94] which interact with both the μ and δ receptors, their affinities being significantly higher for the latter.

Opioid receptors are members of the A (rhodopsin-like) family of GPCRs characterized by the presence of seven transmembrane domains separated by three intracellular and three extracellular loops. Three opioid receptor genes: MOR, DOR, and KOR have been cloned and characterized at the molecular level [95]. The genes encode μ, δ, and κ receptors respectively which have been pharmacologically characterized [96].

Opioid receptors are abundant in the hippocampus and are activated by opioid peptides, coreleased with glutamate, from mossy-fiber and lateral perforant path synapses [97-99]. Opioids are also known to modulate the excitability of hippocampal pyramidal neurons [100, 101], whereas μ-opioid receptor antagonists [102-105] or μ-opioid receptor gene deletions [106-108] block the induction of both mossy-fiber LTP to CA3 and lateral-perforant-path LTP to CA3 and dentate and impair spatial water maze learning.

All three opioid receptors are protypical Gi/Go-coupled receptors because opioid signals are efficiently blocked by pertussis toxin (PTX), a bacterial toxin produced by

Bordetella pertussis that ADP-ribosylates and inactivates the α-subunits of Gi/Go proteins. The opioid receptors have long been known to inhibit adenylyl cyclase [109] and Ca channels [110, 111], as well as stimulate K+ channels [112] and increase intracellular Ca levels [113]. Interestingly, we and others have shown that there are no opioid induced GIRK currents in the hippocampus. In fact, in chapter 3, I present data for an opioid agonist specific inhibition of GIRK currents in hippocampus suggesting coupling to Gq coupled receptors.

Opioid receptor desensitization, resensitization, and trafficking

Like other GPCRs, agonist binding to the receptor results in rapid phosphorylation of the receptor by protein kinases, thereby promoting the association of the cellular protein arrestin [114]. Agonist induced opioid receptor phosphorylation has been demonstrated for the DOR [115], MOR [116] and KOR [117] receptors. These phosphorylations were later shown to be mediated by G-protein coupled receptor kinases (GRKs) [118], or second messenger regulated protein kinases, such as CaMKII [119, 120]. GRKs are a family of serine/threonine protein kinases that specifically recognize agonist activated G-protein coupled receptors as substrates. This association uncouples the receptor from the respective G-protein and blunts the receptor signaling (receptor desensitization) [121-123]. The arrestin also induces an agonist dependent endocytosis into the clathrin-coated vesicles. Endocytosed receptors can be targeted to a rapid recycling path mediating resensitization of the receptors, or to a degradative pathway mediating receptor trafficking to lysosomes and leading to receptor down regulation [124-127]. For continued GPCR signaling, desensitized receptors need to reestablish their responsiveness to extracellular signals through a receptor resensitization process.

One potential mechanism for receptor resensitization is internalization. Desensitized GPCRs are internalized via clathrin-coated pits in early endosomes, where they are thought to be dephosphorylated and recycled back to the cell surface [114, 122].

Down regulation of opioid receptors in response to chronic opioid treatment is a long-term adaptive process that can contribute to opioid tolerance. 35S-GTPγS binding was decreased in several brain areas of rats chronically treated with morphine indicating long term uncoupling of opioid receptors from G proteins [128]. Using the mouse neuroblastoma cell line N4TG, Chang et al. [129] reported a decrease in Bmax after exposure to DOR agonist DADLE. Also, chronic treatment of rats with the selective MOR agonist PLO17 was shown to down regulate MORs [130, 131]. In SHSY5Y human neuroblastoma cell line, which contains both MOR and DOR, morphine was able to down regulate both receptors [132, 133]. In another study, using antibodies directed against MOR, it was shown that chronic treatment with morphine decreases MOR immunoreactivity in mice [134]. Together these studies suggest that receptor desensitization might be a plausible mechanism for opioid tolerance.

The complexity in the signal transduction of the opioid receptor goes beyond the simple involvement of receptor Gi/o proteins and effectors. A universal mechanism could not be applied to all receptor agonists. Of the three opioid receptors, MOR shows the highest affinity for morphine. However, the two MOR receptor agonists, morphine and DAMGO ([d-Ala2,N-Me-Phe4,Gly5-ol]-enkephalin), elicit different cellular responses. Morphine fails to promote endocytosis of MORs, whereas other opiate drugs such as DAMGO readily drive receptor endocytosis [125, 126]. Binding experiments in NG108-15 cells suggested that only full agonists can down regulate opioid receptors. In

these cells, etrophine and other opioid peptides, but not morphine, which is not a full agonist, were able to down regulate opioid receptors [135]. These results were however, not completely supported by in vivo binding studies. For example, only a small reduction in total number of binding sites in brain of guinea pigs treated chronically with morphine was reported [136]. Furthermore, in vivo down regulation of both MOR and DOR has been observed in certain brain areas of rat treated chronically with etrophine, whereas morphine was shown to cause an up regulation [137]. In fact, although endocytic effects of methadone (internalizing) and morphine (non-internalizing) was repeated in cell bodies of nucleus accumbens neurons, in dendrites of the same neurons, morphine-induced internalization of both virally transduced MOR1 and endogenously expressed μ opioid receptors [138]. This result suggests that morphine may indeed induce rapid internalization of opioid receptors in certain populations of CNS neurons, and there may be additional differences in the regulatory effects of opiate drugs occurring in different membrane domains (e.g., axons vs. dendrites) of the same neurons [138]. Together these data suggest that the rate of morphine induced down regulation of opioid receptors is species, brain region, cell type, membrane domain and agonist specific.

Opioid tolerance after chronic treatment requires the continuous presence of opioid agonists as well as a rapid decrease in receptor activity. This dependence on opioids results in withdrawal symptoms after their removal. Early studies revealed that opioids acutely inhibit Adenylate Cyclase (AC) activity in NG108-15 cells, but in the continued presence of morphine, there was an up-regulation of AC activity [139, 140]. To date, the mechanism by which AC activity is regulated by chronic exposure to inhibitory agonists remains unclear. Opioid receptors are predominantly coupled to AC via Gα1.

There are reports that opioid receptor signaling is enhanced via Gβγ during the development of opioid tolerance [75, 141]. Furthermore, an increasing body of results suggest that opioid receptors can also couple to Gs proteins after chronic opioid exposure, leading to an increase in AC activity [142].

Although the up-regulation of AC in cell lines is a clear phenomenon, studies on the effects of chronic morphine treatment on AC activity in brain have produced mixed results [142-144]. Chronic in-vivo administration of morphine has been shown to increase basal GTP and forskolin stimulated AC in locus coeruleus (LC) [144]. NAc and amygdala also show increases in AC and cyclic AMP-dependent protein kinase activity after chronic treatment. In contrast, thalamus showed an increase in cyclic AMP-dependent protein kinase activity only [143]. Further investigation of AC activity in the brain is warranted.

Further complexity arises from opioid receptor oligomerization. Like other GPCRs, the native opioid receptor is believed to exist as an oligomeric complex of the receptor and its G proteins [145-148]. Several lines of evidence, suggest that opioid receptors form homo- and heterodimers/ oligomers [149-153]. Oligomerization of opioid receptors has been suggested to play a role in receptor activation and internalization [149, 154]. The heterodimers also have greatly reduced affinities for their selective ligands. The rank order of agonist affinities for the hetereodimeric receptors is different from that of the individual receptors, suggesting modulation of the binding pocket on dimerization [150, 151, 155, 156].

For example, when μ and δ opioid receptors were coexpressed, the highly selective synthetic agonists for each had reduced potency and altered rank order, whereas

Leu-enkephalin had enhanced affinity, suggesting the formation of a novel binding pocket. Heterodimers exhibit competitive and synergistic interactions in both binding and signaling. Early pharmacological evidence shows that MOR selective ligands inhibit the binding of DOR ligands in both a competitive and a non-competitive way [157, 158]. These results suggest the presence of two types of DORs: those that are associated with MORs and those that are not. On the other hand, in cells expressing μ/ δ heterodimers, low doses of δ selective ligands produce a significant increase in the binding of a μ selective agonist. In addition, DOR selective ligands can potentiate MOR mediated analgesia, suggesting a functional interaction between these two subtypes of the opioid receptor [159-161].

In contrast to the individually expressed MOR and DOR receptors, the hetrodimeric μ-δ complex associates with pertussis toxin insensitive G proteins because pertussis toxin treatment does not completely abolish receptor signaling [150] In chapter 3 we provide evidence for expression of opioid receptor heterodimers in hippocampus which also show selectivity and reduced affinity for opioid agonists and PTX-insensitive signaling.

CaMKII and opioid receptors

CaMKII is a multifunctional calcium regulated second messenger that regulates neuronal activity by modulating ion channels as well as synthesis and release of neurotransmitters [162, 163]. This serine/threonine kinase is highly concentrated in the brain especially in the cerebral cortex and hippocampus [164]. Its autophosphorylation, which is activated by calcium/calmodulin, enables the kinase to phosphorylate its

substrates. CaMKII is encoded by four genes (α ,β, δand γ) with α anḍ βbeing the primarily expressed isoforms in neurons [165]. Among them the ɑsubunit of CaMKII plays a pivotal role in learning and memory, long-term potentiation and neuronal plasticity [165-169].

CaMKII, which is highly enriched in postsynaptic densities of the hippocampus and neocortex, is critical in the regulation of synaptic plasticity such as LTP [170, 171]. Indeed, targeted disruption of the CaMKIIcl gene produces deficits in LTP and severely impairs performance in hippocampal-dependent memory tasks [168,172,173]. Considerable evidence has indicated that opioids can induce long-term potentiation (LTP), a model of neuroplasticity and use-dependent form of learning and memory, in the hippocampus [103,174,175]. Again hippocampal CaMKII has been shown to be involved in the development of morphine addiction. Morphine treatment increases the expression of CaMKII in rat hippocampus [176]**.** In vivo studies revealed that morphine treatment significantly increased activities of CaMKII in the hippocampus of rats [176]. Correspondingly, administration of CaMKII antisense oligonucleotides into the hippocampus attenuates morphine tolerance and dependence [177]. Recently Narita et al. [178] and Fan et al. [177] showed that although inhibiting CaMKII failed to effect antinociception and hyperlocomotion induced by morphine, it significantly attenuated the place preference suggesting that CaMKII contributes to the rewarding effect induced by morphine. Similarly, employing pharmacological treatments, Narita et al. [178] provided evidence that the increase in CaMKII activity in the mouse limbic forebrain may contribute to the rewarding effect, but not the antinociception and the hyperlocomotion, induced by morphine.

Additional evidence for the importance of CaMKII in opioid receptor signaling comes from cellular and biochemical studies. Bruggemann et al. [179] demonstrated that CaMKII colocalizes with MOR in specific regions of the rat brain and thus in a position to phosphorylate the MOR and to contribute to the development of tolerance to the opioid analgesics. Basal phosphorylation of the μ-opioid receptor also appears to involve the CaMKII, as indicated by the CaMKII inhibitor studies. Koch et al. [119] reported that by mutating the two putative phosphorylation sites for CaMKII of the MOR to Alanine, the increase in the rate of receptor desensitization when the CaMKII is over expressed can be blocked. Therefore, it is reasonable to speculate that CaMKII may play a modulatory role in opioid signaling and consequent adaptive changes.

Unfortunately the mechanism by which opioid receptors regulate CaMKII is not yet clear. Considerable evidence has demonstrated that activation of opioid receptors can increase intracellular calcium concentration through activation of phospholipase C in various cell types [180-182], including NG108-15 cells [183, 184]. An increase in intracellular calcium could result in activation of calcium-dependent enzymes such as CaMKII [163, 185-187], and activation of CaMKII may in turn regulate functional responses of opioid receptors (see above).

Gi/Go-coupled receptor activation is commonly associated with preventing the elevation of intracellular free calcium by inhibiting voltage-operated Ca2+ (VOC) channels in the plasma membrane [188]. However, as discussed above, it has been observed in a diverse range of cell types that opioid receptor activation can also elevate intracellular calcium levels. In some instances this is observed during opioid receptor activation alone, but in many cases the opioid receptor-mediated elevation of calcium

only occurs during concomitant activation of Gq-coupled receptors, which themselves stimulate Ca2+ release from intracellular stores via the inositol phosphate pathway [189-193]. This synergy between opioid and Gq-coupled receptors is not unique to opioid receptors, and appears to represent a novel form of coincident signaling that has been reported for a number of other Gi/Go-coupled receptors [194]. These results might be explained by activation of oligomeric receptors (see above) which have been shown to signal through PTX insensitive G proteins such as Gq.

Taken together, these results suggest that CaMKII is critical in the development of opioid tolerance and that inhibition of this kinase may be of therapeutic benefit in the treatment of opioid addiction. In accordance with the above mentioned studies, chapter 3 provides evidence for the significance of CaMKII in opioid receptor signaling.

Neuronal plasticity and the GIRKs

A search of the literature reveals few published reports on the altered gene expression patterns of GIRKs caused by physiological or pharmacological manipulations. Pei et al. [195] showed that a single (acute) electroconvulsive shock increased GIRK2 expression while causing a transient reduction of GIRK1 mRNA in the dentate gyrus. Chronic shock, however, increased mRNA and protein expression of both GIRK1 and 2. He et al. (Neuroscience 2005 abstract) reported that synaptic NMDA receptor activation regulates GIRK channels by increasing surface expression of GIRKs in a protein phosphatase dependent manner. Later, Huang et al [196] showed that common molecular pathways mediate LTP of synaptic excitation and slow synaptic inhibition. The authors showed that inhibiting CaMKII activity abolishes both the excitatory and the slow

inhibitory potentiation. This LTP of slow synaptic inhibition was sensitive both $GABA_B$ and GIRK blockers. However, we still do not understand what regulates this phenomena or what the functional significance might be. One explanation could be that GIRK channels are upregulated upon synaptic activity and trafficked to the cell surface to induce the observed LTPipsp. This upregulation of GIRK2 might underlie the molecular mechanism behind the change in the capacity for LTP withdrawal symptoms associated with chronic exposure to addictive substances. Other pathways including estrogen, muscarinic and metabotropic glutamate receptor signaling have also been shown to be able to uncouples opioid and $GABA_B$ receptors from GIRK channels [197, 198]. In addition, changes in RGS2 have has also been shown to alter the strength of GIRK signaling in the brain [199]. All of the above studies suggest that synaptic activity regulates GIRK channel signaling

In this thesis I describe two studies that advance the field of GIRK channel trafficking. In chapter 2, I describe the first SAP protein regulation of GIRK intracellular trafficking while in chapter 3 I describe the morphine dependent regulation of GIRKs, this is the first report of an agonist dependent pathway. These findings allow for a more elaborate and structured understanding of GIRK trafficking in hippocampal neurons.

References

1. Park, M., et al., *Recycling Endosomes Supply AMPA Receptors for LTP.* Science, 2004. **305**(5692): p. 1972-1975.

2. Kneussel, M. and S. Loebrich, *Trafficking and synaptic anchoring of ionotropic inhibitory neurotransmitter receptors.* Biology of the Cell, 2007. **099**(6): p. 297-309.

3. Barnes, E.M., *Intracellular trafficking of GABAA receptors.* Life Sciences, 2000. **66**(12): p. 1063-1070.

4. Pérez-Otaño, I. and M.D. Ehlers, *Homeostatic plasticity and NMDA receptor trafficking.* Trends in Neurosciences, 2005. **28**(5): p. 229-238.

5. Hille, B., C.M. Armstrong, and R. MacKinnon, *Ion channels: From idea to reality.* Nat Med, 1999. **5**(10): p. 1105-1109.

6. Jan, L.Y. and Y.N. Jan, *Voltage-gated and inwardly rectifying potassium channels.* J Physiol, 1997. **505**(Pt_2): p. 267-282.

7. Jan, L.Y. and Y.N. Jan, *CLONED POTASSIUM CHANNELS FROM EUKARYOTES AND PROKARYOTES.* Annual Review of Neuroscience, 1997. **20**(1): p. 91-123.

8. Heginbotham, L., et al., *Mutations in the K+ channel signature sequence.* Biophys. J., 1994. **66**(4): p. 1061-1067.

9. Doyle, D.A., et al., *The Structure of the Potassium Channel: Molecular Basis of K+ Conduction and Selectivity.* Science, 1998. **280**(5360): p. 69-77.

10. Lipkind, G.M., D.A. Hanck, and H.A. Fozzard, *A Structural Motif for the Voltage-Gated Potassium Channel Pore.* Proceedings of the National Academy of Sciences, 1995. **92**(20): p. 9215-9219.

11. Doupnik, C.A., N. Davidson, and H.A. Lester, *The inward rectifier potassium channel family.* Curr Opin Neurobiol, 1995. **5**(3): p. 268-77.

12. Dascal, N., *Signalling Via the G Protein-Activated K+ Channels.* Cellular Signalling, 1997. **9**(8): p. 551-573.

13. Breitwieser, G.E. and G. Szabo, *Mechanism of muscarinic receptor-induced K+ channel activation as revealed by hydrolysis-resistant GTP analogues.* J. Gen. Physiol., 1988. **91**(4): p. 469-493.

14. Standen, N.B., et al., *Hyperpolarizing vasodilators activate ATP-sensitive K+ channels in arterial smooth muscle.* Science, 1989. **245**(4914): p. 177-80.

15. Signorini, S., et al., *Normal cerebellar development but susceptibility to seizures in mice lacking G protein-coupled, inwardly rectifying K+ channel GIRK2.* Proceedings of the National Academy of Sciences, 1997. **94**(3): p. 923-927.

16. Dascal, N., et al., *Atrial G protein-activated K+ channel: expression cloning and molecular properties.* Proc Natl Acad Sci U S A, 1993. **90**(21): p. 10235-9.

17. Krapivinsky, G., et al., *The G-protein-gated atrial K+ channel IKACh is a heteromultimer of two inwardly rectifying K(+)-channel proteins.* Nature, 1995. **374**(6518): p. 135-41.

18. Kubo, Y., et al., *Primary structure and functional expression of a rat G-protein-coupled muscarinic potassium channel.* Nature, 1993. **364**(6440): p. 802-6.

19. Lesage, F., et al., *Cloning provides evidence for a family of inward rectifier and G-protein coupled K+ channels in the brain.* FEBS Lett, 1994. **353**(1): p. 37-42.

20. Slesinger, P.A., et al., *Functional effects of the mouse weaver mutation on G protein-gated inwardly rectifying K+ channels.* Neuron, 1996. **16**(2): p. 321-31.

21. Schoots, O., et al., *Co-expression of human Kir3 subunits can yield channels with different functional properties.* Cell Signal, 1999. **11**(12): p. 871-83.

22. Jelacic, T.M., S.M. Sims, and D.E. Clapham, *Functional expression and characterization of G-protein-gated inwardly rectifying K+ channels containing GIRK3.* J Membr Biol, 1999. **169**(2): p. 123-9.

23. Silverman, S.K., H.A. Lester, and D.A. Dougherty, *Subunit stoichiometry of a heteromultimeric G protein-coupled inward-rectifier K+ channel.* J Biol Chem, 1996. **271**(48): p. 30524-8.

24. Tucker, S.J., M. Pessia, and J.P. Adelman, *Muscarine-gated K+ channel: subunit stoichiometry and structural domains essential for G protein stimulation.* Am J Physiol, 1996. **271**(1 Pt 2): p. H379-85.

25. Leaney, J.L., *Contribution of Kir3.1, Kir3.2A and Kir3.2C subunits to native G protein-gated inwardly rectifying potassium currents in cultured hippocampal neurons.* European Journal of Neuroscience, 2003. **18**(8): p. 2110-2118.

26. Wischmeyer, E., et al., *Subunit interactions in the assembly of neuronal Kir3.0 inwardly rectifying K+ channels.* Mol Cell Neurosci, 1997. **9**(3): p. 194-206.

27. Kofuji, P., N. Davidson, and H.A. Lester, *Evidence that Neuronal G-Protein-Gated Inwardly Rectifying K+ Channels are Activated by G{beta}{gamma} Subunits and Function as Heteromultimers.* Proceedings of the National Academy of Sciences, 1995. **92**(14): p. 6542-6546.

28. Ma, D., et al., *Diverse Trafficking Patterns Due to Multiple Traffic Motifs in G Protein-Activated Inwardly Rectifying Potassium Channels from Brain and Heart.* Neuron, 2002. **33**(5): p. 715-729.

29. Jelacic, T.M., et al., *Functional and biochemical evidence for G-protein-gated inwardly rectifying K+ (GIRK) channels composed of GIRK2 and GIRK3.* J Biol Chem, 2000. **275**(46): p. 36211-6.

30. Krapivinsky, G., et al., *The cardiac inward rectifier K+ channel subunit, CIR, does not comprise the ATP-sensitive K+ channel, IKATP.* J Biol Chem, 1995. **270**(48): p. 28777-9.

31. Krapivinsky, G., et al., *G beta gamma binds directly to the G protein-gated K+ channel, IKACh.* J Biol Chem, 1995. **270**(49): p. 29059-62.

32. Velimirovic, B.M., et al., *The K+ channel inward rectifier subunits form a channel similar to neuronal G protein-gated K+ channel.* FEBS Lett, 1996. **379**(1): p. 31-7.

33. Chan, K.W., et al., *Specific regions of heteromeric subunits involved in enhancement of G protein-gated K+ channel activity.* J Biol Chem, 1997. **272**(10): p. 6548-55.

34. Vivaudou, M., et al., *Probing the G-protein regulation of GIRK1 and GIRK4, the two subunits of the KACh channel, using functional homomeric mutants.* J Biol Chem, 1997. **272**(50): p. 31553-60.

35. Karschin, C., et al., *IRK(1-3) and GIRK(1-4) inwardly rectifying K+ channel mRNAs are differentially expressed in the adult rat brain.* J Neurosci, 1996. **16**(11): p. 3559-70.

36. Murer, G., et al., *An immunocytochemical study on the distribution of two G-protein-gated inward rectifier potassium channels (GIRK2 and GIRK4) in the adult rat brain.* Neuroscience, 1997. **80**(2): p. 345-357.

37. Drake, Carrie T., et al., *GIRK1 immunoreactivity is present predominantly in dendrites, dendritic spines, and somata in the CA1 region of the hippocampus.* Proceedings of the National Academy of Sciences, 1997. **94**(3): p. 1007-1012.

38. Kennedy, M.E., et al., *GIRK4 confers appropriate processing and cell surface localization to G-protein-gated potassium channels.* J Biol Chem, 1999. **274**(4): p. 2571-82.

39. Brown, A.M., et al., *Gating of atrial muscarinic K+ channels by G proteins.* Prog Clin Biol Res, 1990. **334**: p. 303-12.

40. North, R.A., *Twelfth Gaddum memorial lecture. Drug receptors and the inhibition of nerve cells.* Br J Pharmacol, 1989. **98**(1): p. 13-28.

41. Wickman, K.D., et al., *Recombinant G-protein βγ-subunits activate the muscarinic-gated atrial potassium channel.* Nature, 1994. **368**(6468): p. 255-257.

42. Reuveny, E., et al., *Activation of the cloned muscarinic potassium channel by G protein βγ subunits.* Nature, 1994. **370**: p. 143-146.

43. Reuveny, E., et al., *Activation of the cloned muscarinic potassium channel by G protein beta gamma subunits.* Nature, 1994. **370**(6485): p. 143-6.

44. Clancy, S., et al., *Pertussis-toxin-sensitive Gα subunits selectively bind to C-terminal domain of neuronal GIRK channels: evidence for a heterotrimeric G-protein-channel complex.* Molecular Cellular Neuroscience, 2005. **28**(2): p. 375-389.

45. Zhang, J., et al., *Creating new fluorescent probes for cell biology.*Nat Rev Mol Cell Biol, 2002. **3**(12): p. 906-918.

46. Benians, A., et al., *Regulators of G-protein Signaling Form a Quaternary Complex with the Agonist, Receptor, and G-protein: A NOVEL EXPLANATION FOR THE ACCELERATION OF SIGNALING ACTIVATION KINETICS.* J. Biol. Chem., 2005. **280**(14): p. 13383-13394.

47. Clancy, S.M., et al., *Pertussis-toxin-sensitive G[alpha] subunits selectively bind to C-terminal domain of neuronal GIRK channels: evidence for a heterotrimeric G-protein-channel complex.* Molecular and Cellular Neuroscience, 2005. **28**(2): p. 375-389.

48. Lodish, H.F., et al., *A vesicular intermediate in the transport of hepatoma secretory proteins from the rough endoplasmic reticulum to the Golgi complex.* J. Cell Biol., 1987. **104**(2): p. 221-230.

49. Lundmark, R. and S.R. Carlsson, *The beta-appendages of the four adaptor-protein (AP) complexes: structure and binding properties, and identification of sorting nexin 9 as an accessory protein to AP-2.* Biochem J, 2002. **362**(Pt 3): p. 597-607.

50. Ma, D., et al., *Role of ER Export Signals in Controlling Surface Potassium Channel Numbers.* Science, 2001. **291**(5502): p. 316-319.

51. Stockklausner, C., et al., *A sequence motif responsible for ER export and surface expression of Kir2.0 inward rectifier K+ channels.* FEBS Letters, 2001. **493**(2-3): p. 129-133.

52. Kim, E. and M. Sheng, *PDZ domain proteins of synapses.* Nat Rev Neurosci, 2004. **5**(10): p. 771-781.

53. Morgan Sheng, M.W., *Ion channel targeting in neurons.* BioEssays, 1997. **19**(10): p. 847-853.

54. Craven, S.E. and D.S. Bredt, *PDZ Proteins Organize Synaptic Signaling Pathways.* Cell, 1998. **93**(4): p. 495-498.

55. Fujita, A. and Y. Kurachi, *SAP Family Proteins.* Biochemical and Biophysical Research Communications, 2000. **269**(1): p. 1-6.

56. Blackstone, C. and M. Sheng, *Protein targeting and calcium signaling microdomains in neuronal cells.* Cell Calcium, 1999. **26**(5): p. 181-192.

57. Tezuka, T., et al., *PSD-95 promotes Fyn-mediated tyrosine phosphorylation of the N-methyl-D-aspartate receptor subunit NR2A.* Proceedings of the National Academy of Sciences, 1999. **96**(2): p. 435-440.

58. Wyszynski, M., et al., *Competitive binding of [alpha]-actinin and calmodulin to the NMDA receptor.* Nature, 1997. **385**(6615): p. 439-442.

59. Naisbitt, S., et al., *Shank, a Novel Family of Postsynaptic Density Proteins that Binds to the NMDA Receptor/PSD-95/GKAP Complex and Cortactin.* Neuron, 1999. **23**(3): p. 569-582.

60. Sheng, M. and E. Kim, *The Shank family of scaffold proteins.* J Cell Sci, 2000. **113**(11): p. 1851-1856.

61. Tu, J.C., et al., *Coupling of mGluR/Homer and PSD-95 Complexes by the Shank Family of Postsynaptic Density Proteins.* Neuron, 1999. **23**(3): p. 583-592.

62. Brenman, J.E., et al., *Cloning and Characterization of Postsynaptic Density 93, a Nitric Oxide Synthase Interacting Protein.* J. Neurosci., 1996. **16**(23): p. 7407-7415.

63. Kim, J.H., et al., *SynGAP: a Synaptic RasGAP that Associates with the PSD-95/SAP90 Protein Family.* Neuron, 1998. **20**(4): p. 683-691.

64. Zhang, W., et al., *Citron Binds to PSD-95 at Glutamatergic Synapses on Inhibitory Neurons in the Hippocampus.* J. Neurosci., 1999. **19**(1): p. 96-108.

65. Horio, Y., et al., *Clustering and Enhanced Activity of an Inwardly Rectifying Potassium Channel, Kir4.1, by an Anchoring Protein, PSD-95/SAP90.* J. Biol. Chem., 1997. **272**(20): p. 12885-12888.

66. Hibino, H., et al., *Anchoring proteins confer G protein sensitivity to an inward-rectifier K(+) channel through the GK domain.* Embo J, 2000. **19**(1): p. 78-83.

67. Nehring, R.B., et al., *Neuronal Inwardly Rectifying K+ Channels Differentially Couple to PDZ Proteins of the PSD-95/SAP90 Family.* J. Neurosci., 2000. **20**(1): p. 156-162.

68. Inanobe, A., et al., *Characterization of G-Protein-Gated K+ Channels Composed of Kir3.2 Subunits in Dopaminergic Neurons of the Substantia Nigra.* J. Neurosci., 1999. **19**(3): p. 1006-1017.

69. Daro, E., et al., *Rab4 and cellubrevin define different early endosome populations on the pathway of transferrin receptor recycling.* Proceedings of the National Academy of Sciences, 1996. **93**(18): p. 9559-9564.

70. Worby, C.A. and J.E. Dixon, *Sorting out the cellular functions of sorting nexins.* Nat Rev Mol Cell Biol, 2002. **3**(12): p. 919-931.

71. Gillooly, D.J., et al., *Localization of phosphatidylinositol 3-phosphate in yeast and mammalian cells.* Embo J, 2000. **19**(17): p. 4577-88.

72. Carlton, J., et al., *Sorting Nexins - Unifying Trends and New Perspectives.* Traffic, 2005. **6**(2): p. 75-82.

73. Kurten, R.C., D.L. Cadena, and G.N. Gill, *Enhanced Degradation of EGF Receptors by a Sorting Nexin, SNX1.* Science, 1996. **272**(5264): p. 1008-1010.

74. Wang, Y., et al., *Down-Regulation of Protease-activated Receptor-1 Is Regulated by Sorting Nexin 1.* Mol. Biol. Cell, 2002. **13**(6): p. 1965-1976.

75. Chakrabarti, S., M. Oppermann, and A.R. Gintzler, *Chronic morphine induces the concomitant phosphorylation and altered association of multiple signaling proteins: a novel mechanism for modulating cell signaling.* Proc Natl Acad Sci U S A, 2001. **98**(7): p. 4209-14.

76. Lundmark, R. and S.R. Carlsson, *Sorting nexin 9 participates in clathrin-mediated endocytosis through interactions with the core components.* J Biol Chem, 2003. **278**(47): p. 46772-81.

77. Kajii, Y., et al., *A developmentally regulated and psychostimulant-inducible novel rat gene mrt1 encoding PDZ-PX proteins isolated in the neocortex.* Mol Psychiatry, 2003. **8**(4): p. 434-44.

78. Kajii, Y., et al., *A developmentally regulated and psychostimulant-inducible novel rat gene mrt1 encoding PDZ-PX proteins isolated in the neocortex.* Mol Psychiatry. **8**(4): p. 434-444.

