**Title**
The roles of the novel kinase PKD1 and the AKAP yotiao in NMDA receptor signaling



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**Publication Date**
2007-12-13

Peer reviewed|Thesis/dissertation

**The Roles of the Novel Kinase PKD1 and the AKAP Yotiao**

**in NMDA Receptor Signaling**

by

**Sarah Rosemary Carter**

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

**Neuroscience**

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

For my family,

who always thought that I was smart and my work was interesting,
even when I felt otherwise.

## Acknowledgments

My graduate career has been a very difficult journey for me. The first few years, in particular, were a time when I struggled not only with the regular ups and downs of benchwork science, but also with my own weaknesses and limitations. I had to learn that I could not do everything on my own, and in fact, could hardly do anything without the help of others. First and foremost, I thank my family, especially my husband Brendan without whom, I am certain, I would not have had the perseverance that was required to complete my Ph.D. My parents and siblings were a constant source of support in many ways, and the inquisitive curiosity and respect that I received from them and my extended family was something that I have cherished throughout this process. My family has always been very important to me and the last few years were a time when I was particularly glad to be a part of it.

Steve Finkbeiner, my advisor, is an individual who has been a critical part of my success, and I am very grateful to have worked with him. His scientific intuition and insights were a constant source of inspiration and guidance. He has emphasized that writing is a major part of being a scientist, and to me, his most shining moments were when giving advice and assistance with writing. From my first proposal (my successful NSF Graduate Research Fellowship proposal in 2001) to my most recent publication (most likely being published in 2008), he has been a source of incredible patience and feedback. I believe that writing is one of my strengths, and Steve has helped me develop that skill tremendously. He is also one of the warmest and most caring people I've ever

met; even when his advice or comments were not what I wanted to hear, I could always tell that he cared and had put some thought into what he said. In science, it is a rare thing to find a person who is both a great scientist and a caring mentor, but in Steve, I was lucky enough to work with such a person for over five years.

There were many people in the lab who helped me in innumerable ways. Most recently, Eva LaDow helped me turn an initial observation into a full and interesting story. She has been a great colleague and an even better friend, and I owe much of my success to her. Those who were there early on, including John Bradley, Montse Arrasate, Vikram Rao, Siddhartha Mitra, and Sean Pintchovski were there to help me with my most ignorant questions. John Bradley was really instrumental in getting me off the ground, both in day-to-day labwork and with my first publication. Other members of the lab, while less involved in my projects directly, were an essential part of making my lab experience fun (when things were working) or at least bearable (when things weren't going so well). I am very grateful that I was able to work in an environment that was so supportive.

People at both the Gladstone Institutes and the UCSF Neuroscience program provided me with help, direction, and administrative assistance without which I would have been lost long ago. Carrie Huckaba and Pat Veitch in Neuroscience took care of me so well that I always felt like I was their primary concern. As head of the Neuroscience program, I have always given Louis Reichardt credit for allowing me to come to UCSF, and I know that he has been interested in my progress ever since, and for that I am grateful. At Gladstone, Dr. Mahley has created an environment that is nearly ideal for graduate students, and he has done so apparently through the sheer force of his sincerity.

Kelley Nelson was particularly patient with me when I needed help with each and every form I ever filled out. Gary Howard in Editorial was always a pleasure to talk to and has incredible skills with the written word. Overall, the administrative and non-scientific support at Gladstone is top-notch, and the institutes as a whole would fall apart without them.

My thesis work would not have been possible without the generosity of many other scientists. Nearly every assay that I used during my thesis work was taught to me one-on-one by someone who was more knowledgeable than me, whose time was probably more valuable than mine, and who had very little stake in my success, and I would like to thank each of them. Many of the reagents that I used were gifts, and I could not have done my experiments without them: the GFP-PKD1 construct (from E. Rozengurt); HA-GluR1 and HA-GluR2 (from M. Passafaro); CFP-TGN38 (from A. VanDongen); RFP-mito (from Y. Yoon via J. Brodbeck); the HA-tagged PKD1 constructs (from A. Toker); flag-HDAC5 and flag-HDAC5 SS-AA (from T. McKinsey); yotiao, HA-yotiao, and GFP-yotiao (from M. Sheng); and the GST-tagged fragments of yotiao (from R. Kass, who generously gave me the whole set of seven even though only one was published). Also, I would like to thank T. Curran for the $NR1^{-/-}$ mice, which I have grown pretty attached to after taking care of them for the last five years.

## Contributions of others to the presented work

Portions of Chapter 2, which looks at the role of PKD1 in AMPAR signaling, have been submitted for publication. Experiments for this chapter were done by several people. Vikram Rao created the Venus-PKD1 construct and helped make the initial observations of puncta formation in the dendrites following NMDAR stimulation. Hong Joo Kim created the siRNA constructs and showed their efficacy and selectivity, and so contributed all of the data in Figure 19. Eva LaDow contributed in several ways to this chapter. She made all of the point mutations and domain deletions in the Venus-PKD1 construct and performed most of the experiments to test their effect on puncta formation. This work contributed to Figures 11 and 13. She also made the initial observation of Venus-PKD1 colocalization with HA-GluR2 and did many of the experiments that characterized the effect of PKD1 on GluR2, including all of the colocalization studies and many of the siRNA experiments. Through this work, she contributed to Figures 17, 18A, and 20A.

In Chapter 4, the first figure (Fig. 29) is taken from a previously published study from our lab; the data therein was generated by John Bradley. All other experiments in Chapters 3, 4, and 5 were performed by S.R.C. None of this data has been submitted for publication. Steve Finkbeiner supervised all research and provided direction.

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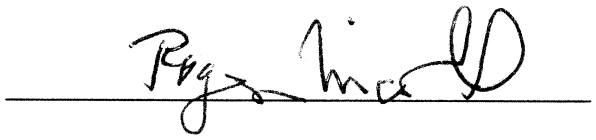

**Roger Nicoll**

**Committee Chair**

**Abstract**

The most striking feature of the brain is its seemingly endless ability to learn, to adapt to new experiences, and to remember. All of this information is encoded in the connections, or synapses, between neurons. These synapses change during learning and new memory formation in a process called synaptic plasticity. While much has been learned about the molecular mechanisms involved in synaptic plasticity, there is a long way to go before these processes are fully understood. In order to better understand these long-term changes, we focus on the roles of the novel kinase PKD1 (PKD1) and the scaffolding protein yotiao, a member of the A-kinase anchoring protein (AKAP) superfamily. In particular, we look at the roles of these proteins in the intracellular signaling pathways that originate with the NMDA-type glutamate receptor (NMDAR), a well-characterized and important glutamate receptor that plays a critical role in many forms of synaptic plasticity. We show that PKD1 is a downstream target of NMDA receptor signaling and

participates in synaptic plasticity by regulating the trafficking of AMPA-type glutamate receptors (AMPARs). Since changes in gene expression are critical for the long-term maintenance of synaptic plasticity, we also look at signaling to the nucleus. In response to certain stimuli, we find that PKD1 is capable of controlling the function of HDACs, a family of transcriptional repressors that regulate gene expression. Finally, we find that yotiao, which binds to NMDARs in a splice variant-dependent way, may play a role in facilitating signaling from the NMDAR to CREB, a transcription factor important to synaptic plasticity. By better understanding the functions of PKD1 and yotiao in neurons, we gain new insights into the molecular mechanisms of synaptic change, and thus, learning and memory.

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# Chapter 1:

## The proteins and signaling cascades that underlie learning and memory

## Synaptic plasticity

Years ago, when I first started working on my PhD, my parents would ask me what my project was about. What they wanted, they explained to me, was something that was accurate but easy to remember so that they could show off to their friends at dinner parties. They are not scientists, so a simple concept and an easy phrase was all they needed. I told them that I worked on "synaptic plasticity." That seemed to do the trick.

Synaptic plasticity is a term that encompasses many different cellular processes. "Synaptic" refers to the junction between two neurons, where a presynaptic neuron sends a signal to the postsynaptic neuron by releasing a chemical neurotransmitter. In the cortex, that neurotransmitter is usually glutamate. "Plasticity" is the ability of that synapse to change by becoming stronger or weaker. The strength of a synapse is defined as the ability of the presynaptic signal to induce a response in the postsynaptic cell. If a synapse is weak, then the presynaptic stimulus may generate a very small response in the postsynaptic cell; conversely, if a synapse is strong, then the presynaptic stimulation will cause a large response in the postsynaptic cell. This dissertation focuses on different intracellular signaling cascades and mechanisms that a postsynaptic neuron employs in order to change the strength of its glutamatergic synapses.

Glutamatergic synapses have three main types of receptors: N-methyl-D-aspartate receptors (NMDARs), α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptors (AMPARs), and metabotropic glutamate receptors (mGluRs) (Fig. 1).

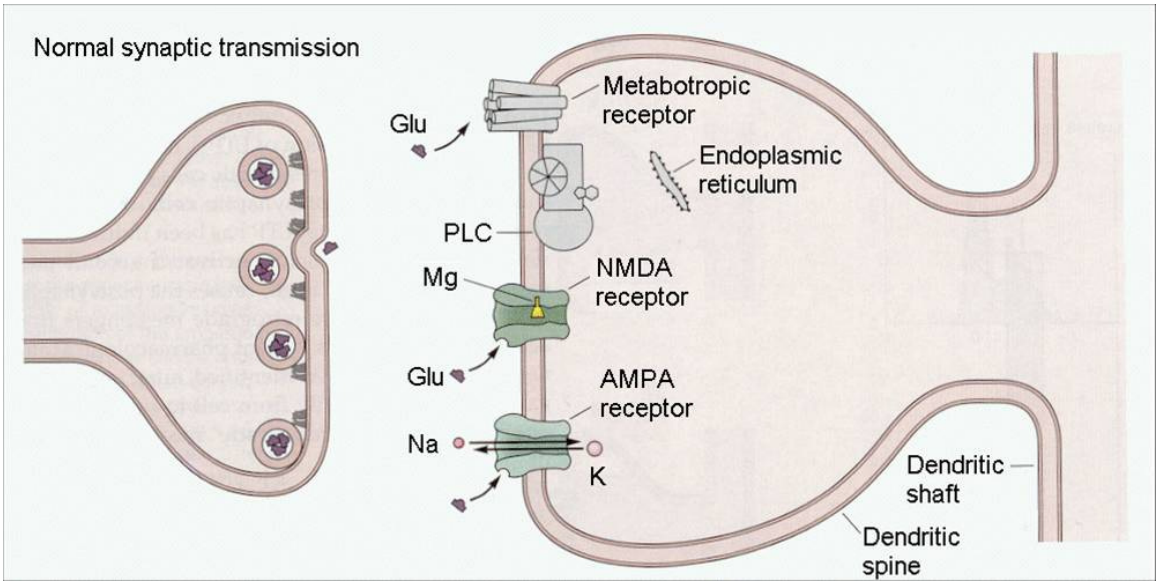


**Figure 1: Glutamate receptors at the synapse**
The three major types of glutamate receptors: mGluRs, NMDARs, and AMPARs. From Kandel, Schwartz, and Jessell (2000)[1].

mGluRs are part of a family of seven-transmembrane, G-protein-coupled receptors. Upon stimulation with glutamate, mGluRs undergo a conformational change that results in the activation of G proteins, which can link to many different enzymatic signaling cascades. Both NMDARs and AMPARs are ion channels that are opened upon stimulation with glutamate. AMPARs are permeable to $Na^+$ ions, which flow into the cell when the channel is opened. While there are a few examples of protein signaling cascades that result from AMPAR channel opening, the primary function of these channels is the depolarization of the membrane resulting from the influx of positively-charged ions. If there is enough depolarization after stimulation, the postsynaptic cell will fire an action potential and release neurotransmitter onto other neurons. NMDARs are permeable to $Ca^{2+}$ as well as $Na^+$, but are blocked by a $Mg^{2+}$ ion under non-stimulated conditions. The $Mg^{2+}$ block is relieved only after the membrane is depolarized (i.e. after AMPARs have been opened). The result is that AMPARs mediate a fast, depolarizing

influx of ions, and NMDARs respond more slowly. When NMDAR channels are opened, the resulting calcium influx triggers many different signaling cascades inside the cell. Nearly all mechanisms of synaptic plasticity start with calcium influx through NMDARs and it will be discussed in more detail in the next section.

The most commonly studied forms of synaptic plasticity are long-term potentiation (LTP) and long-term depression (LTD). Each of these types of synaptic change have both early (within minutes) and late (within hours) phases. The early phases depend on changes in existing proteins, including covalent modifications of those proteins (often phosphorylation) and changes in the location of those proteins within the cell. A major mechanism of synaptic plasticity is the insertion or removal of AMPARs from the synapse[2-9]. During LTP, AMPARs are inserted into the synapse, thereby increasing the magnitude of the postsynaptic response; conversely, during LTD, AMPARs are removed. Chapter 2 will discuss some of the mechanisms that underlie these changes in the trafficking of AMPARs, and more specifically, the role of the novel kinase protein kinase D1 (PKD1).

Late phases of LTP or LTD depend on changes in gene expression and subsequent production of new protein. Gene expression occurs when transcription factors are activated, bind to specific sequences of DNA, and promote transcription of new RNA. The RNA is then translated into new protein. One transcription factor that is known to be important for synaptic plasticity is cAMP/calcium response element binding protein (CREB)[10-15]. CREB is activated upon NMDAR signaling and binds to specific sequences of DNA known as cAMP/calcium response elements (CREs). CREs are known to be upstream of many plasticity-related genes. Chapter 4 looks at one protein,

yotiao, that may promote NMDAR-mediated signaling in the regulation of CRE-mediated gene expression. While there are many transcription factors like CREB that promote transcription, there are also transcriptional repressors that act to decrease the amount of gene expression. Chapter 3 describes the regulation of histone deacetylase complexes (HDACs), which represent one type of transcriptional repressor.

Synaptic plasticity is a critical process with many different players. Understanding all of the implications and all of the signaling pathways that contribute to this phenomenon has been a major scientific undertaking over the past 30 years. While plenty of progress has been made in that time, neuroscientists still have a ways to go before this process if fully elucidated. This dissertation represents one small piece of a very large puzzle.

**NMDAR-mediated signaling pathways**

The NMDAR plays a critical role in many forms of synaptic plasticity, including most forms of LTP and LTD. This glutamate-gated, calcium-permeable channel is a heteromer containing both NR1 and NR2 subunits[16]. The NR1 subunit is essential for functional channel formation and is a single, alternately spliced gene product[17]. There are four different NR2 subunits (NR2A-D) that are developmentally regulated and give the channel different properties and binding partners[18-21]. In addition to these two types of subunits, there exists a separate NR3 subunit, but its function in neurons is unclear; its primary role may be in glial cells[22,23]. At resting membrane potentials, NMDARs are blocked by magnesium ions that occupy the pore of the channel. Only upon depolarization of the membrane (usually accomplished by sodium ion influx through

AMPARs) is this magnesium ion removed. NMDARs are sometimes called a "coincidence detector" since they require both a depolarized membrane and glutamate binding to open and allow calcium into the cell.

Calcium influx through NMDARs triggers signaling through many different kinase pathways, including calcium/calmodulin-dependent kinase (CaMK), protein kinase C (PKC), protein kinase A (PKA), and mitogen-activated protein kinase (MAPK)[24-28] (Fig. 2). CaMKs are a family of kinases that depend on calcium ions binding to calmodulin, a calcium-binding protein that activates several signaling pathways in neurons[29]. There are three major CaMKs: CaMKI, CaMKII, and CaMKIV[30]. The role of CaMKII in NMDAR signaling and synaptic plasticity has been well established[31-36]. In response to calcium influx through NMDARs, it rapidly translocates to synaptic sites, binds to NMDARs[33,35], and has many different synaptic phosphorylation substrates, including AMPARs and NMDARs[30]. CaMKI and CaMKIV have fewer functions at the synapse and instead are thought to regulate gene expression in the nucleus. For CaMKIV, the function of synaptic calcium influx through NMDARs specifically is not clear; nuclear calcium levels may be more important to its regulation[37,38]. CaMKIV phosphorylates several calcium-dependent transcription factors, including CREB[10,30], and regulates HDACs, a family of transcriptional repressors[39-41]. In Chapter 3, we explore the relative roles of PKD1 and CaMKIV in regulating HDACs in neurons. While little is known about CaMKI, it has recently been shown to translocate to the nucleus in response to calcium influx and regulate transcription of CRE-containing genes[42,43].

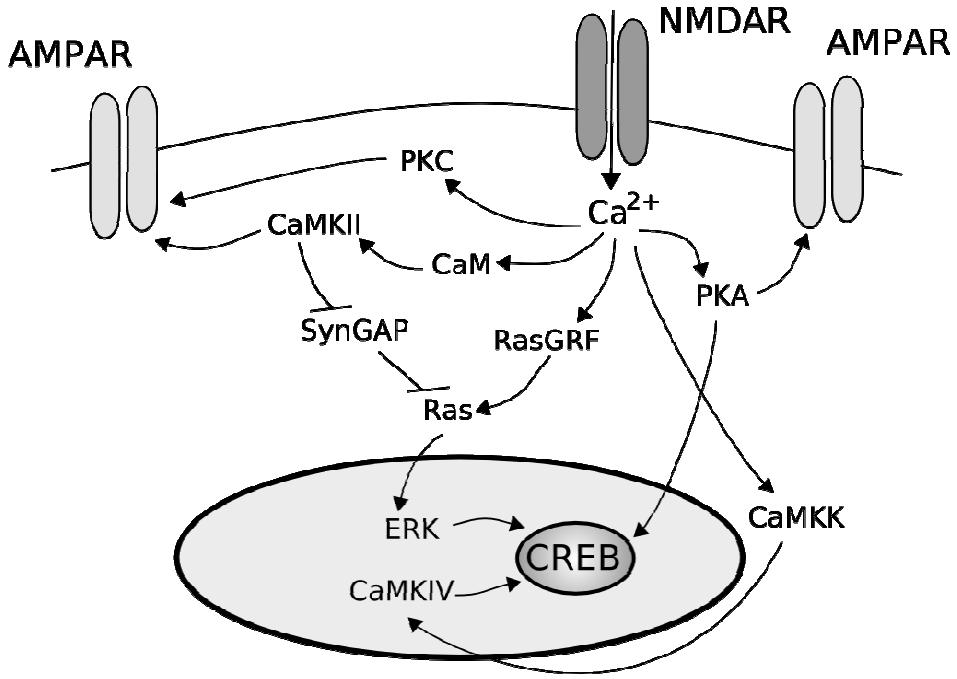


**Figure 2: Signaling pathways downstream of calcium influx through NMDARs**
Calcium influx links NMDARs to CaMKs, PKC, PKA, and MAPK (ERK). Calmodulin (CaM) activates CaMKs upon binding to calcium ions ($Ca^{2+}$). Arrows pointing to AMPARs indicate direct interactions of the kinases with AMPARs and also a role for the kinases in the regulation of the trafficking of AMPARs. For simplicity of the figure, CaM activation of CaMKIV in the nucleus is not drawn. See text for more details.

PKC and PKA are two types of protein kinases that are activated after signaling through NMDARs and function at the synapse. Many forms of PKC are activated by calcium directly[44-46], and the role of PKC in synaptic plasticity and the trafficking of AMPARs have been well characterized[47-50]. A major defining characteristic of PKCs is their diacylglycerol (DAG) binding domains, which promote activation of PKCs upon binding to DAG[51]. DAG is usually produced by phospholipase C (PLC) after stimulation of Gαq-coupled seven-transmembrane proteins, which include type I mGluRs[45]. In this way, PKCs function in mGluR-mediated LTD[4,7]. DAG production is also important for

NMDAR-mediated plasticity, but the mechanisms linking NMDARs to DAG production are less clear[52].

PKA is activated in the presence of cAMP, which is produced by adenylyl cyclase. There are many forms of adenylyl cyclase that are activated by calcium and are thus responsive to NMDAR signaling[53,54]. PKA phosphorylates many synaptic proteins, including the AMPAR subunit GluR1[55,56], as well as nuclear proteins including CREB[10,15]. PKA has two domains: the catalytic domain and the regulatory domain. Upon activation of the kinase by cAMP, the catalytic domain is released from the regulatory domain and free to phosphorylate its substrates. Type II PKA isoforms have regulatory subunits that bind to scaffolding proteins known as A-kinase-anchoring proteins (AKAPs) (see the subsection, "The A-kinase anchoring protein (AKAP) yotiao," below). In Chapter 4, we explore the role of yotiao, an AKAP that binds NMDARs, in transducing calcium influx through NMDARs to CREB-mediated gene expression.

