In vivo disruption of PKA activity through targeted mutations of the RIα regulatory subunit

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Abstract

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The cAMP-dependent protein kinase (PKA) is expressed in all animal cells and plays a key regulatory role in many diverse physiological processes. The PKA holoenzyme is a heterotetramer that exists as a dimer of regulatory (R) subunits that serves to sequester two catalytic (C) subunits in the absence of cAMP. The R-subunit isoforms have the same general domain organization but are not functionally redundant and differ in their biochemical characteristics, expression levels and subcellular localization.

The isolation of mutant R-subunits with useful experimental properties has allowed us to interrogate PKA-dependent signaling *in vivo* in a manner that cannot be accomplished using traditional knockout strategies. Genetic disruption of any of the R-subunits results in a compensatory increase in the RIα subunit, which occurs through reduced turnover of RIα protein when bound to C-subunit in the holoenzyme. This switch to the RIα holoenzyme can confound interpretation of experimental results as basal PKA activity levels are significantly elevated due to increased cAMP sensitivity of the RI-containing holoenzyme. Furthermore, any requirements for subcellular targeting of PKA must be closely scrutinized in light of the ability of a number of A-Kinase Anchoring Proteins (AKAPs) to bind RI, although for most AKAPs this occurs with greatly reduced affinity when compared to RII subunits.

The specific goals of this thesis were to utilize two genetically modified mouse lines harboring mutations in distinct domains of the RIα subunit to: a) assess the specific role of PKA

in fluid homeostasis by disrupting kinase activity through expression of a dominant-negative mutation (RIαB) and, b) examine the role of compartmentalized RIα signaling *in vivo* through the introduction of alanine substitutions (RIα ALA) in the dimerization/docking (D/D) domain, an N-terminal hydrophobic sequence that is required for the vast majority of PKA-AKAP interactions and subcellular targeting of the holoenzyme.

A point mutation (G324D) in the C-terminal cAMP-binding site renders RIαB (B site mutant) insensitive to physiological levels of cAMP while Cre-LoxP technology allowed us to target RIαB to distinct cell populations. Although PKA-dependent phosphorylation is required for apical trafficking and exocytosis of the renal water channel, AQP2, the precise role of PKA in short- and long-term regulation of AQP2 remains unclear. I found that RIαB expression in renal principal cells is sufficient to cause nephrogenic diabetes insipidus while dramatically decreasing total AQP2 protein levels. The effect of RIαB was independent of changes in AQP2 mRNA and persisted with both acute (DDAVP treatment) and chronic (24-hour dehydration) activation of the V2R, suggesting a previously undetermined role for PKA in regulating protein stability and whole cell abundance of AQP2.

To assess the role of RIα-AKAP interactions *in vivo*, we introduced alanine mutations (V22A, I27A) into the D/D that have been shown to disrupt binding to dual-specific AKAPs *in vitro*. RIα ALA abolished binding to the RI-selective AKAP, SKIP; surprisingly, these mutations also resulted in dramatic increases in RIα protein in conjunction with apparent decreases in RIα mRNA. Wild-type RIα protein stability was increased by proteasome inhibition whereas the elevated RIα ALA was unaffected, suggesting that AKAP interactions may be a required part of the proteasome degradation pathway for RIα. RIα ALA expression in MEFs also attenuated CREB phosphorylation and induction of the early response gene, c-Fos in response to elevated cAMP. Mice expressing the RIα ALA mutation had reduced locomotor activity and c-Fos induction was reduced in the striatum in response to the dopamine type 2 receptor antagonist, haloperidol. This work has identified roles for PKA in regulating fluid homeostasis as well as a possible role for the D/D domain in the stability of RIα and its subsequent impact on cAMP-dependent transcriptional regulation.