79. Joubert, L., et al., *New sorting nexin (SNX27) and NHERF specifically interact with the 5-HT4a receptor splice variant: roles in receptor targeting.* J Cell Sci, 2004. **117**(Pt 22): p. 5367-79.

80. Young, R.F., et al., *Release of beta-endorphin and methionine-enkephalin into cerebrospinal fluid during deep brain stimulation for chronic pain. Effects of stimulation locus and site of sampling.* J Neurosurg, 1993. **79**(6): p. 816-25.

81. Pasquini, F., et al., *Electron microscopic localization of photoaffinity-labelled delta opioid receptors in the neostriatum of the rat.* J Comp Neurol, 1992. **326**(2): p. 229-44.

82. Day, R., et al., *Prodynorphin gene expression in the rat intermediate pituitary lobe: gender differences and postpartum regulation.* Endocrinology, 1993. **133**(6): p. 2652-9.

83. Simon, E.J., *In search of the opiate receptor.* Am J Med Sci, 1973. **266**(3): p. 160-8.

84. Simon, E.J., J.M. Hiller, and I. Edelman, *Stereospecific binding of the potent narcotic analgesic (3H) Etorphine to rat-brain homogenate.* Proc Natl Acad Sci U S A, 1973. **70**(7): p. 1947-9.

85. Pert, C.B., G. Pasternak, and S.H. Snyder, *Opiate agonists and antagonists discriminated by receptor binding in brain.* Science, 1973. **182**(119): p. 1359-61.

86. Pert, C.B. and S.H. Snyder, *Properties of opiate-receptor binding in rat brain.* Proc Natl Acad Sci U S A, 1973. **70**(8): p. 2243-7.

87. Pert, C.B. and S.H. Snyder, *Opiate receptor: demonstration in nervous tissue.* Science, 1973. **179**(77): p. 1011-4.

88. Terenius, L., *Stereospecific uptake of narcotic analgesics by a subcellular fraction of the guinea-pig ileum. A preliminary communication.* Ups J Med Sci, 1973. **78**(3): p. 150-2.

89. Terenius, L., *Stereospecific interaction between narcotic analgesics and a synaptic plasm a membrane fraction of rat cerebral cortex.* Acta Pharmacol Toxicol (Copenh), 1973. **32**(3): p. 317-20.

90. Terenius, L., *Characteristics of the "receptor" for narcotic analgesics in synaptic plasma membrane fraction from rat brain.* Acta Pharmacol Toxicol (Copenh), 1973. **33**(5): p. 377-84.

91. Besse, D., et al., *The use of (3H) DAGO to label projections of thin primary afferent fibres at the superficial dorsal horn level of the rat spinal cord.* Prog Clin Biol Res, 1990. **328**: p. 179-82.

92. Besse, D., et al., *Pre- and postsynaptic location of mu, delta and kappa opioid receptors in the superficial layers of the dorsal horn of the rat spinal cord.* Prog Clin Biol Res, 1990. **328**: p. 183-6.

93. Hughes, J., et al., *Purification and properties of enkephalin - the possible endogenous ligand for the morphine receptor.* Life Sci, 1975. **16**(12): p. 1753-8.

94. Hughes, J., et al., *Identification of two related pentapeptides from the brain with potent opiate agonist activity.* Nature, 1975. **258**(5536): p. 577-80.

95. Kieffer, B.L., *Recent advances in molecular recognition and signal transduction of active peptides: receptors for opioid peptides.* Cell Mol Neurobiol, 1995. **15**(6): p. 615-35.

96. Goldstein, A. and A. Naidu, *Multiple opioid receptors: ligand selectivity profiles and binding site signatures.* Mol Pharmacol, 1989. **36**(2): p. 265-72.

97. Stengaard-Pedersen, K., K. Fredens, and L.I. Larsson, *Comparative localization of enkephalin and cholecystokinin immunoreactivities and heavy metals in the hippocampus.* Brain Research, 1983. **273**(1): p. 81-96.

98. Conner-Kerr, T.A., et al., *Evidence for the Corelease of Dynorphin and Glutamate from Rat Hippocampal Mossy Fiber Terminals.* Journal of Neurochemistry, 1993. **61**(2): p. 627-636.

99. Mansour, A., et al., *Autoradiographic differentiation of mu, delta, and kappa opioid receptors in the rat forebrain and midbrain.* J. Neurosci., 1987. **7**(8): p. 2445-2464.

100. Zieglgansberger, W., et al., *Opioid peptides may excite hippocampal pyramidal neurons by inhibiting adjacent inhibitory interneurons.* Science, 1979. **205**(4404): p. 415-7.

101. Simmons, M.L. and C. Chavkin, *Endogenous opioid regulation of hippocampal function.* Int Rev Neurobiol, 1996. **39**: p. 145-96.

102. Breindl, A., et al., *Opioid receptor-dependent long-term potentiation at the lateral perforant path-CA3 synapse in rat hippocampus.* Brain Res Bull, 1994. **33**(1): p. 17-24.

103. Derrick, B.E. and J.L. Martinez, Jr., *Frequency-dependent associative long-term potentiation at the hippocampal mossy fiber-CA3 synapse.* Proc Natl Acad Sci U S A, 1994. **91**(22): p. 10290-4.

104. Derrick, B.E. and J.L. Martinez, Jr., *Opioid receptor activation is one factor underlying the frequency dependence of mossy fiber LTP induction.* J Neurosci, 1994. **14**(7): p. 4359-67.

105. Bramham, C.R. and J.M. Sarvey, *Endogenous activation of mu and delta-1 opioid receptors is required for long-term potentiation induction in the lateral perforant path: dependence on GABAergic inhibition.* J Neurosci, 1996. **16**(24): p. 8123-31.

106. Matthies, H., et al., *Lack of expression of long-term potentiation in the dentate gyrus but not in the CA1 region of the hippocampus of mu-opioid receptor-deficient mice.* Neuropharmacology, 2000. **39**(6): p. 952-60.

107. Jamot, L., et al., *Differential involvement of the mu and kappa opioid receptors in spatial learning.* Genes Brain Behav, 2003. **2**(2): p. 80-92.

108. Jang, C.G., et al., *Impaired water maze learning performance in mu-opioid receptor knockout mice.* Brain Res Mol Brain Res, 2003. **117**(1): p. 68-72.

109. Sharma, S.K., W.A. Klee, and M. Nirenberg, *Opiate-dependent modulation of adenylate cyclase.* Proc Natl Acad Sci U S A, 1977. **74**(8): p. 3365-9.

110. Hescheler, J., et al., *The GTP-binding protein, Go, regulates neuronal calcium channels.* Nature, 1987. **325**(6103): p. 445-7.

111. Surprenant, A., et al., *Inhibition of calcium currents by noradrenaline, somatostatin and opioids in guinea-pig submucosal neurones.* J Physiol, 1990. **431**: p. 585-608.

112. North, R.A., et al., *Mu and delta receptors belong to a family of receptors that are coupled to potassium channels.* Proc Natl Acad Sci U S A, 1987. **84**(15): p. 5487-91.

113. Jin, W., et al., *Dual excitatory and inhibitory effects of opioids on intracellular calcium in neuroblastoma x glioma hybrid NG108-15 cells.* Mol Pharmacol, 1992. **42**(6): p. 1083-9.

114. Lefkowitz, R.J., *G protein-coupled receptors. III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization.* J Biol Chem, 1998. **273**(30): p. 18677-80.

115. Pei, G., et al., *Agonist-dependent phosphorylation of the mouse delta-opioid receptor: involvement of G protein-coupled receptor kinases but not protein kinase C.* Mol Pharmacol, 1995. **48**(2): p. 173-7.

116. Arden, J.R., et al., *Phosphorylation and agonist-specific intracellular trafficking of an epitope-tagged mu-opioid receptor expressed in HEK 293 cells.* J Neurochem, 1995. **65**(4): p. 1636-45.

117. Appleyard, S.M., et al., *Agonist-induced phosphorylation of the kappa-opioid receptor.* J Neurochem, 1997. **69**(6): p. 2405-12.

118. Kovoor, A., et al., *Mu and delta opioid receptors are differentially desensitized by the coexpression of beta-adrenergic receptor kinase 2 and beta-arrestin 2 in xenopus oocytes.* J Biol Chem, 1997. **272**(44): p. 27605-11.

119. Koch, T., et al., *Site Mutation in the Rat μ-Opioid Receptor Demonstrates the Involvement of Calcium/Calmodulin-Dependent Protein Kinase II in Agonist-Mediated Desensitization.* Journal of Neurochemistry, 1997. **69**(4): p. 1767-1770.

120. Mestek, A., et al., *The human mu opioid receptor: modulation of functional desensitization by calcium/calmodulin-dependent protein kinase and protein kinase C.* J. Neurosci., 1995. **15**(3): p. 2396-2406.

121. Lohse, M.J., et al., *G-protein-coupled receptor kinases.* Kidney Int, 1996. **49**(4): p. 1047-52.

122. Ball, A.J. and S. Ferguson, *Analgesia and analgesic drugs in paediatrics.* Br J Hosp Med, 1996. **55**(9): p. 586-90.

123. Koenig, J.A. and J.M. Edwardson, *Endocytosis and recycling of G protein-coupled receptors.* Trends Pharmacol Sci, 1997. **18**(8): p. 276-87.

124. Law, P.Y., D.S. Hom, and H.H. Loh, *Down-regulation of opiate receptor in neuroblastoma x glioma NG108-15 hybrid cells. Chloroquine promotes accumulation of tritiated enkephalin in the lysosomes.* J Biol Chem, 1984. **259**(7): p. 4096-104.

125. Whistler, J.L., et al., *Functional Dissociation of [mu] Opioid Receptor Signaling and Endocytosis: Implications for the Biology of Opiate Tolerance and Addiction.* Neuron, 1999. **23**(4): p. 737-746.

126. Whistler, J.L. and M. von Zastrow, *Dissociation of Functional Roles of Dynamin in Receptor-mediated Endocytosis and Mitogenic Signal Transduction.* J. Biol. Chem., 1999. **274**(35): p. 24575-24578.

127. Tsao, P. and M. von Zastrow, *Downregulation of G protein-coupled receptors.* Curr Opin Neurobiol, 2000. **10**(3): p. 365-9.

128. Sim-Selley, L.J., et al., *Chronic heroin self-administration desensitizes mu opioid receptor-activated G-proteins in specific regions of rat brain.* J Neurosci, 2000. **20**(12): p. 4555-62.

129. Chang, K.J., R.W. Eckel, and S.G. Blanchard, *Opioid peptides induce reduction of enkephalin receptors in cultured neuroblastoma cells.* Nature, 1982. **296**(5856): p. 446-8.

130. Tao, P., *[Clinical study of 8 cases of chronic idiopathic effusive pericarditis].* Zhonghua Xin Xue Guan Bing Za Zhi, 1982. **10**(4): p. 262-4.

131. Tao, P.L., et al., *Immunohistochemical evidence of down-regulation of mu-opioid receptor after chronic PL-017 in rats.* Eur J Pharmacol, 1998. **344**(2-3): p. 137-42.

132. Zadina, J.E., et al., *Mu opiate receptor down-regulation by morphine and up-regulation by naloxone in SH-SY5Y human neuroblastoma cells.* J Pharmacol Exp Ther, 1993. **265**(1): p. 254-62.

133. Zadina, J.E., et al., *Differential regulation of mu and delta opiate receptors by morphine, selective agonists and antagonists and differentiating agents in SH-SY5Y human neuroblastoma cells.* J Pharmacol Exp Ther, 1994. **270**(3): p. 1086-96.

134. Bernstein, M.A. and S.P. Welch, *mu-Opioid receptor down-regulation and cAMP-dependent protein kinase phosphorylation in a mouse model of chronic morphine tolerance.* Brain Res Mol Brain Res, 1998. **55**(2): p. 237-42.

135. Law, P.Y., D.S. Hom, and H.H. Loh, *Opiate receptor down-regulation and desensitization in neuroblastoma X glioma NG108-15 hybrid cells are two separate cellular adaptation processes.* Mol Pharmacol, 1983. **24**(3): p. 413-24.

136. Werling, L.L., P.N. McMahon, and B.M. Cox, *Selective changes in mu opioid receptor properties induced by chronic morphine exposure.* Proc Natl Acad Sci U S A, 1989. **86**(16): p. 6393-7.

137. Tao, P.L., P.Y. Law, and H.H. Loh, *Decrease in delta and mu opioid receptor binding capacity in rat brain after chronic etorphine treatment.* J Pharmacol Exp Ther, 1987. **240**(3): p. 809-16.

138. Haberstock-Debic, H., et al., *Morphine acutely regulates opioid receptor trafficking selectively in dendrites of nucleus accumbens neurons.* J Neurosci, 2003. **23**(10): p. 4324-32.

139. Sharma, S.K., W.A. Klee, and M. Nirenberg, *Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance.* Proc Natl Acad Sci U S A, 1975. **72**(8): p. 3092-6.

140. Sharma, S.K., M. Nirenberg, and W.A. Klee, *Morphine receptors as regulators of adenylate cyclase activity.* Proc Natl Acad Sci U S A, 1975. **72**(2): p. 590-4.

141. Chakrabarti, S., et al., *Chronic morphine augments G(beta)(gamma)/Gs(alpha) stimulation of adenylyl cyclase: relevance to opioid tolerance.* Mol Pharmacol, 1998. **54**(4): p. 655-62.

142. Chakrabarti, S., et al., *Chronic morphine augments adenylyl cyclase phosphorylation: relevance to altered signaling during tolerance/dependence.* Mol Pharmacol, 1998. **54**(6): p. 949-53.

143. Terwilliger, R.Z., et al., *A general role for adaptations in G-proteins and the cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal function.* Brain Res, 1991. **548**(1-2): p. 100-10.

144. Duman, R.S., J.F. Tallman, and E.J. Nestler, *Acute and chronic opiate-regulation of adenylate cyclase in brain: specific effects in locus coeruleus.* J Pharmacol Exp Ther, 1988. **246**(3): p. 1033-9.

145. McLawhon, R.W., J.C. Ellory, and G. Dawson, *Molecular size of opiate (enkephalin) receptors in neuroblastoma-glioma hybrid cells as determined by radiation inactivation analysis.* J Biol Chem, 1983. **258**(4): p. 2102-5.

146. Glasel, J.A., *Physical characterization of native opiate receptors. Additional information from detailed binding analysis of a radiation-inactivated receptor.* Biochim Biophys Acta, 1987. **930**(2): p. 201-8.

147. Otte, A.P., et al., *Production and turnover of cAMP signals by prestalk and prespore cells in Dictyostelium discoideum cell aggregates.* Differentiation, 1986. **32**(3): p. 185-91.

148. Simon, E.J., *Progress in the characterization of the opioid receptor subtypes: peptides as probes. Future directions.* NIDA Res Monogr, 1986. **70**: p. 155-74.

149. Cvejic, S. and L.A. Devi, *Dimerization of the delta Opioid Receptor:. IMPLICATION FOR A ROLE IN RECEPTOR INTERNALIZATION.* J. Biol. Chem., 1997. **272**(43): p. 26959-26964.

150. George, S.R., et al., *Oligomerization of {micro}- and delta -Opioid Receptors. GENERATION OF NOVEL FUNCTIONAL PROPERTIES.* J. Biol. Chem., 2000. **275**(34): p. 26128-26135.

151. Jordan, B.A. and L.A. Devi, *G-protein-coupled receptor heterodimerization modulates receptor function.* Nature, 1999. **399**(6737): p. 697-700.

152. McVey, M., et al., *Monitoring Receptor Oligomerization Using Time-resolved Fluorescence Resonance Energy Transfer and Bioluminescence Resonance Energy Transfer. THE HUMAN DELTA -OPIOID RECEPTOR DISPLAYS CONSTITUTIVE OLIGOMERIZATION AT THE CELL SURFACE, WHICH IS NOT REGULATED BY RECEPTOR OCCUPANCY.* J. Biol. Chem., 2001. **276**(17): p. 14092-14099.

153. Ramsay, D., et al., *Homo- and hetero-oligomeric interactions between G-protein-coupled receptors in living cells monitored by two variants of bioluminescence resonance energy transfer (BRET): hetero-oligomers between receptor subtypes form more efficiently than between less closely related sequences.* Biochem. J., 2002. **365**(2): p. 429-440.

154. He, L., et al., *Regulation of Opioid Receptor Trafficking and Morphine Tolerance by Receptor Oligomerization.* Cell, 2002. **108**(2): p. 271-282.

155. Gomes, I., et al., *Oligomerization of opioid receptors.* Methods, 2002. **27**(4): p. 358-365.

156. Gomes, I., et al., *Heterodimerization of {micro} and delta Opioid Receptors: A Role in Opiate Synergy.* J. Neurosci., 2000. **20**(22): p. 110RC-.

157. Rothman, R.B. and T.C. Westfall, *Allosteric modulation by leucine-enkephalin of [3H]naloxone binding in rat brain.* Eur J Pharmacol, 1981. **72**(4): p. 365-8.

158. Rothman, R. and T.C. Westfall, *Further evidence for an opioid receptor complex.* J Neurobiol, 1983. **14**(5): p. 341-51.

159. Malmberg, A.B. and T.L. Yaksh, *Isobolographic and dose-response analyses of the interaction between intrathecal mu and delta agonists: effects of naltrindole and its benzofuran analog (NTB).* J Pharmacol Exp Ther, 1992. **263**(1): p. 264-75.

160. Heyman, J.S. and F. Porreca, *Evidence for opioid receptor interactions in vivo.* Proc West Pharmacol Soc, 1988. **31**: p. 287-9.

161. Heyman, J.S., et al., *Can supraspinal delta-opioid receptors mediate antinociception?* Trends Pharmacol Sci, 1988. **9**(4): p. 134-8.

162. Schulman, H., *The multifunctional Ca2+/calmodulin-dependent protein kinases.* Curr Opin Cell Biol, 1993. **5**(2): p. 247-53.

163. Schulman, H. and P.I. Hanson, *Multifunctional Ca2+/calmodulin-dependent protein kinase.* Neurochem Res, 1993. **18**(1): p. 65-77.

164. Erondu, N.E. and M.B. Kennedy, *Regional distribution of type II Ca2+/calmodulin-dependent protein kinase in rat brain.* J Neurosci, 1985. **5**(12): p. 3270-7.

165. Hanson, P.I. and H. Schulman, *Inhibitory autophosphorylation of multifunctional Ca2+/calmodulin-dependent protein kinase analyzed by site-directed mutagenesis.* J Biol Chem, 1992. **267**(24): p. 17216-24.

166. Bortolotto, Z.A. and G.L. Collingridge, *Involvement of calcium/calmodulin-dependent protein kinases in the setting of a molecular switch involved in hippocampal LTP.* Neuropharmacology, 1998. **37**(4-5): p. 535-44.

167. Braun, A.P. and H. Schulman, *The multifunctional calcium/calmodulin-dependent protein kinase: from form to function.* Annu Rev Physiol, 1995. **57**: p. 417-45.

168. Mayford, M., et al., *Control of memory formation through regulated expression of a CaMKII transgene.* Science, 1996. **274**(5293): p. 1678-83.

169. De Koninck, P. and H. Schulman, *Sensitivity of CaM kinase II to the frequency of Ca2+ oscillations.* Science, 1998. **279**(5348): p. 227-30.

170. Malinow, R., H. Schulman, and R.W. Tsien, *Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP.* Science, 1989. **245**(4920): p. 862-866.

171. Tan, S.E., R.J. Wenthold, and T.R. Soderling, *Phosphorylation of AMPA-type glutamate receptors by calcium/calmodulin- dependent protein kinase II and protein kinase C in cultured hippocampal neurons.* J. Neurosci., 1994. **14**(3): p. 1123-1129.

172. Silva, A.J., et al., *Impaired spatial learning in alpha-calcium-calmodulin kinase II mutant mice.* Science, 1992. **257**(5067): p. 206-211.

173. Silva, A.J., et al., *Deficient hippocampal long-term potentiation in alpha-calcium-calmodulin kinase II mutant mice.* Science, 1992. **257**(5067): p. 201-206.

174. Xie, C.W. and D.V. Lewis, *Opioid-mediated facilitation of long-term potentiation at the lateral perforant path-dentate granule cell synapse.* J Pharmacol Exp Ther, 1991. **256**(1): p. 289-96.

175. Bramham, C.R., *Opioid receptor dependent long-term potentiation: peptidergic regulation of synaptic plasticity in the hippocampus.* Neurochem Int, 1992. **20**(4): p. 441-55.

176. Lou, L., et al., *Modulation of Ca2+/Calmodulin-Dependent Protein Kinase II Activity by Acute and Chronic Morphine Administration in Rat Hippocampus: Differential Regulation of alpha and beta Isoforms.* Mol Pharmacol, 1999. **55**(3): p. 557-563.

177. Fan, G.-H., et al., *Inhibition of Calcium/Calmodulin-Dependent Protein Kinase II in Rat Hippocampus Attenuates Morphine Tolerance and Dependence.* Mol Pharmacol, 1999. **56**(1): p. 39-45.

178. Narita, M., et al., *Role of the calcium/calmodulin-dependent protein kinase ii (CaMKII) in the morphine-induced pharmacological effects in the mouse.* Neuroscience, 2004. **126**(2): p. 415-421.

179. Brüggemann, I., et al., *Colocalization of the [mu]-opioid receptor and calcium/calmodulin-dependent kinase II in distinct pain-processing brain regions.* Molecular Brain Research, 2000. **85**(1-2): p. 239-250.

180. Heagy, W., M.A. Shipp, and R.W. Finberg, *Opioid receptor agonists and Ca2+ modulation in human B cell lines.* J Immunol, 1992. **149**(12): p. 4074-81.

181. Connor, M., A. Yeo, and G. Henderson, *The effect of nociceptin on Ca2+ channel current and intracellular Ca2+ in the SH-SY5Y human neuroblastoma cell line.* Br J Pharmacol, 1996. **118**(2): p. 205-7.

182. Zimprich, A., T. Simon, and V. Hollt, *Transfected rat mu opioid receptors (rMOR1 and rMOR1B) stimulate phospholipase C and Ca2+ mobilization.* Neuroreport, 1995. **7**(1): p. 54-6.

183. Wandless, A.L., D. Smart, and D.G. Lambert, *Fentanyl increases intracellular Ca2+ concentrations in SH-SY5Y cells.* Br J Anaesth, 1996. **76**(3): p. 461-3.

184. Smart, D. and D.G. Lambert, *delta-Opioids stimulate inositol 1,4,5-trisphosphate formation, and so mobilize Ca2+ from intracellular stores, in undifferentiated NG108-15 cells.* J Neurochem, 1996. **66**(4): p. 1462-7.

185. MacNicol, M. and H. Schulman, *Multiple Ca2+ signaling pathways converge on CaM kinase in PC12 cells.* FEBS Letters, 1992. **304**(2-3): p. 237-240.

186. Fan, G.H., et al., *Modulation by calcium/calmodulin-dependent protein kinase II of functional response of delta opioid receptor in neuroblastoma x glioma hybrid (NG108-15) cells.* Neuropharmacology, 1997. **36**(11-12): p. 1763-1769.

187. Zhong, W., et al., *Opiate withdrawal induces dynamic expressions of AMPA receptors and its regulatory molecule CaMKII[alpha] in hippocampal synapses.* Life Sciences, 2006. **79**(9): p. 861-869.

188. Hille, B., *Modulation of ion-channel function by G-protein-coupled receptors.* Trends in Neurosciences, 1994. **17**(12): p. 531-536.

189. Samways, D.S. and G. Henderson, *Opioid elevation of intracellular free calcium: possible mechanisms and physiological relevance.* Cell Signal, 2006. **18**(2): p. 151-61.

190. Samways, D.S., et al., *Co-incident signalling between mu-opioid and M3 muscarinic receptors at the level of Ca2+ release from intracellular stores: lack of evidence for Ins(1,4,5)P3 receptor sensitization.* Biochem J, 2003. **375**(Pt 3): p. 713-20.

191. Yeo, A., et al., *Coincident signalling between the Gi/Go-coupled delta-opioid receptor and the Gq-coupled m3 muscarinic receptor at the level of intracellular free calcium in SH-SY5Y cells.* J Neurochem, 2001. **76**(6): p. 1688-700.

192. Okajima, F., H. Tomura, and Y. Kondo, *Enkephalin activates the phospholipase C/Ca2+ system through cross-talk between opioid receptors and P2-purinergic or bradykinin receptors in NG 108-15 cells. A permissive role for pertussis toxin-sensitive G-proteins.* Biochem J, 1993. **290 (Pt 1)**: p. 241-7.

193. Connor, M. and G. Henderson, *delta- and mu-opioid receptor mobilization of intracellular calcium in SH-SY5Y human neuroblastoma cells.* Br J Pharmacol, 1996. **117**(2): p. 333-40.

194. Werry, T.D., G.F. Wilkinson, and G.B. Willars, *Mechanisms of cross-talk between G-protein-coupled receptors resulting in enhanced release of intracellular Ca2+.* Biochem J, 2003. **374**(Pt 2): p. 281-96.

195. Pei, Q., et al., *Alteration in expression of g-protein-activated inward rectifier K+-channel subunits girk1 and girk2 in the rat brain following electroconvulsive shock.* Neuroscience, 1999. **90**(2): p. 621-627.

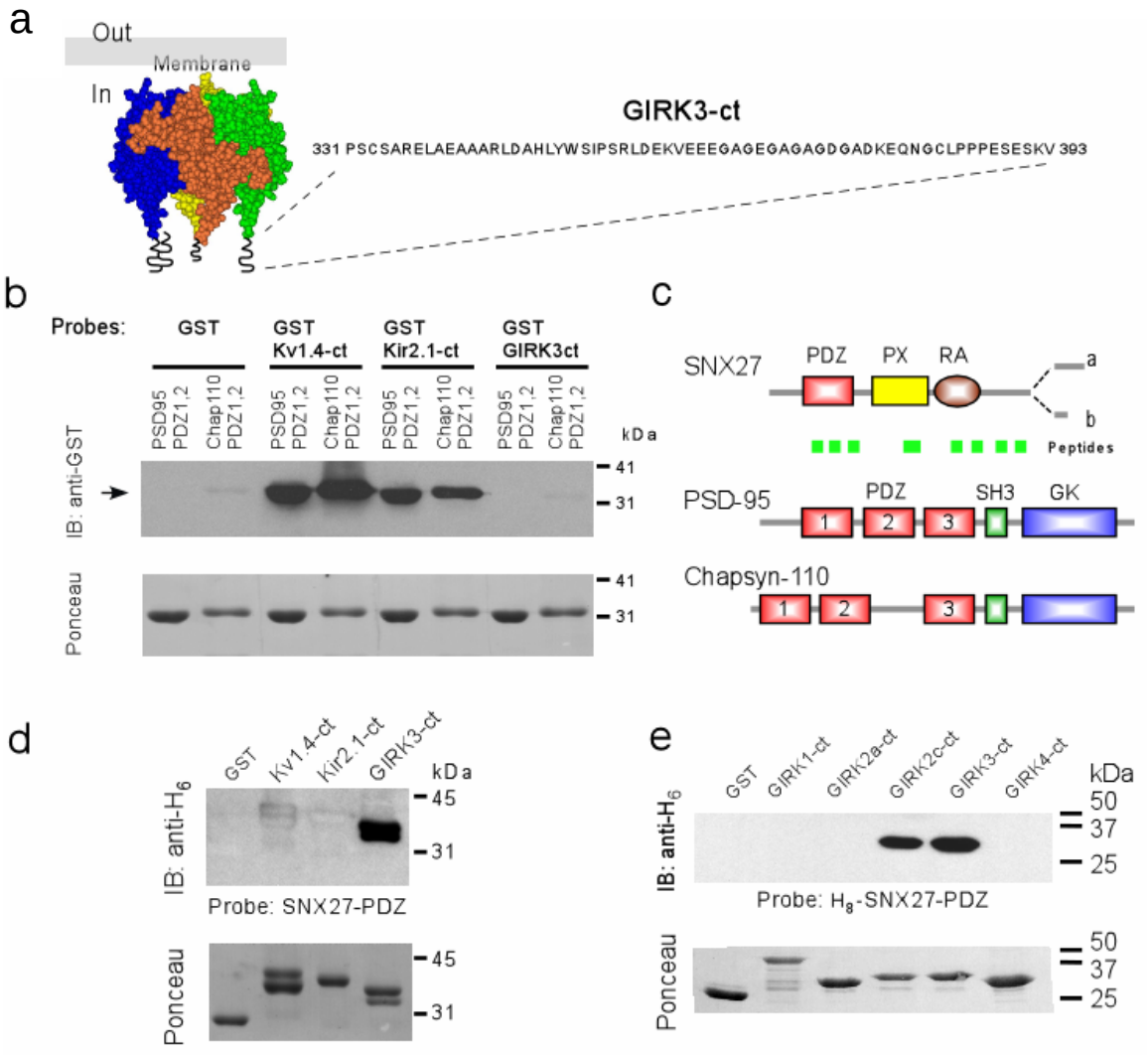
196. Huang, C.S., et al., *Common Molecular Pathways Mediate Long-Term Potentiation of Synaptic Excitation and Slow Synaptic Inhibition.* Cell, 2005. **123**(1): p. 105-118.

197. Kelly, M.J., J. Qiu, and O.K. Ronnekleiv, *Estrogen modulation of G-protein-coupled receptor activation of potassium channels in the central nervous system.* Ann N Y Acad Sci, 2003. **1007**: p. 6-16.

198. Sohn, J.W., et al., *Receptor-specific inhibition of GABAB-activated K+ currents by muscarinic and metabotropic glutamate receptors in immature rat hippocampus.* J Physiol, 2007. **580**(Pt. 2): p. 411-22.

199. Labouebe, G., et al., *RGS2 modulates coupling between GABAB receptors and GIRK channels in dopamine neurons of the ventral tegmental area.* Nat Neurosci, 2007. **10**(12): p. 1559-1568.