MAPK signaling is critical for NMDAR-mediated signaling in synapses[57] and to the nucleus[58], and many complex signaling cascades have been characterized[59]. "MAPKs" refers to a large family of kinases, including JNKs and p38 MAPKs, but the kinase that is best characterized in the context of synaptic plasticity is p42/44 (ERK)[26,60]. While MAPK activation is downstream of many different kinases, including PKC and PKA[27,59], it is also regulated by proteins known as RasGTPase-activating proteins (GAPs) and GDP/GTP exchange factors (GEFs). Ras and Ras family members are G-proteins upstream of ERK and other MAPKs, and their activity is negatively regulated by GAPs and positively regulated by GEFs. SynGAP is a GAP found selectively in synaptic sites[61,62] that is inhibited by CaMKII activity[63], and so promotes signaling to ERK upon

influx of calcium. RasGRF1 is a GEF that binds to NMDARs and is important for linking NMDAR signaling to ERK activation[64] (Fig. 2). Interestingly, both of these Ras effector proteins bind selectively to NR2B subunits[64,65]. This selective binding allows NR2B-containing receptors to couple more closely to those proteins than receptors containing NR2A. By altering the subunit composition of NMDARs, the neuron can regulate the signaling downstream of calcium influx. We explore this notion in the context of splice variants of the NR1 subunit of NMDARs in the next section and in Chapter 4.

The signaling pathways downstream of NMDARs have been extensively studied and characterized. However, there is still more to learn. In Chapter 2, we show evidence that PKD1 is a new and novel downstream signaling target for NMDARs.

**NMDARs and calcium microdomains**

Calcium signaling in neurons is not restricted to NMDARs. There are several different routes for calcium to enter the cell through the plasma membrane, and these different sources of calcium yield distinct effects on gene expression. For example, calcium influx through L-type voltage sensitive calcium channels induces CRE-dependent gene expression much more strongly than signaling through NMDARs, even when cytoplasmic and nuclear calcium levels are the same[66]. A major question in the field is how this specificity is maintained[67]. Our lab and others have proposed the idea of a calcium "microdomain," which holds that calcium entering the cell will be buffered very quickly and that only proteins that are very close to the channel mouth will interact with the calcium ions[13,68-72]. From this "microdomain" idea, we developed the hypothesis that

different subunits of NMDARs, which bind different proteins, may show differences in downstream signaling even while total calcium influx remains the same.

In Bradley, et. al. (2006)[68], we explored this hypothesis by looking at splice variants of NR1, the essential subunit of NMDARs. Two major C-terminal splice variants are formed by alternative splicing of the C1 casette[17], with NR1-1a containing the cassette and NR1-2a lacking it. This C1 cassette is known to bind several proteins, including calmodulin[73], CaMKIIα[74], neurofilament-L[75], and the AKAP yotiao[76] (see below). By alternatively splicing the NR1 subunits that incorporate into NMDARs, the neuron can regulate the complement of proteins available for interaction with incoming calcium. We found that NMDARs with the C1-containing splice variant, NR1-1a, showed more NMDAR-dependent CRE-mediated gene expression than NMDARs with NR1-2a. In Chapter 4, we follow up this result by exploring the role of yotiao in linking the C1 cassette to increased gene expression.

**The novel kinase protein kinase D (PKD)**

There are three isoforms of PKD: PKD1, PKD2, and PKD3[77,78]. PKD1 has been the most extensively studied and is the focus of our studies as well. PKD1 was first cloned as PKCμ[79], and was thought to be a member of the PKC family because it has DAG-binding (C1) domains that show homology with those in PKCs. In addition to the C1 domains, it contains a pleckstrin homology domain, which regulates some protein-protein interactions, a kinase domain, and a Type I PDZ ligand[77,80] (Fig. 3). Despite its C1 domains, PKD1 is more closely related to CaMK isoforms, and is now considered to be a part of the CaMK family of kinases. Phosphorylation of the activation loop within the

kinase domain of PKD1 is usually required for the kinase to be active[77,81]. This activation loop region of PKD1 shows significant homology with checkpoint kinase 2, a CaMK family member that regulates the cell cycle in non-neuronal cell types. Since much is known about checkpoint kinase 2 and its structure[82], it may prove to be a rich resource for understanding the activation and structure of PKD1.

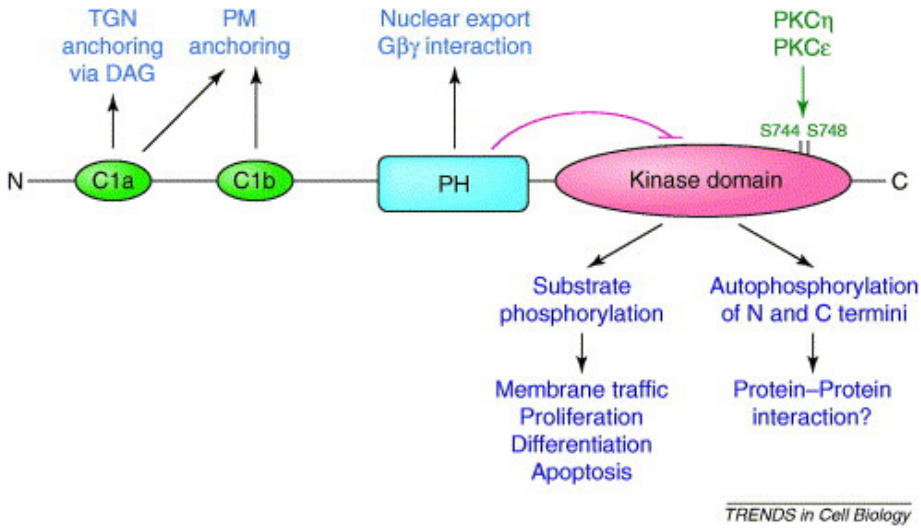


**Figure 3: Primary structure of PKD1**
The structure of PKD1 including the C1 domains, the Pleckstrin homology domain, and the kinase domain. Not shown is a PDZ ligand at the extreme C-terminus. Figure is from Ghanekar and Lowe (2005)[83].

While PKD1 is usually activated by phosphorylation of its activation loop by PKC isoforms[77,81,84], there are several other ways that it can become active. The pleckstrin homology domain is autoinhibitory to the kinase[85], and tyrosine phosphorylation of this domain downstream of oxidative stress stimulation can relieve that inhibition[86]. Caspase-mediated cleavage of the kinase can occur during apoptotic events, leaving the kinase domain uninhibited, and therefore, active[87]. Other PKC-

independent activation of PKD1 has been shown[88,89], including activation in response to calcium influx across the plasma membrane or from intracellular stores[90,91].

There are many ways that PKD1 may affect signaling in neurons. Several studies have shown that PKD1 signals to ERK[92-94] and other MAPKs[89], and there may be cross-talk between PKD1 and PKA signaling as well[95]. One study showed that CREB is a substrate of PKD1[96]. It can also regulate actin remodeling[97], which is important during synaptic plasticity[98-100]. In non-neuronal cell types, many functions for PKD1 have been demonstrated (Fig. 4), but very little is known about its role in neurons, with only a few papers on the topic[80,101-103].

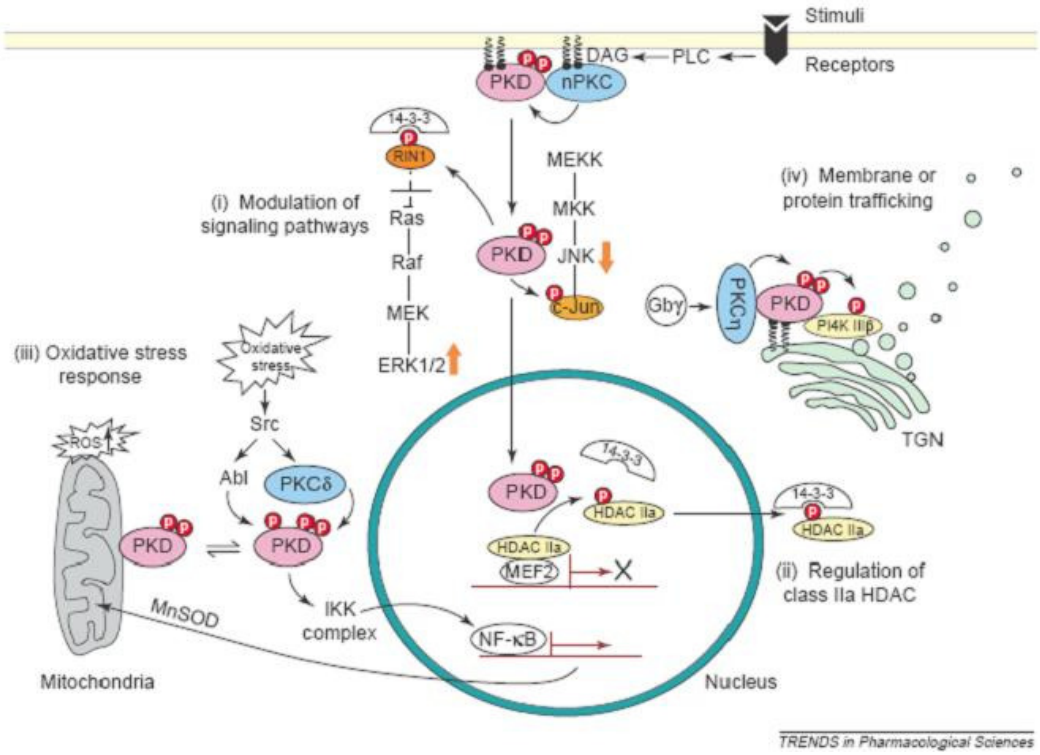


**Figure 4: Some of the functions of PKD1 in non-neuronal cells.**
In non-neuronal cells, PKD1 is activated through a canonical PKC-dependent pathway and is known to play many roles throughout the cell. Figure is from Wang, 2006[77].

In Chapter 2, we look at PKD1 in protein trafficking. In other cell types, regulation of protein trafficking is one of the best-characterized functions of PKD1[77]. While a

trafficking function has previously been shown in neurons[80], we go a step further by demonstrating that PKD1 may take part in synaptic plasticity; it is downstream of NMDAR and mGluR signaling and regulates the localization of AMPAR subunits. In Chapter 3, we shift our focus to HDACs. Class II HDACs are substrates of PKD1, and upon phosphorylation, these HDACs are removed from the nucleus, thereby allowing transcription to occur [104-106]. We show that PKD1 can function in neurons to regulate HDACs in this way.

**The A-kinase anchoring protein (AKAP) Yotiao**

Yotiao is one of a family of scaffolding proteins called A-kinase anchoring proteins (AKAPs). Scaffolding proteins function by binding to proteins and regulating their spatial localization. The defining characteristic of AKAPs is that they bind to Type II regulatory subunits of PKA, but many AKAPs bind to other types of signaling molecules as well. There are several examples of AKAPs as signaling modules that allow for tight regulation of complex cellular processes[56,107-110]. One of the best-characterized roles for an AKAP is that of AKAP79/150 in synaptic plasticity[111]. It has been shown to anchor PKA in synaptic sites close to the AMPAR subunit GluR1, a substrate of PKA[56,112]. NMDAR stimulation causes a redistribution of the AKAP itself out of the synapse[113], decreasing phosphorylation of GluR1, and so promoting the internalization of AMPARs. This mechanism is thought to underlie some forms of LTD[114]. While there have been many studies on AKAP79/150, relatively little is known about yotiao.


**Figure 5: Primary structure of Yotiao**
The PKA binding domain (in red) defines yotiao as an AKAP (see text for more). Yotiao also binds protein phosphatase 1 (PP1) and the NR1 subunit of NMDARs, and has many more potential interactions through its numerous leucine-zipper domains. Image is to scale; yotiao has 1641 amino acids.

Yotiao is a large protein that binds PKA and protein phosphatase 1 (PP1) and targets them to the synapse by binding directly to the alternatively-spliced C1 cassette of the NMDAR subunit NR1 (Fig. 5)[76,115,116]. The N-terminus of yotiao contains several leucine zipper domains, which usually mediate protein-protein interactions. Known binding partners for these domains include the $IP_3$ receptor[117], which, upon activation, mobilizes intracellular calcium stores, and the KCNE1 cardiac potassium channel, which is regulated in multiple ways by binding yotiao[109,118-120]. Yotiao is a splice variant of the much larger AKAP350, which is known to associate with centrosomes in the nucleus[121]. However, yotiao does not contain the domains that localize the AKAP to centrosomes, so its NR1-binding domain is thought to determine its localization.

A function of yotiao in regulating NMDAR currents has been demonstrated[115]. NMDARs are sensitive to PKA and PP1 signaling since they contain a serine that is phosphorylated by PKA and dephosphorylated by PP1. In the absence of yotiao in a heterologous system, NMDARs show an increase in current when treated with a cAMP analog, which activates PKA. In the presence of yotiao, NMDARs are more sensitive to the cAMP analog; since PKA is anchored near its site of action, it is able to more quickly

and completely phosphorylate the NMDARs[115]. While this known function of yotiao is at the channel itself, there is a possibility that it could have some effect on signaling that is downstream of calcium influx. As mentioned above, only splice variants of NR1 containing the C1 cassette of the C-terminus bind to yotiao, and in Chapter 4, we ask if yotiao plays a role in linking NMDAR signaling to gene expression in a splice variant-dependent way.

# Chapter 2:

## The role of the novel kinase PKD1 in glutamatergic signaling and AMPA receptor trafficking

**Abstract**

The trafficking of AMPA-type glutamate receptors (AMPARs) into and out of neuronal synapses is a critical mechanism of synaptic plasticity, which is thought to underlie learning and memory. Both NMDA-type glutamate receptors (NMDARs) and metabotropic glutamate receptors (mGluRs) regulate AMPAR localization, but the mechanisms by which they do so are unclear. The novel kinase PKD1 regulates protein trafficking in other cell types, but its role in neurons is poorly understood. Here we show that PKD1 is a point of convergence for different glutamatergic signaling pathways; its activation depends on mGluRs, while its translocation to AMPAR-containing endosomes depends on NMDARs. Furthermore, altering PKD1 function by overexpression of a kinase-inactive version of the kinase or by siRNA knockdown disrupts AMPAR localization, suggesting that PKD1 plays an important role in the subcellular trafficking of AMPARs. These results demonstrate a novel downstream signaling pathway at glutamatergic synapses and may provide a mechanistic link between NMDAR signaling, mGluR activation, and AMPAR localization.

## Introduction

Synaptic plasticity depends on the dendritic trafficking of AMPA-type glutamate receptors (AMPARs)[8,122]. The localization of AMPARs can be dynamically regulated by both NMDA-type glutamate receptors (NMDARs) and group I metabotropic-type glutamate receptors (mGluRs)[7]. Calcium influx through NMDARs triggers many different signaling cascades that regulate both removal and insertion of AMPARs from the synapse. mGluRs are Gαq-coupled, seven transmembrane receptors that cause activation of phospholipase C (PLC), production of diacylglycerol (DAG) and PKC activation. This signaling controls synaptic strength both by initiating internalization of AMPARs[7] and by promoting specific subunit expression at synaptic sites[123]. While much work has been done to understand the mechanisms and molecules by which NMDARs and mGluRs regulate the localization of AMPARs, they remain incompletely understood.

The novel kinase protein kinase D1 (PKD1) has diverse cellular functions in multiple subcellular compartments including the *trans*-Golgi network (TGN), the nucleus, and the mitochondria[77]. Since PKD1 has numerous targets within cells, its localization to subcellular domains plays a key role in its regulation. The structural domains of PKD1 regulate its localization by binding to different partners. PKD1 contains two cysteine-rich domains (C1a and C1b) that are sensitive to diacylglycerol (DAG) and to phorbol esters[124,125]; a Pleckstrin homology (PH) domain that mediates some protein-protein interactions[126,127]; and a type 1 PDZ ligand at its extreme C-terminus[80]. In response to phorbol esters or Gαq-coupled receptor activation, a two-step translocation has been described in multiple cell types[77,78,128,129]. During this process, the C1b domain has been shown to mediate the first step of the translocation, which is to the

plasma membrane [84,129,130]. Once at the plasma membrane, phosphorylation of PKD1 on two serines in its "activation loop" is required for the second step of the translocation back to the cytosol [84,128].

PKD1 is usually activated after phosphorylation of its activation loop by novel PKC isoforms following DAG production[81]. This signaling pathway is downstream of Gαq-coupled receptors, and there are many examples of PKD1 activation after stimulation of such receptors[84,91,131]. In addition, activation of PKD1 has recently been demonstrated in response to rises in intracellular calcium levels[90,91]. While PKD1 is highly expressed in brain, little is known about its localization or activation in neurons.

The function of PKD1 in protein trafficking has been well established. In several cell types, including neurons[80], dominant-interfering versions of PKD1 prevent trafficking of proteins from the TGN to the plasma membrane. While in some cases it appears that the regulation of this trafficking is dependent on specific interactions between PKD1 and the cargo protein[80,132], PKD1 also regulates the lipid content of the TGN which can directly influence the budding of these intracellular membranes[133-135]. In addition to its trafficking roles at the TGN, PKD1 promotes the recycling of β-integrins in fibroblasts[136]. Since synaptic plasticity depends on protein trafficking and recycling events, we asked if PKD1 functions in neurons during glutamatergic signaling.

Here we show that PKD1 is a point of convergence for different glutamatergic signaling pathways. It is activated by stimulation of mGluRs and responds to calcium influx through NMDARs by translocating to endosomal structures where it colocalizes with the AMPAR subunit GluR2. Furthermore, a dominant-interfering version of PKD1

or knock-down of endogenous PKD1 using siRNA disrupts the localization of GluR2, suggesting that PKD1 plays an important role in the subcellular trafficking of AMPARs.

## Results

### PKD1 activation is downstream of mGluR signaling

While PKD1 is usually activated after phosphorylation by protein kinase C (PKC) isoforms downstream of Gαq-coupled receptors, it can also be activated in response to rises in intracellular calcium[90]. Since glutamatergic activity results in the activation of both Gαq-coupled mGluRs and calcium-permeable NMDARs, we tested whether PKD1 is activated in neurons. We stimulated dissociated mouse cortical cultures with glutamate (30 μM) and examined them by Western blot analysis. PKD1 was phosphorylated at S916, an autophosphorylation site often used as a marker for PKD1 activity[137], and phosphorylation was maintained for up to 1 hour of stimulation. The activation loop of PKD1 also showed some phosphorylation (Fig. 6). To determine what glutamate receptor subtype was responsible for this activation, we used pharmacological tools. The mGluR antagonist MCPG reduced glutamate-induced PKD1 phosphorylation (mean reduction=66%±16%; n=2), and the mGluR-specific agonist ACPD induced phosphorylation, suggesting that mGluRs mediate this activation. Furthermore, APV, an NMDAR antagonist, did not block phosphorylation of PKD1, and NMDA alone did not induce autophosphorylation (Fig. 6), showing that NMDARs do not activate PKD1.

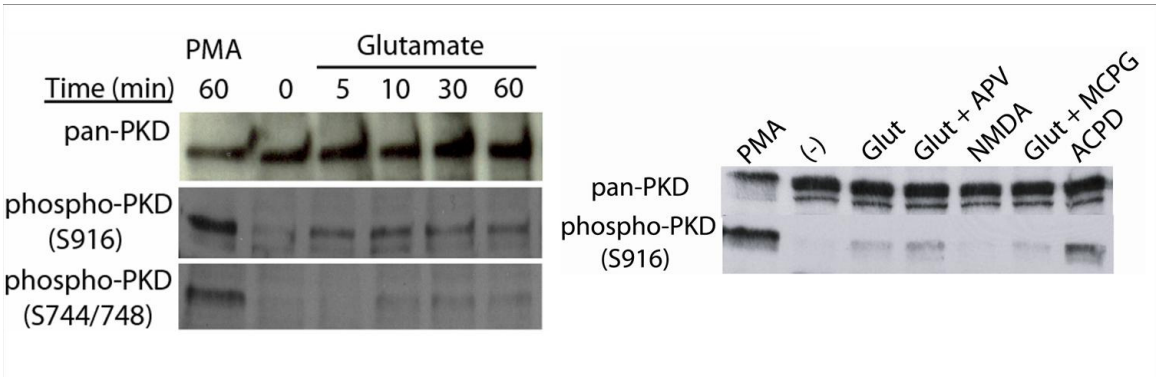

**Figure 6: Glutamate activates PKD1 in a sustained way through stimulation of mGluRs**
Neurons were stimulated with 30 μM glutamate for indicated times (left panels) or with indicated pharmacology (right panels). They were then harvested and lysates were separated by SDS-PAGE and subjected to Western blot. Pan-PKD indicates all PKD1 in the sample, phospho-PKD (S916) stains the phosphorylated auto-phosphorylation site of PKD1, and phospho-PKD (S744/748) stains only the phosphorylated activation loop. The phorbol ester PMA was used as a positive control.