Fig 1.1. **A PDZ-containing sorting nexin, SNX27, associates with GIRK3 and GIRK2c channels.** A) Schematic shows structure of the GIRK1 cytoplasmic domains (Protein Data Base code 1N9P) with the hypothetical location of the flexible C-terminal tail indicated (solid lines). The sequence 331–393 of GIRK3 was fused to GST and used to search for novel GIRK3 binding proteins. B) GIRK3-ct binding to PDZ1,2 of PSD-95 or Chapsyn-110 is not detectable. Overlay binding assay performed with GST-fused C-terminal channel proteins and indicated H_8-tagged PDZ domains. GST-Kv1.4-ct and GST-Kir2.1-ct but not GST or GST-GIRK3-ct bind to PDZ1-2 domains of Chapsyn-110 or PSD-95. H_8-tagged proteins (2 μg) were loaded and probed with GST-fused proteins at 25 nM. *Top*, immunoblot using anti-GST antibody. *Bottom*, Ponceau S stain reveals equivalent levels of protein loaded on SDS-PAGE and transferred to nitrocellulose. C) SNX27 contains three domains; PDZ, PX and RA, and exists in two splice forms. Approximate positions of peptide fragments revealed by tandem mass spectrometry are shown below. PSD-95 and Chapsyn-100 are shown for comparison. D) SNX27 discriminates among channels with similar PDZ-binding motifs. The PDZ domain of SNX27 (H_8-SNX27-PDZ) binds to GST-GIRK3-ct but not to GST-Kir2.1-ct or GST-Kv1.4-ct, or GST alone. Nitrocellulose was probed with H_8-SNX27-PDZ (500 nM). *Top*, immunoblot using anti-H_6 antibody. Ponceau S stain reveals similar levels of loaded protein. E) SNX27 associates with a subset of GIRK channels that contain "–ESKV" in their C-terminus. H_8-SNX27-PDZ binds to GST-Kir3.2c-ct and GST-GIRK3-ct but not to C-terminal domain of GIRK1, GIRK2a or GIRK4.


a
Out
Membrane
In
GIRK3-ct
331 PSCSARELAEAAARLDAHLYWSIPSRLDEKVEEEGAGEGAGAGDGADKEQNGCLPPPESESKV 393
b
Probes:
GST
GST Kv1.4-ct
GST Kir2.1-ct
GST GIRK3ct
PSD95 PDZ1,2
Chap110 PDZ1,2
kDa
IB: anti-GST
41
31
Ponceau
c
SNX27
PDZ
PX
RA
a
b
Peptides
PSD-95
PDZ
SH3
GK
Chapsyn-110
d
GST
Kv1.4-ct
Kir2.1-ct
GIRK3-ct
IB: anti-H6
Probe: SNX27-PDZ
45
31
e
GST
GIRK1-ct
GIRK2a-ct
GIRK2c-ct
GIRK3-ct
GIRK4-ct
IB: anti-H6
Probe: H8-SNX27-PDZ
50
37
25

II. SNX27 regulates expression and intracellular trafficking of neuronal GIRK channels

Abstract

GIRK channels are important for controlling neuronal excitability in the brain and have been shown to play a role in learning, analgesia and drug addiction. Little is known about the cell surface regulation of GIRK channels. Using a proteomics approach, the Slesinger lab recently discovered a unique type of intracellular protein, sorting nexin 27(SNX27), which associates with a subset of GIRK channels. Sorting nexins have been implicated in trafficking of proteins through endosomal compartments. The single PDZ domain of SNX27 binds directly and specifically to the PDZ binding motif of GIRK channels leading to reduced surface levels, increased degradation, and smaller currents. The regulation of endosomal trafficking via sorting nexins reveals a novel mechanism for controlling ion channel surface expression.

Introduction

GIRK channels underlie a slow inhibitory postsynaptic potential (sIPSP) in the brain (1). They have been shown to be important for analgesia (2), limiting hyperexcitability (3) as well as for regulating the response to abused drugs (4) and alcohol (5). There are four different mammalian GIRK channel subunits, GIRK1–4, which assemble into hetero or homo-tetramers (6). GIRK1 coassembles with other GIRK subunits to form functional channels (7) while GIRK2 and GIRK4 also assemble as

functional homo-tetramers (8,9). Three splice variants of GIRK2 (GIRK2a–2c) exist in the brain (10). Neuronal GIRK channels are expressed predominantly in a postsynaptic GPCR/GIRK signaling complex (1,11,12). Targeting and stabilization of GIRK channels in the post synaptic complex is a key but poorly understood step in regulating GIRK channel signaling.

GIRK channels are activated directly by G protein Gβγ dimers, after neurotransmitters, such as GABA and dopamine, bind to Gαi/o (pertussis-toxin sensitive) G protein-coupled receptors (13-16). In addition to the Gβγ dimer, the G□ subunit also interacts directly with the channel, bringing the Gβγ dimer closer to the channel and forming one of the main components in the GPCR/GIRK signaling complex (11,12). Variations in G protein availability or phosphorylation are the only known mechanisms for changing GIRK signaling (17-19).

A well-established mechanism for regulating the surface expression of ion channels is through alterations in channel trafficking. For example, glutamatergic NMDA (and AMPA) receptors and potassium channels, such as Kv1.4 and Kir2.1, are regulated by proteins in the postsynaptic density (PSD) complex that contain PDZ domains (named after PSD-95, discs large, zona occludens) (20). Currently, there are four different members of PSD or synapse-associated proteins (SAP); SAP-97, SAP-102, PSD-93 (Chapsyn-110), and PSD-95 (SAP-90)20. Elevations in PSD-95 can stabilize glutamate receptors in the plasma membrane (20). Alternatively, SAP-97 reduces surface levels of voltage-gated K channels (21). In the inwardly rectifying potassium channel family, Kir2 channels have a C-terminal PDZ-binding motif (ESEI) that interacts directly with SAP-97 (22) and PSD-93 (23), leading to increased stability of Kir2.1 on the cell surface. Both

GIRK2c (–ESKV) and GIRK3 (–ESKV) channels possess a PDZ binding motif, raising the possibility that PDZ-containing proteins might regulate GIRK channels. GIRK2c has been reported to be modulated by PSD-95 and SAP-97 but the association of these PSDs with Kir3.2c channels in native tissues is unclear (10,24,25). GIRK3 does not appear to associate with PSD-95 (24). The failure of the PDZ binding motif in GIRK2/3 channels to interact strongly with PDZ domain of PSD-95 has been perplexing given the similarity in the motif between GIRK3 and Kir2.1 channels (–ESKV vs. –ESEI). In this study, we used a proteomics approach to search for GIRK3 regulatory proteins and now describe the identification and characterization of a novel type of GIRK regulatory protein, sorting nexin 27 (SNX27). SNX27 contains one PDZ domain that appears to distinguish among GIRK3 and Kir2.1 channels and one PX domain that targets SNX27 and channels to the early endosomal pathway. The discovery that SNX27 regulates ion channels highlights an important pathway for regulating excitability in the brain.

Methods

The following channel constructs were used: SNX27-YFP (rat), GIRK1 (rat), GIRK2a (mouse), GIRK2c (mouse), GIRK3 (mouse) (7), GIRK3 lacking the last four amino acids (GIRK3-ΔC), GIRK3-Y350A (GIRK3-Lys*), HA-GIRK1 (HA tag, YPYDVPDYA, inserted between A115 and H116), HA-GIRK2c (52), and HA-GIRK3 (HA inserted between L92 and E93).

Transient transfections

HEK-293 cells were cultured in DMEM supplemented with fetal bovine serum (10%), glutamine (2mM), penicillin (50 Units/ml) and streptomycin (50 μg/ml) in a

humidified 37°C incubator with 95% air and 5% CO2. HEK293 cells were transfected by calcium phosphate precipitation with the following cDNAs: rat $GABA_B$ R1 and R2 receptors (0.08 μg); GIRK channels (0.2 μg); SNXb-27-myc, SNX27b-YFP or SNX27a-YFP (1μg); YFP (0.04 μg) for identifying transfected cells. The cDNA was mixed with 0.25 M $CaCl_2$ made from sterile deionized H2O into which HEPES buffered saline (280 mM NaCl, 10 mM KCl, 1.5 mM Na2HPO4, 12 mM glucose, 50 mM HEPES, pH 7.1) was added drop wise to a final volume of 50 μl per well of 500 μl cell culture. The transfection solution was added to the wells for 18-30 h for electrophysiology and for 48 h for immunocytochemistry.

Immunocytochemistry

Two days following transfection, cells were fixed for 10 min in in ice-cold methanol at -20 ˚C, permeabilized for 10 min in PBS buffer containing 0.25% Triton X-100, blocked in PBS buffer including 3% BSA for 30 min. The cells were incubated with the appropriate combination of primary antibody: anti-myc (A-14) 1:400 (Santa Cruz Biotechnology), anti-GIRK3/GIRK2/GIRK1 1:200, (Alomone), and anti-EEA1 1:400, (BD Biosciences) for 2 hours in PBS buffer containing 2% BSA, washed twice with PBS buffer followed by secondary antibody incubation for 1 hour using Alexa-fluorophore-conjugated anti-rabbit (1:400; for two-color studies rabbit-Alexa-647 for tricolor studies: rabbit-Alexa-568) or anti-mouse (1:400; Alexa-488 for two-color and Alexa-647 for tricolor studies) secondary antibodies (Molecular Probes). The cells were mounted onto glass slides with ProLong Gold (Molecular Probes) and imaged by laser confocal microscopy (63x, oil immersion objective, HeNe and Argon laser) using either the Zeiss LSM5 Pascal or a Leica TCS SP2 AOBS system. For comparison of images, the pinhole,

exposure time, resolution and gain was kept constant. The images were equally scaled in NIH ImageJ.

For surface labeling studies, HEK293 cells were transfected with HA-GIRK2c or HA-GIRK3, in which the HA (YPYDVPDYA) epitope is inserted in the extracellular loop at I126–E127 of GIRK2c and L92–E93 of GIRK3. Cells were washed once with DMEM and incubated with rabbit anti-HA (1:400 in DMEM, Covance) antibody at 4 ºC for 30 min ice. Cells were rapidly rinsed 3x with DMEM and fixed in 100% methanol for 10 min at –20 °C. After blocking non-specific binding with BSA, cells were incubated with anti rabbit secondary antibody conjugated to Alexa-488 (1:500, molecular probes) for 60 min at room temperature in the dark. Cells were washed with PBS and mounted to the microscope slide using Progold antifading reagent (Molecular Probes). The cells were analyzed by confocal fluorescence microscopy. The rabbit 488 conjugated antibody did not show any specific staining (data not shown).

Western blots

For measuring GIRK1 degradation, HEK293 cells were transiently transfected with HA-GIRK1, GIRK3, SNX27b-YFP, and $GABA_B$ R1/R2 receptors. Total protein was extracted 72 hr post transfection using Trizol (Invitrogen, CA). Equal amounts of protein were separated on 12% SDS-PAGE and transferred to nitrocellulose (Bio-Rad). Membranes were blocked overnight at 4˚ C in 5% nonfat dry milk in TBST (25 mM Tris pH 7.4, 150 mM NaCl, 2mM KCl, 0.05% Tween-20). Membranes were incubated with the rabbit anti-HA (1:500: Covance) or rabbit anti-pan actin (1:500: Cell Signaling) for 2 hours at room temperature with constant agitation. After washing, the blots were incubated with HRP-anti-rabbit secondary antibodies (1:5000 dilution) on shaker at RT

for 1 hour. Blots were then rinsed, incubated with SuperSignal ECL reagents (Pierce) and exposed (5–30 sec) to BioMax XAR film (Kodak).

Electrophysiology

HEK-293/T cells were plated onto 12-mm glass coverslips (Warner Instruments) coated with poly-D-lysine (20 μg ml-1, Sigma) and collagen (100 mg ml-1; BD Biosciences) in 24-well plates. Cells were transfected by calcium phosphate precipitation as described above. Whole-cell patch-clamp recordings were performed (55) using borosilicate glass electrodes (Warner, P6165T; 1–3 MΩ), coated with Sylgard and fire polished. Membrane currents were recorded with an Axopatch 200 or 200B (Axon Instruments) amplifier, adjusted electronically for cell capacitance and series resistance (80-100%), filtered at 2 kHz with an 8-pole Bessel filter, digitized at 5 kHz with a Digidata 1200 series interface (Axon Instruments), and stored on a laboratory computer. The extracellular solution contained (in mM): 140 NaCl, 20 KCl, 2 MgCl2, 0.5 CaCl2, and 10 HEPES/NaOH (pH 7.4). (±)-Baclofen (Sigma) was dissolved in water to make a 10 mM stock and then diluted directly in the extracellular solution. The intracellular solution contained (in mM): 20 NaCl, 130 KCl, 5.46 MgCl2, 10 HEPES/NaOH (pH 7.4), and 5 EGTA/KOH (pH 7.4). 2.56 mM K2ATP was added with 300 μM Li2GTP to the intracellular solution on day of recording. Data were acquired at room temperature (22–25 ºC) and analyzed with Clampfit 8.0 (Axon Instruments). Data are presented as mean ± standard error of mean (S.E.M) and evaluated for statistical significance using unpaired Student's t-test, where $P < 0.05$ was considered significant (SigmaStat 3.0).

Cultured hippocampal neurons and Immunohistochemistry

Preparation of the hippocampal cell cultures was according to published protocols (54) with minor modifications. Primary cultures of hippocampal neurons were obtained from 0-2-d-old Sprague Dawley rat pups. The hippocampus was isolated and dissociated with papain, and cells were plated at 50,000 cells cm^2 in Neurobasal-A medium supplemented with B27 and glutamax (Gibco). The media was replaced the day after plating, and cells were fed twice weekly thereafter. Hippocampal neurons were grown on glass coverslips (Warner Instruments) coated with poly-D-lysine (Sigma). All cells were grown at 37 °C and in 5% CO2. For transient transfection, neurons in culture at 3-7 DIV were incubated in Opti-MEM containing 1 μg cDNA plasmid and 2 μl Lipofectamine 2000 (Invitrogen) for 30 min at 37°C. After washing, neurons were fixed with -20°C methanol and permeabilized with 0.1% Triton X-100 in PBS. Neurons were incubated with rabbit anti-GIRK1 or anti-GIRK2 (1:200; Alomone) antibodies overnight at 4°C. We also attempted to use the GIRK3 antibody (Alomone), but unlike GIRK1 and GIRK2, we could not compete the signal with GIRK3 peptide, suggesting high background. Cells were washed and incubated with Alexa 647-conjugated secondary antibodies (Molecular Probes) for 1 hour at room temperature. The coverslips were mounted on microscope slide using Progold antifading reagent (Molecular Probes) and imaged by confocal fluorescence microscopy (Leica).

Results

SNX27 regulates endosomal trafficking of GIRK channels

Among PDZ-containing proteins, SNX27 is the only one, thus far, to contain a putative lipid-binding PX domain (20). The role of the PX domain in SNX27 is not

known. PX domains bind to different types of phosphoinositides, which direct their subcellular localization and endosomal protein trafficking (29). We investigated the ability of the PX domain of SNX27 to interact with different membrane phospholipids and found that it binds strongly to phosphatidyl-inositol-3- phosphate (PI(3)P). Sorting nexins that bind specifically to PI(3)P are enriched in the early Endosome (29). To investigate whether SNX27 is localized in the early endosome, we used an antibody raised against the early endosomal antigen-1 (EEA1) protein, a marker for this endosomal compartment. In transiently transfected HEK cells, YFP-tagged SNX27b, the splice variant regulated by methamphetamine (30), was distributed in puncta throughout the cytoplasm and co-localized with EEA1 (Fig. 1a). If PI(3)P binding to the PX domain of SNX27 is required for localization to the early endosome, then we expected the K220A mutation to alter the pattern of expression of SNX27b. Indeed, SNX27b-K220A-YFP now expressed uniformly throughout the cytoplasm and did not co-localize exclusively with EEA1 (Fig. 1b).

We hypothesized that the PX domain of SNX27 is important for regulating trafficking of GIRK3/GIRK2c channels in the endosomal pathway. Consistent with this, co-expression of GIRK3 with SNX27b resulted in co-localization in the early endosome, evident by small puncta containing SNX27b-YFP, GIRK3 and EEA1 (Fig. 1c). We next examined whether GIRK1 would also cluster with GIRK3 and SNX27b. In HEK293 cells coexpressing SNX27b-YFP, GIRK1 and GIRK3, small puncta were visible that contained all three proteins (Fig. 2a). To determine the functional consequence of SNX27 expression in the surface localization of GIRK channels, we used whole-cell patch-clamping on HEK cells transiently transfected with GIRK3 and GIRK1, which promotes

formation of heteromeric channels on the surface (7), along with $GABA_B$ R1 and R2 receptors. The $GABA_B$ receptor-activated GIRK1/GIRK3 currents were significantly reduced in HEK293 cells coexpressing SNX27b (Fig. 2b–c). A reduction in current could arise from a defect in gating or from expression of fewer channels on the plasma membrane surface. To examine the latter possibility, HEK293 cells were transfected with GIRK3 containing an extracellular HA tag, GIRK1 and SNX27b-YFP and surface channels detected by live antibody staining at 4º C. (Fig. 2d). The levels of HA-GIRK3 channels on the surface decreased significantly in cells coexpressing SNX27b (Fig. 2e).

To explore the role of the C-terminal PDZ-binding domain further, we measured the baclofen-induced currents in cells coexpressing GIRK1 and a mutant of GIRK3 lacking the last four amino acids (3.3ΔC). Surprisingly, the baclofen-induced currents were significantly smaller than with GIRK1/GIRK3 channels (Fig. 3a). Thus, removing the PDZ binding motif appeared to alter trafficking on its own, possibly allowing the lysosomal targeting motif to dominate the expression. To test this, we created a double mutant containing the C-terminal deletion and a mutation in the lysosomal targeting motif - YxxΦ (Y350A; lys*) (35). In contrast to GIRK1+GIRK3ΔC, large, baclofen-activated currents were now evident in cells expressing GIRK1+GIRK3-lys*ΔC (Fig. 3a), which were indistinguishable from GIRK1+GIRK3-lys*. These data suggest that altered targeting of GIRK3ΔC occurs from enhanced degradation. If the C-terminal PDZ binding motif is important for regulation by SNX27, then we predicted that the decrease in current would be abrogated in cells expressing GIRK3-lys*ΔC. Indeed, SNX27b-myc

coexpression did not significantly alter the amplitude of baclofen-activated currents in cells coexpressing GIRK3-lys*ΔC plus GIRK1 (Fig. 3a).

Because GIRK3 channels contain a lysosomal targeting motif (35) and coassembles with GIRK1, we wondered whether SNX27b promoted degradation of GIRK1 via GIRK3. We examined the levels of GIRK1 total protein ~3 days post transfection using Western blot analysis. In cells transfected with GIRK1/GIRK3 and SNX27b-YFP, the levels of total GIRK1 protein were consistently lower (Fig. 3b). Thus, the loss of GIRK current with coexpressed SNX27b results from decreased surface expression and subsequent degradation of GIRK channels.

Because GIRK1 lacks a PDZ binding motif, we predicted that GIRK1 expressed alone in HEK293 cells would not be affected by coexpressed SNX27. We transfected HEK cells with cDNA for GIRK1 and SNX27b and then carried out immunostaining for GIRK1. Standard immunostaining and imaging techniques were used as described previously (11). Figure 4 shows that GIRK1 expresses along long cytoplasmic strands, shown previously to be associated with intermediate filaments, rope-like structures similar to microtubules (19). Coexpression of SNX27b did not alter the protein levels or trafficking pattern of GIRK1. Coexpressing GIRK3 channels caused a marked redistribution of GIRK1 channels (Figure 4b). The cytoskeletal expression pattern of GIRK1 now appears more punctate in the presence of GIRK3. Interestingly, in cells transfected with GIRK1/3, coexpression of SNX27b dramatically reduced the total protein levels of GIRK1 (Figure 4c, 4d), similar to the change in protein measured by Western blot analysis (Figure 3b). In contrast to GIRK1, GIRK2c (HA-GIRK2c) channels expressed on the cell membrane and within internal compartments (Figure 4e).

Coexpression of GIRK3 did not apparently alter the distribution or total protein for GIRK2c (Figure 4f). However, coexpression of SNX27b with GIRK3 reduced GIRK2c channels on the membrane and total protein (Figure 4g, 4h). Together, these data suggest that SNX27 can regulate surface expression of GIRKX/GIRK3 channels by promoting endocytosis and degradation of GIRK channels.

We predicted that SNX27b-YFP expression would lead to smaller GIRK2c currents; however, baclofen-induced currents for GIRK2c channels were not significantly different in HEK293 cells coexpressing SNX27b-YFP (Figure 5c). Similarly, surface levels of GIRK2c channels were not significantly different with coexpressed SNX27b (Figure 6a–b). One possible explanation for this result is that a strong forward trafficking signal in GIRK2c promotes efficient recycling back to the plasma membrane (35). We therefore studied the effect of coexpressing GIRK2c with GIRK3, whose lysosomal targeting motif might dominant and lead to degradation. Using a HA-tagged GIRK2c, we found that expression levels of HA-GIRK2c channels on the plasma membrane were significantly decreased in cells coexpressing GIRK3 and SNX27b (Fig. 6c–d), in contrast to GIRK2c alone. Consistent with a reduction of channels on the plasma membrane surface, the baclofen-induced currents were also smaller in cells coexpressing GIRK3 and SNX27b (Figure 6e–f). Taken together, these experiments demonstrate that SNX27b regulates trafficking of GIRK1/3 and GIRK2c/3 heteromeric channels via its association with GIRK3.

Comparison of GIRK channel and SNX27 expression in brain

GIRK3 channels are widely expressed in the brain (36), while the expression pattern of SNX27 still remains to be examined. Given the association of SNX27 with

GIRK3 channels, we investigated whether SNX27 and GIRK3 expression exhibited cell-specific overlap. An in situ hybridization analysis was carried out with radioactive cRNA probes for both SNX27 and GIRK3 transcripts in rat brain. SNX27 mRNA exhibited widespread expression at relatively low levels throughout gray matter regions of the brain and was preferentially enriched in certain brain regions, including the medial septum, hippocampus, some precerebellar relays (red and lateral reticular nuclei, pontine gray), cerebellar cortex and cranial nerve motor nuclei. GIRK3 mRNA expression was concentrated in olfactory bulb, dorsal thalamus, hippocampus, isocortex and cerebellum, similar to previous studies (36). In some regions of the brain (e.g. thalamus, red nucleus, VII and XII nuclei), there was little overlap between GIRK3 and SNX27. We next examined whether SNX27b expressed in hippocampal neurons is targeted to subcellular compartments containing GIRK channels. Dissociated cultures of hippocampal neurons were transfected after 3–7 days with the cDNA for SNX27b-YFP and then immunostained for endogenous GIRK1 or GIRK2 channels. SNX27b-YFP is expressed in discrete puncta located in the soma and dendrites (Fig. 7a–b, f–g). Immunostaining for GIRK1 or GIRK2 channels also reveals discrete puncta in the dendrites of hippocampal neurons (Fig. 7c,h). Examination of areas of colocalization of SNX27 puncta with either GIRK1 or GIRK2 puncta shows numerous regions of overlap (Fig. 7d–e, i–j), as well as other regions of non-overlap. Thus, these experiments demonstrate that SNX27b is expressed in the same puncta that contain GIRK channels

Discussion

In this study, we searched for novel proteins that could potentially regulate GIRK channels and discovered a new GIRK regulatory protein, SNX27. We show for the first

time that a sorting nexin (SNX27) regulates the cell surface expression of an ion channel. Furthermore, the ability of SNX27 to regulate the GIRK channel depends on the combination of GIRK subunits expressed. Both SNX27 and GIRK3 exhibited overlapping expression patterns in the rodent brain, and especially the hippocampus.

Trafficking of GIRK channels is modulated by SNX27

In the endosomal pathway, plasma membrane proteins internalize via endocytotic vesicles (both clathrin-dependent and independent) that fuse with early endosomes, at which point proteins are sorted and targeted to multivesicular bodies and late endosomes/lysosomes (degradation pathway), to perinuclear endosomes (trans-Golgi network), or back to the plasma membrane via recycling Endosomes (39). Sorting nexins are generally involved in facilitating movement of proteins through these endosomal compartments (39). Currently, 29 sorting nexins have been identified, each possessing a Phox (PX) lipid binding domain. Among these, SNX27 is the only SNX that also contains a PDZ domain (29). Pioneering studies conducted with SNX1 suggested a role in trafficking and degradation of EGF receptors (40). Consistent with this, mice lacking SNX1 or SNX2 are viable but display severe deficits in endosomal trafficking in a variety of cell types (41). In addition, SNX1/2 mutant mice exhibit severe growth retardation. Thus, SNX1 and SNX2 are both involved in regulating lysosomal targeting of EGF receptor but neither protein is essential (42). SNX1 was initially proposed to enhance degradative sorting of EGF receptor (40) but then subsequent genetic evidence pointed to a role in retrieval of cargo away from Golgi (44). Overexpression of sorting nexins can have secondary effects on trafficking by inducing tubulation of endosomal networks (29). We consider this possibility unlikely for GIRK channels, since tubulation of endosomal

networks would have been expected to alter trafficking of all GIRK channels, yet SNX27 affected surface expression of GIRK3 but not GIRK2c channels.

Expression of SNX27 revealed colocalization with GIRK3 and with GIRK1/3 channels in the early endosome. The functional consequence of expressed SNX27 was a reduction in GIRK1 protein and smaller GIRK currents. These findings suggest that SNX27 promotes or stabilizes GIRK1/3 channels in the early endosome, where trafficking is then further specified by the channel's targeting motifs (Figure 8). A previously identified lysosomal targeting motif in GIRK3 channels, YxxΦ (35), raised the possibility that reduced GIRK1/3 currents occurred from SNX27-mediated increase in endocytosis and subsequent degradation. Consistent with this, surface expression of GIRK channels decreased dramatically, along with reduced levels of GIRK1 protein, in cells coexpressing SNX27. Conversely, mutation of the lysosomal motif in GIRK3 led to larger GIRK1/3 currents. Previous studies suggest that GIRK3 channels enter the lysosomal degradation (35) pathway, though other pathways such as ubiquitination could be involved. Taken together, SNX27 appears to regulate GIRK1/3 channels directly by affecting their surface expression.

Surprisingly, deletion of the last four amino acids in the C-terminal tail of GIRK3 led to smaller GIRK1/3 currents. The C-terminal deletion mutant, however, could be rescued by mutating the GIRK3 lysosomal motif. These findings suggest the C-terminal domain of GIRK3 is important for both the surface membrane targeting and subsequent regulation by SNX27 – lack of this PDZ binding may lead to direct or accelerated transport to a degradation pathway. Interestingly, when GIRK3 was first cloned, there were conflicting reports on whether GIRK3 functionally expressed (7,45,46). Inspection

of the cDNAs used in these studies reveals that the C-terminal PDZ binding motif, –ESKV, was absent in the original cDNA clone (–GRWS). Our data combined with previous studies emphasize an important functional role for the C-terminal tail in GIRK3 channels.

We also observed that SNX27 colocalized strongly with GIRK2c channels in the early endosome but did not significantly alter the amplitude of GIRK current or the levels of GIRK2c protein on the plasma membrane. If both GIRK3 and GIRK2c channels interact with the PDZ domain of SNX27, then how do the levels of GIRK2c channels remain stable on the plasma membrane? One possibility is that GIRK2 channels contain a strong forward trafficking signal, in addition to lacking the lysosomal targeting motif found in GIRK3 (35). Accordingly, the change in endosomal trafficking produced by SNX27 would be compensated by a fast reinsertion of channels on the plasma membrane. A similar effect was reported for PSD93 regulation of Kir2.1 channels, where PSD93δ attenuated internalization but did not lead to a significant increase in Kir2.1 current (23). In the presence of both GIRK3 and SNX27, however, the forward trafficking of GIRK2c channels is dominated by the lysosomal targeting motif of GIRK3, leading to down regulation of GIRK2c/3 heteromultimers. Thus, expression of different combinations of GIRK subunits in neurons, along with varying levels of specific trafficking proteins, such as SNX27, will dictate the ultimate expression levels of GIRK channel protein on the plasma membrane, and therefore strength of inhibitory signaling in the brain. This difference in regulation is particularly intriguing since GIRK2 channels are exclusively expressed in dopamine neurons of the substantia nigra (10), while dopamine neurons in ventral tegmental area express GIRK2c and GIRK3 (4).

The role of other PDZ containing proteins in regulating GIRK2c has been unclear. For example, GIRK2c was shown to associate directly with PDZ_2 of SAP-97 in pull-down assays (25) and with PSD-95 in immunoprecipitation assays (10). By contrast, coimmunoprecipitation of PSD-95 did not bring down GIRK2c in transfected cells (24). Furthermore, neither PSD-95 nor SAP-97 colocalized with GIRK2 in the substantia nigra (10). Conceivably, binding of SNX27 to GIRK2c could antagonize the association of other PDZ domains to the channel. It will be interesting to explore the interaction of different PDZ containing proteins with GIRK channels endogenously expressed in neurons.

A role for sorting nexins in the brain

Activation of neurotransmitter receptors that couple to GIRK channels generates a slow inhibitory postsynaptic potential (sIPSP) (1), which is important for electrical signaling in the brain (2,3,50). If SNX27 channels regulate GIRK channels, then we would expect to find that GIRK3 channels expressed in the same neurons as SNX27. In situ hybridization indicated that SNX27 is expressed in many brain regions which also express GIRK3 channels. In fact, SNX27 colocalizes with GIRK1 and GIRK2 channels in the dendrites of hippocampal neurons. Interestingly, SNX27 is also expressed in areas lacking GIRK3, where SNX27 could associate with GIRK2c or with other proteins that contain a PDZ-binding motif similar to GIRK. For example, 5-HT4 (a) receptors, which couple to Gs G proteins, were recently shown to be regulated by SNX27 (31). Similar to its effect on GIRK3 channels, SNX27 redirected 5-HT4 (a) receptors to the early endosome. For excitatory glutamatergic synapses, movement through the endosome is essential for long-term potentiation (51). SNX27 expression may provide a key pathway

for regulating inhibitory synapses in the brain. Inhibitory GIRK currents were also recently reported to be potentiated (50). SNX27 may be one of the cytoplasmic proteins involved in regulating levels of GIRK channels on the plasma membrane. SNX27 transcripts in neurons have been reported to be upregulated by psychostimulants, such as cocaine and methamphetamine (30). Thus, changes in SNX27 expression may establish an important link between trafficking of potassium channels and drug actions in the brain, possibly opening up new avenues for treating drug addictions.

Acknowledgments

We thank C. Vandenberg and D. Leonoudakis for their invaluable advice for the proteomics study, D. Clapham for providing GIRK3 and GIRK4 cDNAs, M. Sheng for providing PSD-95 and Chapsyn-110 cDNAs, L. Jan and E. Cooper for providing Kv1.4 cDNA, I. Verma for the use of his confocal microscope, and C. Luscher, M. Montminy and members of the Slesinger lab for reading versions of the manuscript. This work was made possible by financial support from the McKnight Endowment for Neuroscience (P.A.S.), the Hearst Foundation (P.A.S.) and the National Institute of Neurological Disorders and Stroke (NIH R01 NS37682, P.A.S.).