Since PKD1 activation downstream of Gαq-coupled receptors is usually dependent on DAG production and PKC activation, we asked if mGluR-mediated PKD1 activation works through the same mechanism. Indeed, we found that ACPD-induced PKD1 activation was blocked by U73122, a PLC inhibitor that blocks DAG production, by GF109203X, an inhibitor of novel PKC isoforms, by Gö6983, another PKC inhibitor, or by Gö6976, an inhibitor of many PKC isoforms as well as PKD1 (Fig. 7).

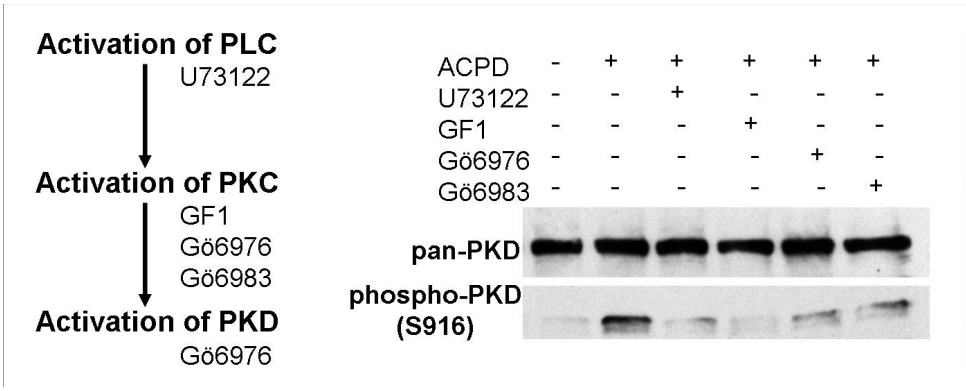


**Figure 7: PKD1 activation downstream of mGluRs follows canonical pathway for PKD1 activation.**
Left: activation pathway of PKD1. Drugs that inhibit each step of the pathway are listed under their targets. Right: pharmacology was applied as shown at the top. ACPD-induced PKD1 phosphorylation is blocked by each drug.

**PKD1 translocation is downstream of NMDAR signaling**

Since the translocation of PKD1 plays a key role in its regulation, we determined its localization with a PKD1 construct tagged at its N-terminus with Venus, a YFP variant.

When transfected into neurons, Venus-PKD1 showed a diffuse, nuclear-excluded distribution, as expected. When stimulated with glutamate or with a field stimulator, Venus-PKD1 translocated to discrete puncta throughout the dendrites (Fig. 8). Co-transfected mCherry, a diffuse fluorescent protein, showed the unchanged, healthy morphology of the neurons during puncta formation.

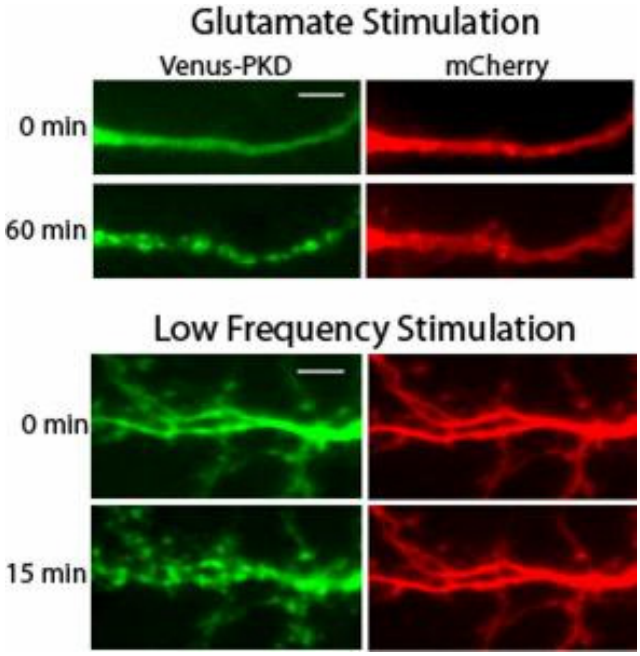


**Figure 8: Venus-PKD1 forms puncta in dendrites upon stimulation with glutamate or with a field stimulator.**
Neurons transfected with Venus-PKD1 and mCherry were imaged both before and after stimulation. Top panels show stimulation with 30 μM glutamate; bottom panels show stimulation at 5 Hz with a field stimulator. Both types of stimulation show dendritic translocation of Venus-PKD1 with minimal effect on diffuse mCherry. Scale bars indicate 10 μm in this and future images. Images are representative (n>30 neurons, >3 experiments).

We next identified the receptor subtype responsible for translocation of Venus-PKD1. Puncta formation after glutamate stimulation was unaffected by MCPG, and high doses of ACPD (500 μM) did not induce translocation. In contrast, puncta formation was induced by NMDA and was blocked by APV or the absence of extracellular calcium

(Fig. 9). To further confirm the role of NMDARs in Venus-PKD1 puncta formation, we cultured neurons from NR1–/– embryos, which lack functional NMDARs[68]. These neurons showed no Venus-PKD1 translocation after stimulation (Fig. 9). To determine if the source of calcium influx was important, we depolarized the neurons with high $K^+$ (55 mM) in the presence of APV, which should activate voltage-sensitive calcium channels[68], but we saw no puncta formation (Fig. 9). These data show that, while mGluRs activate PKD1 after glutamatergic stimulation, calcium influx specifically through NMDARs mediates its translocation to dendritic puncta. Since PKD1 has distinct responses to mGluRs and to NMDARs, PKD1 may act as an integration point for these different glutamatergic signaling cascades.

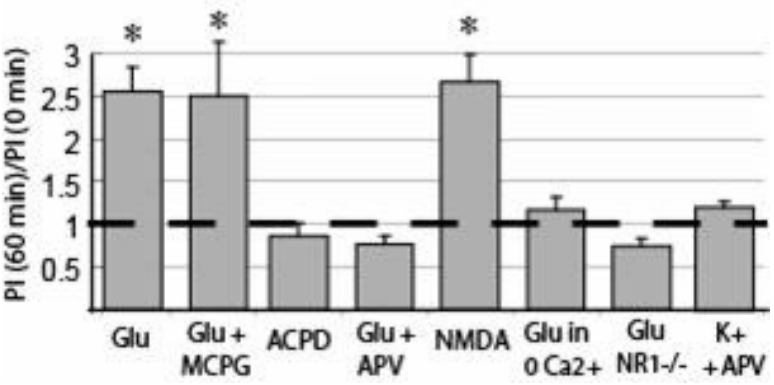


**Figure 9: Venus-PKD1 translocation depends on calcium influx specifically through NMDARs.**
Puncta were quantified using a Puncta Index (PI), which is defined as the standard deviation of Venus-PKD1 fluorescence along a region of dendrite divided by the standard deviation of mCherry along the same region (see Materials and Methods). * significant difference ($p<0.05$) between PI at 60 min and PI at 0 min. $n>4$ dendrites from at least 3 neurons in at least 2 experiments.

**PKD1 translocation is a two-step process**

Since a two-step translocation process has been shown in other cell types[77,78,128,129], we looked at earlier time points during stimulation. We saw that puncta formation is indeed a two-step process; after 10 minutes of stimulation, many neurons showed Venus-PKD1 localization to the plasma membrane both in the cell body and dendrites (Fig. 10). Only after 45-60 minutes did Venus-PKD1 form puncta.

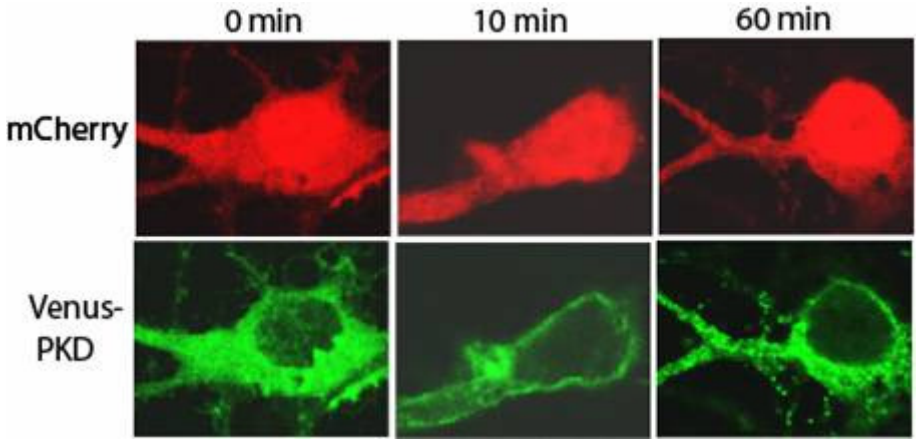


**Figure 10: Venus-PKD1 undergoes a two-step translocation in response to glutamate.**
Neurons transfected with Venus-PKD1 and mCherry were stimulated for 0 min, 10 min or 60 min with glutamate, fixed and imaged using a confocal microscope. At 10 min, Venus-PKD1 shows clear plasma membrane localization in approximately 20-25% of neurons. At 60 min, nearly every neuron shows Venus-PKD1 puncta.

In order to better understand the mechanisms underlying this two-step process, we made mutations in the kinase that are known to affect the two steps of translocation. The C1b domain has been shown to be critical for translocation of PKD1 to the plasma membrane after stimulation of Gαq-coupled receptors and after treatment with phorbol esters[84,129,131]. When we mutated the critical proline in the C1b domain to a glycine (Venus-PKD1 P287G) and tested its translocation, we found that it remained diffuse throughout the stimulation (Fig. 11). Since this domain functions by binding to

DAG[84,125,131], we next asked if the translocation of Venus-PKD1 was dependent on DAG production. Indeed, addition of U73122, an inhibitor of PLC, blocked glutamate-induced puncta formation (Fig. 12), further supporting a role for the C1b domain in Venus-PKD1 translocation.

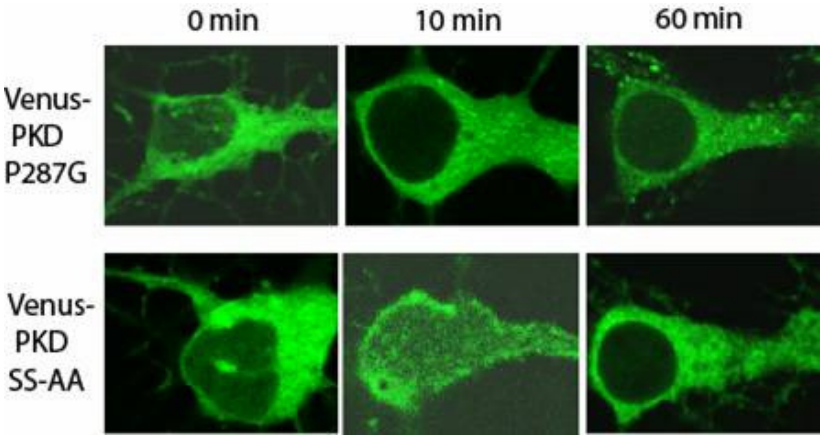


**Figure 11: Mutations in the C1b domain or the activation loop block puncta formation at different steps of the translocation.**
Neurons were stimulated, fixed, and imaged as above. Neurons transfected with either Venus-PKD1 P287G or with Venus-PKD1 SS-AA showed no puncta formation at 60 min. For Venus-PKD1 P287G, the first step of the translocation to the plasma membrane was blocked, while for Venus-PKD1 SS-AA, puncta formation was blocked during the second step of the translocation.

The phosphorylation of the two serines in the activation loop (Ser744/748) has been postulated to release PKD1 back to the cytosol after translocation to the plasma membrane[78,84,128]. We tested to see if it was required for PKD1 puncta formation. We found that a mutant form of PKD1 that is unable to be phosphorylated (Venus-PKD1 SS-AA) shows good translocation to the plasma membrane at early time points, but does not form puncta (Fig. 11). These observations are consistent with a role for the C1b domain in the first step of the translocation and for the activation loop in the second. Since the activation loop is usually phosphorylated by PKC isoforms[77], we used inhibitors of PKC

to block translocation of Venus-PKD1. Surprisingly, PKC inhibitors GF109203X, Gö6976, and Gö6983 had no effect on puncta formation (Fig. 12) (see Discussion).

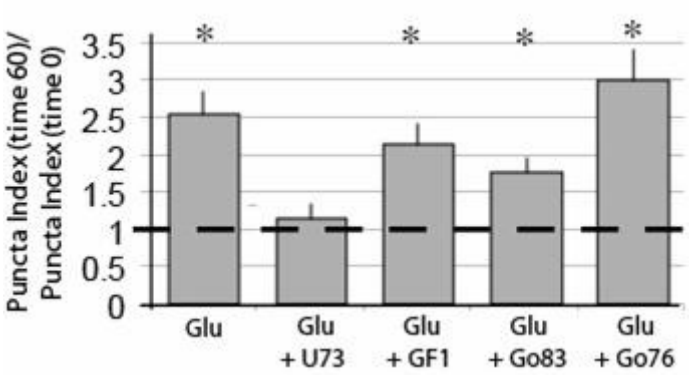


**Figure 12: A PLC inhibitor blocks Venus-PKD1 translocation, but PKC inhibitors do not.**
Neurons transfected with Venus-PKD1 and mCherry were stimulated with glutamate (plus indicated drugs) and their puncta indices were calculated as above. Only U73122, the PLC inhibitor blocks puncta formation. PKC inhibitors GF109203X, Gö6976, and Gö6983 had no effect. Doses of drugs were the same as those used in Fig. x. * significant difference ($p<0.05$) between PI at 60 min and PI at 0 min. $n>4$ dendrites from at least 3 neurons in at least 2 experiments.

To better understand the relationship between the structure of PKD1 and its translocation, we made several deletions and mutations in the kinase to determine their effects on puncta formation (Fig. 13). While the Venus-PKD1 SS-AA mutant showed no puncta formation, the activation loop mutant that should mimic phosphorylation (Venus-PKD1 SS-EE) showed robust puncta formation. We saw no effect after deletion of either the PH domain (Venus-PKD1 ΔPH) or the C-terminal PDZ ligand (Venus-PKD1 ΔPDZ) (Fig. 13). Studies have found that the C1a domain is important for PKD1 localization to endosomal structures[124,138]. However, we found that full deletion of the domain (Venus-PKD1 ΔC1a) or a point mutation that disrupts its structure (Venus-PKD1 P155G)[124,138] has no effect on puncta formation (Fig. 13). A major difference between previous work

and ours is that these previous studies have only studied the localization of kinase-inactive versions of PKD1 while our experiments show an agonist-induced translocation of wild-type Venus-PKD1 to these structures.

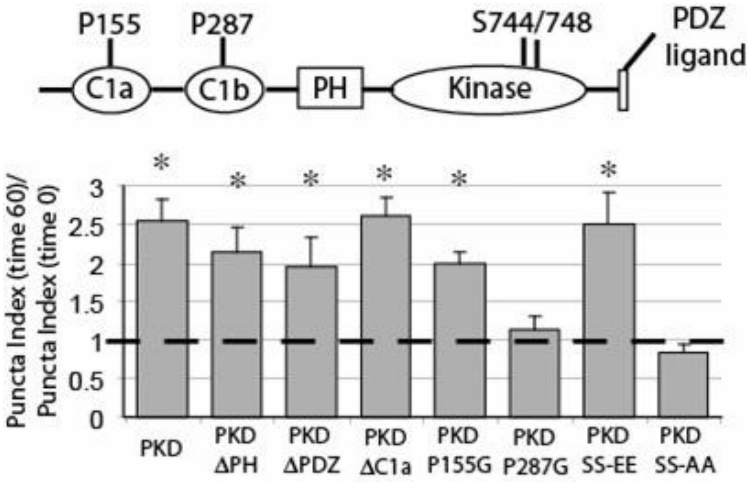


**Figure 13: Disruption of domains of Venus-PKD1 other than the C1b domain or the activation loop has no effect on puncta formation.**
Top panel shows the primary structure of PKD1 with critical amino acids highlighted. Bottom shows puncta indices for each construct in response to 30 μM glutamate. Only the Venus-PKD1 P287G and the Venus-PKD1 SS-AA constructs do not form puncta. * significant difference ($p<0.05$) between PI at 60 min and PI at 0 min. $n>4$ dendrites from at least 3 neurons in at least 2 experiments.

**Translocation of PKD1 is not an excitotoxic response**

While bath applied glutamate can have excitotoxic effects, we do not believe that puncta formation is due to cellular death or dysfunction for several reasons. First, we have been able to reproduce Venus-PKD1 puncta formation after field stimulation, which more closely mimics physiological, synaptic stimulation (Fig. 8). Furthermore, we have been able to see washout of Venus-PKD1 puncta after stimulation with the field stimulator (Fig. 14A). Second, only healthy neurons were included in all of our analyses; in all

translocation experiments, mCherry was co-transfected as a morphology marker, and neurons that had dendritic blebbing or other hallmarks of apoptosis were excluded. Third, a single point mutation (Venus-PKD1 P287G) was able to block puncta formation (Figs. 11,13), indicating that Venus-PKD1 likely participates in a specific interaction rather than a non-specific aggregation. Fourth, by following single neurons over time, we have been able to see instances of spontaneous Venus-PKD1 puncta formation followed by washout of those puncta and long-term survival of the neurons (Fig. 14B). Finally, when we cotransfected Venus-PKD1 along with an RFP-tagged portion of isovaleryl coenzyme A (RFP-mito)[139], a marker for mitochondria, we saw puncta formation in response to glutamate even while the RFP-mito remained elongated throughout the dendrites, indicating that the neurons were healthy[140] (Fig. 14C).

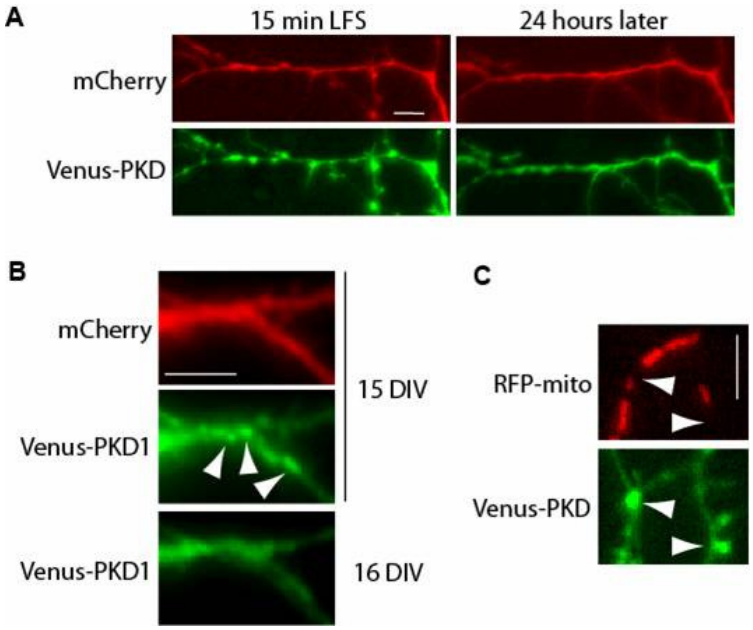


**Figure 14: Venus-PKD1 puncta formation is not an excitotoxic response.**
A) Neurons were transfected with Venus-PKD1 and mCherry and imaged as above. Left panels show Venus-PKD1 puncta induced by low-frequency stimulation with a field

stimulator. Right panels show the same neuron 24 hours later. Puncta have disappeared, indicating that cell death does not follow puncta formation. B) Neurons transfected with Venus-PKD1 and mCherry were incubated without stimulation and imaged every 24 hours. Images show Venus-PKD1 puncta at 15 days *in vitro* (DIV) (arrowheads), but none at 16 DIV. This neuron lived at least until 21 DIV. Of 92 neurons followed, 19 formed Venus-PKD1 puncta, and of those, 14 lived more than one day after puncta formation. C) Neurons were transfected with RFP-mito and Venus-PKD1 and stimulated with glutamate for 60 min, as above. Venus-PKD1 puncta do not colocalize with the mitochondrial marker. Furthermore, the RFP-mito shows elongated, healthy mitochondria. Images are representative (n=8 neurons).