This Chapter is a revised version of Lunn and Nassirpour et al. 2007. A unique sorting nexin regulates trafficking of potassium channels via a PDZ domain interaction. Nature Neuroscience. Oct;10(10):1249-1259 and Nassirpour and Slesinger. 2007. Subunit-specific regulation of Kir3 channels by sorting nexin 27. 2007. Channels. Sept/Oct 1(5):331-333. The data included in Chapter 2 only comprise those for which the thesis author was primarily responsible.

References

1. Lüscher, C., Jan, L.Y., Stoffel, M., Malenka, R.C. & Nicoll, R.A. G-protein-coupled inwardly rectifying K+ channels (GIRKs) mediate postsynaptic, but not presynaptic transmitter actions in hippocampal neurons. Neuron 19, 687-695 (1997).

2. Marker, C.L., Lujan, R., Loh, H.H. & Wickman, K. Spinal G-Protein-Gated Potassium Channels Contribute in a Dose-Dependent Manner to the Analgesic Effect of {micro}- and {delta}- But Not {kappa}-Opioids. J. Neurosci. 25, 3551-3559 (2005).

3. Signorini, S., Liao, Y.J., Duncan, S.A., Jan, L.Y. & Stoffel, M. Normal cerebellar development but susceptibility to seizures in mice lacking G protein-coupled, inwardly rectifying K+ channel GIRK2. Proc. Natl. Acad. Sci. USA 94, 923-927 (1997).

4. Cruz, H.G., et al. Bi-directional effects of GABAB receptor agonists on the mesolimbic dopamine system. Nat Neurosci 7, 153-159 (2004).

5. Kobayashi, T., et al. Ethanol opens G-protein-activated inwardly rectifying K+ channels. Nat. Neurosci. 2, 1091-1097 (1999).

6. Stanfield, P.R., Nakajima, S. & Nakajima, Y. Constitutively active and G-protein coupled inward rectifier K+ channels: Kir2.0 and Kir3.0. Rev Physiol Biochem Pharmacol 145, 47-179 (2002).

7. Jelacic, T.M., Sims, S.M. & Clapham, D.E. Functional expression and characterization of G-protein-gated inwardly rectifying K+ channels containing GIRK3. J Membr Biol 169, 123-129 (1999).

8. Schoots, O., et al. Co-expression of human Kir3 subunits can yield channels with different functional properties. Cell Signal 11, 871-883 (1999).

9. Slesinger, P.A., et al. Functional effects of the mouse weaver mutation on G protein-gated inwardly rectifying K+ channels. Neuron 16, 321-331 (1996).

10. Inanobe, A., et al. Characterization of G-protein-gated K+ channels composed of Kir3.2 subunits in dopaminergic neurons of the substantia nigra. J. Neurosci. 19, 1006-1017 (1999).

11. Clancy, S., et al. Pertussis-toxin-sensitive Ga subunits selectively bind to C-terminal domain of neuronal GIRK channels: evidence for a heterotrimeric G-protein-channel complex. Mol. Cell. Neurosci. 28, 375-389 (2005).

12. Peleg, S., Varon, D., Ivanina, T., Dessauer, C.W. & Dascal, N. Gai controls the gating of the G protein-activated K+ channel, GIRK. Neuron 33, 87-99 (2002).

13. Logothetis, D.E., Kurachi, Y., Galper, J., Neer, E.J. & Clapham, D.E. The bg subunits of GTP-binding proteins activate the muscarinic K+ channel in heart. Nature 325, 321-326 (1987).

14. Pfaffinger, P.J., Martin, J.M., Hunter, D.D., Nathanson, N.M. & Hille, B. GTP-binding proteins couple cardiac muscarinic receptors to a K channel. Nature 317, 536-538 (1985).

15. Reuveny, E., et al. Activation of the cloned muscarinic potassium channel by G protein bg subunits. Nature 370, 143-146 (1994).

16. Wickman, K.D., et al. Recombinant G-protein bg-subunits activate the muscarinic-gated atrial potassium channel. Nature 368, 255-257 (1994).

17. Wiser, O., et al. Modulation of Basal and Receptor-Induced GIRK Potassium Channel Activity and Neuronal Excitability by the Mammalian PINS Homolog LGN. Neuron 50, 561-573 (2006).

18. Chuang, H.-H., Yu, M., Jan, Y.N. & Jan, L.Y. Evidence that the nucleotide exchange and hydrolysis cycle of G proteins causes acute desensitization of G-protein gated inward rectifier K+ channels. Proc. Natl. Acad. Sci. USA 95, 11727-11732 (1998).

19. DiMagno, L., Dascal, N., Davidson, N., Lester, H.A. & Schreibmayer, W. Serotonin and protein kinase C modulation of a rat brain inwardly rectifying K+ channel expressed in Xenopus oocytes. Pflügers Arch. -- Eur. J. Physiol. 431, 335-340 (1996).

20. Kim, E. & Sheng, M. PDZ domain proteins of synapses. Nat Rev Neurosci 5, 771-781 (2004).

21. Tiffany, A.M., et al. PSD-95 and SAP97 Exhibit Distinct Mechanisms for Regulating K+ Channel Surface Expression and Clustering. J. Cell Biol. 148, 147-158 (2000).

22. Leonoudakis, D., Conti, L.R., Radeke, C.M., McGuire, L.M. & Vandenberg, C.A. A multiprotein trafficking complex composed of SAP97, CASK, Veli, and Mint1 is associated with inward rectifier Kir2 potassium channels. J. Biol. Chem. 279, 19051-19063 (2004).

23. Leyland, M.L. & Dart, C. An alternatively spliced isoform of PSD-93/chapsyn 110 binds to the inwardly rectifying potassium channel, Kir2.1. J. Biol. Chem. 279, 43427-43436 (2004).

24. Nehring, R.B., et al. Neuronal inwardly rectifying K+ channels differentially couple to PDZ proteins of the PSD-95/SAP90 Family. J. Neurosci. 20, 156-162 (2000).

25. Hibino, H., et al. Anchoring proteins confer G protein sensitivity to an inward-rectifier K(+) channel through the GK domain. Embo J 19, 78-83 (2000).

26. Washburn, M.P., Wolters, D. & Yates, J.R., 3rd. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nat Biotechnol 19, 242-247 (2001).

27. Pegan, S., et al. Kir2.1 and Kir3.1 cytoplasmic domain structures reveal sites for modulating gating and rectification. Nat Neurosci 8, 279-287 (2005).

28. Leonoudakis, D., et al. Protein trafficking and anchoring complexes revealed by proteomic analysis of inward rectifier potassium channel (Kir2.x)-associated proteins. J. Biol. Chem. 279, 22331-22346 (2004).

29. Carlton, J., Bujny, M., Rutherford, A. & Cullen, P. Sorting Nexins - Unifying Trends and New Perspectives. Traffic 6, 75-82 (2005).

30. Kajii, Y., et al. A developmentally regulated and psychostimulant-inducible novel rat gene mrt1 encoding PDZ-PX proteins isolated in the neocortex. Mol Psychiatry 8, 434-444 (2003).

31. Joubert, L., et al. New sorting nexin (SNX27) and NHERF specifically interact with the 5-HT4a receptor splice variant: roles in receptor targeting. J Cell Sci 117, 5367-5379 (2004).

32. Hung, A.Y. & Sheng, M. PDZ Domains: Structural Modules for Protein Complex Assembly. J. Biol. Chem. 277, 5699-5702 (2002).

33. Zimmermann, P., et al. PIP2-PDZ Domain Binding Controls the Association of Syntenin with the Plasma Membrane. Molecular Cell 9, 1215-1225 (2002).

34. Bravo, J., et al. The crystal structure of the PX domain from p40(phox) bound to phosphatidylinositol 3-phosphate. Mol Cell 8, 829-839 (2001).

35. Ma, D., et al. Diverse trafficking patterns due to multiple traffic motifs in G protein-activated inwardly rectifying potassium channels from brain and heart. Neuron 33, 715-729. (2002).

36. Karschin, C., Dissmann, E., Stuhmer, W. & Karschin, A. IRK(1-3) and GIRK(1-4) inwardly rectifying K+ channel mRNAs are differentially expressed in the adult rat brain. J. Neurosci. 16, 3559-3570 (1996).

37. Slesinger, P.A., Stoffel, M., Jan, Y.N. & Jan, L.Y. Defective g-aminobutyric acid type B receptor-activated inwardly rectifying K+ currents in cerebellar granule cells isolated from weaver and Girk2 null mutant mice. Proc. Natl. Acad. Sci. USA 94, 12210-12217 (1997).

38. Sun, Q.-Q., Huguenard, J.R. & Prince, D.A. Neuropeptide Y receptors differentially modulate G-protein-activated inwardly rectifying K+ channels and high-voltage-activated Ca2+ channels in rat thalamic neurons. J. Physiol. 531, 67-79 (2001).

39. Worby, C.A. & Dixon, J.E. Sorting out the cellular functions of sorting nexins. Nat Rev Mol Cell Biol 3, 919-931 (2002).

40. Kurten, R.C., Cadena, D.L. & Gill, G.N. Enhanced Degradation of EGF Receptors by a Sorting Nexin, SNX1. Science 272, 1008-1010 (1996).

41. Schwarz, D.G., Griffin, C.T., Schneider, E.A., Yee, D. & Magnuson, T. Genetic Analysis of Sorting Nexins 1 and 2 Reveals a Redundant and Essential Function in Mice. Mol. Biol. Cell 13, 3588-3600 (2002).

42. Gullapalli, A., et al. A Role for Sorting Nexin 2 in Epidermal Growth Factor Receptor Down-regulation: Evidence for Distinct Functions of Sorting Nexin 1 and 2 in Protein Trafficking. Mol. Biol. Cell 15, 2143-2155 (2004).

43. Gillooly, D.J., et al. Localization of phosphatidylinositol 3-phosphate in yeast and mammalian cells. Embo J 19, 4577-4588 (2000).

44. Horazdovsky, B., et al. A sorting nexin-1 homologue, Vps5p, forms a complex with Vps17p and is required for recycling the vacuolar protein-sorting receptor. Mol. Biol. Cell 8, 1529-1541 (1997).

45. Lesage, F., et al. Molecular properties of neuronal G-protein-activated inwardly rectifying K+ channels. J. Biol. Chem. 270, 28660-28667 (1995).

46. Kobayashi, T., et al. Molecular cloning of a mouse G-protein-activated K+ channel (mGIRK1) and distinct distributions of three GIRK (GIRK1, 2 and 3) mRNAs in mouse brain. Biochem. Biophys. Res. Commun. 208, 1166-1173 (1995).

47. Long, J.F., et al. Supramodular structure and synergistic target binding of the N-terminal tandem PDZ domains of PSD-95. J Mol Biol 327, 203-214 (2003).

48. Doyle, D.A., et al. Crystal structures of a complexed and peptide-free membrane protein-binding domain: molecular basis of peptide recognition by PDZ. Cell 85, 1067-1076 (1996).

49. Songyang, Z., et al. Recognition of Unique Carboxyl-Terminal Motifs by Distinct PDZ Domains. Science 275, 73-77 (1997).

50. Huang, C.S., et al. Common Molecular Pathways Mediate Long-Term Potentiation of Synaptic Excitation and Slow Synaptic Inhibition. Cell 123, 105-118 (2005).

51. Park, M., Penick, E.C., Edwards, J.G., Kauer, J.A. & Ehlers, M.D. Recycling endosomes supply AMPA receptors for LTP. Science 305, 1972-1975 (2004).

52. Finley, M., Arrabit, C., Fowler, C., Suen, K.F. & Slesinger, P.A. bL-bM loop in the C-terminal domain of GIRK channels is important for Gbg activation. J. Physiol. 555, 643-657 (2004).

53. Hall, R.A., et al. The b2-adrenergic receptor interacts with the Na+/H+-exchanger regulatory factor to control Na+/H+ exchange. Nature 392, 626-630 (1998).

54. Brewer, G.J., Torricelli, J.R., Evege, E.K. & Price, P.J. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. J. Neurosci. Res. 35, 567-576 (1993).

55. Hamill, O.P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F.J. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflügers Arch. 391, 85-100 (1981).

56. Tabb, D.L., McDonald, W.H. & Yates, J.R., 3rd. DTASelect and Contrast: tools for assembling and comparing protein identifications from shotgun proteomics. J Proteome Res 1, 21-26 (2002).

57. Link, A.J., et al. Direct analysis of protein complexes using mass spectrometry. Nat Biotechnol 17, 676-682 (1999).

58. Eng, J.K., McCormack, A.L. & Yates, I., John R. An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. Journal of the American Society for Mass Spectrometry 5, 976-989 (1994).

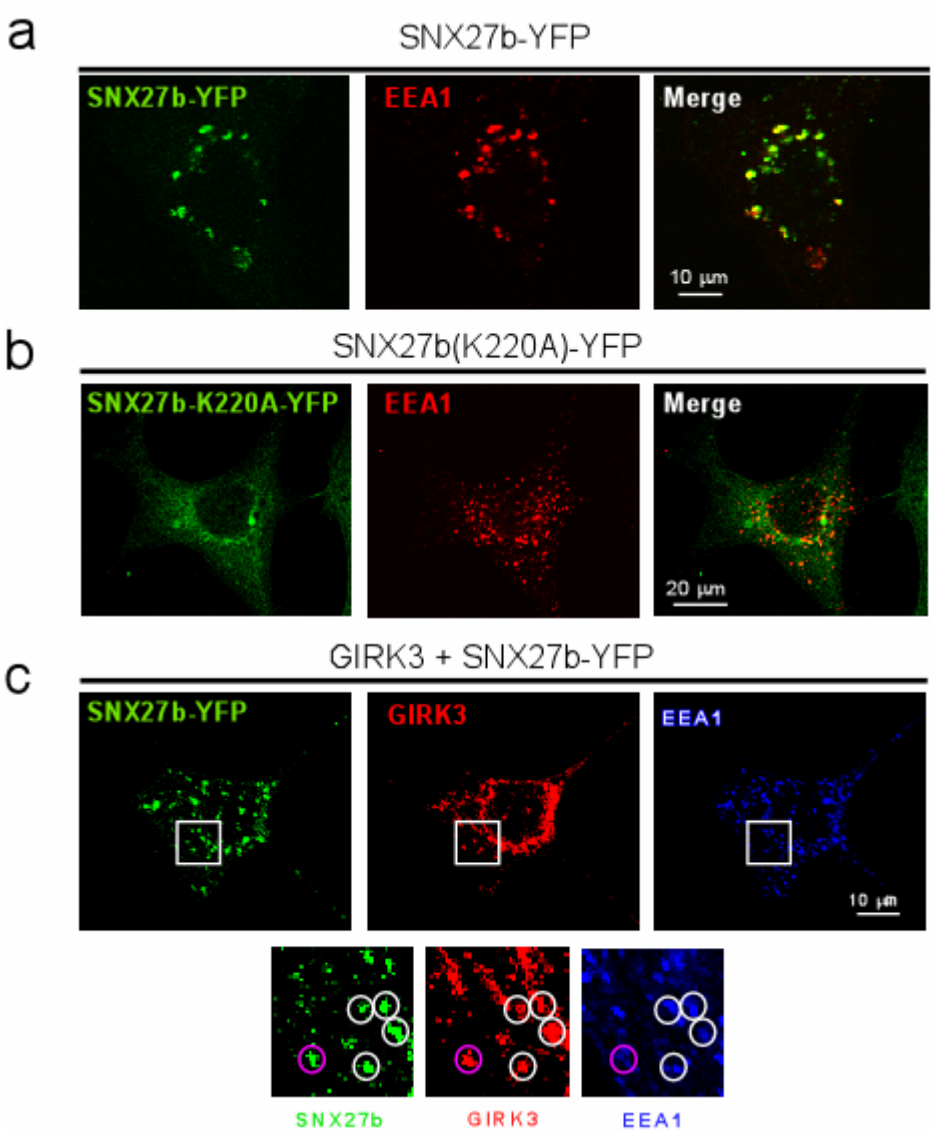
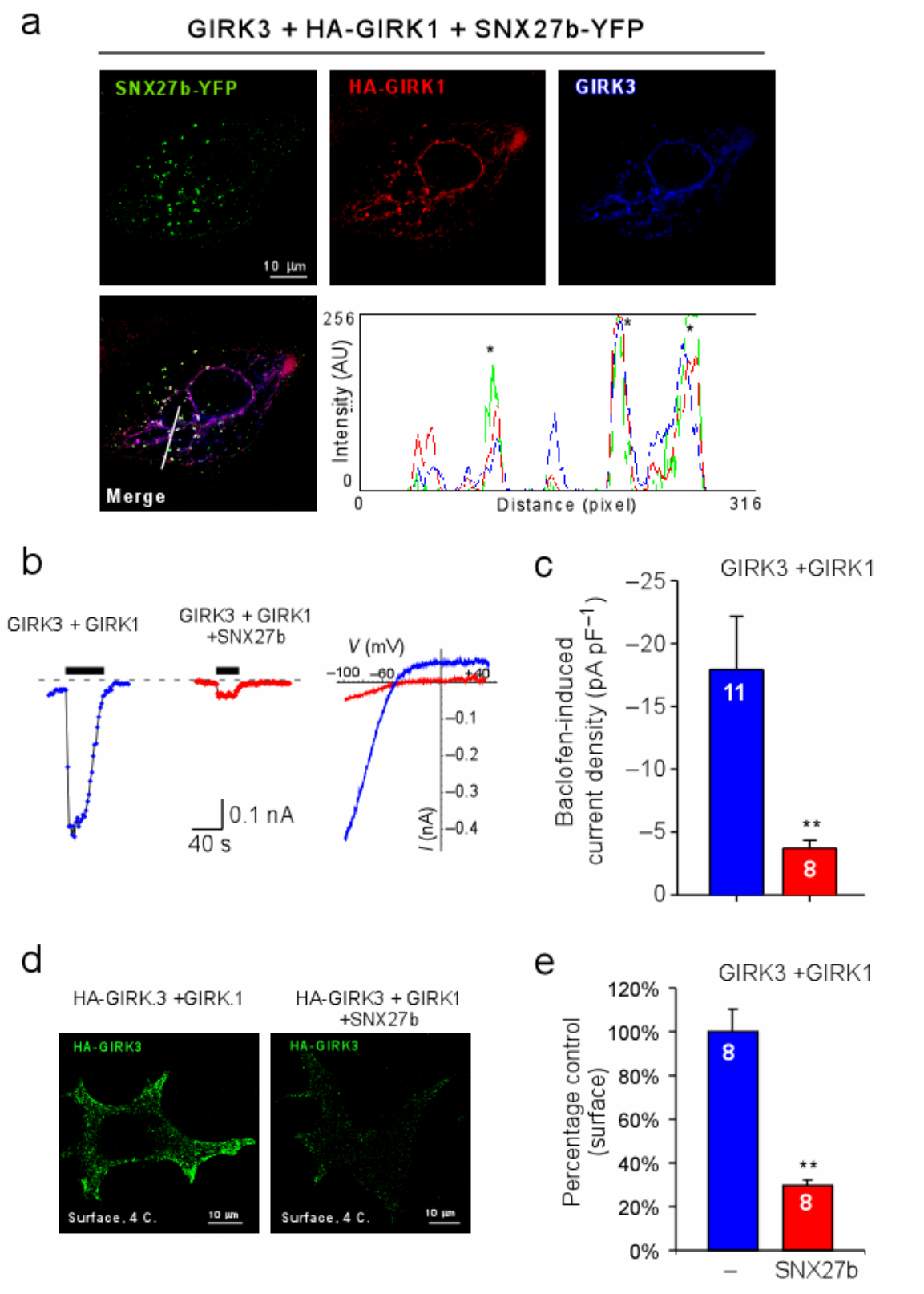


Fig 2.1. **SNX27 contains a lipid binding motif (PX domain) that co-localizes GIRK3 in the early endosome.** **A**, SNX27b co-localizes in early endosome (identified by anti-EEA1 immunostaining) in HEK293 cells transfected with SNX27b-YFP. **B**, K220A mutation in PX lipid binding domain alters expression pattern of SNX27b [SNX27b-K220A-YFP] in HEK293 cells, indicating that PI(3)P binding is essential for targeting to the early endosome. Inset shows green channel for SNX27b-K220A-YFP. **C**, SNX27b-YFP and GIRK3 colocalize in the early endosome. HEK293 cells were transfected with SNX27b-YFP and GIRK3. SNX27 was visualized by YFP tag, GIRK3 by anti-GIRK3 antibody and early endosome by anti-EEA1 antibody. Images below show merges of two channels. Inset, magnification of endosomal compartments containing all three proteins (white circle) or two of three (purple).

Fig 2.2. **SNX27 regulates cell surface expression of GIRK3 channels.** (a) GIRK3 colocalized with GIRK1 and SNX27b-YFP in discrete puncta. HEK293 cellswere transfected with GIRK3, HA-GIRK1 and SNX27b-YFP, permeabilized and immunostained with antibody to HA (red) or to GIRK3 (blue). Graph shows fluorescence intensity for the indicated line highlighting puncta containing all three proteins (asterisks). (b) Continuous recording at –100 mV shows the response to 100 mM baclofen in HEK293 cells expressing GIRK3/1 in the absence or presence of coexpressed SNX27b-myc. Current-voltage relation for the baclofen-induced current is shown on the right. (c) SNX27b expression markedly reduced the average baclofen-induced current density. HEK293 cells were transfected with the cDNAs for $GABA_B$ R1/R2 receptors, GIRK1 and GIRK3, with or without SNX27b-myc. Current density is the current induced at –100 mV divided by the cell capacitance. (d) Surface expression of GIRK3 decreased with coexpression SNX27b. HEK293 cells were transfected with cDNA for HA-GIRK3 and GIRK1, or HA-GIRK3, GIRK1 and SNX27b-myc. Surface HA-GIRK3 channels were detected using an antibody to HA at 4 ˚C (see Methods). (e) Quantification of total fluorescence in each cell is normalized to the mean fluorescence for control, and plotted in the absence or presence of SNX27b. **Student's t-test for significance ($P < 0.05$).


a
GIRK3 + HA-GIRK1 + SNX27b-YFP
SNX27b-YFP
HA-GIRK1
GIRK3
10 μm
Merge
256
Intensity (AU)
0
0
Distance (pixel)
316
b
GIRK3 + GIRK1
GIRK3 + GIRK1
+SNX27b
V (mV)
−100
−60
+40
−0.1
−0.2
−0.3
−0.4
I (nA)
0.1 nA
40 s
c
GIRK3 +GIRK1
Baclofen-induced
current density (pA pF−1)
−25
−20
−15
−10
−5
0
11
8
**
d
HA-GIRK.3 +GIRK.1
HA-GIRK3 + GIRK1
+SNX27b
HA-GIRK3
HA-GIRK3
Surface, 4 C.
10 μm
Surface, 4 C.
10 μm
e
GIRK3 +GIRK1
Percentage control
(surface)
120%
100%
80%
60%
40%
20%
0%
8
8
**
–
SNX27b

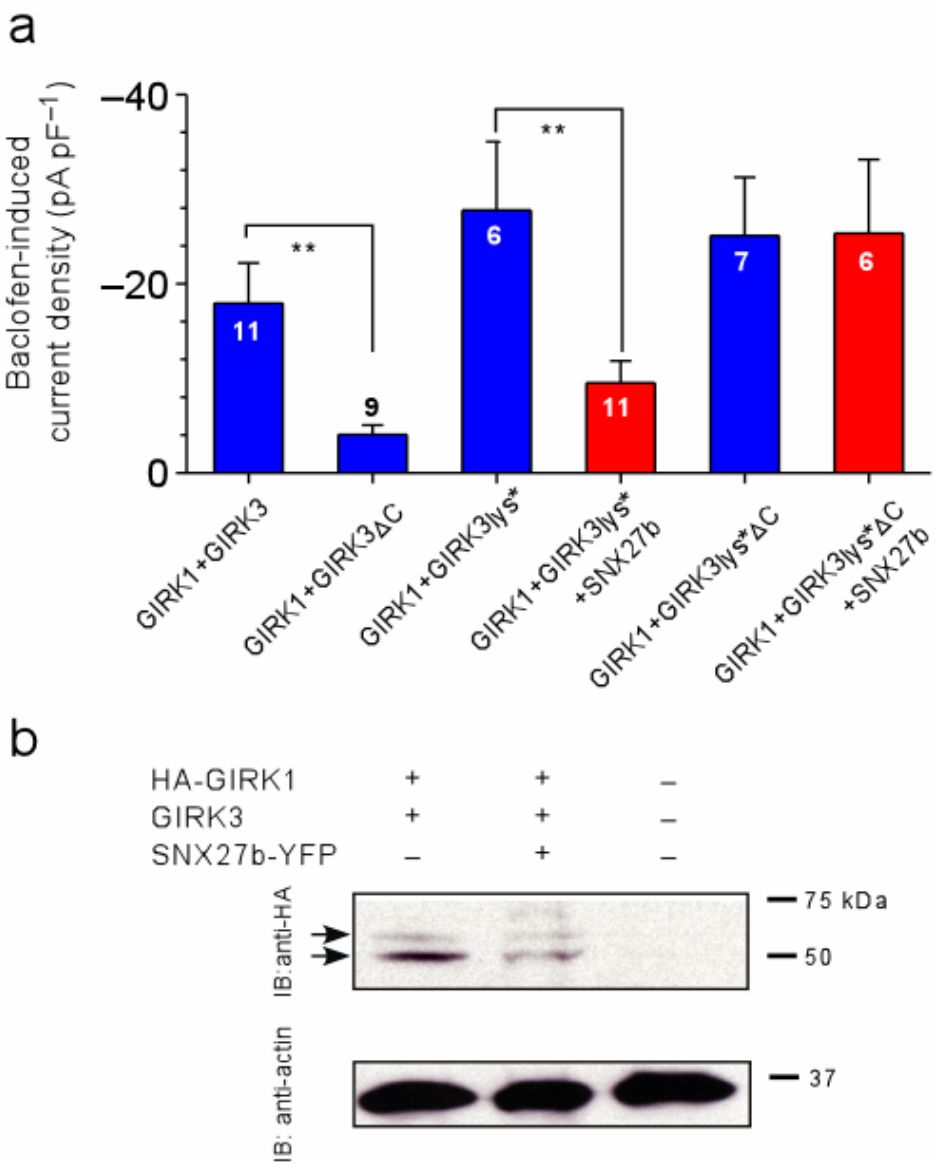


Fig 2.3. **Downregulation of GIRK channels by SNX27 requires lysosomal targeting and PDZ-binding motifs in GIRK3.** (a) Average baclofen-induced current density for HEK293 cells transfected with the cDNA for the indicated channel constructs coexpressed with GABAB R1/R2 receptors is shown. **Student's t-test ($P < 0.05$). (b) Western blotting showed reduced levels of GIRK1 protein in HEK293 cells coexpressing SNX27b-YFP and GIRK3. HEK293 cells were transfected with HA-GIRK1 and GIRK3, or HA-GIRK1, GIRK3 and SNX27b-YFP for ~3 d. Arrows show unglycosylated and glycosylated (50–55 kDa and 455 kDa) forms of GIRK1. Similar results were obtained with untagged GIRK1/3 and SNX27b-YFP (data not shown).

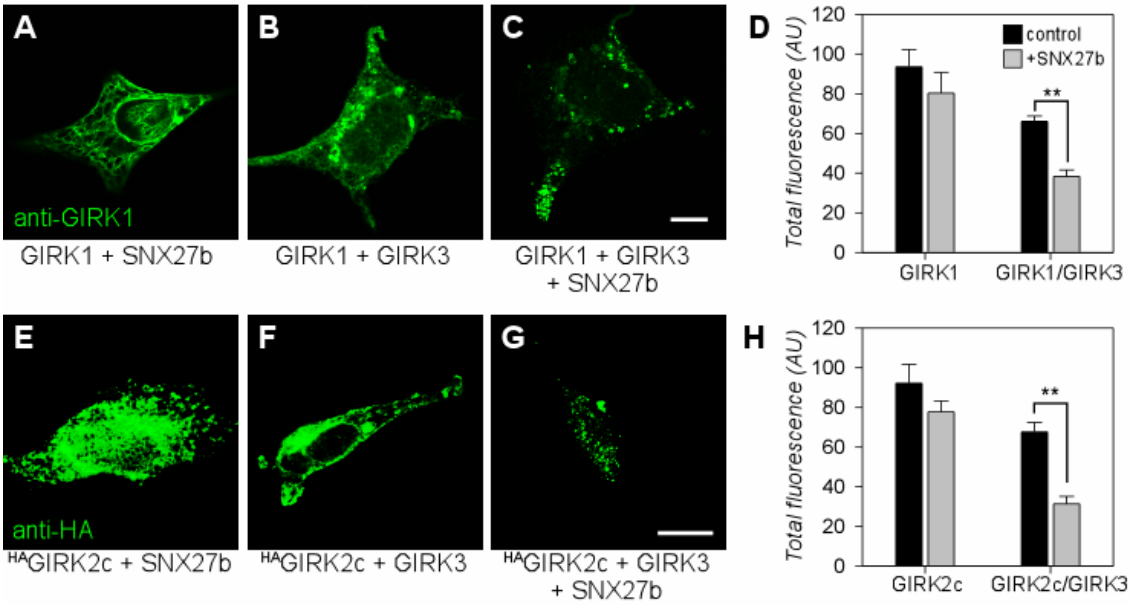


Fig 2.4. **Subunit-specific down regulation of GIRK channels by SNX27**. HEK293 cells were transfected with GIRK1, HA-GIRK2c, GIRK3 and/or SNX27b as indicated. Immunofluorescence staining with anti-GIRK1 and anti-HA (for GIRK2c) antibodies reveals distribution of proteins. a) GIRK1 retains its cytoskeletal expression pattern in the presence of SNX27b. b) Coexpression of GIRK3 redistributes GIRK1 to intracellular vesicles and plasma membrane. c) SNX27b alters distribution of GIRK1 when coexpressed with GIRK3. Scale bar, 10 ⌈m. d) Quantification (average intensity ± SEM) of the total fluorescence shows reduced levels of GIRK1 staining in cells coexpressing SNX27b and GIRK3. e) SNX27b does not alter the expression of GIRK2c. f) GIRK3 expression does not appear to alter the pattern of expression for GIRK2c. g) In the presence of GIRK3, SNX27 now dramatically reduces the levels and pattern of expression for GIRK2c. h) Quantification of total fluorescence as described above. ** $P < 0.05$ one-way ANOVA on ranks followed by Dunn's post hoc test for significance ($N = 7$-15).