**PKD1 colocalizes with AMPAR subunit GluR2 after stimulation**

We next sought to identify these Venus-PKD1 puncta. We first tested for colocalization of Venus-PKD1 and the *trans*-Golgi network (TGN), since PKD1 has an established role in these structures[77]. We tested for colocalization with the TGN using a CFP-tagged version of TGN38, a marker for the TGN that has been used in other studies of PKD1[80,132]. As predicted, CFP-TGN38 showed some overlap with glutamate-induced Venus-PKD1 puncta (Fig. 15).

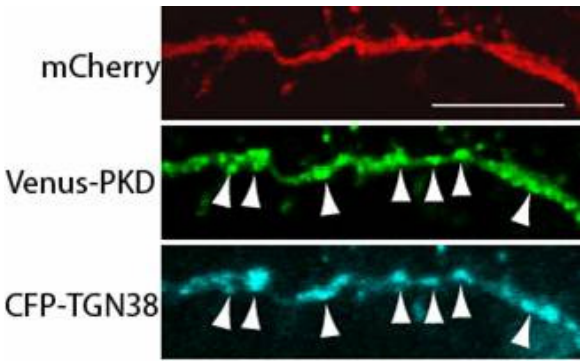


**Figure 15: Venus-PKD1 partly colocalizes with the *trans*-Golgi network marker CFP-TGN38**
Neurons were co-transfected with mCherry, Venus-PKD1, and CFP-TGN38 and stimulated with glutamate for 60 min. Arrowheads show colocalizing puncta. Images are representative (n=16 neurons, 4 experiments).

Since TGN38 is sometimes found in other dendritic endosomes[141], we used antibodies against endogenous protein markers to test for colocalization with glutamate-induced Venus-PKD1 puncta. Venus-PKD1 puncta colocalized best with Rab5, a marker for the early endosome, and less well with Syntaxin13, a recycling endosome marker, and Lamp1, a lysosomal marker (Fig. 16). These data are the first to show an association between PKD1 and these endosomal structures.

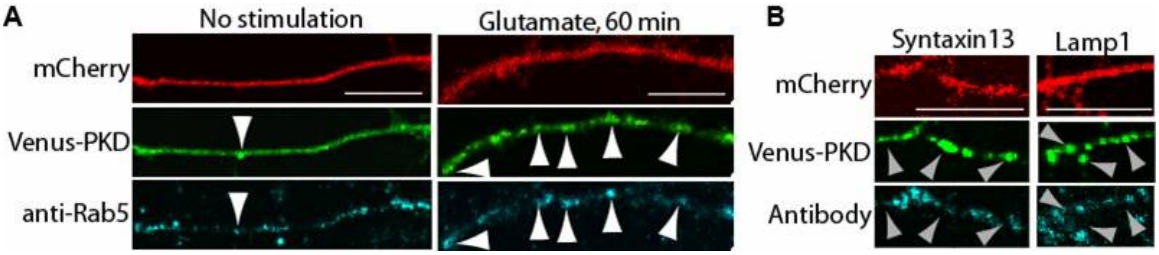


**Figure 16: Venus-PKD1 puncta colocalize with Rab5, an early endosome marker**
Neurons transfected with mCherry and Venus-PKD1 were stimulated with glutamate for 60 min and stained with antibodies against endosomal proteins. A) Venus-PKD1 puncta colocalize with Rab5 immunofluorescence. Left panels show a spontaneous punctum (arrowhead). Right panels show overlapping puncta after glutamate stimulation (arrowheads). B) Neurons were stained with antibodies against syntaxin13 (left panels) or Lamp1 (right panels). Arrowheads show Venus puncta that do not colocalize with antibody immunofluorescence. Images are representative (n>10 neurons, 3 experiments).

Since AMPARs are found in this endosomal pathway[5,8], and since AMPARs internalize in response to glutamatergic activity, we reasoned that PKD1 may colocalize with AMPAR subunits. When Venus-PKD1 was coexpressed with an HA-tagged GluR2 subunit, both proteins were diffuse throughout the dendrites, as expected. When stimulated by either glutamate or by a field stimulator, the two proteins formed puncta that colocalized (Fig. 17).

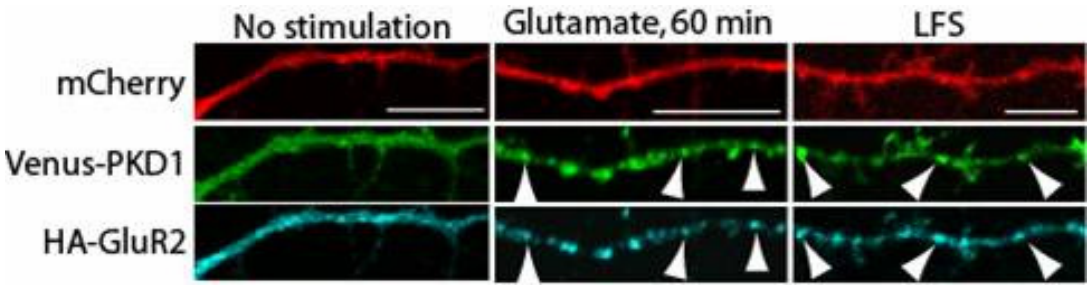


**Figure 17: Venus-PKD1 puncta induced by glutamate or low-frequency stimulation colocalize with HA-tagged AMPAR subunit GluR2.**
Neurons were transfected with Venus-PKD1 and HA-GluR2, stimulated with glutamate or a field stimulator, and stained with anti-HA antibodies. Left panels show non-stimulated neurons, in which all three fluors are diffuse, middle panels are glutamate-stimulated neurons, and right panels are neurons stimulated by a field stimulator. Arrowheads show colocalizing puncta. Images are representative (n>9 neurons, >2 experiments).

**Interfering with PKD1 function disrupts AMPAR trafficking**

To determine if PKD1 is involved in regulating the localization of AMPARs, we first used Venus-PKD1 K618N along with overexpression of AMPAR subunits, which promotes formation of homomeric AMPARs[142]. Venus-PKD1 K618N is a kinase-inactive, dominant-interfering version of PKD1 that prevents certain proteins from trafficking to the plasma membrane, trapping them in PKD1-positive puncta[80,132,133]. In our neurons, the localization of overexpressed HA-GluR2 was altered when it was co-expressed with Venus-PKD1 K618N, with HA-GluR2 showing puncta that colocalized with Venus-PKD1 K618N (Fig. 18A, left panels). This PKD1 construct does not disrupt all protein trafficking; the localization of HA-GluR1 was not altered and did not colocalize with Venus-PKD1 K618N (Fig. 18A, right panels). To test if these Venus-PKD1 K618N puncta were similar to glutamate-induced Venus-PKD1 puncta, we stained

neurons containing Venus-PKD1 K618N with antibodies against Rab5 and found that these puncta colocalized with each other very well (Fig. 18B).

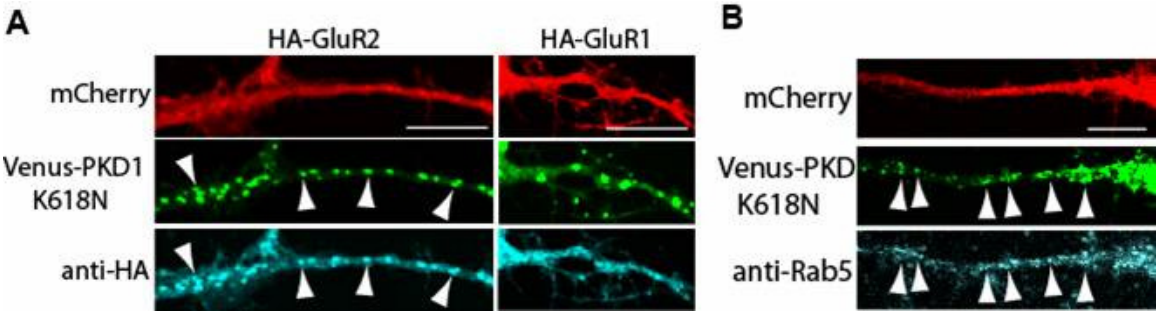


**Figure 18: Kinase-inactive Venus-PKD1 traps GluR2 in PKD1- and Rab5-positive puncta.**
A) Neurons were transfected with Venus-PKD1 K618N and HA-GluR2 (left panels) or HA-GluR1 (right panels) and were stained with anti-HA antibodies. Arrowheads show colocalizing puncta. Images are representative (n>9 neurons, 4 experiments). B) Neurons transfected with Venus-PKD1 K618N were stained with antibodies against early endosomal marker Rab5. Arrowheads show colocalizing puncta.

To determine more specifically if this effect was due to the disruption of PKD1 function and to avoid the confounds of protein overexpression, we used an siRNA construct targeted to PKD1. First, we confirmed that our siRNA sequence strongly and selectively reduced the levels of PKD1 (Fig. 19).

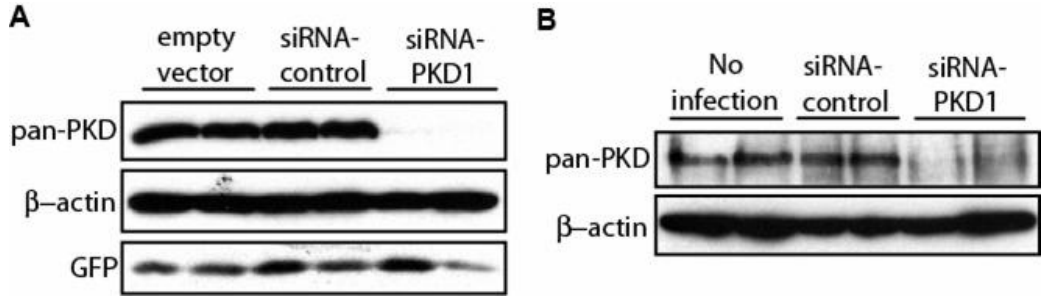


**Figure 19: siRNA directed against PKD1 reduces PKD1 expression in heterologous cells and in neurons.**
A) HEK293FT cells were transfected with plasmid DNA encoding siRNA constructs (or empty vector), PKD1 and GFP, harvested, and subjected to Western blot. siRNA-PKD1 shows a decrease in PKD1 levels. A blot of GFP expression shows that cells in each

condition were comparably transfected. Blots are representative (n>3). B) The same siRNA sequences were subcloned into lentiviral vectors. Neurons were infected with these lentiviruses, harvested 7 days later, and subjected to Western blot for staining against endogenous PKD1. siRNA-PKD1 shows a decrease in endogenous PKD1 levels in these neurons. Blots are representative (n=2). β-actin immunostaining shows equal protein loading across wells.

When this siRNA construct was transfected into neurons and stained with antibodies against endogenous GluR2, we found that the localization of GluR2 was altered; instead of its normal, more diffuse pattern, it was found in punctate structures (Fig. 20A). These data show that PKD1 affects the trafficking of GluR2-containing AMPARs. These siRNA-induced GluR2-containing puncta contained GluR1 as well, but GluR1 is more punctate even under baseline conditions[143] (Fig. 20B), making it difficult to tell if siRNA knock-down of PKD1 affects the trafficking of GluR1 subunits.

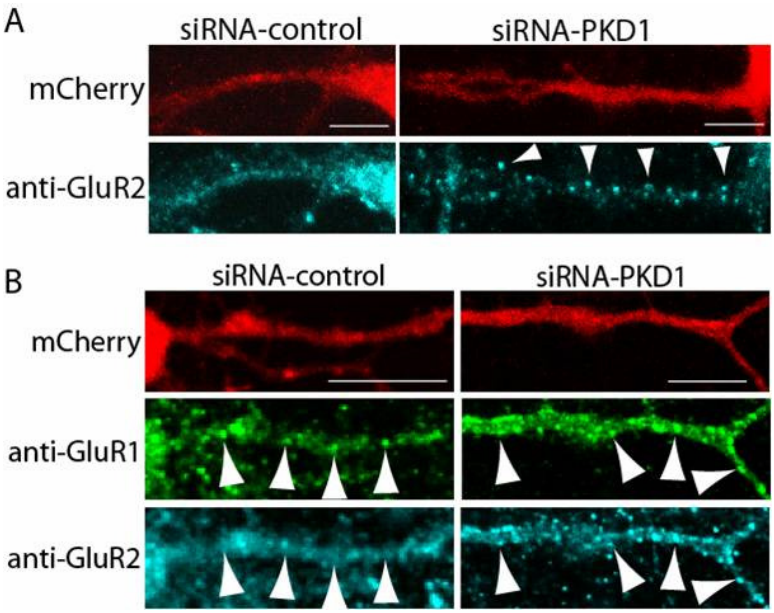


**Figure 20: siRNA knockdown of PKD1 disrupts regulation of AMPAR subunit GluR2.**
A) Neurons were transfected with siRNA-control or siRNA-PKD1, incubated for 7 days, and stained with antibodies against endogenous GluR2. Arrowheads show puncta. Images are representative (n=20 neurons, 5 experiments). B) GluR1 is punctate even

under control conditions, and colocalizes with siRNA-PKD1-induced GluR2 puncta. Neurons transfected with siRNA-control or siRNA-PKD1 were stained with antibodies against both endogenous GluR1 and endogenous GluR2. Arrowheads show GluR1 puncta, which overlap with GluR2 in the siRNA-PKD1 images. Images are representative (n>8 neurons, 2 experiments).

## Discussion

We show here that PKD1 is activated after mGluR activation and translocates to early endosomes containing AMPAR subunits in response to NMDAR signaling. Interfering with the function of PKD1 by using a dominant-interfering form or by siRNA knock-down of endogenous PKD1, disrupts the localization of AMPAR subunit GluR2. Our results showing accumulation of GluR2 in endosomal structures after interfering with PKD1 function suggest that PKD1 normally acts to promote forward trafficking of the AMPAR subunit out of these endosomes. This model (Fig. 21) is consistent with the known functions of PKD1 in protein trafficking[77].

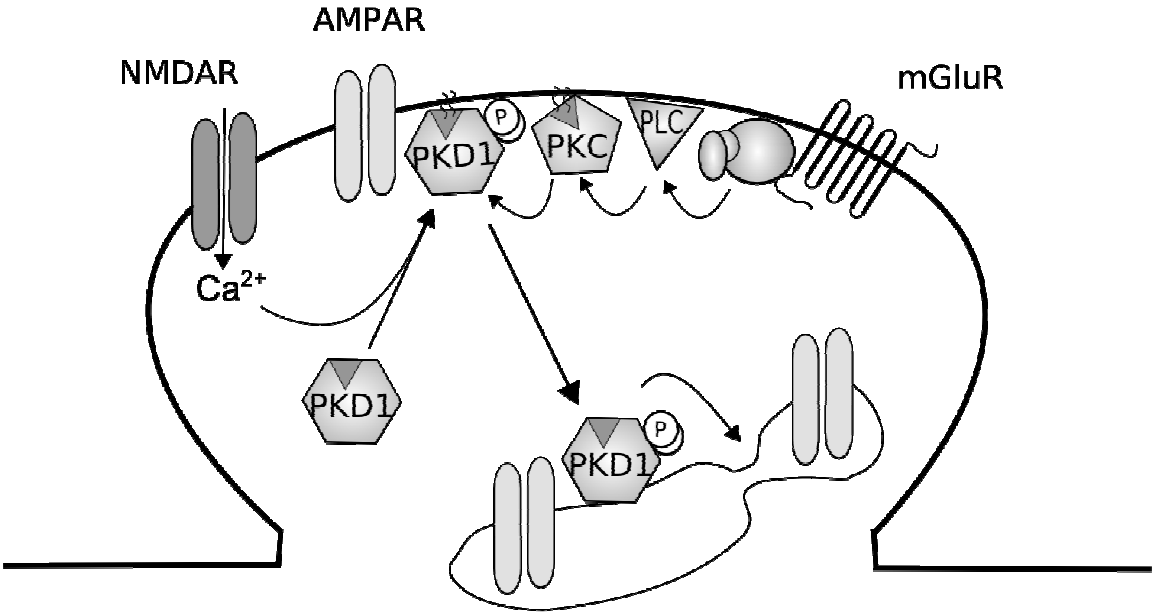


**Figure 21: A model of PKD1 activity and regulation in glutamatergic synapses.** PKD1 undergoes a two-step translocation in response to calcium influx through NMDARs. The first step of this translocation is to the plasma membrane and depends on DAG binding to the C1b domain of PKD1. The second step depends on the activation loop, but the role of phosphorylation of this loop is unclear (see text). The target of this translocation is endosomal structures containing GluR2 and colocalizing with Rab5, an early endosomal marker. At the same time as this translocation, signaling through mGluRs activates PKD1 through the canonical, Gαq-coupled receptor PKD1 activation pathway; this pathway includes PLC activation, subsequent DAG production, and PKC

activation. Once at these endosomal sites, PKD1 functions to promote trafficking of AMPARs.

**Mechanisms of PKD1 regulation of AMPARs**

The mechanisms by which PKD1 regulates AMPAR trafficking remain unclear. Since we found that PKD1 colocalizes well with Rab5, an early endosome marker, and since AMPARs are internalized and recycled through this endosomal pathway[5,8], PKD1 may function to increase protein recycling, as reported in fibroblasts[136]. In that study, it was not clear if the effect of PKD1 was specific to β-integrins or if PKD1 was working through a more general mechanism. To test if PKD1 regulates the endosomal recycling pathway in a non-specific way, we determined if altering the function of PKD1 altered the uptake or recycling of fluorescently-tagged transferrin. This method has been used by several groups to study synaptic recycling mechanisms[5,144]. We found that PKD1 constructs that interfere with the function of PKD1 or siRNA knock-down of PKD1 had no effect on transferrin recycling (Fig. 22). While these data would dispute a non-specific role for PKD1 in the endosomal recycling pathway, it is possible that PKD1 may regulate AMPARs more selectively, as is the case for the endosomal protein NEEP21[144].

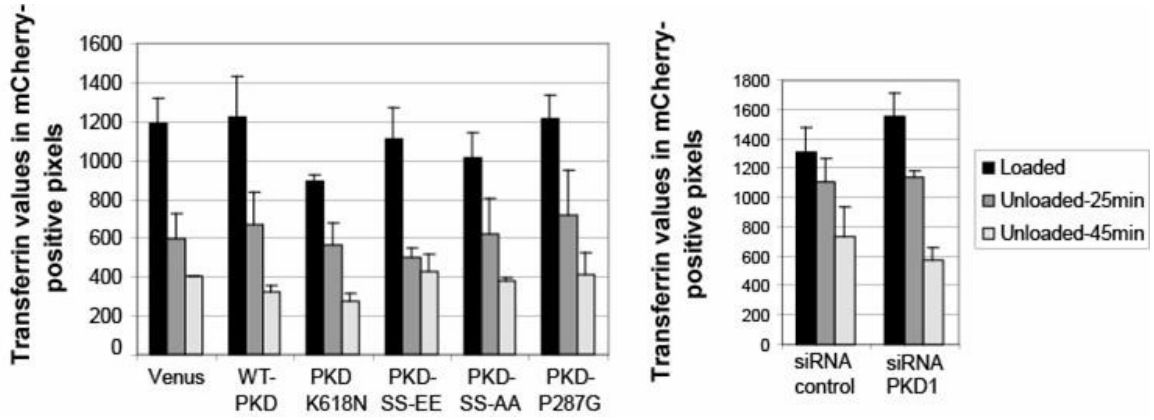

**Figure 22: PKD1 has no effect on transferrin recycling in neurons.**
Fluorescently-labeled transferrin was loaded into neurons transfected with mCherry and PKD1 constructs. After reaching equilibrium, neurons were unloaded with an excess of unlabelled transferrin for either 25 min or 45 min and fixed. The amount of transferrin fluorescence remaining in the dendrites was compared across constructs. No significant differences were found among Venus-PKD1 constructs (left panel) or between siRNA and control constructs (right panel).

In other cell lines, PKD1 effects trafficking from the TGN by regulating its lipid content, thereby promoting budding and trafficking of many transmembrane proteins to the plasma membrane[134,135]. In neurons[80], as well as in MDCK cells[132], it has been shown that PKD1 selectively binds and regulates the trafficking of specific cargoes. Our transferrin experiments (Fig. 22), along with our data showing that a dominant-interfering PKD1 disrupts overexpressed GluR2, but not GluR1 (Fig. 18), indicate some degree of specificity and may support a selective trafficking mechanism. In the previous study of PKD1 in neurons, Kidins220, the protein regulated by PKD1, was a substrate for PKD1 phosphorylation[80]. It is not yet clear if AMPAR subunits are substrates of PKD1 or if PKD1 directly binds the AMPAR complex.