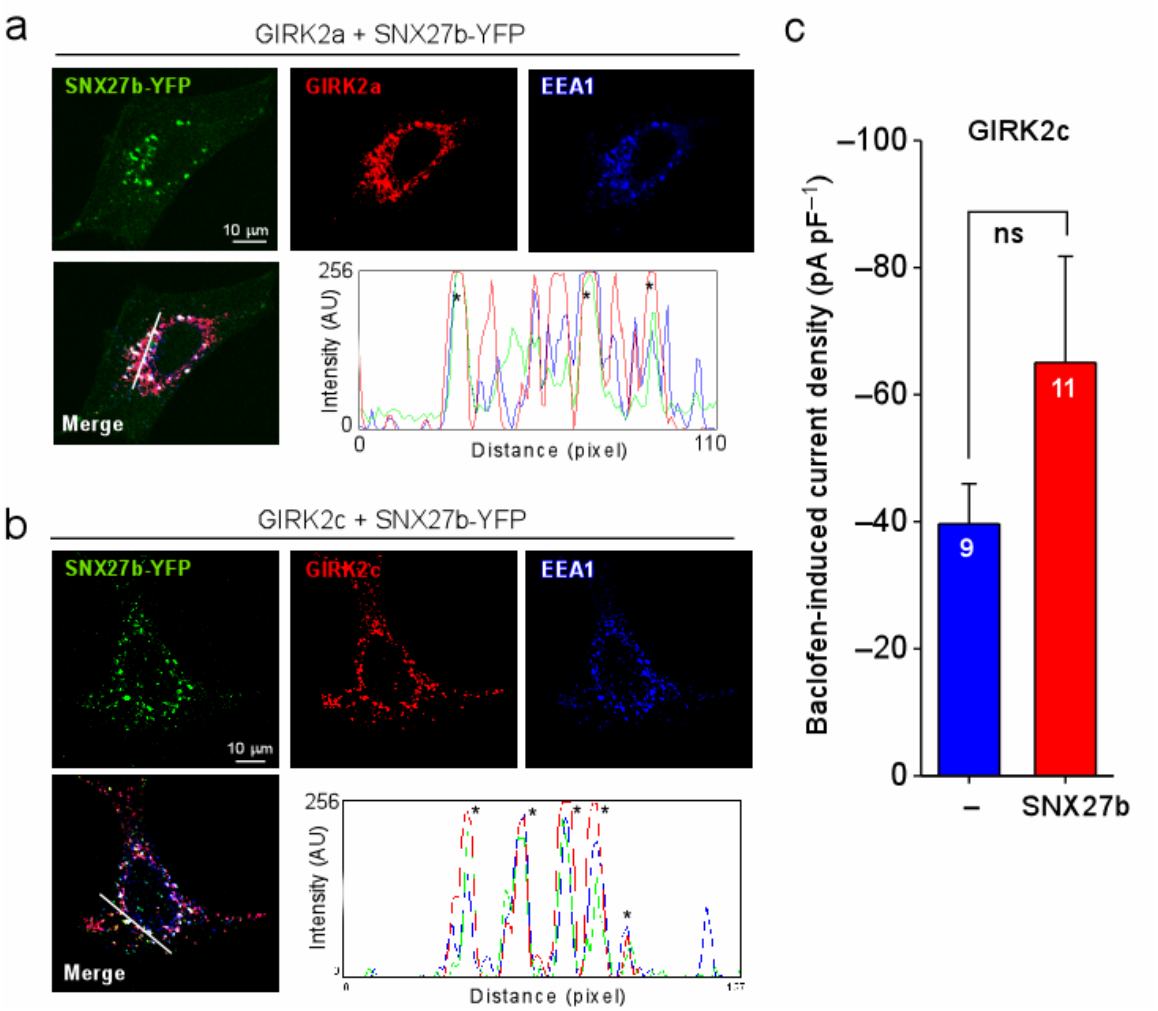
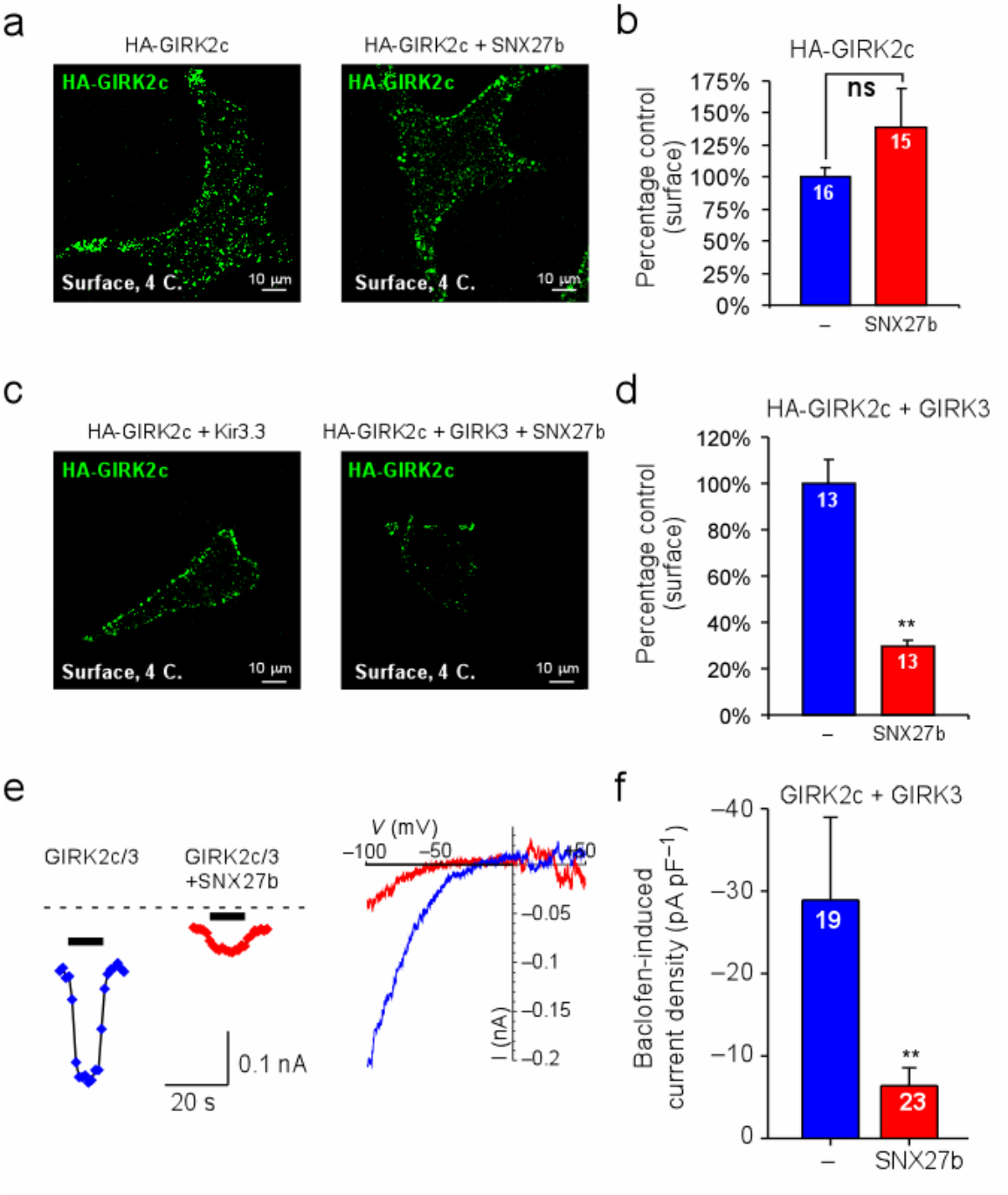


Fig 2.5. **GIRK2c channels colocalize with SNX27 in the early endosomes, but show no change in cell surface expression.** (a,b) Comparison of GIRK2a and GIRK2c expression in the absence or presence of SNX27b is shown. Immunostaining for GIRK2a (red) showed some overlap with SNX27-YFP (green) and EEA1 (blue), shown in a. Immunostaining for GIRK2c showed more extensive colocalization with SNX27-YFP in the early Endosome (EEA1), shown in b. HEK293 cells were transfected with GIRK2a or GIRK2c (visualized with antibody to GIRK2) and SNX27b-YFP, and co-stained with antibody to EEA1 to show the early endosomal compartment. Graphs show the fluorescence intensity for the indicated line. Note that there were puncta containing all three proteins (asterisks). (c) Bar graph shows no significant change in the baclofen induced current density in cells coexpressing SNX27b-myc. HEK293 cells were transfected with the cDNAs for GABAB R1/R2 receptors and GIRK2c, alone or with SNX27b-myc.

Fig 2.6. **GIRK3 promotes downregulation of GIRK2c channels by SNX27.** (a–d) We examined HA-GIRK2c channel expression on the membrane surface. Cells were immunostained with antibody to HA at 4 ˚C to detect HA-GIRK2c channels expressed on the surface. Coexpression of SNX27b-myc did not change surface levels of HA-GIRK2c (a,b). In cells coexpressing both GIRK3 and GIRK2c channels, however, SNX27b-myc reduced surface expression of HA-GIRK2c (c,d). Quantification of total fluorescence in each cell is normalized to the mean fluorescence for control, and plotted in the absence or presence of SNX27b (b,d). (e) A continuous current recording at –100 mV shows the response to 100 mM baclofen in HEK293 cells expressing GIRK3/2c in the absence or presence of coexpressed SNX27b-myc. Current-voltage relations for the baclofen-induced currents are shown on the right. (f) Bar graph shows average baclofen-induced current density for HEK293 cells expressing $GABA_B$ R1/R2 and GIRK2c/3, alone or with SNX27b-myc. Consistent with a decrease in the levels of GIRK channels on the plasma membrane, SNX27b expression led to smaller baclofeninduced GIRKcurrents. **Student's t-test for significance ($P < 0.05$).


a
HA-GIRK2c
HA-GIRK2c + SNX27b
HA-GIRK2c
HA-GIRK2c
Surface, 4 C.
10 µm
Surface, 4 C.
10 µm
b
HA-GIRK2c
Percentage control (surface)
175%
150%
125%
100%
75%
50%
25%
0%
ns
16
15
–
SNX27b
c
HA-GIRK2c + Kir3.3
HA-GIRK2c + GIRK3 + SNX27b
HA-GIRK2c
HA-GIRK2c
Surface, 4 C.
10 µm
Surface, 4 C.
10 µm
d
HA-GIRK2c + GIRK3
Percentage control (surface)
120%
100%
80%
60%
40%
20%
0%
13
**
13
–
SNX27b
e
GIRK2c/3
GIRK2c/3 +SNX27b
V (mV)
–100
–50
+50
–0.05
–0.1
–0.15
–0.2
I (nA)
0.1 nA
20 s
f
GIRK2c + GIRK3
Baclofen-induced current density (pA pF−1)
–40
–30
–20
–10
0
19
**
23
–
SNX27b

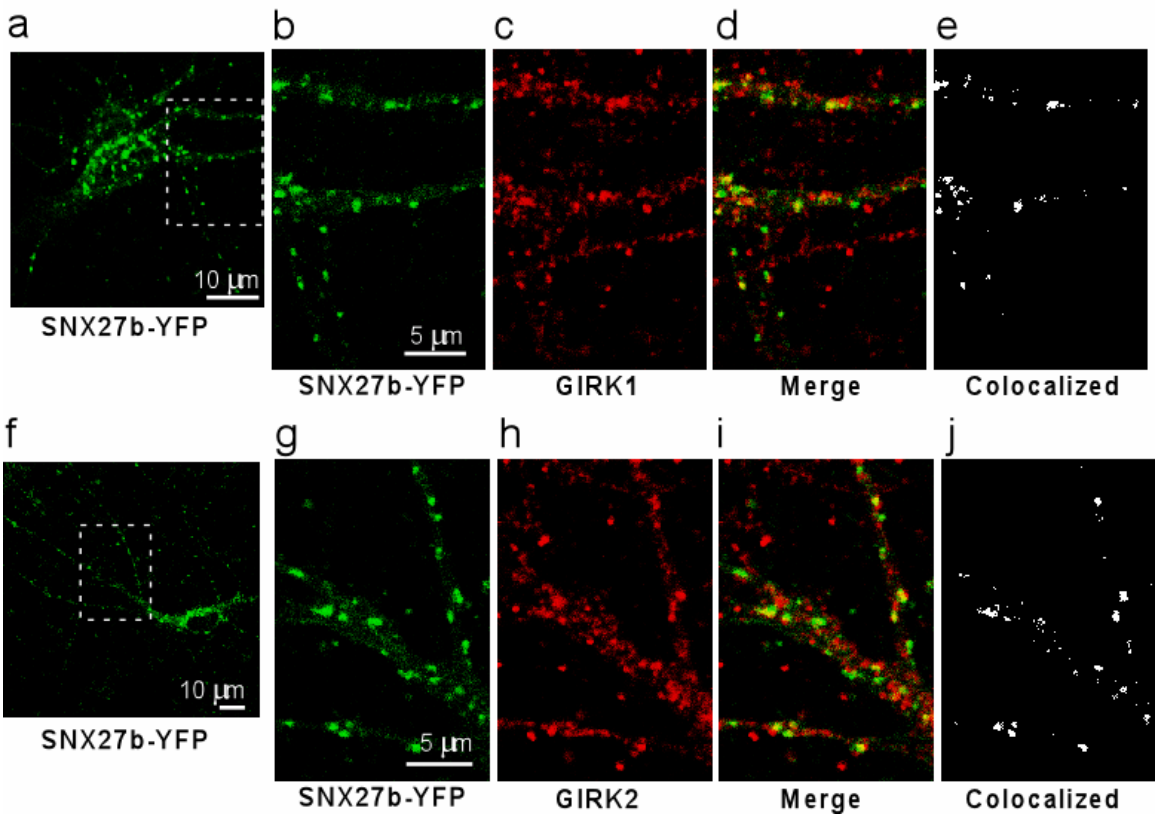


Fig 2.7. **Colocalization of SNX27b and GIRK channels in hippocampal neurons.** (a–e) Dissociated cultures of hippocampal neurons (3–7 d) were transfected with the cDNA for SNX27b-YFP and compared to endogenously expressed GIRK1 and GIRK2 channel proteins. Low-power image of a neuron transfected with SNX27b-YFP is shown in a. Dashed box indicates area of magnification shown in b–e. Image shows dendritic localization of endogenous GIRK1 (c) and ectopically expressed SNX27b-YFP (b). Colocalization of the GIRK1 and SNX27b-YFP fluorophores are indicated in the merged (yellow) and colocalized (white, using ImageJ plugin) images (d,e). (f–j) Low-power image of different neuron transfected with SNX27b-YFP. Dashed box indicates area of magnification shown in g–j. Images show dendritic localization of endogenous GIRK2 (h) and ectopically expressed SNX27b-YFP (g). Colocalization of the GIRK2 and SNX27b-YFP as described above (i,j).

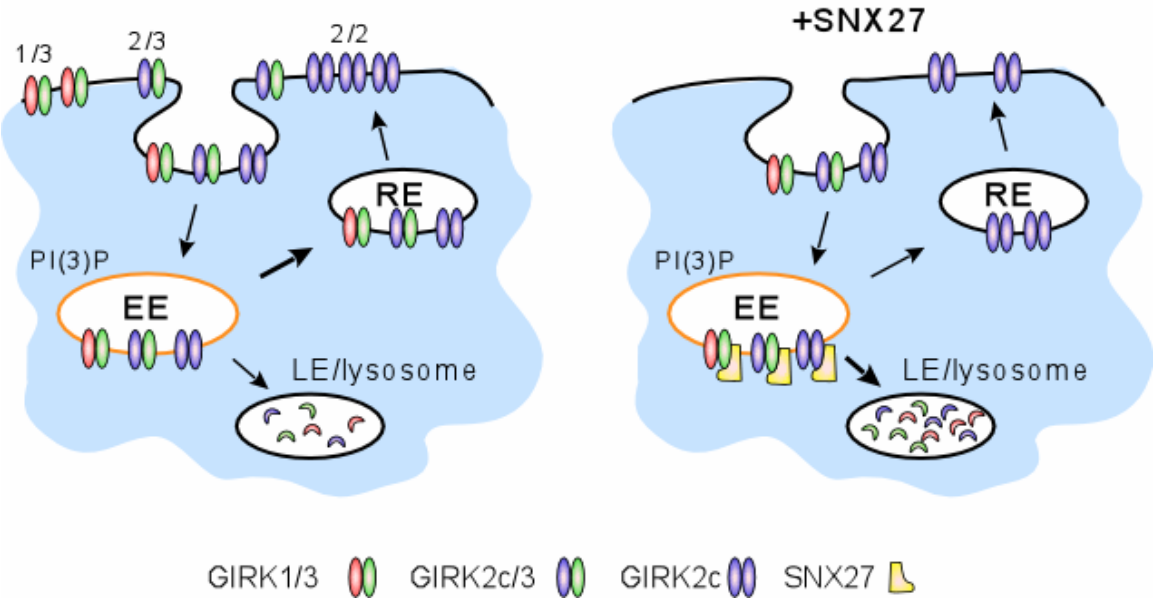


Fig 2.8. **Proposed pathway for subunit-specific down regulation of GIRK channels by SNX27.** Movement of channels through endosomal compartments, including the early Endosome (EE), recycling endosome (RE), late endosome (LE) and lysosome (Lys). SNX27 is anchored to membranes enriched in PtdIns(3)P (PI3P) and facilitates trafficking of channels containing GIRK3 or GIRK2c into the early endosome, where channels are sorted further and return back to the plasma membrane via the recycling endosome (GIRK2c) or are targeted to the LE/lysosome (GIRK1/3) for degradation.

III. CaMKII dependent opioid modulation of GIRK channels alters inhibitory input in hippocampal neurons

Abstract

Balancing inhibitory and excitatory inputs is essential for proper signaling in the brain. A significant component of inhibition involves the G protein-gated inwardly rectifying potassium (GIRK/Kir3) channels. GIRK channels have been shown to play a role in learning, pain sensation and drug addiction. Inhibitory and excitatory inputs generally occur in distinct morphological regions of the dendrite, the shaft and spine. Inhibitory GIRK channels, however, appear to be expressed in both the spine and shaft. Little is known about mechanisms for regulating the trafficking of GIRK channels. In mature hippocampal neurons, the majority of GIRK2 does not colocalize with spine markers, such as PSD95 or NMDA receptors. Using immunohistochemistry at both light and electron microscopic levels, we discovered that chronic (24 hr) morphine treatment increases the colocalization of GIRK2 and PSD95 within dendritic spines. This change in expression requires activation of CaMKII and is mimicked by constitutively active form of CaMKII. The net effect of morphine treatment and CaMKII activation is to reduce the amplitude of $GABA_B$ receptor-activated GIRK currents. Our results demonstrate that stimulation of opioid receptors produces a form of CaMKII-dependent plasticity for the $GABA_B$ receptor–GIRK channel inhibitory pathway in the hippocampus.

Introduction

Electrical signaling in the brain between neurons requires a proper balance between inhibitory and excitatory inputs [1]. Too much inhibition leads to unexcitable neurons, while excessive excitation can trigger cell death. In some regions of the brain, the balance between excitation and inhibition are not equal. For example, in the hippocampus and neocortex, excitatory synapses greatly outnumber inhibitory synapses [1]. Electron microscopy (EM) studies demonstrate that excitatory inputs preferentially form on dendritic spines, whereas inhibitory synapses typically form directly on dendritic shafts [2, 3]. Thus, fast excitatory and inhibitory synapses appear in spatially distinct regions of the dendrite. The suppressive effects of the inhibitory synapses are more effective when excitatory and inhibitory inputs are separated by less than 20 μm on the same dendritic branch [4]. Fast excitation and inhibition are typically produced by neurotransmitter stimulation of ionotropic NMDA/AMPA and $GABA_A$/glycine receptors, respectively.

A second pathway for synaptic inhibition utilizes neurotransmitter receptors that signal to G proteins. Activation of this slower pathway leads to presynaptic inhibition of voltage-gated Ca^{2+} channels [5] and to postsynaptic inhibition by activation of G protein-gated inwardly rectifying potassium (GIRK or Kir3) channels [6]. Postsynaptic activation of GIRK channels, which are members of the inwardly rectifying potassium channel family, produces a slow inhibitory postsynaptic potential (sIPSP) [6]. The slow potential arises, in part, from the pooling of GABA that is released into the synaptic cleft and spillovers to activate G protein-coupled $GABA_B$ receptors [7, 8]. $GABA_A$ channels are located beneath the inhibitory terminal. By contrast, EM immunohistochemical studies

have revealed that $GABA_B$ receptors and GIRK channels are localized to the dendritic shaft near GABA terminals as well as to the dendritic spine [9, 10].

In the hippocampus, the dendritic spine is well known for its role in learning and memory, long-term potentiation (LTP), long-term depression (LTD) and involvement in diseases such as epilepsy [11-15]. Interestingly, the structure of the dendritic spine may be heterogeneous in the hippocampus. Each spine is connected to the dendritic shaft via a narrow neck. Diffusion across the neck can limit the chemical and electrical contribution of the spine to the shaft. Recent two-photon imaging studies have shown that neuronal activity regulates the diameter of the spine neck, and therefore diffusion, in hippocampal neurons [16]. Regulating the diffusion across the neck could be an important factor for permitting the accumulation of key second messenger proteins involved in synaptic plasticity. The function and regulation of $GABA_B$ receptors and GIRK channels in the spine is not well understood. Expression of GIRK channels in the spine could serve to regulate the excitability of spines. In support of this, glutamate-dependent potentiation has been recently shown to augment the sIPSP in hippocampal neurons [17].

In the current study, we investigated the effect of opioids on natively expressed receptors, G proteins and GIRK channels in dissociated cultures of mature hippocampal neurons. We selected morphine because opiates have been shown to significantly alter glutamatergic transmission [18], neurogenesis [19], dendritic stability [20], and long term potentiation [21-24], suggesting that morphine might also modulate the function of GIRK channels in dendritic spines. Using a combination of electrophysiological, immunohistochemical and pharmacological approaches, we report that GIRK channels are regulated upon application of opioids. Morphine stimulation leads to upregulation of

GIRK2 in the dendritic spines. The activity of CaMKII is both necessary and sufficient for change in GIRK2 expression pattern. Interestingly, the net effect of morphine stimulation and CaMKII activation is to decrease the amplitude of $GABA_B$ receptor activated GIRK currents. We discuss the results in terms of a model for regulating synaptic plasticity in the hippocampus.

Methods

Hippocampal neuronal cultures

Preparation of the hippocampal cell cultures was according to published protocols with minor modifications [85]. Primary cultures of hippocampal neurons were obtained from 0-2 day-old Sprague Dawley rat pups. The hippocampus was isolated and dissociated with papain (Worthington), and cells were plated at 50,000 cells cm-2 in Neurobasal-A medium supplemented with B27 and glutamax (Invitrogen: Gibco). The media was replaced the day after plating, and cells were fed twice weekly thereafter. Hippocampal neurons were plated onto 12-mm glass coverslips (Warner Instruments) coated with poly-D-lysine (Sigma). All cells were grown at 37 °C and in 5% CO2.

For overexpression experiments, neurons in culture at 20 DIV were infected with Sindbis viruses expressing actin-YFP or CaMKII(1–290)-EGFP (ΔCaMKII-GFP) and processed 20 hours later for confocal imaging. Whole-cell patch-clamp recordings were made 20-30 hrs after infection. Recordings from infected (determined by the GFP fluorescence) and uninfected cell were performed from the same infected cultures.

Immunocytochemistry and confocal imaging

At 20 DIV, cultures were incubated with morphine sulfate salt pentahydrate (Sigma) at 100 μM for 20 hours. For experiments with pertussis toxin, PTX (Sigma; 250 ng/ml) was added to the culture medium and incubated for 6 h at 37˚C in the presence of morphine. KN-92, 93 (10 uM Calbiochem) were applied during the last two hours of the morphine treatment. DMSO (Sigma) was used as the vehicle control for the CaMKII inhibitor experiments. Naloxone (100 uM, Sigma Aldrich), DAMGO (100 uM, Tocris Ellisville, Missouri), 6-cyano-7-nitroquinoxaline-2,3-dione, NBQX (10 uM), picrotoxin (50 uM, Sigma Aldrich), GABA (100 uM), and DL-2-Amino-5-phosphonovaleric acid lithium salt, APV (100 uM Sigma Aldrich), Tetrodotoxin, TTX (2 uM), α-Methyl-(4-carboxyphenyl)glycine, MCPG (300 uM) were applied for 20 hours in the presence and absence of morphine. After washing, 21DIV neurons were fixed with -20°C methanol and permeabilized with 0.2% Triton-X in PBS. Blocking solution [2% bovine serum albumin (Jackson immuno Research), 5% goat serum (Sigma) , 0.2% Triton X (Sigma) in PBS] was then applied for 1 h at room temperature. Neurons were incubated with rabbit anti-GIRK2 (1:200; Alomone) and anti-PSD95 (1:300, Signal Transduction) antibodies overnight at 4°C. Cells were washed and incubated with Alexa 488 or 647-conjugated secondary antibodies (1:300, Molecular Probes) for 1 hour at room temperature. Primary and secondary antibodies were diluted in blocking solution. The coverslips were mounted on microscope slide using Progold antifading reagent (Molecular Probes) and imaged by confocal fluorescence microscopy (Leica).

Images were processed using Image J. Colocalization was visualized by merging red-green channels into RGB and using *RGB Colocalization* plug in (with default values) to determine the Pearson's coefficient. To compare different preparations of cultures and

different experimental conditions, we converted the Pearson's coefficient to a colocalization ratio, where the Pearson's coefficient for the experimental groups was normalized to the untreated control group. Dendritic fields containing many dendrites from multiple neurons were analyzed for most experiments. For actin-YFP experiments, one set of infected cultures was treated with morphine for 20 hours while another set from the same neuronal prep was used as control. Regions of isolated dendrites were studied for colocalization using the YFP fluorescence as the green channel and the endogenous PSD95 or GIRK2 as red. For the ΔCaMKII-GFP neurons, isolated infected and uninfected dendrites were used to quantify colocalization between PSD95 and GIRK2. For these images, GFP was captures at 488 nm, PSD95 at 568 nm, and GIRK2 at 647 nm (all bleed through was eliminated before images were obtained). For the purposes of the RGB colocalization quantification, GIRK2 was used as the red channel and PSD95 as the green. Data are presented as mean ± S.E.M. and evaluated for statistical significance using unpaired Student's t-test for groups of two, or one-way ANOVA for multiple groups using untreated as a control. For all analyses, $p \leq 0.05$ was considered statistically significant (SigmaStat 3.0; Systat Software).

Whole-cell patch-clamp electrophysiology

Patch-clamp recordings were made from hippocampal neurons which had the morphological features of pyramidal neurons as previously described [86-88]. Whole-cell patch-clamp recordings were carried out using borosilicate glass electrodes (Warner) with resistances of 5-8 MΩ when filled with the intracellular solution contained (in mM): 20 NaCl, 130 KCl, 5.46 $MgCl_2$, 10 HEPES/NaOH (pH 7.4), and 5 EGTA/KOH (pH 7.4). On the day of recording, 2.56 mM K_2ATP was added with either 300 μM Li_2GTP to the

intracellular solution. Macropscopic membrane currents were recorded using an Axopatch 200B (Axon Instruments) amplifier, adjusted electronically for cell capacitance and series resistance (80-100%), filtered at 2 kHz with an 8-pole Bessel filter, digitized at 5 kHz with a Digidata 1200 series interface (Axon Instruments), and stored on a laboratory computer. Glass patch pipettes Giga-ohm seals were formed and the whole-cell configuration obtained in extracellular solution contained (in mM): 156 NaCl, 4 KCl, 2 $MgCl_2$, 0.5 $CaCl_2$, and 10 HEPES/NaOH (pH 7.4). A different external solutions was perfused during recordings where NaCl was replaced with an equimolar concentration of KCl (140, 20 mM respectively) and also contained 100 μM APV, 50 μM Picrotoxin, and 2 μM TTX. This extracellular solution and other drugs were applied directly to the patched cell using a localized perfusion system. (±)-baclofen (Sigma Aldrich) was dissolved in water to make a 10 mM stock and then diluted directly into the extracellular solution. Data were acquired at room temperature (22–25º C) and analyzed with Clampfit 8.0 (Axon Instruments). Data are presented as mean ± S.E.M. and evaluated for statistical significance using unpaired Student's t-test, where $P \leq 0.05$ was considered significant (SigmaStat 3.0; Systat Software). All drugs used were obtained from Sigma Aldrich unless otherwise stated.

Immuno-gold electron microscopy and quantification:

The subcellular localization of GIRK2 in hippocampal cultures in control conditions and after treatment with morphine was analyzed using the pre-embedding immunogold method [89]. Briefly, hippocampal cultures were fixed using 4% paraformaldehyde + 0.2% glutaraldehyde + 15% picric acid in 0.1 PM (pH 7.4), during 4 hours. Because these technical conditions are not suitable for studying GIRK2 expression

in the post-synaptic density, we cannot make any conclusions about possible expresion of GIRK2 in PSD. Hippocampal cultures were incubated in 10% NGS diluted in TBS for 1 h at room temperature, and then they were incubated for 24 h in anti-GIRK2 antibodies (Alomone, Israel) at a final protein concentration of 1-2 μg ⁄ mL diluted in TBS containing 1% NGS. After several washes in TBS, sections were incubated for 3 h in goat anti-rabbit IgG coupled to 1.4 nm gold (Nanoprobes Inc., Stony Brook, NY, USA) diluted 1:100 in TBS containing 1% NGS. The cultures were then washed in phosphate-buffered saline and postfixed in 1% glutaraldehyde diluted in the same buffer for 10 min. They were washed in double-distilled water, followed by silver enhancement of the gold particles with an HQ Silver kit (Nanoprobes Inc.). This processing was followed by treatment with osmium tetraoxide (1% in 0.1 m PB), block-staining with uranyl acetate, dehydration in graded series of ethanol and embedding in Durcupan (Fluka) resin. Hippocampal cultures were cut at 70–90 nm on an ultramicrotome (Reichert Ultracut E; Leica, Austria) and collected on 200-mesh nickel grids. Staining was performed on drops of 1% aqueous uranyl acetate followed by Reynolds's lead citrate. Ultrastructural analyses were performed in a Jeol-1010 electron microscope.