**Convergence of NMDAR and mGluR signaling**

As a regulator of AMPAR trafficking that is downstream of NMDARs, PKD1 may represent a link between NMDARs and synaptic plasticity. NMDARs are known to regulate AMPAR trafficking in multiple ways, with stimulation of the receptors a critical step in both the removal of AMPARs from the synapse and their reinsertion[7,8]. Since PKD1 functions to increase trafficking of proteins, it may play a role in NMDAR-mediated regulation of insertion of AMPARs into the plasma membrane. Interestingly,

the translocation of Venus-PKD1 is calcium-dependent, but is specific to calcium influx through NMDARs; calcium influx induced by high $K^+$ shows no Venus-PKD1 translocation (Fig. 9). This result may indicate that calcium-sensitive signaling proteins close to the channel mouth are more important to PKD1 signaling than bulk calcium flow[58,70] and underscores the specific importance of NMDARs in this signaling pathway.

Although many forms of synaptic plasticity are dependent on NMDARs, some forms of long-term depression depend on mGluRs[7]. Most studies on the role of mGluRs in AMPAR trafficking have focused on their role in the removal of AMPARs from the synapse, but there is also evidence that they promote the specific insertion of AMPARs containing GluR2[123]. Since PKD1 shows some degree of specificity for GluR2 over GluR1 (Fig. 18), PKD1 may be suited to effect this type of synaptic change. As PKD1 is activated upon stimulation of mGluRs, PKD1 may provide a mechanistic link between mGluR signaling and AMPAR localization.

Since PKD1 has distinct responses to mGluRs and to NMDARs, PKD1 may act as an integration point for these different glutamatergic signaling cascades. While NMDAR signaling causes translocation to GluR2-containing endosomes, it does not activate the kinase. Conversely, stimulation of mGluRs activates PKD1, but does not effect its translocation. Convergence of these two signaling cascades may be required for PKD1 to have an effect on GluR2 localization. This independent regulation of the activity and the localization of PKD1 provides the neuron with exquisite control over the kinase with two critical receptors cooperating to regulate its function.

**Implications for the molecular biology of PKD1**

Independent regulation of the activation and localization of PKD1 has not been demonstrated previously. While translocation to endosomes has not been shown by other groups, our observed NMDAR-mediated two-step translocation for PKD1 is similar to what has been described previously for the translocation to other intracellular targets[77,78,84,128-130]. However, in each of those previous studies, translocation and activation coincided. Our results suggest that translocation and activation of PKD1 are fully independent and that the two-step translocation may be a more general behavior of PKD1 and not a response to or precursor of activation. Furthermore, by separately regulating these two aspects of PKD1 function, cells may be able to control PKD1 in a combinatorial way during complex processes such as synaptic plasticity.

With our data demonstrating translocation of PKD1 to GluR2-containing endosomes, we add endosomes to the list of subcellular targets to which PKD1 translocates in response to stimulation. Dynamic translocation of wild-type PKD1 to the plasma membrane[84,129], to the nucleus[78], and to mitochondria[145] has been shown by other groups. However, previous studies of PKD1 function in protein trafficking have depended on kinase-inactive versions of the kinase that constitutively localize to the TGN and other organelles[80,132,133,135]. Here we show that calcium influx through NMDARs is a stimulus that effects translocation of Venus-PKD1 to endosomal structures.

Interestingly, we found that while PKD1 is not active during its translocation, phosphorylation on the activation loop appears to be required since puncta formation is blocked by the activation loop mutant Venus-PKD1 SS-AA (Figs. 11,13). It is possible that activation loop phosphorylation is not sufficient to activate the kinase, as has been

shown previously for certain cell types[146]. Conversely, conformational changes associated with activation loop phosphorylation[147] may regulate the function or binding partners of PKD1 independently of its kinase activity. However, inhibitors of PKC do not block translocation (Fig. 12), indicating that a canonical phosphorylation of the activation loop may be not be a critical step in the translocation. It is possible that the structure of the activation loop is required for a protein-protein interaction and that the SS-AA mutation of Venus-PKD1 alters the structure enough to block the interaction. The crystal structure of checkpoint kinase 2, the closest homolog of PKD1, shows a highly structured activation loop, and so may support the hypothesis of the activation loop of PKD1 as a structural domain[82]. The binding partners of PKD1 and the structural determinants of such interactions in neurons has not yet been explored.

This study is the first to implicate PKD1 in glutamatergic signaling in neurons. We showed that PKD1 is downstream of NMDAR and mGluR signaling and that these two signaling pathways evoke independent responses from PKD1, suggesting convergent control of the function of this kinase. Furthermore, our results show that PKD1 regulates the localization of the AMPAR subunit GluR2. This signaling pathway may represent a novel mechanism by which NMDARs and mGluRs cooperate to regulate the trafficking of AMPARs and thus, synaptic strength.

## Materials and Methods

### Cortical Cultures and Pharmacology

Embryonic mouse primary cortical neurons were cultured and NR1–/– neurons were genotyped as described[68]. For experiments not including NR1–/– neurons, wild-type C57/BL6 mice (Charles River, Wilmington, MA) were used. Neurons from E18–19 embryos were plated at a density of 0.6 x $10^6$ cells/$cm^2$ on 12 mm glass coverslips coated with poly D-lysine and maintained in Neurobasal medium supplemented with B27 (Invitrogen, Carlsbad, CA). Experiments were done at 8–14 DIV. Neurons were transfected using the calcium phosphate method[14] and used 1–2 days post-transfection except for siRNA experiments, in which neurons were used 7 days after transfection. All stimulations were performed at room temperature in HEPES-buffered saline (HBS: 119 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM HEPES, 30 mM glucose, 1 μM tetrodotoxin, 10 μM NBQX, pH 7.4). For all stimulations except those using high $K^+$, 25 μM Nimodipine was included in HBS. Neurons were pre-incubated in HBS for 30–60min before stimulation. Doses used (unless otherwise indicated): PMA (100 nM), Glutamate (30 μM with 10 μM glycine), APV (100 μM), NMDA (100 μM with 10 μM glycine), MCPG (1 mM), ACPD (100 μM), U73122 (5 μM), Gö6976 (3.5 μM), Gö6983 (3.5 μM), GF109203X (2 μM), and $K^+$ (55 mM). Stimulation with 0 calcium indicates nominally zero extracellular calcium.

**Imaging and analysis**

For puncta analysis, images were taken using an inverted epifluorescence microscope (Nikon, Japan) with a cooled CCD digital camera (Hamamatsu Orca II, Japan)[148]. Venus-PKD1 and mCherry images of the same field were taken before and after stimulation. Calculations of puncta indices (PIs) were based on a previous study[68]. Using MetaMorph software (Molecular Devices, Downingtown, PA), PIs were calculated as the standard deviation (SD) of Venus fluorescence along a region of dendrite divided by the SD of mCherry fluorescence along the same region. PIs were calculated both before and after a 60–min stimulation with glutamate and expressed as the ratio of PI (time 60)/PI (time 0). Dendrites for which the SD of mCherry intensities showed a change after stimulation were excluded from the analysis. For spontaneous puncta formation, longitudinal analysis was adapted from a previous study[148]. Locations of fields of neurons were memorized on the first day of imaging and then revisited each day thereafter using a robotic microscope. Images were taken beginning at 13 DIV and ending at 21 DIV. For colocalization studies, a Zeiss LSM 510 laser scanning confocal microscope was used to obtain images. Images were minimally processed using GIMP freeware. When necessary, identical manipulations were used in control and experimental conditions.

**Constructs**

GW1-Venus-PKD1 was created from a Clontech vector containing GFP-PKD1 described previously[84]. A 46–amino acid linker links Venus to the N-terminus of mouse PKD1. The point mutation in Venus-PKD1 was generated using a site-directed mutagenesis kit

from Stratagene (La Jolla, CA); mutation was confirmed by DNA sequencing. CFP-TGN38 has been described[141], as have the HA-GluR1 and HA-GluR2 constructs[149] and the RFP-mito construct[139]. The siRNA-PKD1 was constructed as a short hairpin RNA in mammalian expression vector pSilencer 2.0 using the target PKD1 sequence AAAGAGTGTTTGTTGTTATGG, followed by a 9–bp linker, followed by the inverse 21–bp sequence. The control siRNA used the same target sequence with four mutations (underlined): AAAGTGTGATTGTTGTTTAGG. For experiments looking at endogenous GluR subunits, neurons were transfected with siRNA constructs and analyzed 7 days later. To generate lentiviral constructs, PCR amplification of pSilencer 2.0 constructs containing the U6 promoter and short hairpin RNAs was used to subclone sequences into viral vector FUGW2. FUGW2 was expressed in HEK293FT cells (Invitrogen) along with viral packaging proteins Δ8.9 and VSVG. Viruses were harvested, titered, and used for infection as described previously[150]. Neurons were harvested and analyzed 7 days after infection.

**Field Stimulation**

At 14 DIV, coverslips containing transfected neurons were transferred to a custom-designed field stimulator with two parallel platinum electrodes suspended in the medium approximately 1 mm from the cell monolayer. Neurons were stimulated at 5 Hz for 15 min with a 1–ms pulse width using a D330 MultiStim stimulation apparatus (Digitimer, England). This frequency has been previously shown to induce LTD in hippocampal slices[151].

**Immunocytochemistry**

Cells were fixed in 4% paraformaldehyde for 10 min, permeabilized in 0.1% Triton X-100, blocked (3% fetal bovine serum or donkey serum, 2% bovine serum albumin, 0.1% Triton X-100), and then incubated with primary antibody against HA (1:1000; Cell Signaling, Danvers, MA), GluR2 (1:300; Millipore, Billerica, MA), GluR1 (1:200; Millipore), Rab5 (1:100; BD Biosciences, San Jose, CA), LAMP1 (1:200; Stressgen, Ann Arbor, MI), or Syntaxin13 (1:50; AbCam, Cambridge, MA). Coverslips were then washed in PBS, incubated with secondary antibodies, washed again, and mounted on glass slides for analysis by confocal microscopy. For all staining except for endogenous GluR1, an Alexa647-conjugated anti-mouse secondary antibody (1:250; Molecular Probes, Carlsbad, CA) was used. For GluR1 staining, a Cy2-conjugated anti-rabbit secondary (1:250; Jackson Immunoresearch, West Grove, PA) was used. Images are shown in pseudocolor.

**Western Blot**

After stimulation, neurons were lysed by addition of ice-cold RIPA buffer (1% Triton X-100, 0.1% SDS, 50 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, 10 mM NaF, and protease inhibitor cocktail (Roche, Switzerland)). Samples were then centrifuged and supernatants were loaded onto gels, separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies against pan-PKD1 (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA), phospho-S916 PKD1 (1:1000; Cell Signaling), β-actin (1:4000; Sigma, St. Louis, MO), GFP (1:5000; Millipore), or tubulin (1:500,000; Sigma). Anti-rabbit or anti-mouse secondary antibodies conjugated to horseradish

peroxidase were used on all blots and were imaged by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) or using SuperSignal West Femto substrate (Pierce, Rockford, IL). For quantification of MCPG reduction of glutamate-induced PKD1 activation, integrated intensity of phospho-S916 PKD1 bands were calculated using MetaMorph software, background subtracted, and normalized to the intensity of corresponding tubulin bands, which were used as loading controls.

**Transferrin Assay**

Assay was adapted from a previous study[5]. Neurons were incubated with Alexa 647-conjugated transferrin (50 μg/mL; Invitrogen) for 60 minutes in HBS (containing no antagonists) to achieve a steady-state concentration of internalized fluorescently conjugated transferrin. The extracellular fluorescent transferrin was then washed out with an excess of unlabeled transferrin (5 mg/mL, Sigma) and cells were fixed at various time points for analysis by confocal microscopy. Neurons were selected based on mCherry images and the experimenter was blind to the Alexa 647 content of the cells. Confocal images were taken of in the mCherry channel, and Alexa 647 images were taken at identical laser settings for each cell to allow for quantitative comparisons between cells. The average pixel intensity of transferrin in mCherry positive pixels was determined using the Zeiss LSM 510 software. Cell bodies were excluded from analysis, and thresholds for mCherry pixels were set for each image using the software's default algorithm.

# Chapter 3:

## PKD1 regulates HDAC removal from the nucleus in primary neurons

**Abstract**

Class II histone deacetylase complexes (HDACs) function in the regulation of gene expression downstream of neuronal synaptic activity. In other cell types, these transcriptional repressors are regulated by the novel kinase PKD1, but little is known about the role of PKD1 in neurons. Here we show that HDAC5 can be regulated by multiple signaling pathways in neurons. In response to the phorbol ester PMA, HDAC5 localization depends on PKD1. However, in response to stimulation of NMDA-type glutamate receptors (NMDARs), regulation of HDAC5 depends on CaMK isoforms and not on PKD1.

## Introduction

In neurons, changes in gene expression play a critical role in long-term adaptive change. In response to synaptic activity, new transcription is initiated through the activation and recruitment to the nucleus of transcription factors that promote transcriptional activity. In addition, repressive elements, such as histone deacetylase complexes (HDACs) are removed from the nucleus. HDACs act as transcriptional repressors by deacetylating histones, which keeps these chromatin structures in a closed state and unable to bind transcriptional machinery[40,152]. The localization, and therefore the function, of class II HDACs (including HDAC4, 5, 7 and 9) is regulated by phosphorylation. Upon phosphorylation, they bind to the cytoplasmic scaffolding protein 14-3-3. This protein masks the nuclear localization sequence of the HDAC and prevents it from returning to the nucleus, thereby eliminating its transcriptional repression[41,104,153,154]. In this way, class II HDACs provide a mechanism through which synaptic activity and its downstream kinases can regulate gene expression.

The class II HDACs HDAC4 and HDAC5 are known to be downstream of synaptic activity in neurons[39,155,156]. The kinases known to phosphorylate class II HDACs include protein kinase D1 (PKD1) and the superfamily of calcium-calmodulin dependent kinases (CaMKs)[40,41,157]. However, it is not clear which kinases effect the regulation of HDAC5 in neurons. Previous work on the role of CaMKs has been ambiguous[158] or has shown some discrepancies; while one study found that nucleo-cytoplasmic shuttling of HDAC5 was regulated by CaMKs[39], another found that a constitutively active CaMK was unable to induce HDAC5 removal from the nucleus[159]. Furthermore, the role of PKD1 in the regulation of HDAC5 in neurons has not been

studied. We show here that HDAC5 in neurons can be regulated by multiple signaling pathways. While PKD1 regulates HDAC5 removal from the nucleus in neurons after stimulation with the phorbol ester PMA, it does not regulate HDAC5 removal downstream of NMDAR stimulation.

## Results

### PKD1 regulates phorbol ester-induced HDAC removal from the nucleus

Since it has been shown in other cell types that PKD1 regulates class II HDAC removal from the nucleus, we wanted to determine if the same is true in neurons. In cardiac myocytes, lymphocytes, and thymocytes, it has been shown that phorbol esters activate PKD1 and thereby effect HDAC removal from the nucleus[104-106]. We first tested to see if PMA had the same effect in neurons. We co-transfected flag-tagged HDAC5 and GFP into neurons and stimulated with PMA. We then stained the cells with an anti-HDAC5 antibody and looked at its localization. Under non-stimulated conditions, HDAC5 was almost exclusively nuclear, while after stimulation with PMA, HDAC5 was predominantly cytoplasmic and nuclear-excluded (Fig. 23).

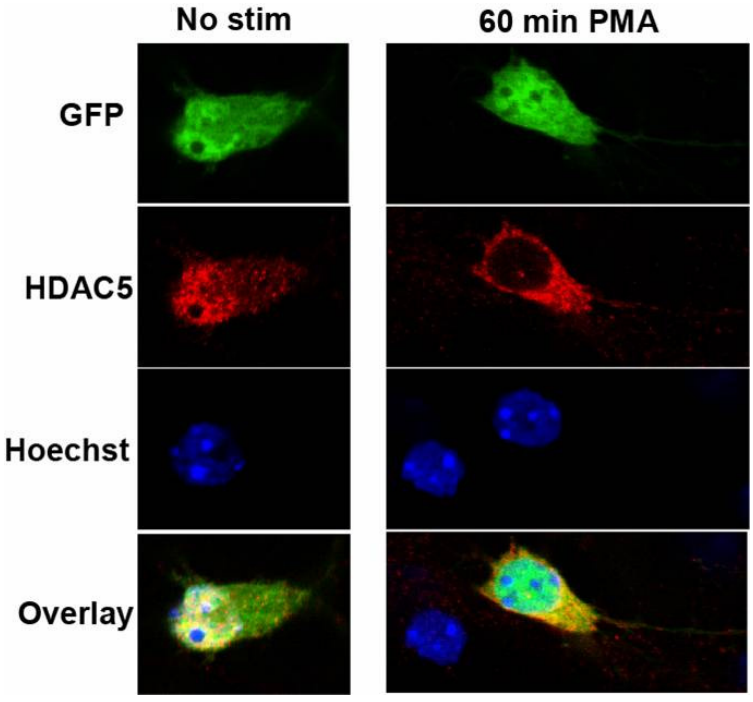


**Figure 23: The phorbol ester PMA induces HDAC5 removal from the nucleus in neurons.**

Neurons were transfected with GFP and flag-HDAC5, stimulated for 60 min with 100 nM PMA and stained with antibodies against HDAC5 as well as a Hoechst stain to label the nucleus. Upon stimulation, HDAC5 switches from almost completely nuclear to almost completely cytoplasmic.

To test for the role of PKD1 in PMA-induced HDAC removal from the nucleus, we used activation loop mutants of PKD1. Mutating the two serines to alanines (PKD1-SS-AA) should prevent activation of the kinase; other studies have shown that this mutant acts as a dominant negative[81,106]. Conversely, mutating the serines to glutamates (PKD1-SS-EE) should create a constitutively active kinase[104]. We first tested the effects of these PKD1 mutants on HDAC5 in HEK293 cells. We co-transfected HA-tagged PKD1 constructs with GFP and flag-HDAC5 into HEK293 cells, and again stained the cells with an anti-HDAC5 antibody. Our quantification method was based on a previous study[39]; the HDAC5 localization for each GFP-positive cell was scored as nuclear, cytoplasmic, or both. Under basal conditions, we found that wild-type PKD1 showed an even distribution of nuclear and cytoplasmic localization of HDAC5. Upon transfection of PKD1-SS-AA, we saw a significant increase in nuclear-localized HDAC5 with a concomitant decrease in cytoplasmic HDAC5 (Fig. 24). This result shows that PKD1-SS-AA is acting to decrease HDAC5 removal from the nucleus, which is consistent with its role as a dominant negative form of PKD1. While the PKD1-SS-EE mutant showed some increase in cytoplasmic HDAC5 ($p=0.04$ using a Student's t-test), the results were not as dramatic as with the PKD1-SS-AA mutant.

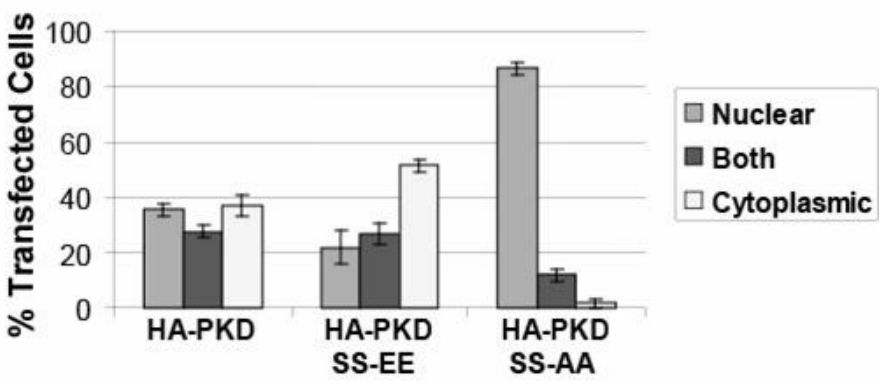


**Figure 24: PKD1 activation loop mutants determine HDAC5 localization in HEK293 cells.**
HEK293 cells were transfected with HA-PKD1 constructs, GFP, and flag-HDAC5 and stained with antibodies against HDAC5. Mutations in the activation loop of PKD1 affect the localization of HDAC5. HA-PKD SS-AA overexpression shows a dramatic increase in the nuclear localization of HDAC5 and a decrease in cytoplasmic localization. (n=3 experiments.)

To test if the dominant negative PKD1 SS-AA mutant could prevent PMA-induced HDAC5 removal from the nucleus in neurons, we co-expressed wild type PKD1 or PKD1-SS-AA with flag-HDAC5 and GFP. We found that cells expressing PKD1-SS-AA showed a complete inhibition of PMA-induced HDAC5 removal from the nucleus (Fig. 25), indicating that PKD1 regulates HDAC5 localization downstream of PMA stimulation.