Immunogold labeling was evaluated quantitatively to test differences in GIRK2 in control conditions and after treatment with morphine. Quantification was carried out on three ultra thin sections randomly chosen from each of four different coverslips. Electron microscopic serial ultrathin sections were cut close to the surface of each block because immunoreactivity decreased with depth. In hippocampal culture neurons, randomly selected areas were captured at a final magnification of 35,000X, and measurements covered a total section area of ~4000μm^2. Dendritic shafts, dendritic spines and axon

terminals were assessed for the presence of immunoparticles. In each reference area, the numbers of gold particles attached to the plasma membrane of dendritic spines (n=60 spines) were counted, and the length of the extrasynaptic membrane from all immunopositive spines was measured using a digitizing tablet and appropriate software (Sigma-Scan Pro; Jandel Scientific, Ekzath, Germany). Then, the density of immunoparticles for GIRK2 at dendritic spines, measured as number of immunoparticles per micron of spine plasma membrane, was calculated in control conditions and after treatment with morphine, and statistically compared using a Student's t-test. The background labeling was evaluated in the same way calculating the density of GIRK2 over mitochondria, nuclei and empty resin.

Results

To investigate the regulation of natively expressed GIRK channels in response to opioid receptor stimulation, we used mature (~3 weeks) cultures of rat hippocampal neurons [25]. Dissociated cultures of neurons are amenable to high resolution imaging and whole-cell patch-clamping. Importantly, hippocampal neurons develop dendritic spines, become electrically active, and express GIRK channels, G proteins and a variety of GPCRs *in vitro*. Dendritic spines were identified by immunohistochemistry using antibodies against PSD95 or NMDA receptors. Both NMDA and PSD95 colocalize in the dendritic spines of ~3 week old hippocampal neurons, while GIRK2 channels reside largely on the dendritic shaft, overlapping less with NMDA or PSD95 (Figure 1). The degree of colocalization was quantified by comparing the two fluorophores and deriving the Pearson's coefficient (see Methods for details). The Pearson's coefficient was

approximately 0.1 for GIRK2 vs. NMDA and GIRK2 vs. PSD95, as compared to ~0.45 for NMDA and PSD95 (Figure 1D).

We next examined the effect stimulating hippocampal neurons with a supra-maximal concentration of morphine (100 μM) for ~24 hrs. We chose a supra-maximal dose to ensure that all receptors including homo- and hetero-dimeric opioid receptors are fully activated. Morphine (100 μM) stimulation for ~24 hrs produced a dramatic change in GIRK2 expression, increasing the intensity of GIRK2 staining and increasing the colocalization of GIRK2 with PSD95 nearly 2-fold (Figure 2). The competitive opioid receptor antagonist naloxone (100 μM) prevented the change in GIRK2 expression with morphine (Figure 2C, 2E). Furthermore, a supra-maximal concentration of DAMGO (100 μM) did not alter the expression of GIRK2 channels (Figure 2D, E). Taken together, these results suggest the change in GIRK2 with a singe dose of morphine is specifically through opioid receptors. To facilitate the comparison of the change in colocalization among different neuronal preparations and experimental conditions, we calculated the change in colocalization relative to control (no morphine treatment) cultures that were paired with the experiment (Figure 2E). For example, morphine but not morphine plus naloxone increased the colocalization factor to nearly 2-fold. Morphine also increased the colocalization between GIRK2 and NMDA (data not shown) another marker for dendritic spines.

The increase in colocalization between GIRK2 and PSD95 proteins suggested that GIRK2 channels trafficked to the dendritic spine in response to morphine. However, a shift in the targeting of PSD95 to the dendritic shaft was also possible. To examine this directly, we infected hippocampal cultures with Sindbis virus encoding actin-YFP, which

enables visualization of the dendritic spines [26]. In these hippocampal cultures, infected neurons exhibited intense fluorescence in the spines. We examined the colocalization of PSD95 and actin-YFP in the absence or presence of morphine (100 μM). The colocalization ratio changed little with morphine stimulation (Figure 3A, B). By contrast, the colocalization of GIRK2 channels with actin-YFP increased in cultures treated with morphine (Figure 3C, D), supporting with the conclusion that GIRK2 channels are upregulated in the spine following morphine stimulation.

To investigate the localization of GIRK2 in the spine more directly, we carried out immuno-electron microscopic (EM) studies. To study the subcellular distributions of GIRK2 in hippocampal cultured neurons, control and morphine-treated cultures were subjected to pre-embedding immuno-gold techniques (Figure 4). Labeling for GIRK2 in control conditions was found primarily along the plasma membrane of dendritic spines and dendritic shafts of hippocampal cultured cells (Figure 4A), in addition to sites on intracellular membranes. The subcellular distribution pattern of GIRK2 in morphine treated neurons was similar to that described in control neurons, but labeling for GIRK2 was more frequently observed along the plasma membrane of dendritic spines (Figure 4B, D) and dendritic shafts (Figure 4B). To gain further insights into the up-regulation of GIRK2 in dendritic spines of morphine treated neurons, we measured the density of GIRK2 immuno-gold particles (see methods). The mean density for GIRK2 in control neurons was 2.15 ± 0.69 immuno-gold particles/μm of spine plasma membrane (Figure 4E), while that in morphine treated neurons was 4.59 ± 0.87 immuno-gold particles/μm of spine plasma membrane (Figure 4E, $p<.05$). Thus, morphine treatment increased more than 2-fold the GIRK2 protein in dendritic spines (Figure 4E).

If morphine stimulation promoted release of GABA or glutamate, then receptor antagonists would be expected to attenuate the increase in colocalization between GIRK2 and PSD95. Inclusion of antagonists for $GABA_A$, or $GABA_B$, or both $GABA_A$ and $GABA_B$ did not prevent the morphine-dependent change in colocalization (Figure 5A, B, D). Like GABA, inclusion of both ionotropic and metabotropic glutamate receptor antagonists did not prevent the morphine-induced increase in colocalization of GIRK2 and PSD95 (Figure 5C, E). Thus, morphine does not appear to act presynaptically by promoting release of neurotransmitters. If morphine produced disinhibition, then $GABA_A$ antagonist might mimic the effect of morphine. We did not observe any change in the colocalization of GIRK2 and PSD95 with bicuculline (Figure 5). Conversely, direct stimulation of postsynaptic GABA receptors with GABA also did not mimic the effect of morphine (Figure 5D). Finally, inhibiting intrinsic electrical activity with 2 μM TTX did not prevent the morphine-induced increase in colocalization between GIRK2 and PSD95 (Figure 5D). Taken together, these results suggest that the morphine-induced change in GIRK2 localization does not involve presynaptic terminals.

Opioid receptors commonly signal through PTX-sensitive G proteins but also signal via PTX-insensitive G proteins [27-32]. We investigated this possibility by examining the effect of morphine in the absence or presence of PTX for 6 hrs (Figure 5F). Inhibiting PTX-sensitive G proteins did not occlude the morphine-dependent increase in colocalization, suggesting that PTX-insensitive G proteins mediate the effect of morphine (Figure 5F).

If morphine stimulation of opioid receptors activates PTX-insensitive G proteins, which can activate IP3 and increase intracellular Ca^{2+}, then morphine might activate

CaMKII in hippocampal neurons. Consistent with this, previous studies have shown that morphine stimulates the activity of CaMKII [33-35]. To investigate this possibility, we incubated neurons in a membrane permeant inhibitor of CaMKII (KN-93; [36]. Colocalization of GIRK2 and PSD95 in morphine treated hippocampal neurons exposed to 10 μM of KN-93 were indistinguishable from control cultures (Figure 6A-D). By contrast, the inactive peptide, KN-92, did not block the morphine-dependent increase in GIRK2/PSD95 colocalization (Figure 6B, D). These results implicate the CaMKII pathway in the morphine-dependent increase in GIRK2 and PSD95 colocalization.

To examine the role of CaMKII directly and bypass the opioid receptor, hippocampal neurons were infected with a Sindbis virus encoding the constitutively active form of CaMKII fused to GFP (ΔCaMKII-GFP). ΔCaMKII-GFP was found previously to be required for insertion of GluR1 receptors into the dendritic spine [37]. Similar to the effect of morphine, expression of ΔCaMKII-GFP significantly increased the colocalization of GIRK2 and PSD95 as compared to uninfected dendrites (Figure 7). Thus, either stimulating with morphine, or expressing activated CaMKII in hippocampal neurons resulted in an upregulation of GIRK2 channels in the dendritic spines.

We next investigated the physiological consequence of altered GIRK2 expression in hippocampal neurons. Several attempts to record whole-cell patch-clamp recordings from ~3 week old neurons failed, perhaps due to age of the neuron and fragile membranes. To circumvent this problem, we examined younger (11-14 DIV) cultures of hippocampal neurons, which are more amenable to patch-clamp recordings. We thus established that chronic (24 hr) morphine (100 μM) stimulation of opioid receptors increased the colocalization of GIRK2 and PSD95 in younger hippocampal neuronal

cultures (Figure 8A-C). Like the ~ 3 week old neurons, we observed a nearly 2-fold increase in the colocalization of GIRK2 and PSD95. Macroscopic current recordings from untreated hippocampal neurons revealed large $GABA_B$ receptor-activated currents (Figure 9A). Activated currents show inward rectification and reversed near the potassium equilibrium potential, indicating activation of inwardly rectifying potassium channels.

Interestingly, morphine treatment significantly decreased the amplitude of baclofen-activated GIRK currents (Figure 9B, C). A similar decrease was observed for adenosine and somatostatin receptor activated GIRK currents (data not shown). Consistent with an increase in GIRK expression, the Ba-sensitive basal current was also increased in morphine treated hippocampal neurons (Figure 9D). Because the CaMKII pathway was implicated in the change in trafficking of GIRK2 with morphine, we reasoned the decrease in $GABA_B$ receptor activated GIRK current might be mimicked by expression of ΔCaMKII. To examine this possibility, we measured the macroscopic currents in neurons infected with ΔCaMKII-GFP containing virus. Like morphine, the amplitudes of $GABA_B$ receptor activated currents were >50% smaller in neurons coexpressing ΔCaMKII-GFP (Figure 9E, F).

Treating the hippocampal cultures with a selective MOR agonist, DAMGO, did not mimic the morphine induced trafficking of GIRK2 into the dendritic spine (Figure 2). We thus investigated the agonist dependency of this effect. To our surprise met-enkephalin also increased colocalization between GIRK2 and PSD-95 (Figure 10A-D). This increase in spine trafficking for the GIRKs was dose dependent (Figure 10E). In fact met-emkephalin seems to be a more potent agonist than morphine as 10 uM was

sufficient to increase the colocalization between GIRK2 and PSD-95. However, other selective MOR or DOR agonists (endomorphin1, DPDPE) did not change GIRK2 expression pattern.

If an increase in colocalization between GIRK2 and PSD-95 elicits a decrease in $GABA_B$ mediated GIRK currents, we should expect agonist that don't change GIRK trafficking not to elicit the same electrophysiological effects. Consistent with the immunocytochemistry studies, treating the dissociated cultures with 100 uM of DAMGO did not cause a decrease in baclofen induced GIRK currents.

Discussion

GIRK channels provide an important inhibitory pathway in the brain [6]. In the current study, we present new evidence for a selective GPCR and CaMKII-dependent pathway for regulating natively expressed neuronal GIRK channels. Chronic (24 hr) morphine stimulation of cultured hippocampal neurons increases the colocalization between GIRK2 and PSD95 in the dendritic spine. Consistent with this, morphine treatment increased GIRK2 channel colocalization with a spine-specific marker, actin-YFP, and ultrastructural analyses demonstrated a >2-fold immuno-gold labeling of GIRK2 channels in the dendritic spines of morphine-treated neurons. These morphine-dependent changes in GIRK expression required activation of CaMKII. Remarkably, both morphine treatment and CaMKII activation reduced the amplitude of $GABA_B$ receptor GIRK macroscopic currents. Taken together, our results suggest that stimulation of opioid receptors reveals a form of CaMKII-dependent plasticity for $GABA_B$ receptor–GIRK channel inhibition.

Opioid regulation of signaling in hippocampus

Opioids have been shown to modulate synaptic transmission by altering glutamatergic transmission, long term potentiation, and dendritic stability [21-24, 38-52]. These effects are achieved by suppressing presynaptic release in GABAergic inhibitory neurons [53, 54] or by activating opioid receptors expressed postsynaptically on glutamatergic neurons [31, 52]. Three types of opioid receptors (MOR, DOR and KOR) are expressed in the hippocampus. Each is activated by alkaloids and opioid peptides coreleased with glutamate from mossy-fiber and lateral perforant path synapses [55-57]. MORs are expressed at high levels on interneurons where they regulate presynaptic release of GABA [58]. However, MORs are also expressed in dendritic shaft and spines of pyramidal neurons, where they have been shown to be involved in spine morphology [31, 52]. In addition to MOR, KORs are also expressed postsynaptically in pyramidal neurons [59-61]. Antagonists for GABA/glutamate receptors and TTX treatment did not prevent the morphine-dependent upregulation of GIRK2 in the dendritic spine. These results the postsynaptic action of morphine is required for the upregulation of GIRK2.

The inhibition of morphine-dependent upregulation of GIRK2 with naloxone confirms the involvement of opioid receptors. DAMGO, however, did not mimic the effect of morphine. One possible explanation for this difference is that DAMGO but not morphine promotes receptor internalization [62, 63]. Thus, the sustained activation of opioid receptors could be essential for the postsynaptic activation. Alternatively, morphine acts on multiple types of opioid receptors, while DAMGO is a selective agonist for MOR. Interestingly, opioid receptors can form both homo- and heterodimers [27, 29,

64-66]. Oligomerization of opioid receptors has been suggested to play a role in receptor activation and desensitization [27, 29, 64-67]. Furthermore, hetero-oligomerization of opioid receptors can generate novel ligand binding properties, such as lower agonist affinities, and coupling to PTX-insensitive G Proteins [27-29, 68-70]. In the current study, we found that a supra-maximal concentration (100 μM) of morphine, which would stimulate all combinations of opioid receptors, was insensitive to PTX. Consistent with this, heterologous expression studies with KOR/MOR have shown that PTX does not shift the receptors to a low-affinity unbound state (see above). In support of this, acute application of morphine inhibited basal GIRK currents (data not shown), which can occur because of activation of Gq G proteins and depletion of PIP_2 [71, 72]. Thus, a tenable model is that morphine stimulates heterodimers, leading to activation of CaMKII and upregulation of GIRK2 channels in dendritic spines.

The regulation of GIRK channels by CaMKII could underlie some morphine-dependent changes in synaptic plasticity. CaMKII is well known for its involvement in modifying synaptic plasticity is CaMKII [73, 74]. Targeted disruption of CaMKII produces deficits in LTP and severely impairs performance in hippocampal-dependent memory tasks [75-77]. Morphine treatment significantly increases CaMKII activity in the hippocampus of rats [35, 78]. Furthermore, administration of CaMKII antisense oligonucleotides into the hippocampus attenuates morphine tolerance and dependence [79]. Recently, Narita et al. [80] and Fan et al. [79] showed that although inhibiting CaMKII significantly attenuated the place preference induced by morphine. Finally, CaMKII is highly enriched in postsynaptic densities of hippocampus and neocortex where it colocalizes with opioid receptors and could therefore be involved in the opioid-

induced plasticity [81]. Taken together, these studies suggest CaMKII may be a common site for convergence for opioid receptor-induced and glutamate receptor-induced changes in synaptic plasticity.

Mechanism for reduced $GABA_B$ receptor-activated current and increased GIRK2 expression in dendritic spines

Both morphine treatment and CaMKII activity increased GIRK2 expression in the dendritic spines coincident with decreased amplitude of $GABA_B$ receptor-activated GIRK currents. Alternatively, the increase in GIRK2 protein expression and upregulation of GIRK channels in the dendritic spine could have resulted in an increase in $GABA_B$ receptor-activated current. There are several possible mechanisms to explain the reduction in GIRK current. One possibility is that GIRK2 channels internalize at dendritic shafts and are re-targeted to the dendritic spine. Because whole-cell recordings with bath applied baclofen are dominated by somatodendritic $GABA_B$ receptors and GIRK channels, it might be difficult to monitor an increase of GIRK2 channels in the dendritic spine. GIRK channels are clearly expressed in the somatodendritic regions of the neuron, as evidenced by recordings of basally active dendritic GIRK channels [82] and large baclofen-activated GIRK currents in dissociated hippocampal neurons [83]. Thus, it remains possible that morphine-treatment and CaMKII activation of GIRK channels leads to larger synaptically-evoked GIRK currents. Consistent with this explanation, over expression of □CaMKII in hippocampal neurons potentiates the sIPSP [17].

Another possibility is that GIRK2 is upregulated in a subset of spines that are electrically and chemically isolated from the shaft [16]. Alternatively, GIRK2 channels

expressed in the dendritic spine may couple to a different GPCR. For example, stimulation of adenosine and somatostatin receptors activates GIRK channels in hippocampal neurons [83]. However, both adenosine and somatostatin activated GIRK currents are reduced like $GABA_B$-activated currents (data not shown). Finally, increasing the physical distance between $GABA_B$ receptors and GIRK channels, either by free diffusion or by internalization, would lead to smaller $GABA_B$ receptor activated currents. Immuno-EM studies indicate that GIRK channels and $GABA_B$ receptors are spatially segregated on the dendritic shaft [9, 84]. Increasing the separation between $GABA_B$ and GIRKs, or selectively internalizing $GABA_B$ receptors would lead to a net reduction in the amplitude of $GABA_B$ receptor activated current.

Acknowledgments

We are grateful to Dr. Jose A. Esteban for his generosity in providing the ΔCaMKII-GFP virus and to Dr. Gentry Patrick and his lab for the actin-YFP virus, technical input and providing some of the neuronal cultures. This work was made possible by financial support from a Spanish Ministry of Education and Science (BFU-2006-01896, RL) grant, and to McKnight Endowment for Neuroscience (PAS) and the National Institute of Neurological Disorders and Stroke (NIH R01 NS37682, PAS).

This chapter is a modified version of the material as it appears in Nassirpour, Lujan and Slesinger. 2008 (in preparation). It also includes additional data not included in the manuscript. The dissertation author was the primary author of this paper.

References

1. Trevelyan, A.J. and O. Watkinson, *Does inhibition balance excitation in neocortex?* Progress in Biophysics and Molecular Biology, 2005. **87**(1): p. 109-143.

2. Gulyas, A.I., et al., *Total Number and Ratio of Excitatory and Inhibitory Synapses Converging onto Single Interneurons of Different Types in the CA1 Area of the Rat Hippocampus.* J. Neurosci., 1999. **19**(22): p. 10082-10097.

3. MegIas, M., et al., *Total number and distribution of inhibitory and excitatory synapses on hippocampal CA1 pyramidal cells.* Neuroscience, 2001. **102**(3): p. 527-540.

4. Liu, G., *Local structural balance and functional interaction of excitatory and inhibitory synapses in hippocampal dendrites.* Nat Neurosci, 2004. **7**(4): p. 373-379.

5. Ikeda, S.R., *Voltage-dependent modulation of N-type calcium channels by G-protein beta gamma subunits.* Nature, 1996. **380**(6571): p. 255-8.

6. Lüscher, C., et al., *G Protein-Coupled Inwardly Rectifying K+ Channels (GIRKs) Mediate Postsynaptic but Not Presynaptic Transmitter Actions in Hippocampal Neurons.* Neuron, 1997. **19**(3): p. 687-695.

7. Scanziani, M., *GABA spillover activates postsynaptic GABA(B) receptors to control rhythmic hippocampal activity.* Neuron, 2000. **25**(3): p. 673-81.

8. Isaacson, J.S., J.M. Solis, and R.A. Nicoll, *Local and diffuse synaptic actions of GABA in the hippocampus.* Neuron, 1993. **10**(2): p. 165-75.

9. Kulik, A., et al., *Compartment-dependent colocalization of Kir3.2-containing K+ channels and GABAB receptors in hippocampal pyramidal cells.* J Neurosci, 2006. **26**(16): p. 4289-97.

10. Kulik, A., et al., *Subcellular localization of metabotropic GABA(B) receptor subunits GABA(B1a/b) and GABA(B2) in the rat hippocampus.* J Neurosci, 2003. **23**(35): p. 11026-35.

11. Harris, K.M. and S.B. Kater, *Dendritic Spines: Cellular Specializations Imparting Both Stability and Flexibility to Synaptic Function.* Annual Review of Neuroscience, 1994. **17**(1): p. 341-371.

12. Hering, H. and M. Sheng, *Dentritic spines : structure, dynamics and regulation.* Nat Rev Neurosci, 2001. **2**(12): p. 880-888.

13. Kennedy, M.B., *The postsynaptic density at glutamatergic synapses.* Trends in Neurosciences, 1997. **20**(6): p. 264-268.

14. Kennedy, M.B., *Signal-Processing Machines at the Postsynaptic Density.* Science, 2000. **290**(5492): p. 750-754.

15. Nimchinsky, E.A., B.L. Sabatini, and K. Svoboda, *STRUCTURE AND FUNCTION OF DENDRITIC SPINES.* Annual Review of Physiology, 2002. **64**(1): p. 313-353.

16. Bloodgood, B.L. and B.L. Sabatini, *Neuronal Activity Regulates Diffusion Across the Neck of Dendritic Spines.* Science, 2005. **310**(5749): p. 866-869.

17. Huang, C.S., et al., *Common Molecular Pathways Mediate Long-Term Potentiation of Synaptic Excitation and Slow Synaptic Inhibition.* Cell, 2005. **123**(1): p. 105-118.

18. Xu, N.-J., et al., *Morphine Withdrawal Increases Glutamate Uptake and Surface Expression of Glutamate Transporter GLT1 at Hippocampal Synapses.* J. Neurosci., 2003. **23**(11): p. 4775-4784.

19. Eisch, A.J., et al., *Opiates inhibit neurogenesis in the adult rat hippocampus.* Proceedings of the National Academy of Sciences, 2000. **97**(13): p. 7579-7584.

20. Liao, D., et al., *Mu-opioid receptors modulate the stability of dendritic spines.* Proceedings of the National Academy of Sciences, 2005. **102**(5): p. 1725-1730.

21. Bao, G., et al., *Morphine and Heroin Differentially Modulate In Vivo Hippocampal LTP in Opiate-Dependent Rat.* Neuropsychopharmacology, 2007. **32**(8): p. 1738-1749.

22. Pu, L., et al., *Hippocampal Long-Term Potentiation Is Reduced by Chronic Opiate Treatment and Can Be Restored by Re-Exposure to Opiates.* J. Neurosci., 2002. **22**(5): p. 1914-1921.

23. Salmanzadeh, F., et al., *Dependence on morphine impairs the induction of long-term potentiation in the CA1 region of rat hippocampal slices.* Brain Research, 2003. **965**(1-2): p. 108-113.

24. Salmanzadeh, F., et al., *Dependence on morphine leads to a prominent sharing among the different mechanisms of long-term potentiation in the CA1 region of rat hippocampus.* Brain Research, 2003. **963**(1-2): p. 93-100.

25. Papa, M., et al., *Morphological analysis of dendritic spine development in primary cultures of hippocampal neurons.* J Neurosci, 1995. **15**(1 Pt 1): p. 1-11.

26. Honkura, N., et al., *The Subspine Organization of Actin Fibers Regulates the Structure and Plasticity of Dendritic Spines.* Neuron, 2008. **57**(5): p. 719-729.

27. George, S.R., et al., *Oligomerization of {micro}- and delta -Opioid Receptors. GENERATION OF NOVEL FUNCTIONAL PROPERTIES.* J. Biol. Chem., 2000. **275**(34): p. 26128-26135.

28. Gomes, I., et al., *Oligomerization of opioid receptors.* Methods, 2002. **27**(4): p. 358-365.

29. Jordan, B.A. and L.A. Devi, *G-protein-coupled receptor heterodimerization modulates receptor function.* Nature, 1999. **399**(6737): p. 697-700.

30. Law, P.-Y., et al., *Heterodimerization of {micro}- and {delta}-Opioid Receptors Occurs at the Cell Surface Only and Requires Receptor-G Protein Interactions.* J. Biol. Chem., 2005. **280**(12): p. 11152-11164.

31. Liao, D., et al., *Distinct effects of individual opioids on the morphology of spines depend upon the internalization of mu opioid receptors.* Molecular and Cellular Neuroscience, 2007. **35**(3): p. 456-469.

32. Wang, D., et al., *Opioid Receptor Homo- and Heterodimerization in Living Cells by Quantitative Bioluminescence Resonance Energy Transfer.* Mol Pharmacol, 2005. **67**(6): p. 2173-2184.

33. Fan, G.H., et al., *Modulation by calcium/calmodulin-dependent protein kinase II of functional response of delta opioid receptor in neuroblastoma x glioma hybrid (NG108-15) cells.* Neuropharmacology, 1997. **36**(11-12): p. 1763-1769.

34. Liu, J.-G. and K.J.S. Anand, *Protein kinases modulate the cellular adaptations associated with opioid tolerance and dependence.* Brain Research Reviews, 2001. **38**(1-2): p. 1-19.

35. Lou, L., et al., *Modulation of Ca2+/Calmodulin-Dependent Protein Kinase II Activity by Acute and Chronic Morphine Administration in Rat Hippocampus: Differential Regulation of alpha and beta Isoforms.* Mol Pharmacol, 1999. **55**(3): p. 557-563.

36. Margrie, T.W., J.A.P. Rostas, and P. Sah, *Presynaptic long-term depression at a central glutamatergic synapse: a role for CaMKII.* Nat Neurosci, 1998. **1**(5): p. 378-383.

37. Hayashi, Y., et al., *Driving AMPA Receptors into Synapses by LTP and CaMKII: Requirement for GluR1 and PDZ Domain Interaction.* Science, 2000. **287**(5461): p. 2262-2267.

38. Sarkaki, A., et al., *Effect of parental morphine addiction on hippocampal long-term potentiation in rats offspring.* Behavioural Brain Research, 2008. **186**(1): p. 72-77.

39. San Nan Yang, L.-T.H.C.-L.W.W.-F.C.C.-H.Y.S.-Z.L.M.-C.L.S.-J.C.P.-L.T., *Prenatal administration of morphine decreases CREB,Serine-133 phosphorylation and synaptic plasticity range mediated by glutamatergic transmission in the hippocampal CA1 area of cognitive-deficient rat offspring.* Hippocampus, 2003. **13**(8): p. 915-921.

40. Ito, Y., et al., *Acute and chronic intracerebroventricular morphine infusions affect long-term potentiation differently in the lateral perforant path.* Pharmacology Biochemistry and Behavior, 2001. **70**(2-3): p. 353-358.

41. Akaishi, T., et al., *Morphine augments excitatory synaptic transmission in the dentate gyrus through GABAergic disinhibition.* Neuroscience Research, 2000. **38**(4): p. 357-363.

42. Terman, G.W., C.L. Eastman, and C. Chavkin, *Mu Opiates Inhibit Long-Term Potentiation Induction in the Spinal Cord Slice.* J Neurophysiol, 2001. **85**(2): p. 485-494.

43. Velísek, L., et al., *Prenatal morphine exposure enhances seizure susceptibility but suppresses long-term potentiation in the limbic system of adult male rats.* Brain Research, 2000. **869**(1-2): p. 186-193.

44. Terman, G.W., et al., *Opioid Modulation of Recurrent Excitation in the Hippocampal Dentate Gyrus.* J. Neurosci., 2000. **20**(12): p. 4379-4388.

45. Mansouri, F.A., F. Motamedi, and Y. Fathollahi, *Chronic in vivo morphine administration facilitates primed-bursts-induced long-term potentiation of Schaffer collateral-CA1 synapses in hippocampal slices in vitro.* Brain Research, 1999. **815**(2): p. 419-423.

46. Yu, T.-P. and C.-W. Xie, *Orphanin FQ/Nociceptin Inhibits Synaptic Transmission and Long-Term Potentiation in Rat Dentate Gyrus Through Postsynaptic Mechanisms.* J Neurophysiol, 1998. **80**(3): p. 1277-1284.

47. Williams, S.H. and D. Johnston, *Actions of Endogenous Opioids on NMDA Receptor-Independent Long-Term Potentiation in Area CA3 of the Hippocampus.* J. Neurosci., 1996. **16**(11): p. 3652-3660.

48. Terman, G.W., J.J. Wagner, and C. Chavkin, *Kappa opioids inhibit induction of long-term potentiation in the dentate gyrus of the guinea pig hippocampus.* J. Neurosci., 1994. **14**(8): p. 4740-4747.

49. Wagner, J.J., G.W. Terman, and C. Chavkin, *Endogenous dynorphins inhibit excitatory neurotransmission and block LTP induction in the hippocampus.* Nature, 1993. **363**(6428): p. 451-454.

50. Xie, C.W. and D.V. Lewis, *Opioid-mediated facilitation of long-term potentiation at the lateral perforant path-dentate granule cell synapse.* J Pharmacol Exp Ther, 1991. **256**(1): p. 289-96.

51. Li, Y., et al., *Chronic morphine exposure alters the dendritic morphology of pyramidal neurons in visual cortex of rats.* Neuroscience Letters, 2007. **418**(3): p. 227-231.

52. Liao, D., et al., *Agonist-Dependent Postsynaptic Effects of Opioids on Miniature Excitatory Postsynaptic Currents in Cultured Hippocampal Neurons.* J Neurophysiol, 2007. **97**(2): p. 1485-1494.

53. Simmons, M.L. and C. Chavkin, *Endogenous opioid regulation of hippocampal function.* Int Rev Neurobiol, 1996. **39**: p. 145-96.

54. Zieglgansberger, W., et al., *Opioid peptides may excite hippocampal pyramidal neurons by inhibiting adjacent inhibitory interneurons.* Science, 1979. **205**(4404): p. 415-7.

55. Conner-Kerr, T.A., et al., *Evidence for the Corelease of Dynorphin and Glutamate from Rat Hippocampal Mossy Fiber Terminals.* Journal of Neurochemistry, 1993. **61**(2): p. 627-636.

56. Mansour, A., et al., *Autoradiographic differentiation of mu, delta, and kappa opioid receptors in the rat forebrain and midbrain.* J. Neurosci., 1987. **7**(8): p. 2445-2464.

57. Stengaard-Pedersen, K., K. Fredens, and L.I. Larsson, *Comparative localization of enkephalin and cholecystokinin immunoreactivities and heavy metals in the hippocampus.* Brain Research, 1983. **273**(1): p. 81-96.

58. Arvidsson, U., et al., *Distribution and targeting of a mu-opioid receptor (MOR1) in brain and spinal cord.* J. Neurosci., 1995. **15**(5): p. 3328-3341.

59. Arvidsson, U., et al., *The {kappa}-Opioid Receptor is Primarily Postsynaptic: Combined Immunohistochemical Localization of the Receptor and Endogenous Opioids.* Proceedings of the National Academy of Sciences, 1995. **92**(11): p. 5062-5066.

60. George, S.R., et al., *Distinct Distributions of Mu, Delta and Kappa Opioid Receptor mRNA in Rat Brain.* Biochemical and Biophysical Research Communications, 1994. **205**(2): p. 1438-1444.

61. Tempel, A. and R.S. Zukin, *Neuroanatomical Patterns of the {micro} , delta , and kappa Opioid Receptors of Rat Brain as Determined by Quantitative in vitro Autoradiography.* Proceedings of the National Academy of Sciences, 1987. **84**(12): p. 4308-4312.

62. von Zastrow, M., *Opioid receptor regulation.* NeuroMolecular Medicine, 2004. **5**(1): p. 51-58.

63. Whistler, J.L., et al., *Functional Dissociation of [mu] Opioid Receptor Signaling and Endocytosis: Implications for the Biology of Opiate Tolerance and Addiction.* Neuron, 1999. **23**(4): p. 737-746.

64. Cvejic, S. and L.A. Devi, *Dimerization of the delta Opioid Receptor:. IMPLICATION FOR A ROLE IN RECEPTOR INTERNALIZATION.* J. Biol. Chem., 1997. **272**(43): p. 26959-26964.

65. McVey, M., et al., *Monitoring Receptor Oligomerization Using Time-resolved Fluorescence Resonance Energy Transfer and Bioluminescence Resonance Energy Transfer. THE HUMAN delta -OPIOID RECEPTOR DISPLAYS CONSTITUTIVE OLIGOMERIZATION AT THE CELL SURFACE, WHICH IS NOT REGULATED BY RECEPTOR OCCUPANCY.* J. Biol. Chem., 2001. **276**(17): p. 14092-14099.

66. Ramsay, D., et al., *Homo- and hetero-oligomeric interactions between G-protein-coupled receptors in living cells monitored by two variants of bioluminescence resonance energy transfer (BRET): hetero-oligomers between receptor subtypes form more efficiently than between less closely related sequences.* Biochem. J., 2002. **365**(2): p. 429-440.

67. He, L., et al., *Regulation of Opioid Receptor Trafficking and Morphine Tolerance by Receptor Oligomerization.* Cell, 2002. **108**(2): p. 271-282.

68. Connor, M. and M.J. Christie, *OPIOID RECEPTOR SIGNALLING MECHANISMS.* Clinical and Experimental Pharmacology and Physiology, 1999. **26**(7): p. 493-499.

69. Gomes, I., et al., *Heterodimerization of {micro} and delta Opioid Receptors: A Role in Opiate Synergy.* J. Neurosci., 2000. **20**(22): p. 110RC-.

70. Jordan, B.A., S. Cvejic, and L.A. Devi, *Opioids and Their Complicated Receptor Complexes.* Neuropsychopharmacology, 2000. **23**(4, Supplement 1): p. S5-S18.

71. Cho, H., et al., *Receptor-induced depletion of phosphatidylinositol 4,5-bisphosphate inhibits inwardly rectifying K+ channels in a receptor-specific manner.* Proceedings of the National Academy of Sciences, 2005. **102**(12): p. 4643-4648.

72. Sohn, J.-W., et al., *Receptor-specific inhibition of GABAB-activated K+ currents by muscarinic and metabotropic glutamate receptors in immature rat hippocampus.* J Physiol, 2007. **580**(2): p. 411-422.

73. Malinow, R., H. Schulman, and R.W. Tsien, *Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP.* Science, 1989. **245**(4920): p. 862-866.

74. Tan, S.E., R.J. Wenthold, and T.R. Soderling, *Phosphorylation of AMPA-type glutamate receptors by calcium/calmodulin- dependent protein kinase II and protein kinase C in cultured hippocampal neurons.* J. Neurosci., 1994. **14**(3): p. 1123-1129.

75. Mayford, M., et al., *CaMKII regulates the frequency-response function of hippocampal synapses for the production of both LTD and LTP.* Cell, 1995. **81**(6): p. 891-904.

76. Silva, A.J., et al., *Impaired spatial learning in alpha-calcium-calmodulin kinase II mutant mice.* Science, 1992. **257**(5067): p. 206-211.

77. Silva, A.J., et al., *Deficient hippocampal long-term potentiation in alpha-calcium-calmodulin kinase II mutant mice.* Science, 1992. **257**(5067): p. 201-206.

78. Zhong, W., et al., *Opiate withdrawal induces dynamic expressions of AMPA receptors and its regulatory molecule CaMKII[alpha] in hippocampal synapses.* Life Sciences, 2006. **79**(9): p. 861-869.

79. Fan, G.-H., et al., *Inhibition of Calcium/Calmodulin-Dependent Protein Kinase II in Rat Hippocampus Attenuates Morphine Tolerance and Dependence.* Mol Pharmacol, 1999. **56**(1): p. 39-45.

80. Narita, M., et al., *Role of the calcium/calmodulin-dependent protein kinase ii (CaMKII) in the morphine-induced pharmacological effects in the mouse.* Neuroscience, 2004. **126**(2): p. 415-421.

81. Brüggemann, I., et al., *Colocalization of the [mu]-opioid receptor and calcium/calmodulin-dependent kinase II in distinct pain-processing brain regions.* Molecular Brain Research, 2000. **85**(1-2): p. 239-250.

82. Chen, X. and D. Johnston, *Constitutively Active G-Protein-Gated Inwardly Rectifying K+ Channels in Dendrites of Hippocampal CA1 Pyramidal Neurons.* J. Neurosci., 2005. **25**(15): p. 3787-3792.

83. Leaney, J.L., *Contribution of Kir3.1, Kir3.2A and Kir3.2C subunits to native G protein-gated inwardly rectifying potassium currents in cultured hippocampal neurons.* European Journal of Neuroscience, 2003. **18**(8): p. 2110-2118.

84. Koyrakh, L., et al., *Molecular and Cellular Diversity of Neuronal G-Protein-Gated Potassium Channels.* J. Neurosci., 2005. **25**(49): p. 11468-11478.

85. Brewer, G.J., et al., *Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination.* J Neurosci Res, 1993. **35**(5): p. 567-76.

86. Shah, M. and D.G. Haylett, *Ca2+ Channels Involved in the Generation of the Slow Afterhyperpolarization in Cultured Rat Hippocampal Pyramidal Neurons.* J Neurophysiol, 2000. **83**(5): p. 2554-2561.

87. Sodickson, D.L. and B.P. Bean, *GABAB Receptor-Activated Inwardly Rectifying Potassium Current in Dissociated Hippocampal CA3 Neurons.* J. Neurosci., 1996. **16**(20): p. 6374-6385.

88. Sodickson, D.L. and B.P. Bean, *Neurotransmitter Activation of Inwardly Rectifying Potassium Current in Dissociated Hippocampal CA3 Neurons: Interactions among Multiple Receptors.* J. Neurosci., 1998. **18**(20): p. 8153-8162.

89. Lujan, R., et al., *Perisynaptic location of metabotropic glutamate receptors mGluR1 and mGluR5 on dendrites and dendritic spines in the rat hippocampus.* Eur J Neurosci, 1996. **8**(7): p. 1488-500.

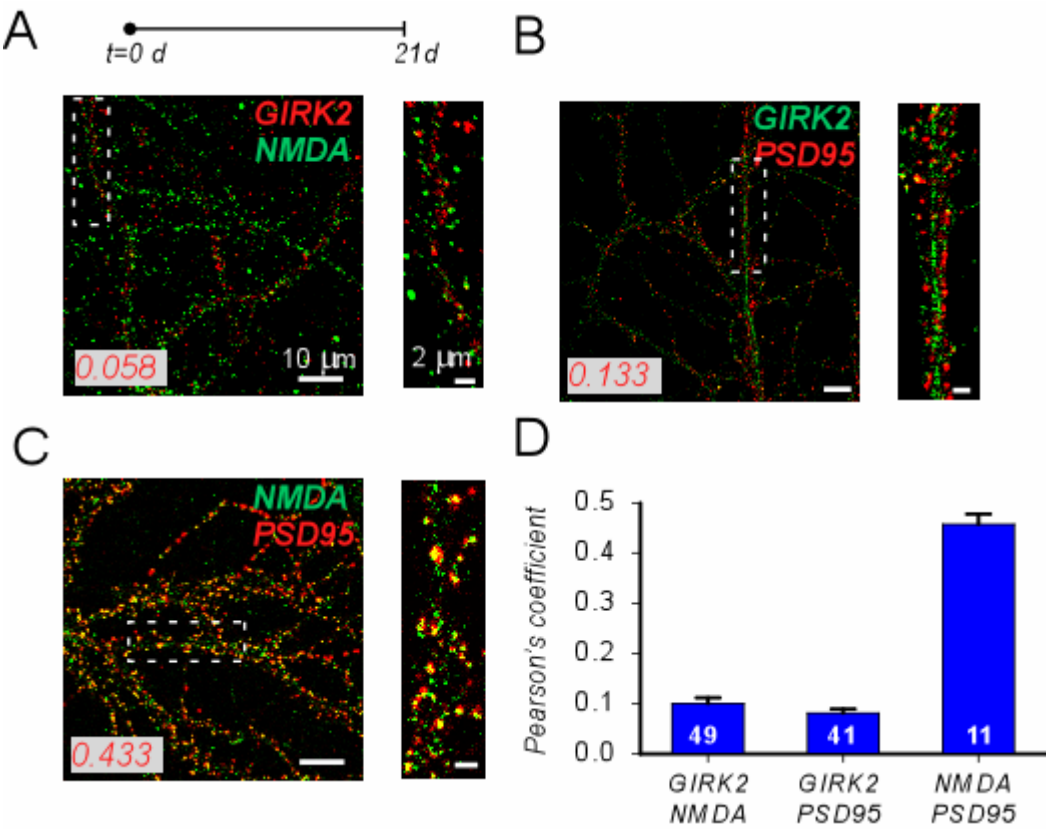


Fig 3.1. **GIRK2 is highly expressed in the dendritic shaft of dissociated hippocampal neurons and overlaps little with dendritic spine markers (PSD95 and NMDA receptors).** 21DIV cultures were stained for GIRK2 and spine markers NMDA receptors (A), PSD95 (B). Little colocalization of GIRK2 with the dendritic spine markers is observed, suggesting GIRK channels are expressed mainly on the dendritic shaft. C) Comparison of PSD95 and NMDA receptors. As expected, PSD95 and NMDA exhibited high degree of colocalization (evident by yellow in merged image). The extent of colocalization was determined by deriving the Pearson's coefficient for red-green images. The Pearson's coefficient is shown for each image. The zooms show magnification of a single dendritic branch. D) Bar graph shows the average (± SEM) Pearson's coefficient for GIRK2/NMDA, GIRK2/PSD95 and NMDA/PSD95 (with the number of dendritic fields indicated).

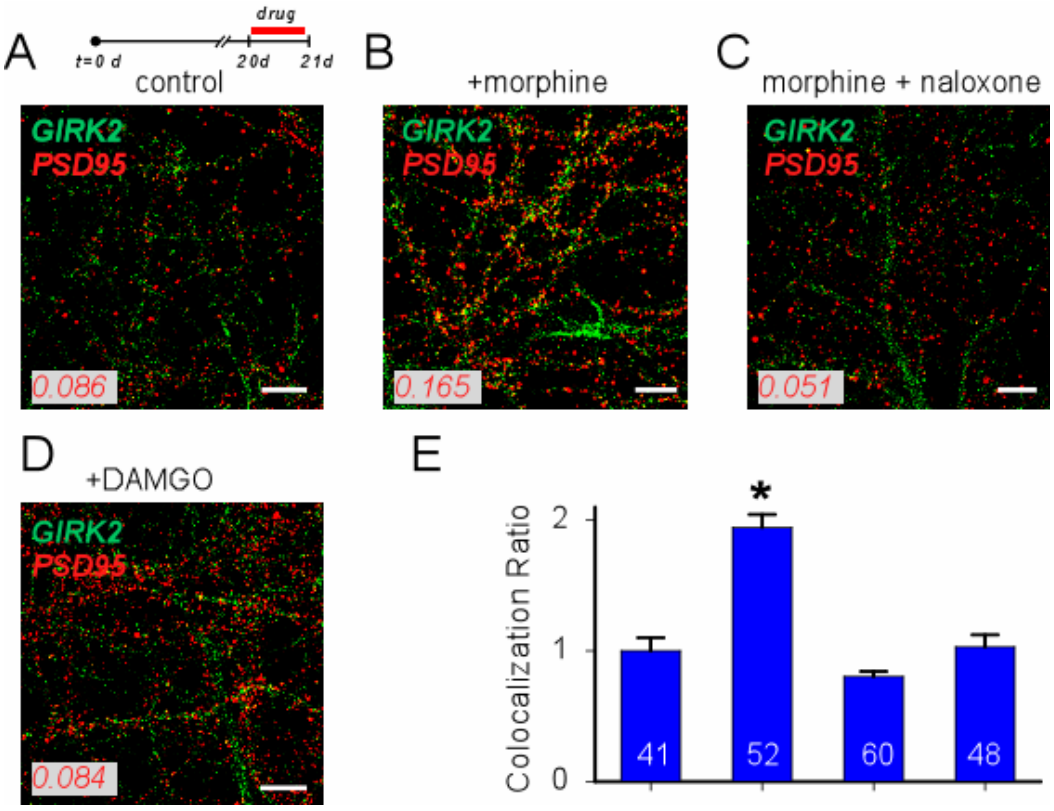


Fig 3.2. **Stimulation of opioid receptors with morphine alters GIRK2 localization in hippocampal neurons.** A-B) Mature dissociated hippocampal neurons were exposed to morphine for ~ 1 day (inset). Morphine (100 μM) increases the colocalization of GIRK2 with PSD95. C) This increase in colocalization between GIRK2 and PSD95 is occluded by the non-selective opioid receptor antagonist, naloxone. D) Treatment with the MOR selective agonist, DAMGO, did not change the expression pattern for GIRK2. E) Pearson's coefficient for individual images are shown and the mean values normalized to the control group (colocalization ratio ± SEM) are shown for each group. N denotes number of dendritic fields analyzed. *One-way ANOVA followed by post hoc test for significance ($P < 0.05$).

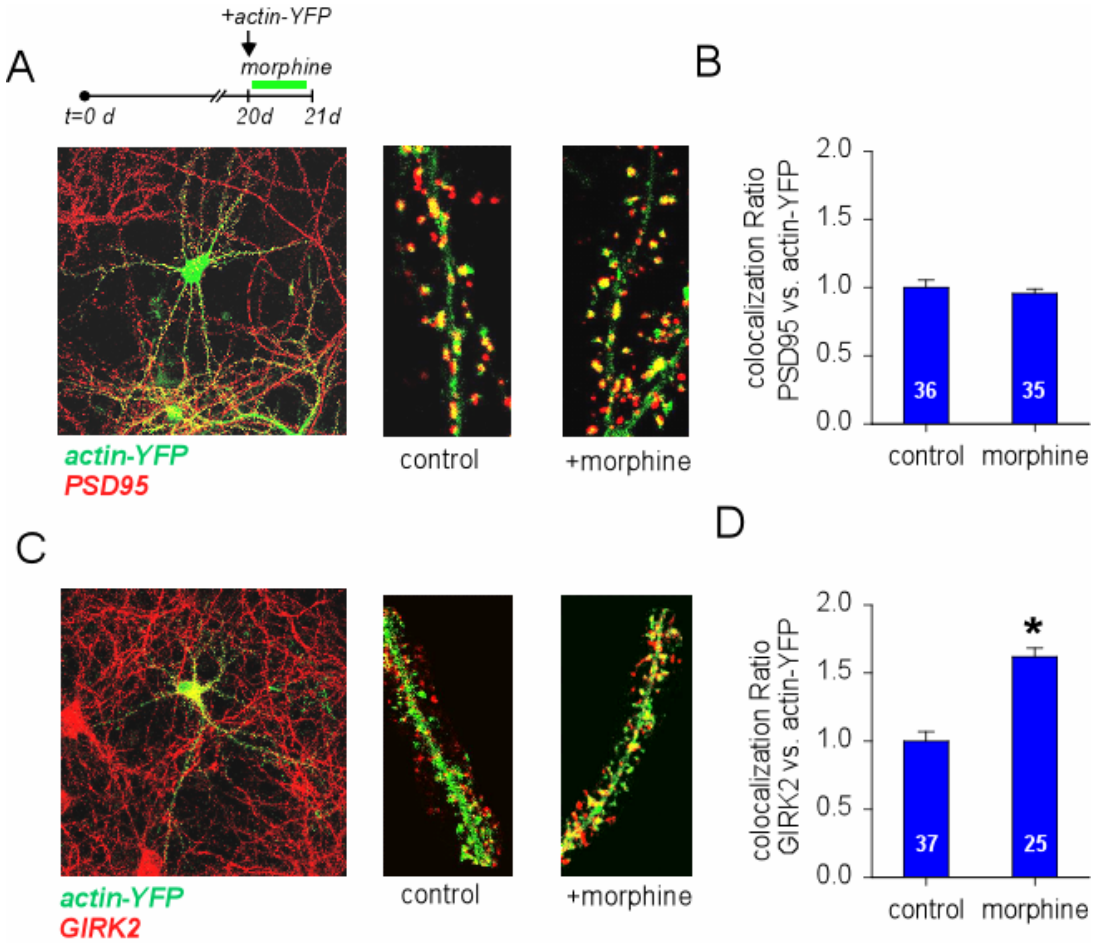
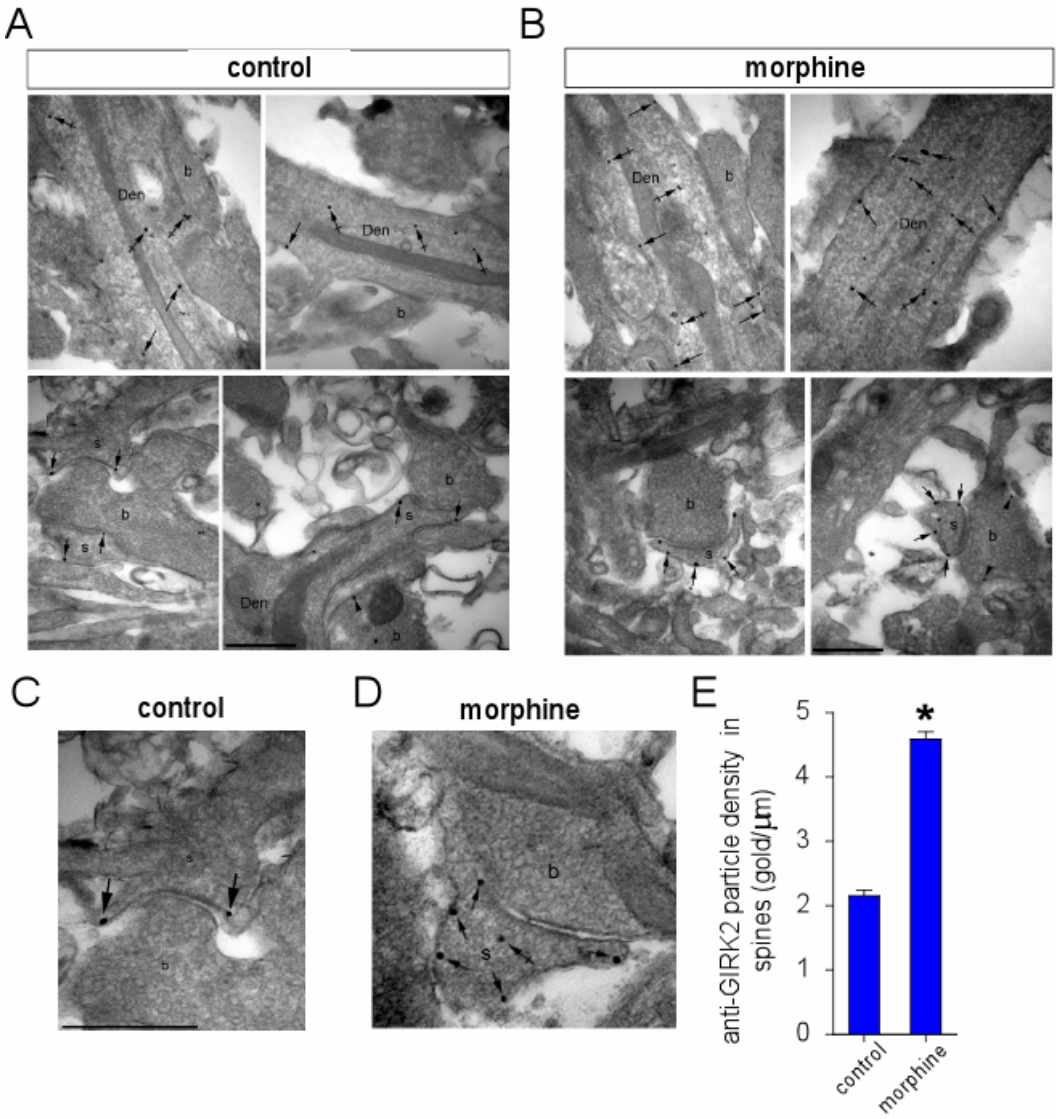


Fig 3.3. **Morphine increases colocalization of GIRK2 with actin-labeled dendritic spines.** Dissociated hippocampal cultures were infected with Sindbis actin-YFP virus at 20 DIV and either untreated (control) or exposed to morphine for ~1 day. A-D) Cultures were immunostained for PSD95 (A, red) or GIRK2 (C, red) and compared to actin-YFP expression (green) at 21DIV. Zoom shows examples of dendrites analyzed for control and morphine treated cultures. Morphine treatment did not alter PSD95 colocalization with spine specific actin-YFP. By contrast, morphine increased colocalization of GIRK2 with actin-YFP. B, D) Bar graphs show average colocalization for PSD95/actin-YFP and GIRK2/actin-YFP in the absence (control) or presence of morphine. *Student's t-test for significance ($P < 0.05$). N represents dendritic regions analyzed.

Fig 3.4. **Morphine increases the expression of GIRK2 along the plasma membrane of hippocampal cells**. Immunoreactivity for GIRK2 in control conditions (A and C) and after treatment with morphine (B and D) in hippocampal cultured neurons, as detected using a pre-embedding immunogold method at EM level. (A) In control conditions, immunoparticles for GIRK2 were detected in dendritic shafts (Den) of cultured cells, both along the plasma membrane (e.g. arrows) and at intracellular sites (e.g. crossed arrows), and especially along the plasma membrane of dendritic spines (s) (e.g. arrows) establishing asymmetrical synapses with axon terminals (b). Few immunoparticles for GIRK2 were also detected in axon terminals (b) (e.g. arrowheads). (B and D) After the treatment with morphine, we detected an increase in the immunoreactivity for GIRK2 along the plasma membrane of dendritic shafts (Den) (e.g. arrows) and dendritic spines (s) (e.g. arrows), as well as at intracellular sites (e.g. crossed arrows). (E) Quantitative analysis confirmed the increase in immunoreactivity for GIRK2 after treatment with morphine. Indeed, we observed around a 2-fold increase in the density of immunoparticles for GIRK2 after morphine treatment, measured as immunoparticles per μm of spines plasma membrane. Scale bars: 0.5 μm. These densities were statistically significant (*t-test*, $P<0.001$), were different from background labeling of 0.06 ± 0.21 immunoparticles/□m in control neurons and 0.06 ± 0.17 immunoparticles/μm in morphine treated neurons. *Student's t-test for significance ($P < 0.05$).


A
control
B
morphine
Den
b
s
C
control
D
morphine
E
anti-GIRK2 particle density in spines (gold/μm)
0
1
2
3
4
5
*
control
morphine

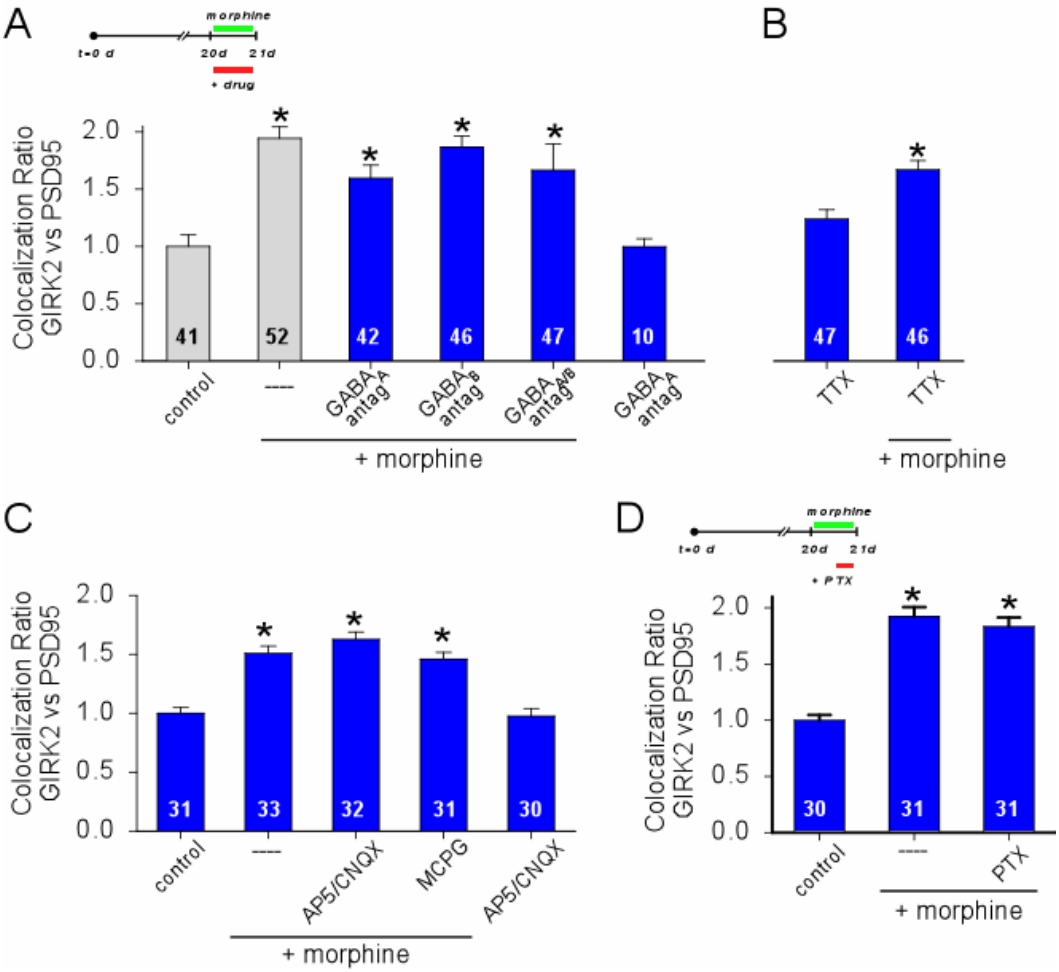


Fig. 3.5. **Morphine-dependent increase in GIRK2 and PSD95 colocalization does not require activity dependent release of presynaptic glutamate and GABA, or activation of PTX-sensitive G proteins.** 21DIV hippocampal neurons were treated with indicated agonists or toxins in the presence of morphine. A, B) Dissociated hippocampal neurons were treated with morphine and antagonists for $GABA_A$ and/or $GABA_B$. These blockers did not prevent the morphine-dependent increase in colocalization between GIRK2 and PSD95. GABA alone did not alter colocalization of GIRK2 and PSD95. C) Dissociated hippocampal neurons were treated with morphine and antagonists for NMDA R and AMPA R Cultures were immunostained for GIRK2 (green) and PSD95 (red). D, E) Bar graphs show the mean colocalization ratio (± SEM) with the N indicating the number of dendritic regions analyzed. Inhibiting $GABA_A$ channels or inhibiting spontaneous activity with TTX did not block the effect of morphine. F) Morphine-dependent change in GIRK2/PDS95 colocalization was insensitive to PTX treatment, suggesting a PTX-insensitive G-protein pathway. *One-way ANOVA followed by xx post hoc test for significance ($P < 0.05$).

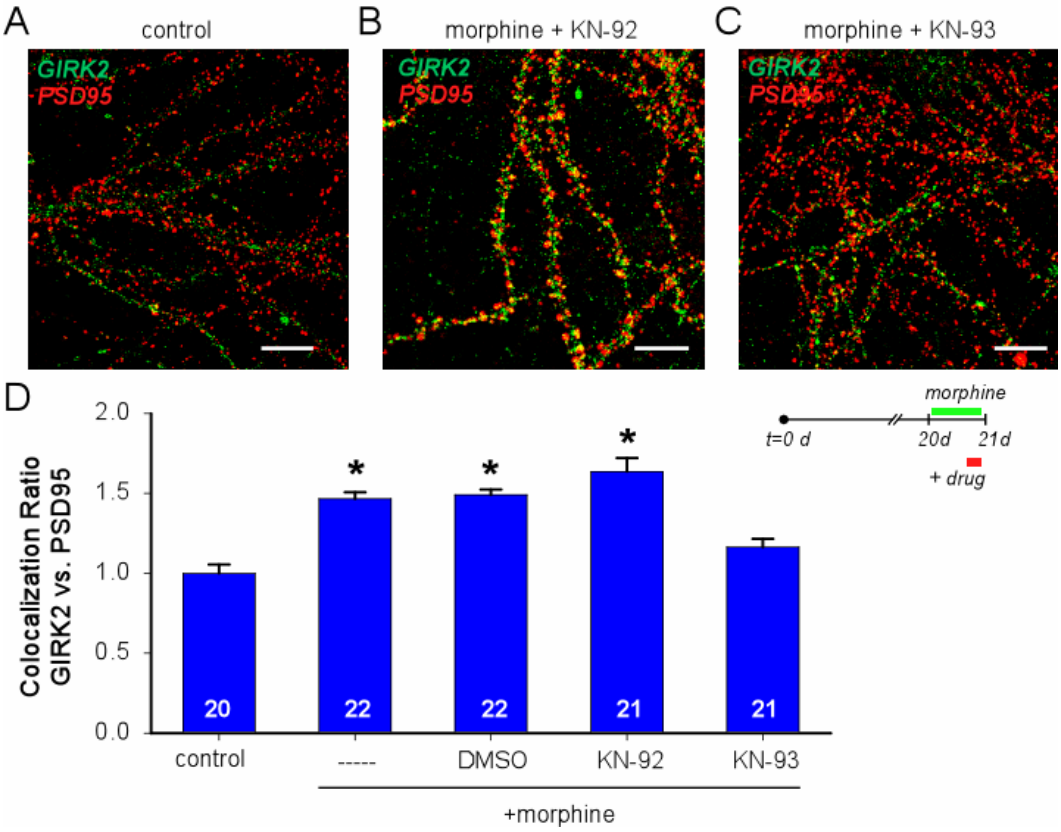


Fig. 3.6: **Morphine-dependent increase in colocalization of GIRK2 and PSD95 requires activation of CaMKII.** A-D) Selective inhibiting of CaMKII activity with KN-93 during the last 2 hrs of morphine treatment reversed the morphine-dependent increase of GIRK2 in the dendritic spines. KN-92, the inactive analogue of KN-93, and DMSO did not block the morphine-dependent change in GIRK2 expression. D) Bar graph shows the mean colocalization ratio (± SEM) with the N indicating the number of dendritic regions analyzed. *One-way ANOVA followed by xx post hoc test for significance ($P < 0.05$).