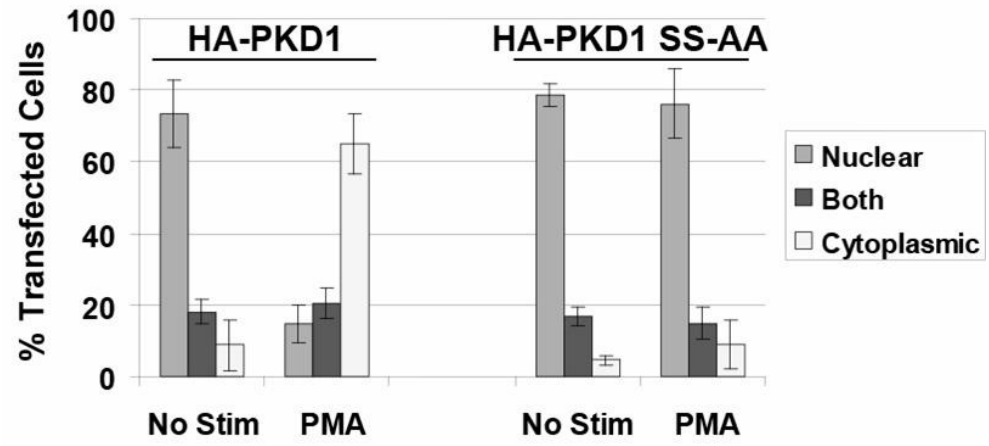

**Figure 25: HA-PKD1 SS-AA blocks PMA-induced HDAC removal from the nucleus.**
In neurons expressing HA-PKD1, PMA induces a redistribution of HDAC5 from the nucleus to the cytoplasm (left). However, when the dominant-negative HA-PKD1 SS-AA is expressed in these neurons, the PMA-induced HDAC removal from the nucleus is completely blocked. (n=5 experiments with at least 50 cells per condition.)

**NMDAR-induced HDAC regulation is downstream of CaMK isoforms, not PKD1**

Since PKD1 is capable of regulating the localization of HDAC5 in neurons, and since HDAC5 is downstream of NMDAR stimulation, we next asked if PKD1 regulates HDAC5 after stimulation with NMDA. We found that while NMDAR stimulation effects HDAC5 removal from the nucleus, PKD1-SS-AA does not block the translocation (Fig. 26).

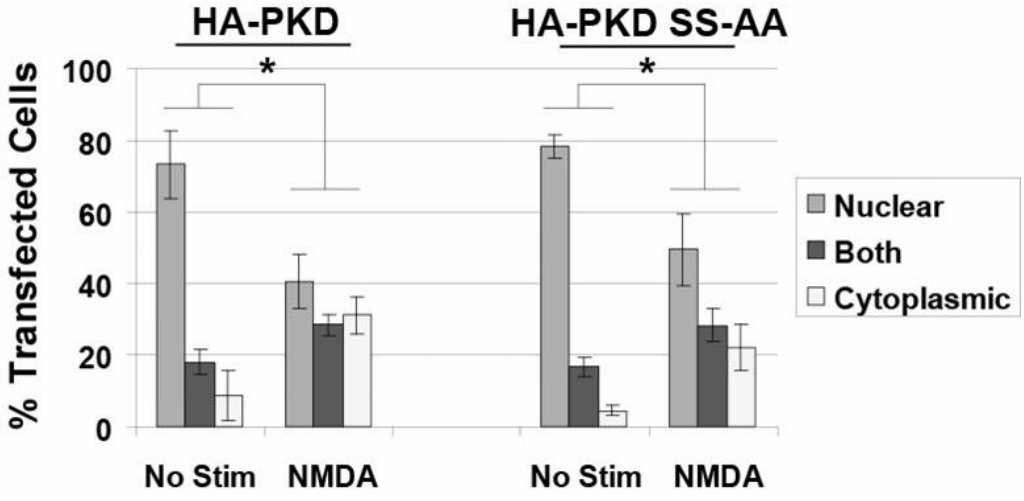


**Figure 26: HA-PKD1 SS-AA does not block NMDAR-induced HDAC removal from the nucleus.**
Stimulation of neurons with 30 μM NMDA for 60 min causes a significant redistribution of HDAC5 from the nucleus to the cytoplasm. In contrast to the results using PMA (Fig. 25), overexpression of HA-PKD SS-AA has no effect on this translocation. * indicates significant difference ($p<0.05$) between non-stimulated and NMDA-stimulated conditions in both the nuclear and cytoplasmic values. (n=5 experiments with at least 50 cells per condition.)

Since other studies have shown that the CaMK superfamily regulate class II HDACs[41,157,160] and that NMDAR-mediated HDAC removal from the nucleus is at least partially dependent on CaMK[39], we used CaMK inhibitors to determine if they are involved in our observed effect. Inhibitors including KN93, which is selective for CaMKII, KN62, which inhibits a wider range of CaMKs, and STO-609, which inhibits CaMK-kinase activity, all blocked NMDA-induced HDAC5 removal from the nucleus (Fig. 27). Together, these data show that PKD1 does not play a role in NMDAR-mediated HDAC5 regulation and that CaMKs are more important for this form of NMDAR signaling to the nucleus.

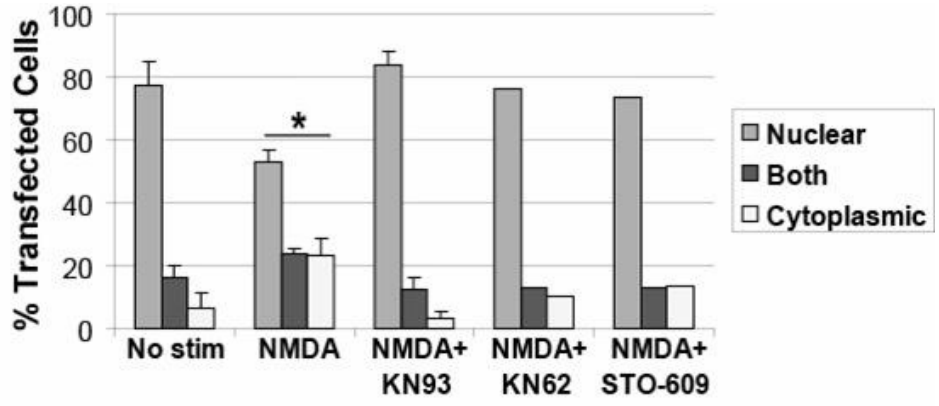


**Figure 27: Inhibitors of CaMK signaling blocks NMDAR-mediated HDAC removal from the nucleus.**
Neurons were stimulated with NMDA alone or with NMDA plus indicated pharmacology. Inhibiting CaMK activity blocks NMDA-induced HDAC redistribution. * indicates significantly different ($p<0.05$) from non-stimulated and from NMDA + KN93 conditions in both the nuclear and cytoplasmic values. For non-stimulated, NMDA, and NMDA + KN93 conditions, n=3 experiments with at least 45 cells per experiment. For NMDA + KN62 and NMDA + STO-609 conditions, n=1.

**PKD1 does not effect NMDA-induced CRE-mediated gene expression**

To further test if PKD1 plays any role in signaling from NMDARs to the nucleus, we performed CRE-luciferase assays. We first determined if CREB-mediated transcription

was sensitive to regulation by HDAC5 using a version of flag-HDAC5 that can not be phosphorylated on the two serines responsible for nuclear exclusion (HDAC5 SS-AA)[104,157]. When we transfected this construct into neurons along with CRE-luciferase constructs, we found that it decreased NMDA-induced, CRE-dependent gene expression (Fig. 28A). To test if PKD1 affects NMDA-induced gene expression, we used pharmacological tools. When we transfected neurons with CRE-luciferase constructs and stimulated them with NMDA in the presence of Gö6976, a drug that should block PKD1 as well as many PKC isoforms, we found no effect on NMDA-induced gene expression (Fig. 28B). This result indicates that PKD1 is not part of the signaling pathway linking NMDARs to CREB-dependent transcription.

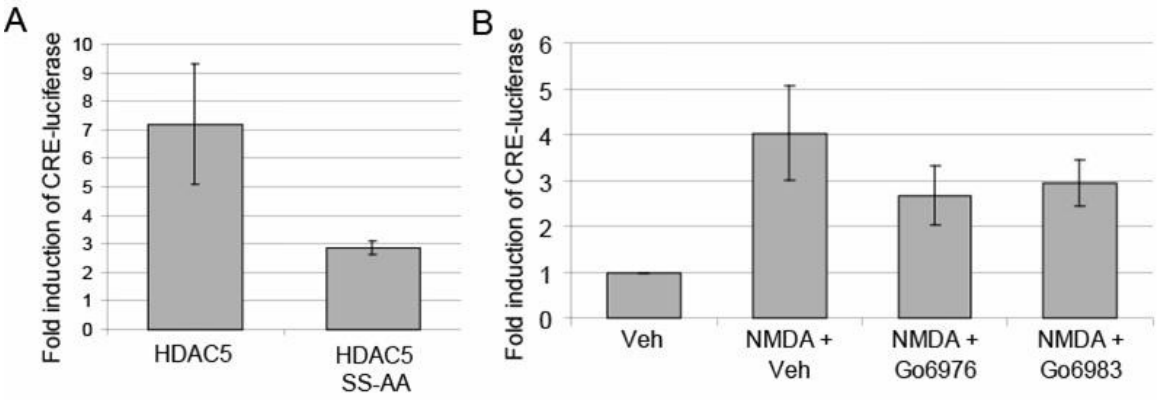


**Figure 28: PKD1 does not affect NMDA-induced, CRE-mediated gene expression.** Neurons were transfected with GFP, CRE-luciferase, and EF-renilla. They were stimulated with 30 μM NMDA (with 10 μM glycine) for 8 hours, harvested, and subjected to a dual luciferase assay. Values are expressed here at fold induction over non-stimulated control. A) Neurons were additionally transfected with either HDAC5 or with HDAC5 SS-AA. HDAC5 SS-AA shows lower gene expression, but it is not quite significant ($p=0.07$; n=6 embryos from 3 experiments). B) Neurons were stimulated with NMDA is the presence of indicated pharmacology. Go6976 blocks PKD1 and several PKC isoforms; Go6983 blocks the same PKC isoforms but not PKD1 and is used here as a control. There are no significant differences between NMDA + Vehicle, NMDA + Go6976, and NMDA + Go6983 (n=7 embryos from 4 experiments).

## Discussion

We show here that PKD1 is capable of regulating class II HDACs in neurons; in response to PMA, a dominant-negative version of PKD1 blocked HDAC5 nucleo-cytoplasmic shuttling. We also show that NMDARs regulate HDAC5 translocation by signaling through CaMK isoforms and not through PKD1. Furthermore, inhibitors of PKD1 do not block NMDA-induced, CRE-dependent gene expression. While PMA is often used to stimulate PKD1, Gαq-coupled, seven-transmembrane receptors are more physiological activators of PKD1 activity[84,91,131]. During synaptic activity, metabotropic-type glutamate receptors (mGluRs), which are Gαq-coupled, are stimulated. In Chapter 2, we showed that mGluRs activate PKD1 robustly, so it is possible that mGluRs regulate HDACs by this mechanism. We also showed in Chapter 2 that NMDARs do not activate PKD1, but instead direct its translocation to endosomal structures. In this case, it is perhaps not surprising that the HDAC regulation and CRE-dependent gene expression downstream of NMDARs is not mediated by PKD1. It will be interesting to determine if mGluRs or other neuronal Gαq-coupled receptors regulate HDAC localization or gene expression via PKD1 activation.

In our system, it appears that NMDAR-mediated regulation of HDAC5 localization is completely blocked by CaMK inhibitors, which is consistent with a previous study[39]. In the same study, it was shown that activity-dependent translocation of another class II HDAC, HDAC4, was not as sensitive to inhibitors of CaMKs[39]. It remains a possibility that HDAC4 is responsive to PKD1 following glutamatergic signaling. HDAC4 also differs from HDAC5 in other ways; for example, under non-

stimulated conditions, its localization is more cytoplasmic than nuclear[39]. This may suggest that HDAC4 and HDAC5 play distinctive roles in neurons.

Given that HDACs are not regulated by PKD1 downstream of NMDARs, it is not surprising that NMDA-induced gene expression does not depend on PKD1. Even so, CRE-mediated gene expression may not be the most sensitive system for studying the effect of HDACs on gene expression. While we found that CRE-luciferase expression was slightly decreased when stimulated in the presence of HDAC5 SS-AA, the effect was not dramatic and not statistically significant ($p=0.07$). For future experiments into the role of HDAC regulation of gene expression in neurons, it may be more informative to look at transcriptional targets and promoters known to be regulated by this mechanism, including Nur77[105,161] and MEF-2[106,160,162].

This study is the first to explore the role of PKD1 in gene expression and HDAC regulation in neurons. PMA-induced HDAC5 removal from the nucleus was fully blocked upon overexpression of a dominant-negative PKD1 construct, suggesting that class II HDAC regulation in neurons is analogous to other cell types. However, HDAC5 regulation downstream of NMDARs is not mediated by PKD1 and instead is dependent on CaMK isoforms. These data suggest that HDAC5 can be regulated by multiple signaling pathways in a single cell type. Thus, HDACs may be uniquely situated to respond to a variety of signals to regulate gene expression downstream of synaptic plasticity.

## Materials and Methods

### HDAC5 localization assay

Neurons were cultured, transfected, stained, and confocally imaged as described in Chapter 2. pcDNA3 HA-PKD, HA-PKD SS-AA and HA-PKD SS-EE constructs were created by A. Toker and have been previously described[163]. Flag-tagged HDAC5 and HDAC5 SS-AA were obtained from T. McKinsey[164]. Neurons were stimulated in HBS (described in Chapter 2) with the following doses: PMA (100 nM); NMDA (30 μM + 10 μM glycine); STO-609 (10 μM); KN-93 (5 μM); KN-92 (5 μM); KN-62 (10 μM). For immunocytochemistry, anti-HDAC5 antibody (Cell Signaling) was used at 1:500 dilution followed by a Cy3-conjugated anti-rabbit secondary antibody (Jackson Immunoresearch; 1:250). A Hoechst stain was used to label the nucleus. Quantification of HDAC localization was based on a previous study[39]. Transfected neurons were identified by GFP fluorescence, evaluated based on anti-HDAC5 fluorescence in combination with the Hoechst stain, and scored as nuclear, cytoplasmic or both. The percentage of cells in each category was calculated for each experiment. During all quantifications, the experimenter was blind to the conditions used.

### Luciferase Assay

Luciferase assays were performed using a dual luciferase kit (Promega), as described[68]. For each well of a luciferase experiment, CRE-driven firefly luciferase was normalized to constitutively-driven renilla luciferase values. This normalization accounts for small differences in transfection efficiency and cell health. Wells with abnormally low renilla

values were thought to indicate unhealthy cultures and were excluded from analyses. All stimulations were performed at 37°C in Basal Medium Eagle (BME; Gibco). Neurons were pre-incubated in BME containing 1 μM tetrodotoxin, 25 μM Nimodipine, and 10 μM NBQX for 30 minutes and stimulated with NMDA (30 μM) and glycine (10 μM) for 8 hours in the presence of antagonists.

# Chapter 4:

## The role of the AKAP yotiao in NMDA receptor signaling

**Abstract**

Calcium influx through N-methyl-D-Aspartate receptors (NMDARs) initiates many signaling pathways that effect gene expression during synaptic plasticity. The proteins that are available at the channel mouth often determine which signaling pathways are activated and to what extent. We found previously that alternative splicing of the C-terminus of NR1, the essential subunit of NMDARs, differentially induces NMDA-mediated gene expression. Here we show that yotiao, an A-Kinase Anchoring Protein (AKAP) that selectively binds to one C-terminal NR1 splice variant, may play a role in linking calcium signaling through NMDARs to CRE-mediated gene expression.

## Introduction

Signaling through NMDARs is critical for many aspects of synaptic plasticity, including long-term changes in gene expression. The proteins that are a part of the large NMDAR complex (~185 proteins) determine the signaling pathways downstream of the receptor[165]. The C-terminus of NR1, the essential subunit of NMDARs, undergoes highly regulated splicing that changes the complement of proteins that it brings to the channel mouth[17]. It contains a C0 cassette, which is present in all splice variants, a C1 cassette that can be removed by splicing, and a C2 cassette that can be alternatively spliced to create a C2' cassette. In a previous study, we found that two C-terminal splice variants of NR1 differentially effect NMDAR-dependent, CRE-mediated gene expression[68], with NR1-1a showing significantly more gene expression than NR1-2a (Fig. 29). These two splice variants were indistinguishable in their electrophysiological properties and in the amount of calcium that flowed into the cell in response to NMDA. The only difference is the presence (NR1-1a) or absence (NR1-2a) of the C1 cassette, which suggests that the C1 cassette is mediating some interaction that increases the efficacy of NMDAR signaling. There are only a few known binding partners for the C1 cassette: calmodulin[73], calcium/Calmodulin-dependent protein kinase IIα (CaMKIIα)[74], neurofilament-L[75], and yotiao[76].

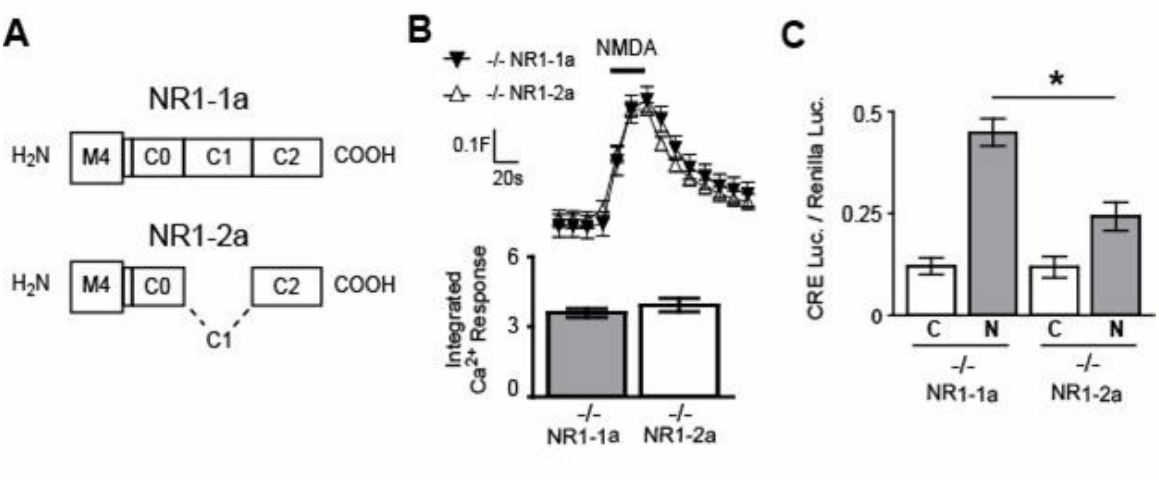


**Figure 29: Splice variants of NR1 differentially induce CRE-dependent gene expression, but show similar calcium influx.**
Data and figures are from Bradley, et. al. (2006)[68]. A) Structure of the C-terminus of NR1, showing the two splice variants NR1-1a and NR1-2a. The only difference is the presence of absence of the C1 cassette. B) These two splice variants show comparable amounts of calcium influx. Top panel shows averaged calcium traces of $NR1^{-/-}$ neurons transfected with each splice variant and stimulated with NMDA (30 μM) for 30 sec. Bottom panel shows quantification of total calcium response (total area under the curve). There is no significant difference (each condition represents the mean ± SEM for at least 14 neurons from at least four embryos). C) CRE-dependent luciferase gene expression was significantly less in $NR1^{-/-}$ neurons transfected with NR1-2a than with NR1-1a (mean ± SEM, n=4). *$P < 0.01$; unpaired t-test.

To follow up on this result, we wanted to determine what interactions are important for linking the NR1-1a splice variant to gene expression. As a scaffolding protein with many potential interaction partners, yotiao was an attractive candidate. Yotiao is a part of the superfamily of proteins known as A-Kinase Anchoring Proteins (AKAPs), which are defined by their binding to protein kinase A (PKA), but act as scaffolding proteins for a wide variety of kinases, phosphatases, and other enzymes[107,116,166]. Yotiao binds to protein phosphatase 1 (PP1) as well as PKA, bringing them into close proximity to the NMDAR channel. It has been shown to regulate NMDARs by making them more sensitive to PKA activity stimulated by cAMP

analogs[115]. In addition, yotiao has several large leucine zipper domains which bind to $IP_3$ receptors[117] and to a cardiac potassium channel, where it affects the properties of the channel[109,119,120]. These leucine zipper domains are likely to mediate other protein-protein interactions as well.