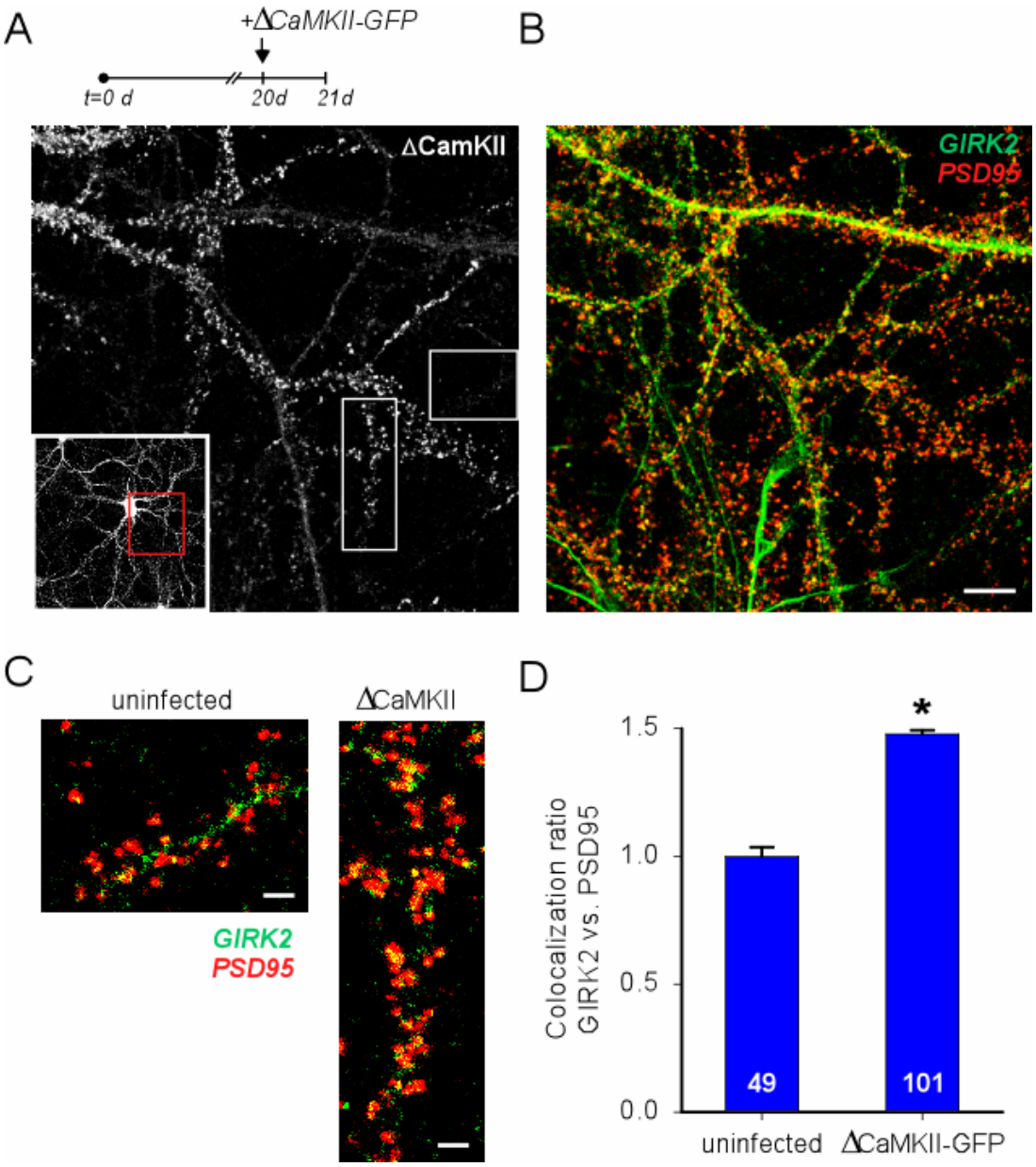
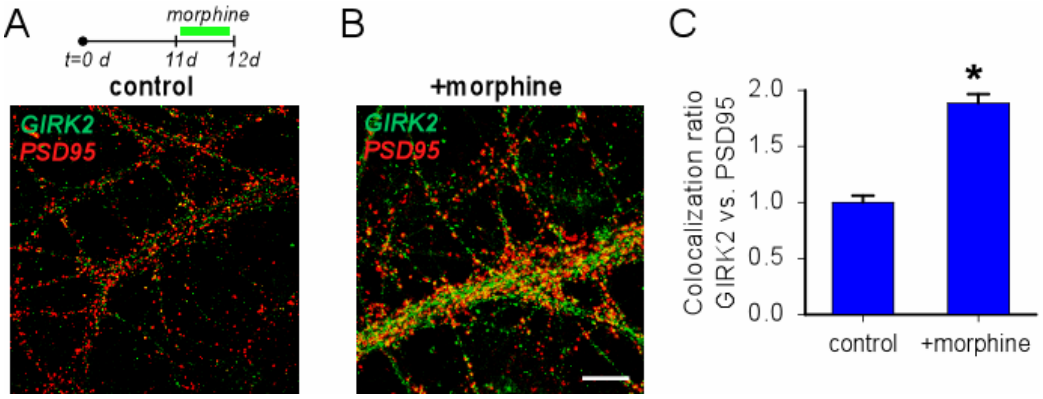
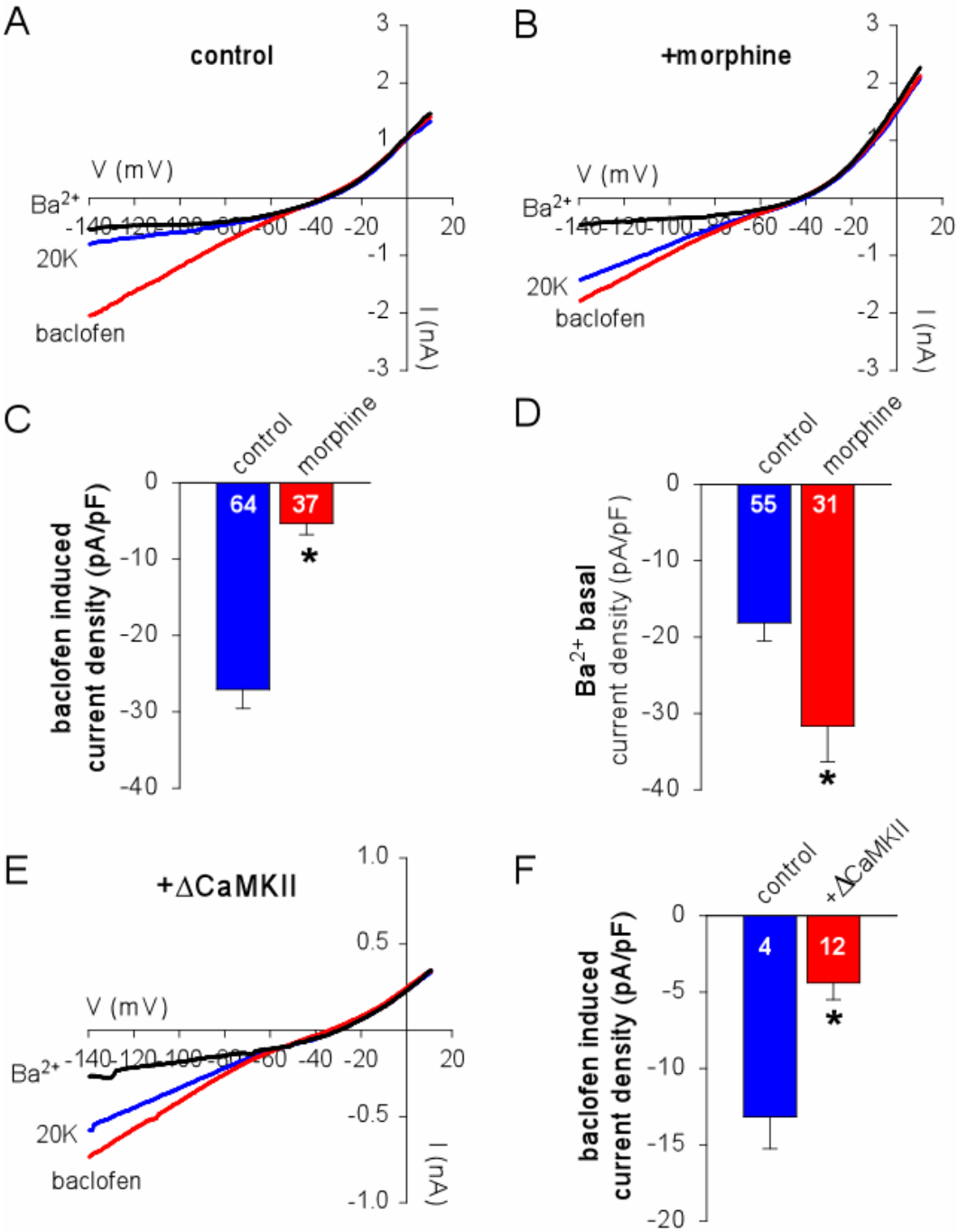


Fig. 3.7: **Expression of activated CaMKII increases colocalization of GIRK2 and PSD95 in hippocampal neurons.** A-D) 20DIV dissociated hippocampal neurons infected with viral vector expressing constitutively active CaMKII (ΔCaMKII-GFP, inset) for 1 day. A) Image shows ΔCaMKII-GFP (grey scale). B) Immunostaining for GIRK2 (green) and PSD95 (red) in ΔCaMKII-GFP infected cultures. C) Zoom images show dendrites immunostained for GIRK2 and PSD95 in infected and uninfected dendrites. D) Quantification of the colocalization ratio for GIRK2 and PSD95 in ΔCaMKII-GFP infected and uninfected neurons. The mean colocalization ratio (± SEM) with the N indicating the number of dendritic regions analyzed. *Student's t-test for significance ($P < 0.05$).

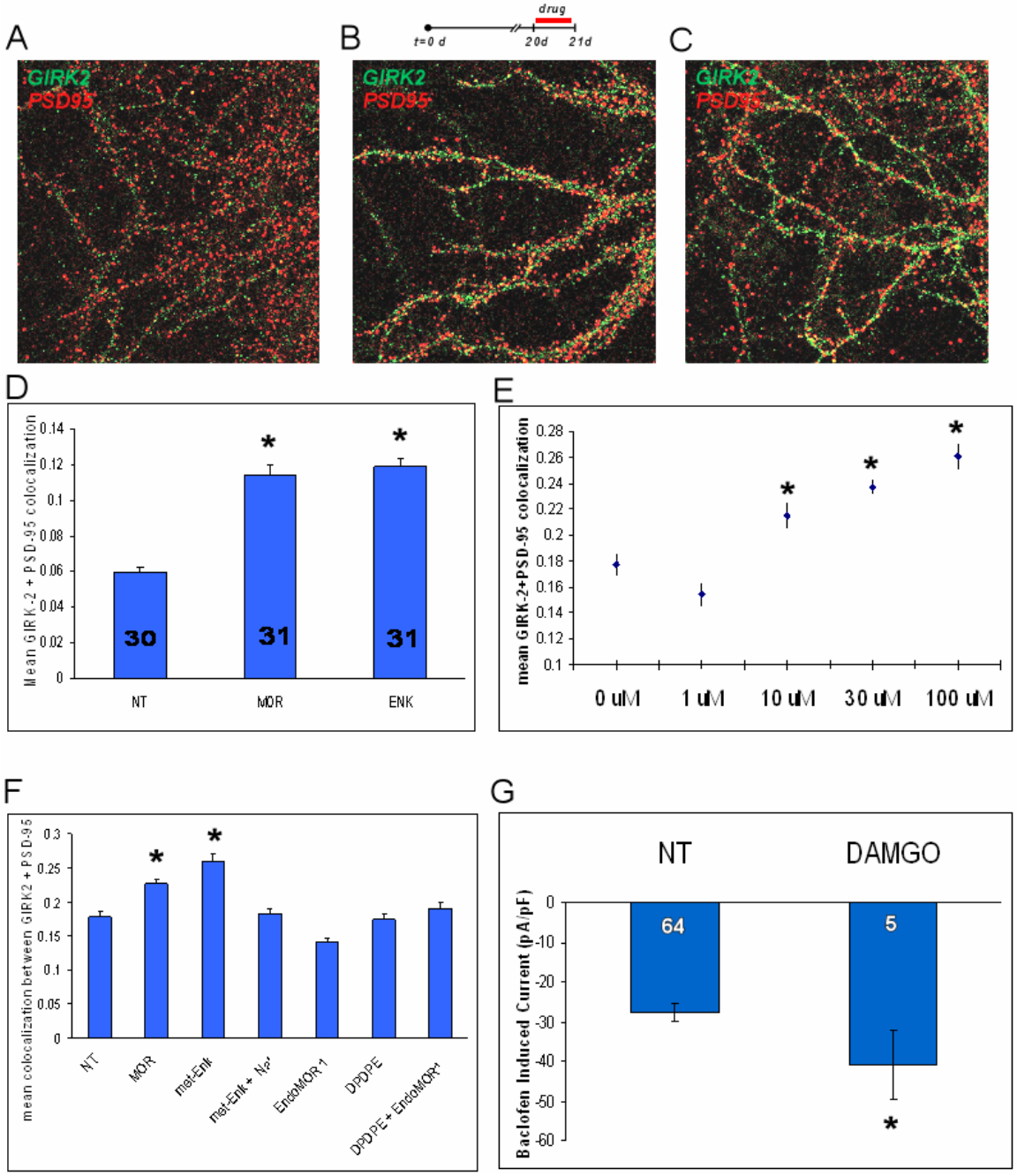


3.8: **Morphine-dependent increase in colocalization of GIRK2 and PSD95 in young neurons**. Immunostaining of GIRK2 and PSD95 in younger hippocampal neurons (10-14 DIV) grown in the absence or presence of chronic (24 hr) morphine (100 μM). A, B). Morphine increased colocalization between GIRK2 and PSD95, suggesting the 10-14 DIV cultures are similar to the 21 DIV cultures. C) The colocalization ratio was calculated as described in Methods and plotted as mean ± SEM (number of dendritic fields indicated). *Student's t-test for significance ($P < 0.05$).

Fig. 3.9: **Morphine treatment and expression of activated CaMKII in hippocampal neurons reduce amplitude of $GABA_B$ receptor-activated GIRK currents.** 10-12 DIV dissociated hippocampal neurons were exposed to morphine for 1 day and then whole-cell patch-clamp used to measure the $GABA_B$ receptor-activated GIRK currents. Representative current-voltage plots show the macroscopic current recorded in 20K, 20K + 1 mM Ba^{2+} or 20K + 100 μM baclofen for control (A) and morphine (B) treated neurons. Note smaller baclofen-activated current. C) Bar graph shows a significant decrease in average baclofen-induced currents in morphine treated neurons. D) Barium sensitive basal currents were not significantly different in morphine treated neurons. E) Current voltage relationship for constitutively active CaMKII infected neurons shows reduced baclofen induced GIRK current. F) Quantification of baclofen induced current densities for infected and uninfected neurons. *Student's t-test for significance ($P < 0.05$). N represents the number of neuronal recordings.


A
control
V (mV)
Ba2+
20K
baclofen
I (nA)
B
+morphine
C
baclofen induced current density (pA/pF)
control
morphine
64
37
D
Ba2+ basal current density (pA/pF)
55
31
E
+ΔCaMKII
F
+ΔCaMKII
4
12

3.10. **Agonist-dependent changes in GIRK channel trafficking**. A-d) GIRK2 and PSD95 colocalize in 21 DIV hippocampal neurons treated with supramaximal doses of morphine and met-enkephalin (24 hr). E). Met-enkephalin is a more potent agonist than morphine as 10 uM is sufficient to induce trafficking of GIRKs into the dendritic spines. F) Other selective DOR (DPDPE) and MOR (Endomorphin 1) aganists can not increase colocalization between GIRK2 and PSD95, suggesting the opioid receptor activity is agonist dependent G) Interstingly, although treatment with DAMGO (another MOR selective agonist, see figure 2) does not induce trafficking of GIRKs into the spines, it seems to increase the baclofen-induced GIRK currents in treated cultures The changes in GABA B induced currents are opposite to those observed in morphine treated cultures. The colocalization ratio was calculated as described in Methods and plotted as mean ± SEM (number of dendritic fields indicated). *Student's t-test for significance ($P < 0.05$).


A
B
C
drug
t=0 d
20d
21d
GIRK2
PSD95
D
Mean GIRK-2 + PSD-95 colocalization
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
30
31
31
NT
MOR
ENK
E
mean GIRK-2+PSD-95 colocalization
0.28
0.26
0.24
0.22
0.2
0.18
0.16
0.14
0.12
0.1
0 uM
1 uM
10 uM
30 uM
100 uM
F
mean colocalization between GIRK2 + PSD-95
0.3
0.25
0.2
0.15
0.1
0.05
0
NT
MOR
met-Enk
met-Enk + Np'
EndoMOR-1
DPDPE
DPDPE + EndoMOR+
G
NT
DAMGO
Baclofen Induced Current (pA/pF)
0
-10
-20
-30
-40
-50
-60
64
5

IV. Conclusion

Synaptic plasticity and addiction

Substance abuse ranks as one of the most expensive health problems of modern America and the world. Rates of drug-induced deaths have been increasing in the US for the past 25 years despite efforts to control drug misuse [1]. Substance abuse can affect people throughout life, in utero to old age, as prenatal exposure to drugs is linked to low birth weight and to developmental disorders [2]. Although the neurobiological basis of drug addiction has been under investigation for several decades, much remains to be done.

All drugs of abuse, including opioids and psychostimulants (such as cocaine and methamphetamine), activate the mesolimbic (reward) pathway, which includes the ventral midbrain, nucleus accumbens, and the frontal cortex (Figure 1)[3]. Dopamine release is associated with reward and as a result increases in the levels of dopamine form the basis of most addictions. However, the euphoric and addictive effects of these drugs could also be mediated by dopamine-independent mechanisms in other brain regions including the hippocampus, the hypothalamus and the amygdala [4, 5]. The challenge for biologists interested in understanding the consequences of chronic exposure to drugs is to establish which actions at the molecular and cellular levels are important for producing cellular tolerance and synaptic adaptation.

The hippocampus plays a central role in declarative and spatial learning and memory. Recently the hippocampus has received attention for its potential role in the initiation, maintenance, and treatment of addiction. The hippocampus is a major source

of glutamatergic afferents to the nucleus accumbens. Vorel et al. [6] reported that stimulation of the hippocampus at theta frequency caused reinstatement of drug-taking behavior and caused addicted rats to relapse. The authors thus suggested that the hippocampus is the anatomical relapse circuit of the brain and can play a significant role in the phenomena of addiction.

The same neuronal changes that underpin learning and memory are also important in addictive behavior. Both of these behaviors seem to be dependent on the induction of LTP and other forms of synaptic plasticity. For example, in the hippocampus LTP involves insertion of GluR1 containing AMPA receptors into synapses while repeated exposure to morphine and cocaine results in an increase of GluR1 protein at synapses in the VTA [7]. Furthermore, the AMPA/NMDA ratio is significantly increased in the morphine or cocaine treated animals as it is during hippocampal LTP [8].

There are however many forms of LTP. In the hippocampal perforant path/dentate gyrus pathway and the Schaffer collateral/CA1 synapses, LTP is dependent on the activation of NMDA receptors. However, at the hippocampal mossy fiber/CA3 synapses, induction of LTP is NMDA independent and is instead mediated by metabotropic glutamate receptors [9] and opioid receptors [10]. The mechanisms involved in hippocampal LTP are not unique. Similar mechanisms operate in many other forms of plasticity, such as LTP in basal ganglia [11], as well as LTD in the hippocampus, cerebellum or basal ganglia [11, 12].

GIRKs and addiction

One common cellular mechanism in all these forms of plasticity is the modulation of the spatial dynamics of excitation and inhibition. All forms of plasticity modulate the excitability of neurons by altering inhibitory transmission via suppression of presynaptic neurotransmitter release [13, 14] or by activating receptors and ion channels expressed postsynaptically. The synergistic effect of these pre- and postsynaptic components of plasticity could be readily modulated by GIRK channels, which are involved in both. Activation of neurotransmitter receptors that couple to GIRK channels generates an sIPSP [15], which is important for modulating excitability in the brain. There is evidence of GIRK involvement in NMDA mediated LTP [16] as well as other forms of plasticity. Due to their coupling to a variety of GPCRs, GIRK channels may also be involved in the NMDA independent forms of plasticity. Thus, revealing regulatory mechanisms involved in localization and function of these important modulators of synaptic excitability is crucial to a better understanding of many forms of plasticity.

This thesis describes novel mechanisms for regulation of GIRK channels. I provide evidence, although somewhat indirect, for the modulation of GIRKs by two different drugs of abuse. First, I provide evidence for the modulation of GIRK channels by a stimulant regulated transcript (SNX27), and secondly I describe the direct modulation of these channels by opioids. Finally, I also provide evidence for a more general type of regulation for the GIRKs by CaMKII.

In chapter 2, I describe for the first time, that an SNX regulates the cell surface expression of an ion channel. Specifically, I show that SNX27 regulates GIRK channel

intracellular trafficking and function in a subunit dependent manner. SNX27 expression may provide a key pathway for regulating inhibitory synapses in the brain. Thus, unlike other classical SAP proteins, SNX27 may be one of the only cytoplasmic proteins involved in regulating the amount of GIRK channels on the plasma membrane. Since SNX27 can be upregulated by psychostimulants such as cocaine and methamphetamine, changes in SNX27 expression may establish an important link between trafficking of GIRK channels and drug actions in the brain, possibly opening up new avenues of treatment for drug addiction.

In chapter 3, I show that a single dose of morphine alters inhibitory transmission in rat hippocampal cultures. Morphine alters inhibitory transmission by altering the spatial location of GIRK channels thus positioning them where they can more locally modulate synaptic excitability. This is the first evidence for an agonist dependent modulation of GIRK channels and elucidates a new mechanism for their neuronal trafficking. This study may shed light on the many unanswered questions related to opioid addiction and invites further studies on the role of GIRKs in addiction and other forms of synaptic plasticity.

Lastly, I show that CaMKII activation can regulate inhibitory transmission in hippocampus by altering GIRK trafficking. CaMKII is a necessary component of the cellular machinery underlying learning, memory and addiction. In chapter 3, I show that CAMKII is both necessary and sufficient for trafficking of GIRK channels into dendritic spines, attesting to the key role of this enzyme in signaling and synaptic plasticity.

I provide a direct link for modulation of GIRK channels by opioids, CaMKII and SNX27. Morphine increased the potency of neurotransmitter dependent modulation of

neuronal excitability by upregulating CaMKII and increasing the proportion of GIRK2 channels that are incorporated into the dendritic spines. I believe it is likely that opioid receptors also regulate SNX27 and that opioids may be able to alter SNX27 expression in a similar way to mechanism by which SNX27 is upregulated by stimulants. I speculate that the opioid induced alterations in GIRK trafficking might be mediated by changes in SNX27b expression. Increases in SNX27b protein may affect GIRK channel expression in morphine treated cultures. Consistent with this, we find that SNX27 mRNA is expressed in regions of the brain reward pathway (Padgett and Slesinger, unpublished data). I did not get the opportunity to investigate this hypothesis here. However, our data showing subunit specific regulation by SNX27 leads to several interesting possible outcomes. For example, in a neuron that expresses only GIRK2c and GIRK3, upregulation of SNX27b could selectively down-regulate the heterotetramers of GIRK2c/3 but leave GIRK2c homotetramers, altering the ratio of heterotetramers and homotetramers. Alternatively, in neurons expressing GIRK1 and GIRK3 channels, upregulated SNX27 could dramatically decrease the size of GIRK current. Further work is needed to elucidate the subunit-specific regulation in neurons and potentially the role for SNX27b and GIRK channels in opioid addiction.

It is my hope that the work I have presented in this thesis will provide the scientific community with a cellular model for the possible roles of GIRK channel trafficking in addiction and synaptic plasticity.

References

1. Paulozzi, L.J. and J.L. Annest, *US data show sharply rising drug-induced death rates.* Inj Prev, 2007. **13**(2): p. 130-132.

2. Huestis, M.A. and R.E. Choo, *Drug abuse's smallest victims: in utero drug exposure.* Forensic Sci Int, 2002. **128**(1-2): p. 20-30.

3. Kauer, J.A. and R.C. Malenka, *Synaptic plasticity and addiction.* Nat Rev Neurosci, 2007. **8**(11): p. 844-58.

4. Vos, P.E., et al., *Short and long term plasticity after lesioning of the cell body or terminal field area of the dopaminergic mesocorticolimbic system in the rat.* Brain Res, 1999. **831**(1-2): p. 237-47.

5. Leshner, A.I. and G.F. Koob, *Drugs of abuse and the brain.* Proc Assoc Am Physicians, 1999. **111**(2): p. 99-108.

6. Vorel, S.R., et al., *Relapse to cocaine-seeking after hippocampal theta burst stimulation.* Science, 2001. **292**(5519): p. 1175-8.

7. Fitzgerald, L.W., et al., *Drugs of abuse and stress increase the expression of GluR1 and NMDAR1 glutamate receptor subunits in the rat ventral tegmental area: common adaptations among cross-sensitizing agents.* J. Neurosci., 1996. **16**(1): p. 274-282.

8. Saal, D., et al., *Drugs of abuse and stress trigger a common synaptic adaptation in dopamine neurons.* Neuron, 2003. **37**(4): p. 577-82.

9. Conquet, F., et al., *Motor deficit and impairment of synaptic plasticity in mice lacking mGluR1.* Nature, 1994. **372**(6503): p. 237-43.

10. Morris, B.J. and H.M. Johnston, *A role for hippocampal opioids in long-term functional plasticity.* Trends Neurosci, 1995. **18**(8): p. 350-5.

11. Kombian, S.B. and R.C. Malenka, *Simultaneous LTP of non-NMDA- and LTD of NMDA-receptor-mediated responses in the nucleus accumbens.* Nature, 1994. **368**(6468): p. 242-6.

12. Nakazawa, K., et al., *The conjunctive stimuli that cause long-term desensitization also predominantly induce c-Fos and Jun-B in cerebellar Purkinje cells.* Neuroreport, 1993. **4**(11): p. 1275-8.

13. Zieglgansberger, W., et al., *Opioid peptides may excite hippocampal pyramidal neurons by inhibiting adjacent inhibitory interneurons.* Science, 1979. **205**(4404): p. 415-7.

14. Simmons, M.L. and C. Chavkin, *Endogenous opioid regulation of hippocampal function.* Int Rev Neurobiol, 1996. **39**: p. 145-96.

15. Lüscher, C., et al., *G Protein-Coupled Inwardly Rectifying K+ Channels (GIRKs) Mediate Postsynaptic but Not Presynaptic Transmitter Actions in Hippocampal Neurons.* Neuron, 1997. **19**(3): p. 687-695.

16. Huang, C.S., et al., *Common Molecular Pathways Mediate Long-Term Potentiation of Synaptic Excitation and Slow Synaptic Inhibition.* Cell, 2005. **123**(1): p. 105-118.

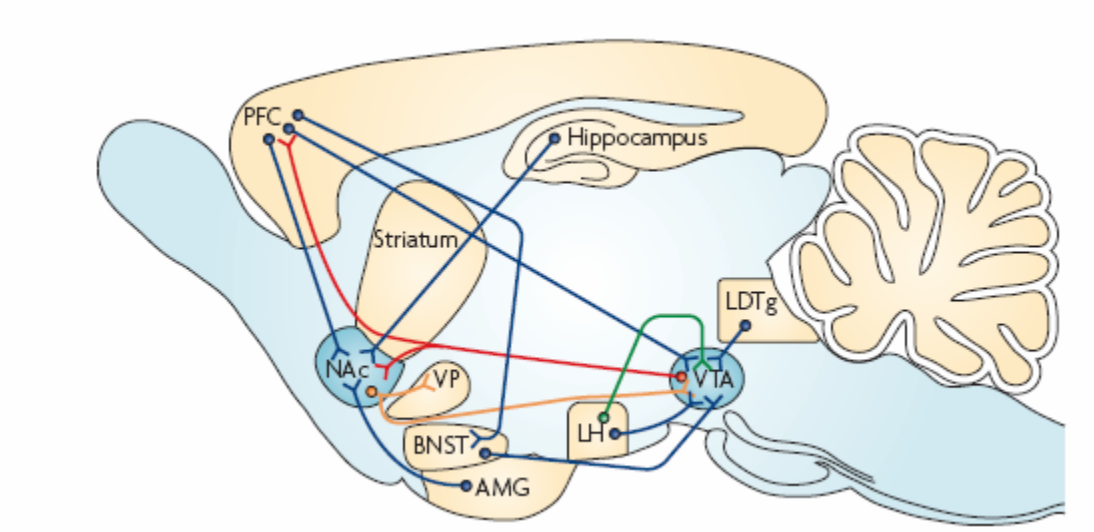


Fig 4.1. **Mesolimbic dopamine system circuitry.** Simplified schematic of the circuitry of the mesolimbic dopamine system in the rat brain highlighting the major inputs to the nucleus accumbens (NAc) and ventral tegmental area (VTA) (glutamatergic projections, blue; dopaminergic projections, red; GABAergic projections, orange; orexinergic projections, green). Glutamatergic synapses excite postsynaptic neurons and GABAergicsynapses inhibit postsynaptic neurons. AMG, amygdala; BNST, bed nucleus of the stria terminalis; LDTg, laterodorsal tegmental nucleus; LH, lateral hypothalamus; PFC, prefrontal cortex; VP, ventral pallidum. (Taken from Kauer and Malenka, 2007).