In this study, we use the same $NR1^{-/-}$ culture and NMDAR reconstitution system used previously[68]. These cortical neurons lack NR1, and show no functional NMDAR responses. By transfecting NR1 splice variants into these neurons, we reconstitute NMDARs that are similar to wild-type in their electrophysiology, calcium flux, intracellular signaling, and CRE-dependent gene expression. Using this system, we explore the role of yotiao in the regulation of calcium flow through NMDARs, and we show that yotiao may play a role in splice variant-specific gene expression.

## Results

### Yotiao increases the magnitude of calcium influx

The only known function for yotiao in NMDAR signaling was shown electrophysiologically; yotiao is known to affect the properties of NMDARs by bringing PKA and PP1 into close proximity with the channel, thereby increasing the sensitivity of the channel to 8-Br-cAMP, a PKA activator[115]. We were able to repeat this result in our lab (Fig. 30).

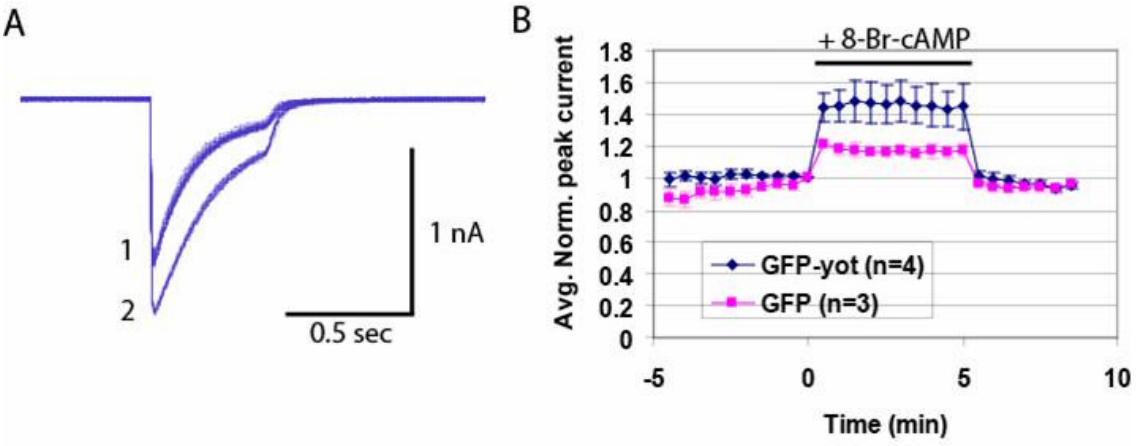


**Figure 30: Yotiao increases NMDAR sensitivity to 8-Br-cAMP as measured electrophysiologically.**
HEK293 cells were transfected with NR1-1a, NR2A, and GFP or GFP-yotiao. Transfected cells were patched and held at -70 mV in a voltage clamp configuration. Cells were given 0.5 sec pulses of 1 mM glutamate every 30 sec. After 5 min, the perfusion medium was switched to one containing 8-Br-cAMP, and after 5 min, was switched back. A) Overlaid traces from one cell. Traces at (1) are baseline traces and traces at (2) were in the presence of 8-Br-cAMP. After washout of 8-Br-cAMP, traces returned immediately to position (1). B) Average peak currents for several cells. In agreement with a previous study, the presence of GFP-yotiao increased responses upon addition of 8-Br-cAMP relative to GFP alone.

Since signaling downstream of the receptor depends on calcium influx, we asked if yotiao had a similar effect on bulk calcium flow. In order to address this question, we

used the NMDAR NR1$^{-/-}$ reconstitution system to look at the splice variant-specific effects of yotiao in neurons. We first determined if yotiao overexpression had any effect on calcium influx. We co-transfected yotiao along with NR1-1a or NR1-2a into NR1$^{-/-}$ neurons, stimulated them with NMDA for 30 seconds (30 μM with 10 μM glycine) and imaged the calcium influx using the ratiometric calcium indicator Fura-2. We found that yotiao increased calcium influx significantly for NMDARs reconstituted with NR1-1a, the variant that contains the yotiao-binding C1 cassette (Fig. 31A). This effect is specific to NR1-1a-containing receptors, since NMDARs containing NR1-2a did not show an increase. We saw no difference in basal calcium levels (not shown) or in the shape of the curve of calcium influx (Fig. 31B).

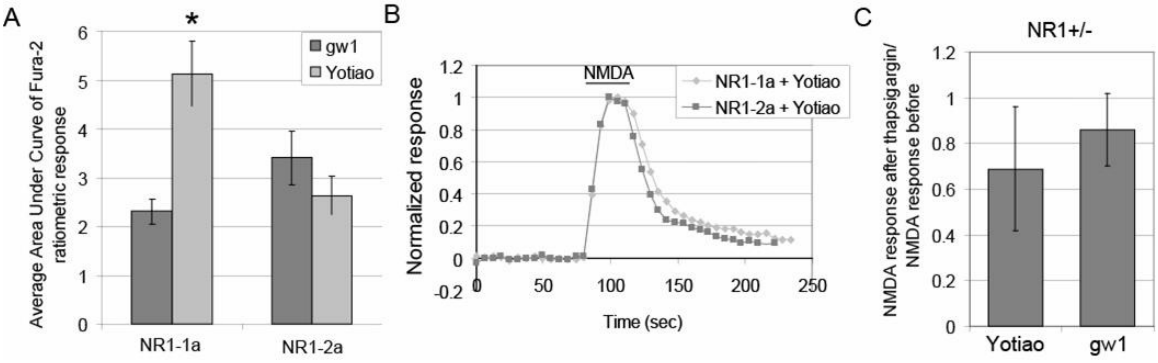


**Figure 31: Yotiao increases bulk calcium flow in a splice variant-dependent way.** NR1$^{-/-}$ neurons were transfected with NR1 splice variants plus yotiao or the empty vector gw1 and stimulated with 30 μM NMDA for 30 sec. A) The area under the curve of the Fura-2 calcium response was calculated. For neurons containing NR1-1a, but not for those containing NR1-2a, yotiao significantly increased calcium influx. * $p<0.01$, students t-test; graph shows SEM for $n>14$ neurons from at least 2 embryos. Difference between NR1-1a and NR1-2a in gw1-transfected neurons is not significant. B) Averaged Fura-2 traces normalized to the peak response show similar curves for NR1-1a and NR1-2a-containing neurons. C) NR1$^{+/-}$ neurons were transfected with yotiao or gw1 and stimulated with 30 μM NMDA for 30 sec. They were then treated with thapsigargin for 15 min and stimulated with 30 μM NMDA for 30 sec again. Note that both yotiao and gw1-containing neurons show a slight decrease after thapsigargin treatment. However, there is no significant difference between neurons transfected with yotiao and gw1 (n=4 neurons).

NMDARs are known to couple to intracellular calcium stores through both calcium-activated ryanodine receptors and through $IP_3$ receptors[167-169]. Since yotiao binds to $IP_3$ receptors[117], we next asked if a difference in the mobilization of intracellular calcium could explain the observed increase in NMDA-elicited calcium levels. We used thapsigargin (1 μM), which blocks the endoplasmic reticulum calcium pump, to deplete intracellular calcium stores, and stimulated with NMDA. While we saw a small decrease in NMDA-elicited calcium levels after thapsigargin treatment, we found that the effect of transfection of yotiao into $NR1^{+/-}$ neurons on calcium levels was no different than the empty vector gw1 (Fig. 31C). Since yotiao had no effect on calcium mobilization from intracellular stores, we concluded that the effect of yotiao on calcium levels is mediated by influx through the NMDAR itself.

The electrophysiological result above[115] (Fig. 30) shows that yotiao increases the sensitivity of NMDARs to cAMP signaling in heterologous cells. To determine if that result is reproducible in the context of bulk calcium influx in neurons, we stimulated $NR1^{-/-}$ neurons transfected with yotiao and NR1 subunits in the presence of forskolin, the adenylyl cyclase activator. As expected, forskolin treatment (100 μM) increased NMDA-elicited calcium influx (Fig. 32). However, the increase for NR1-1a was not significantly different than for NR1-2a, indicating that yotiao overexpression does not have a splice variant-specific effect.

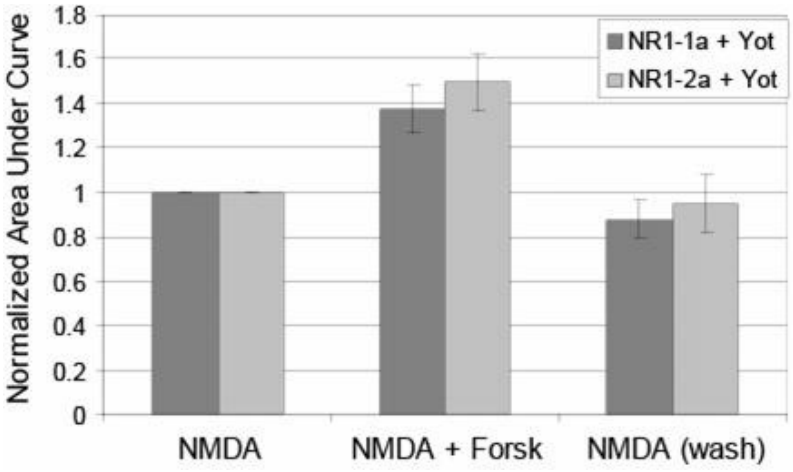


**Figure 32: Yotiao does not increase sensitivity of NMDA-induced calcium influx to forskolin.**
$NR1^{-/-}$ neurons were transfected with NR1-1a or NR1-2a and yotiao. They were stimulated with 30 μM NMDA for 30 sec, then stimulated again in the presence of forskolin. Washout of forskolin shows NMDA-induced responses are back to baseline. Both NR1-1a and NR1-2a-containing neurons show an increase in the presence of forskolin with no significant difference. Graph shows normalized average and SEM (n>12 neurons from at least 3 embryos).

**Yotiao affects level of CRE-mediated, NMDAR-induced gene expression.**

Since the C1 cassette of the NR1 subunit is responsible for increased CRE-dependent gene expression[68], and since yotiao binds to that cassette[76], we reasoned that yotiao may link NMDARs to gene expression. To test this hypothesis, we again used overexpression of yotiao by transfecting yotiao into $NR1^{-/-}$ cultures along with NR1 splice variants. We found that neurons containing NR1-1a showed a significant decrease in gene expression, while those containing NR1-2a did not (Figure 33). These data show that overexpressed yotiao interferes with NMDAR signaling only when the receptor contains the binding site for yotiao. Since neurons containing NR1-2a were not affected by yotiao overexpression, it is unlikely that nonspecific, non-NMDAR-mediated effects can account for the observed decrease in gene expression.

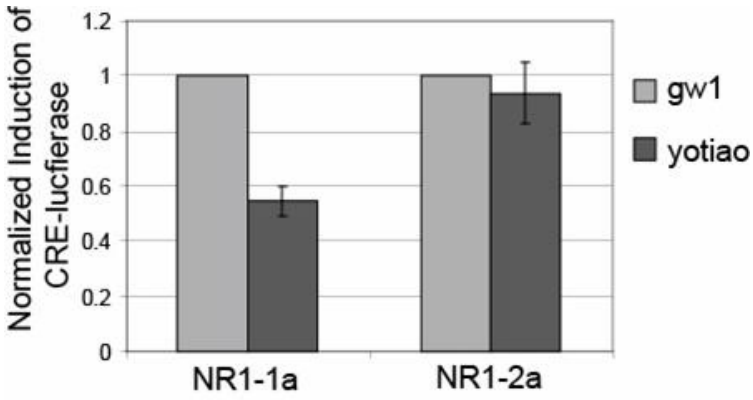


**Figure 33: Overexpression of yotiao decreases CRE-mediated, NMDAR-dependent gene expression in a splice variant-dependent way.**
$NR1^{-/-}$ cultures were transfected with NR1 splice variants, yotiao or empty vector gw1, and luciferase constructs, including CRE-luciferase and EF-renilla. Cultures were then stimulated with 30 μM NMDA for 8 hours, harvested, and subjected to a dual luciferase assay. Both firefly luciferase (driven by CRE promoter) and renilla luciferase (driven constitutively) were measured. Firefly luciferase was normalized to renilla luciferase to control for transfection efficiency and cell health. For this graph, the effect of yotiao is normalized to gw1 for each experiment (n>3 embryos). Data is expressed this way to avoid variability issues (see Discussion).

**PKA is critical for NMDAR signaling to the nucleus.**

To determine the role of PKA and PKA anchoring in CRE-mediated gene expression in our neurons, we first used the PKA inhibitor H89. We found that H89 significantly decreased NMDA-induced CRE-dependent gene expression in $NR1^{+/-}$ neurons (Fig. 34A). Gene expression downstream of potassium depolarization, which depends on calcium influx through voltage-sensitive calcium channels, was not affected by H89 at this dose, indicating a degree of specificity in the signaling pathways. To test whether anchoring of PKA was involved in the signaling pathway, we used a stearated version of Ht-31, a peptide that interferes with the interaction between AKAPs and PKA. However, we did not see any effect of this peptide on NMDA-induced CRE-dependent gene expression (Fig. 34B).

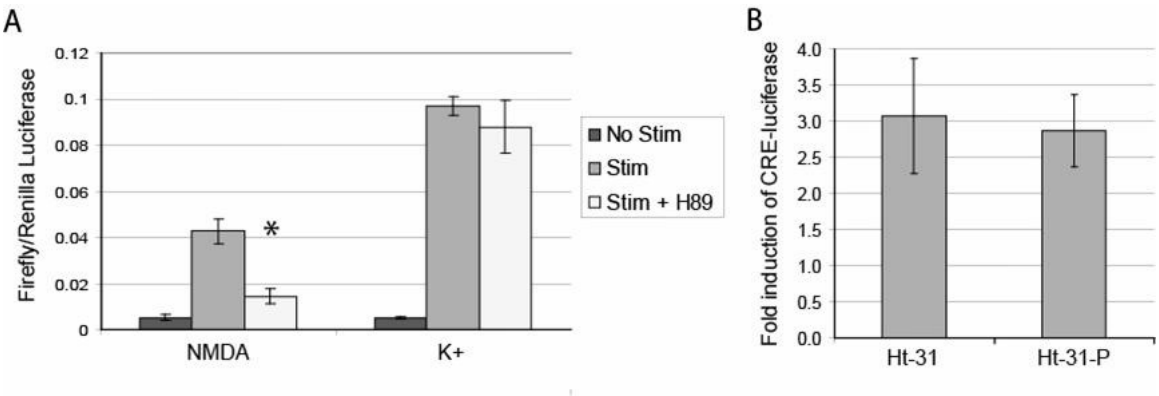


**Figure 34: A PKA inhibitor blocks NMDA-induced gene expression, but an AKAP-PKA interfering peptide does not.**
$NR1^{+/-}$ neurons were transfected with CRE-luciferase constructs and stimulated as indicated. A) Neurons were stimulated with either 30 μM NMDA or with 55 mM $K^+$ in the presence or absence of 1 μM H89. H89 blocked NMDA-induced, but not $K^+$-induced, gene expression (* $p<0.05$; n=at least 3 embryos from at least 2 experiments). B) Neurons were stimulated with NMDA plus either Ht-31 or a control peptide with a point mutation that disrupts the structure of the peptide (Ht-31-P). There is no significant difference.

## Discussion

We show here that yotiao overexpression significantly increases calcium influx through NMDARs and decreases CRE-mediated gene expression. Both effects were specific to the NR1-1a splice variant which contains the C1 cassette and binds yotiao; the NR1-2a variant that lacks the cassette showed no effect from yotiao overexpression. Since yotiao is a large (~280 KDa) scaffolding protein, it is likely that its overexpression causes a disruption in the interactions between its binding partners. In that case, our model would predict that endogenous yotiao would facilitate gene expression and its binding to the C1 cassette may explain our previously published observation that NR1-1a-containing NMDARs yield more CRE-mediated gene expression than NR1-2a[68].

### AKAPs and gene expression

The role of AKAPs in facilitating gene expression and signaling to the nucleus is not unprecedented. AKAP79/150, a brain-enriched AKAP that is known to play a role in synaptic plasticity[111,114], has been shown to facilitate cAMP and PKA signaling to the nucleus in a variety of cell lines and in cortical and cerebellar neurons[170-172]. In these studies, it was shown that this AKAP increases gene expression. Yotiao may have an analogous role in signaling downstream of NMDARs. This function could be restricted to cAMP and PKA signaling, which is downstream of NMDAR activation[27,53,54] (Fig. 34A), or may involve other potential binding partners of yotiao. Since we did not see an effect of Ht-31, the peptide that should interfere with PKA anchoring to AKAPs, other interactions may be more important. For example, one such protein may be PKD1 (see

Chapter 5, Figs. 37,38). Alternatively, while the Ht-31 peptide was stearated and so should have been able to cross the plasma membrane, it is possible that it could not reach its site of action.

**The effect of yotiao on calcium influx and comparison to previous studies**

In addition to effects on gene expression, we found that yotiao overexpression increased calcium influx through NMDARs in a splice variant-dependent way. Since we believe that yotiao overexpression interferes with its normal function, this result suggests that endogenous yotiao decreases calcium influx. Since phosphorylation by PKA increases calcium influx through NMDARs, it may seem paradoxical that an AKAP would decrease calcium flow. However, it has been shown previously that yotiao brings PP1 into proximity with NR1-1a containing NMDARs as well as PKA[115]. Since PP1 is tonically active, it may negatively regulate the channel by keeping it in a non-phosphorylated state. Upon overexpression of yotiao, this tonic dephosphorylation is lifted.

Interestingly, we found that forskolin increased calcium influx through NR1-1a-containing NMDARs and NR1-2a-containing channels to a similar extent. This would appear to contradict the electrophysiological result showing that current through NMDARs containing NR1-1a are more sensitive to cAMP analogs. However, we have previously observed a similar disconnect between channel properties and bulk calcium flow; in our previous study, we saw that an NR1 mutant that lacked much of the C-terminus showed no channel desensitization, but yielded bulk calcium levels similar to NR1-1a and NR1-2a in response to NMDA[68]. One explanation for this effect is that

calcium buffers, pumps and stores throughout the neuron is more important to the global calcium levels that we measure than the calcium influx at the channel mouth [173]. Another explanation for the discrepancy may be that the observed electrophysiological result from HEK293 cells depends on the properties of HEK293 cells and would not be true in cortical neurons. However, for technical reasons we were not able to test the effects of yotiao on channel properties in neurons.

**Alternative approaches and Caveats**

Since it is difficult to determine how overexpression of yotiao affects its function, we attempted to develop a more targeted dominant negative construct that we could use to interfere with the function of yotiao. We created Yotiao-DN, an HA-tagged construct of yotiao containing only amino acid residues 971-1241, which contains the full NR1-binding domain of yotiao, but does not contain the N-terminal leucine zipper domains or the PKA binding site (see Chapter 1, Fig. 5). We reasoned that this construct would act as a dominant negative since it will bind to the C1 cassette of NR1, but will not bring many of its binding partners with it. When transfected into $NR1^{+/-}$ neurons, Yotiao-DN decreased gene expression (Fig. 35A). However, we found that the effect of Yotiao-DN was not splice variant-specific. While $NR1^{-/-}$ neurons transfected with NR1-1a showed a decrease in CRE-mediated gene expression, we also saw a decrease in gene expression in neurons transfected with NR1-2a (Fig. 35B). The decrease is slightly more pronounced for NR1-1a-containing neurons, but not significantly so.

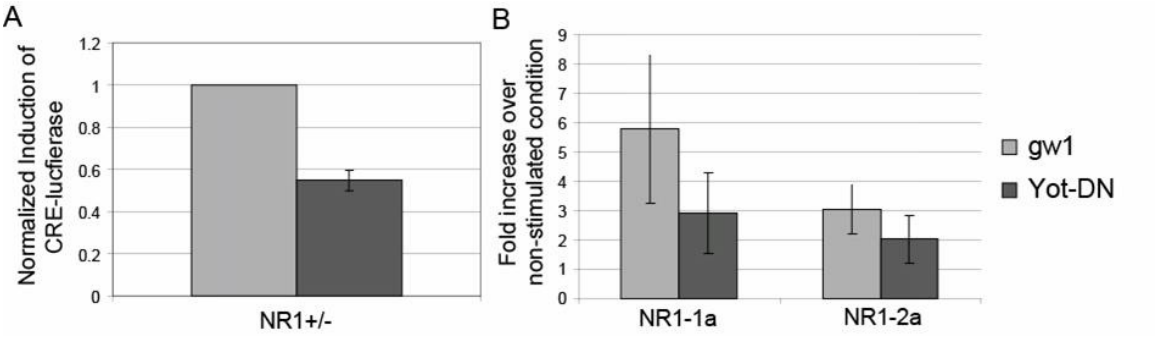


**Figure 35: The effect of a dominant-negative yotiao construct on gene expression is not splice variant-specific.**
A) Yotiao-DN or gw1 was transfected into $NR1^{+/-}$ neurons along with CRE-luciferase construct. Neurons were stimulated with NMDA as above, and luciferase values for yotiao-DN are normalized to gw1 values within each experiment. (n=4 embryos in two experiments.) B) To test whether the effect of yotiao-DN is splice variant-specific, it was transfected into $NR1^{-/-}$ neurons and stimulated. Graph shows fold increase over non-stimulated condition (n=3 embryos per condition). Note the variability (see Discussion).

The fact that this "dominant negative" construct does not have a splice variant-specific effect, while yotiao overexpression does, is puzzling. It is possible that Yotiao-DN interferes with some other function of yotiao. For example, yotiao is a splice variant of AKAP350, which is known to associate with centrosomes in the nucleus[121]. Since Yotiao-DN is much smaller than full-length yotiao, it is possible that its overexpression is more pronounced and therefore, more likely to interfere with gene expression in a non-specific way.

A confounding factor for these luciferase experiments is the amount of variability that we experience. This variability can be seen in Figure 35B, in which even the fold increase over the non-stimulated condition showed wide standard error bars. While a small amount of this variability is well-to-well within a single experiment, the biggest source of concern was experiment-to-experiment differences. One source of variability is most certainly in the variable nature of calcium phosphate transfections. By normalizing

CRE-driven firefly luciferase to constitutive EF-renilla luciferase, we can normalize for some of this inconsistency, but we have found anecdotally that poor transfections yield poor NMDAR-mediated responses even after normalization to EF-renilla. For Figure 33, in which we show a splice variant-specific effect of overexpressed yotiao on CRE-dependent gene expression, we got around this problem by normalizing the responses within experiments and expressing the effect of yotiao as a fold reduction. While we believe this effect is real, especially since we found that it is splice variant-specific within single experiments, we would be more comfortable with the data if it had shown more reproducibility in the magnitude of the response.

In this study, we have shown that yotiao may play a role in the regulation of NMDAR signaling in neurons. Its overexpression both increases calcium influx through NMDARs and decreases CRE-mediated gene expression. Since both of these effects are restricted to NR1-1a, the NR1 splice variant that contains the C1 cassette that binds to yotiao, these effects are unlikely to be mediated by a non-specific interaction. Furthermore, yotiao binding to the NR1-1a subunit may explain our previously described observation that NR1-1a-containing NMDARs yield more CRE-dependent gene expression than those containing NR1-2a.

## Materials and Methods

### Constructs, stimulations, and luciferase assay

Neurons were cultured and transfected as described previously[68] and in Chapter 2. Yotiao[76], HA-yotiao, and GFP-yotiao[115] constructs were gifts from M. Sheng. DN-yotiao was generated by restriction digest and ligation. NR1 constructs were described previously[68]. Stimulations for luciferase assays and the assay itself was described in Chapter 3.

### Calcium Imaging

Imaging was performed as described[68] using a Nikon inverted epifluorescence microscope and MetaFluor software (Universal Imaging). Neurons were loaded with 4 μM Fura-2 for 45-60 min at 37$^{o}$C and then imaged in a Warner perfusion chamber. Stimulations were done in HEPES-buffered saline (HBS: 119mM NaCl, 2.5mM KCl, 2mM $MgCl_2$, 2mM $CaCl_2$, 25mM HEPES, 30mM glucose, 1uM tetrodotoxin, 25uM Nimodipine, 10uM NBQX, pH 7.4). For each experiment, neurons were stimulated with 30 μM NMDA (plus 10 μM glycine) for 30 sec. In thapsigargin experiments, neurons were stimulated with NMDA, then incubated for 10 min in 1 μM thapsigargin and stimulated again. For forskolin experiments, an initial response to NMDA was obtained and cells were allowed to recover for 5 min, then neurons were stimulated with NMDA plus forskolin (100 μM). The neurons were again allowed to recover for 5 min, and were then stimulated once more with NMDA for "washout" responses.

**Electrophysiology**

Experiments were based on previous work[68,115]. HEK293 cells were maintained in DMEM (VWR) supplemented with 10% calf serum (Gibco), Glutamaxx (Invitrogen), and Pen/Strep (Invitrogen), and were transfected by the calcium phosphate method. GFP-yotiao or GFP alone were used in all experiments. GFP fluorescence was detected using an upright epifluorescence microscope (Zeiss). Whole cell recordings were performed at room temperature with cells voltage clamped to -60mV using Multiclamp 700A amplifier (Axon Instruments). Software was used to automatically measure and compensate for membrane capacitance. Series resistance was compensated 10-20%. Recording electrodes were filled with intracellular solution (in mM: 140 Cs Methanesulfonate, 10 HEPES, 5 Na-ATP, 10 BAPTA, 5 $MgCl_2$) and typically had a resistance of 2-4 MΩ when held in extracellular solution (in mM: 140 NaCl, 5 KCl, 1.8 $CaCl_2$, 10 HEPES, 10 glucose, 0.1 glycine). Once a gigaohm seal was obtained, the cell was lifted off of the glass coverslip and held in front of the fast-step perfusion system (Warner Instruments, SF-77B). This system allows for streams of solutions to run in parallel and changes solutions by a motorized shift of the apparatus controlled by the software. Cells were stimulated with glutamate (1 mM) for .5 seconds every 30 seconds for 5 minutes. After 5 minutes, solutions were changed to those containing 8-Br-cAMP (100 μM). After 5 minutes with 8-Br-cAMP, solutions were changed back to control solutions. Only cells showing both a stable baseline and a return to baseline after 8-Br-cAMP treatment were included in the analysis.

# Chapter 5:

# Connections and Conclusions

**Multiple locations and functions for PKD1 in neurons depending on stimulation**

Many studies of PKD1 have shown that the kinase translocates in response to stimulation and can have functions in several different subcellular compartments. However, very few studies have looked in the same cell type for multiple functions of PKD1. For example, most studies of the role of PKD1 in protein trafficking have used HeLa[133,135,138] or MDCK cells[132], while the role of PKD1 in regulation of HDACs has been studied in lymphocytes[105,106] or cardiac myocytes[104]. In our studies in neurons, we have demonstrated two separate functions for PKD1 in the same cell type, depending on the stimulation. In Chapter 2, we showed that PKD1 downstream of NMDAR and mGluR signaling regulates protein trafficking, while in Chapter 3, PKD1 was downstream of PMA stimulation in its regulation of HDAC localization.

These two functions for PKD1 in neurons depend on the localization of the kinase. In response to NMDAR signaling, Venus-PKD1 forms endosomal puncta throughout neuronal dendrites. In response to PMA stimulation, Venus-PKD1 translocates to the plasma membrane (Fig. 36). It may seem paradoxical that an effect in the nucleus is mediated by a kinase at the plasma membrane. However, while some reports have suggested nuclear localization of PKD1 following stimulation[78,128], PKD1 translocation to the nucleus itself is controversial. Since HDACs undergo constitutive cycling, it is thought that PKD1 may phosphorylate HDACs while they are in the cytoplasmic fraction, thereby preventing their return to the nucleus.

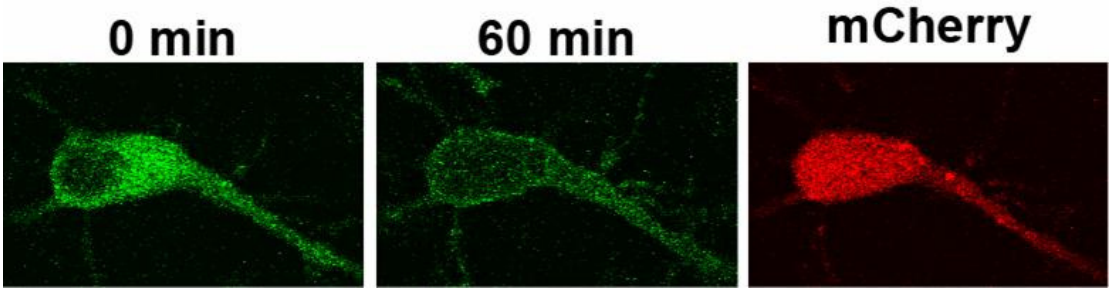


**Figure 36: PMA stimulation causes plasma membrane translocation of Venus-PKD1.**
A neuron transfected with Venus-PKD1 and mCherry was stimulated with 100 nM PMA for 60 min. Confocal images were taken every few minutes. At 60 min, plasma membrane localization of Venus-PKD1 is apparent both in the cell body and the major proximal dendrite, while mCherry remains diffuse (right panel image was taken at 60 min).

A possible role for mGluRs in regulating HDACs through PKD1 activation was mentioned in the Discussion in Chapter 3. It will be interesting to determine if stimulation of mGluRs leads to plasma membrane localization of Venus-PKD1, as this may help to understand the link between PKD1 localization and function.

**Connecting PKD1 and yotiao**

A relationship between AKAPs and PKD1 was first established by Carnegie, et. al. (2006)[110] when they reported that PKD1 binds to AKAP-Lbc. A previous study had shown an interaction of PKD1 with 14-3-3, another scaffolding protein[174]. While 14-3-3 appears to inhibit PKD1 activity[174], interaction between PKD1 and AKAP-Lbc promotes activation of the kinase[110]. We wondered if binding of PKD1 to scaffolding proteins was a more general phenomenon, and more specifically, if PKD1 bound to yotiao. To determine if there was an interaction between these two proteins, we first performed co-immunoprecipitation experiments. When we overexpressed HA-yotiao and HA-PKD1 in

HEK293 cells and incubated the lysates with anti-PKD1 antibodies, we were able to pull down yotiao (Fig. 37A). To determine which regions of yotiao were important for this interaction, we used a GST pull-down assay with GST-tagged fractions of yotiao (Fig. 37C), which we obtained from Dr. R. Kass. We found that pulling down GST-Y1, which contains the first 205 amino acids of yotiao, also pulled down PKD1 (Fig. 37B), while other fractions were unable to bind PKD1.

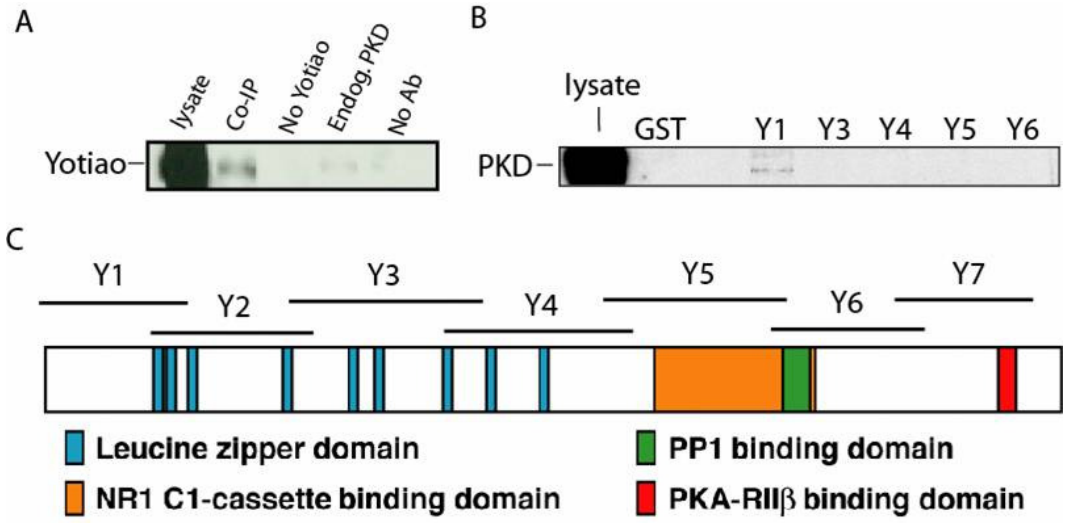


**Figure 37: Yotiao and PKD1 co-immunoprecipitate and a GST-tagged, N-terminal fraction of yotiao pulls down PKD1.**
A) HEK293 cells were transfected with both HA-yotiao and HA-PKD1. Cells were harvested in RIPA buffer, spun down, and supernatants were collected, pre-cleared with protein G beads, and incubated with anti-PKD1 antibodies at 4$^{o}$C overnight. Antibodies were then pulled down with protein G beads. Samples were then separated by SDS-PAGE, transferred to a nitrocellulose membrane and subjected to Western blot using anti-HA antibodies. "Co-IP" lane shows that yotiao was co-immunoprecipitated. In "No yotiao" condition, cells were not transfected with HA-yotiao (and so show no yotiao immunofluorescence). In "Endog. PKD" lane, cells were not transfected with HA-PKD1; yotiao immunofluorescence remains due to the endogenous PKD1 in these cells. B) GST and GST-tagged fractions of yotiao in pGEX4T1 vectors were expressed in BL-21 bacteria and purified. They were then incubated with lysates from HEK293 cells transfected with HA-PKD1. Proteins bound to GST were then pulled down using GST purification columns, run on SDS-PAGE gels and subjected to anti-HA Western blots. Y1 shows some immunofluorescence while GST alone and other fractions of yotiao do not. C) A map of GST constructs and what regions of yotiao correspond to each.

We next asked if yotiao may be a substrate of PKD1, and if so, what fraction of yotiao was phosphorylated by PKD1. Full-length yotiao that was overexpressed and immunoprecipitated from HEK293 cells was phosphorylated by purified PKD1 (Fig. 38A). Using the GST-tagged fractions of yotiao, we found that GST-Y1 was phosphorylated by PKD1, while the remaining six fractions were not (Fig. 38B). These data suggest that there is a real interaction between PKD1 and yotiao and that yotiao is a kinase substrate of PKD1.

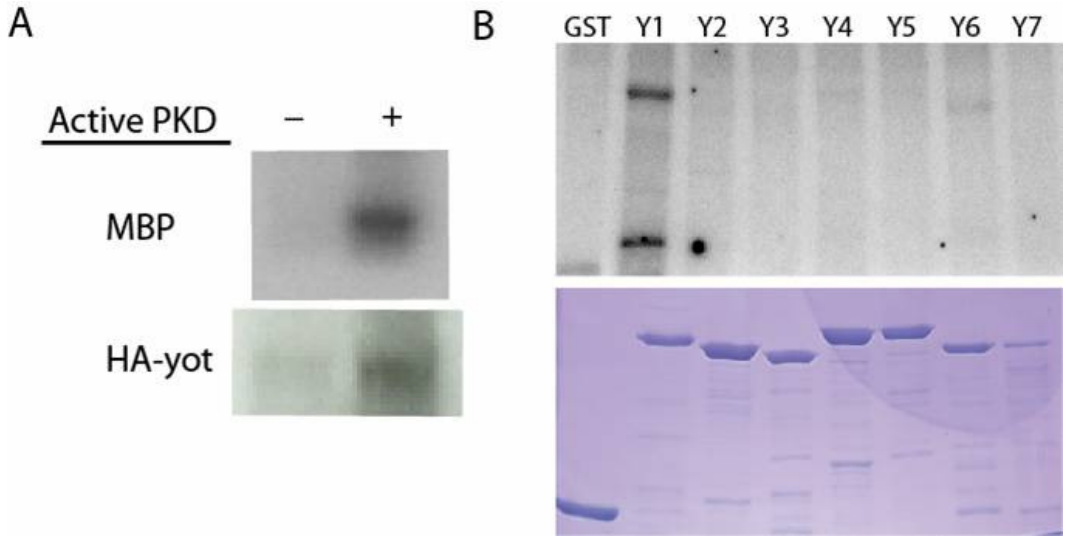


**Figure 38: PKD1 phosphorylates yotiao on an N-terminal fraction.**
Purified, recombinant active PKD1 was used to test if yotiao was a substrate of PKD1 kinase activity. The autoradiographical kinase assay was based on a previous study[175]. Briefly, active PKD1 (Upstate) was added to lysates or purified protein mixes including γ-$^{32}$P-labelled ATP (Amersham) and incubated at 30$^{o}$C for 30 min. The reaction was stopped by addition of 2X LSB, boiled for 5 min, and run on an SDS-PAGE gel. Gels were then dried and exposed. A) HEK293 cells transfected with HA-yotiao were harvested and immunoprecipitated with anti-yotiao antibodies. Samples were then subjected to the kinase assay. Purified myelin basic protein (MBP), a known substrate of PKD1, was used as a positive control. B) GST and GST-tagged fractions of yotiao were expressed and purified as above, and tested for phosphorylation by purified active PKD1. Top panel shows the result with only Y1 showing incorporation of $^{32}$P-labelled ATP. Bottom panel is a Coomassie-stained gel run in parallel showing that each GST-tagged fraction of yotiao was purified to a similar extent.

This interaction may have implications for PKD1-mediated signaling downstream of NMDARs. By being anchored close to the NMDAR channel mouth, PKD1 may be able to respond more quickly and efficiently to calcium influx. In Chapter 2, we showed that PKD1 responds to NMDAR signaling by translocating to endosomes containing AMPAR subunit GluR2. Since yotiao binds only to NR1 splice variants containing the C1 cassette, we would predict that PKD1 would be anchored only near NMDARs containing those splice variants. Using the NMDAR reconstitution system described in Chapter 4, we tested if Venus-PKD1 responds to NMDARs containing NR1-1a better than those containing NR1-2a. We found that PKD1 responds to NMDARs containing either subunit equally well. However, it is possible that there are subtle differences that we are unable to detect using our live-cell imaging puncta analysis, or that translocation to endosomes does not represent the full response of PKD1 to NMDAR signaling.

There are several interesting possibilities to be explored about the interaction between yotiao and PKD1. First, we found that yotiao is a substrate for PKD1 phosphorylation. It has been shown previously that AKAPs use phosphorylation events to regulate binding of proteins to the AKAP[110,176]. While in each previous case, the AKAP was phosphorylated by PKA, it is possible that the PKD1 phosphorylation site on yotiao plays a similar role. Second, in its interactions with other scaffolding proteins, the kinase activity of PKD1 has been affected, with 14-3-3 blocking it[174] and AKAP-Lbc promoting it[110]. By binding PKD1, yotiao may bring the kinase into synaptic sites and closer to mGluRs. Since mGluRs activate PKD1, this proximity may promote activation. And third, it will be interesting to determine if binding of PKD1 to AKAPs is a common theme. In particular, AKAP79/150 would be an intriguing AKAP to test for association

with PKD1. As discussed in Chapters 1 and 4, AKAP79/150 is found at synaptic sites near AMPARs and promotes PKA regulation of AMPAR localization[56,111,113,114]. Since we now know that PKD1 plays a role in AMPAR trafficking (Chapter 2), a link between PKD1 and AKAP79/150 may provide a novel signaling module during synaptic plasticity.

**Summary of Findings**

Synaptic plasticity is a complex process involving many different signaling molecules and pathways. While a few important players have been discovered, many more will probably be revealed in the coming years. With this dissertation, we add PKD1 to the list of molecules that play some role in long-term adaptive change. In Chapter 2, we showed that PKD1 is downstream of both NMDAR and mGluR signaling, with NMDARs regulating its localization and mGluRs regulating its activation state. Furthermore, the activity of PKD1 was shown to be important for proper localization of AMPARs. In Chapter 3, we determined a role for PKD1 in the regulation of neuronal gene expression. We found that the activation of PKD1 could robustly regulate the localization and therefore the function of HDAC5, a transcriptional repressor. While PKD1 did not function in this way downstream of NMDARs, other stimuli that activate PKD1, such as metabotropic signaling, may regulate HDACs through PKD1.

The AKAP yotiao is another protein that may be involved in synaptic plasticity. In Chapter 4, we explored its role in NR1 splice variant-dependent signaling to CRE-mediated gene expression. We found that overexpression of yotiao, which we believe interferes with its function, decreased gene expression in a splice variant-dependent way.

While we found that PKA was important for NMDAR signaling, we were unable to determine the signaling pathways or interactions that were most important for the effect of yotiao on gene expression. Our work on the roles of PKD1 and yotiao in synaptic plasticity brings us one step closer to understanding the mechanisms underlying learning and memory.

# Chapter 6:

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