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GLOSSARY

AC: Adenylyl cyclase

ACTH: Adrenocorticotropic hormone

AKAP: A-kinase Anchoring Protein

AQP2: Aquaporin-2

AVP: Arginine vasopressin

BAPTA: 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid

CYCLIC-AMP (cAMP): 3′-5′ cyclic adenosine monophosphate

COOH: Carboxy terminus

CRE: cAMP-response element

CREB: cAMP-response element binding protein

CSK: C-terminal Src kinase

D/D DOMAIN: Docking/Dimerization domain

DDAVP: 1-desamino-8-D-arginine vasopressin

D2R: Dopamine 2 Receptor

FRET: Fluorescence resonance energy transfer

GPCR: G protein-coupled receptor

IEG: Immediate early gene

LTF: Long-term facilitation

MAP2: Microtubule-associated protein 2

MEF: Mouse Embryonic Fibroblasts

MLCK: Myosin Light Chain Kinase

MSN: Medium spiny neurons

NFAT: Nuclear factor of activated T cells

ORE: Osmotic response element

PDE: Phosphodiesterase

PEPCK: Phosphoenolpyruvate carboxykinase

PKI: Protein Kinase Inhibitor

PTPD1: Protein tyrosine phosphatase D1

PVN: Paraventricular nucleus

RGS: Regulatory of G-protein signaling domain

SKIP: Sphingosine Kinase-Interacting Protein

SON: Supraoptic nucleus

TONEBP: Tonicity Element Binding Protein

V2R: Vasopressin receptor 2 (*Avpr2*)

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DEDICATION

to my wife, Michelle, for all her love and support

Chapter 1

INTRODUCTION

1.1 Historical perspective

Signal transduction in eukaryotic cells is highly specific and exquisitely tuned to allow for rapid responses to changes in the extracellular environment. Signaling molecules, including hormones, growth factors, and neurotransmitters, act through cell surface receptors expressed at the plasma membrane, and are used to convey information to the cell that results in changes in many diverse processes including gene transcription, enzymatic activity, and metabolism. These changes in cellular activity are often affected in response to the increased generation of so-called second messengers, of which cAMP is the prototypical molecule[157]. Studies on the actions of glucagon and epinephrine and the generation of cAMP[12, 129, 156], combined with the later identification of an intracellular receptor for cAMP, discussed below, laid the foundation for advancement in numerous scientific fields including biochemistry, physiology, and neuroscience for decades to come.

In 1968, the Krebs lab announced the purification of an enzyme from rabbit skeletal muscle that was dependent on adenosine 3′- 5′-monophosphate (cAMP) for activation[169]. The cAMP-dependent Protein Kinase identified in this report catalyzed the transfer of a phosphate moiety to phosphorylase kinase, as well as casein and protamine, and its enzymatic activity was detected in a variety of mammalian tissues and invertebrate phyla[92]. Later renamed as Protein Kinase A (PKA), separate regulatory and catalytic components of the enzyme were identified, and this holoenzyme was further separated into two distinct pools (Peak I and Peak II) of kinase activity that differentially eluted by ion exchange chromatography[130, 131].

Corbin and colleagues observed that the Type I (Peak I) kinase was largely cytoplasmic while the Type II (Peak II) kinase was maintained in the particulate fraction[37]. Continued work in isolated perfused rat hearts clearly demonstrated differential effects of epinephrine

and prostaglandin E1 on the development of ventricular force and the cAMP-dependent phosphorylation of cardiac substrates[20, 61, 86, 87]. Both hormones stimulated PKA activity, cAMP production, and modulated the activation states of phosphorylase kinase and glycogen synthase. However, only epinephrine increased the development of force within the ventricle (dP/dt) as well as the phosphate content of troponin I, both measures of the positive inotropy and lusitropy, respectively, associated with the cardiac response to catecholamines. These seminal findings established the concept of compartmentalized cAMP generation and targeted, rather than global, PKA activation in response to distinct physiological stimuli or pharmacological cues.

Parallel work in two primary research areas built upon the concept that the Type I and Type II kinases are not functionally redundant. First, the development of molecular cloning techniques allowed for the identification of four regulatory (R) subunits (RIα, RIIα, RIβ, RIIβ)[36, 80, 96, 143] and two catalytic (C) subunits (Cα, Cβ)[44, 55, 164, 165] that are differentially expressed across cell types and tissues. Mutational analysis and overexpression studies using the newly identified R and C subunits greatly expanded our knowledge of R:C interactions, holoenzyme formation, and the *in vivo* role of PKA[107]. The arrival of gene-disruption technology in mice prompted further analysis of the physiological role of each of the PKA isoforms as individual R[4, 17, 22, 39] and C[71, 77, 128, 149] subunits were completely removed from the total complement of PKA holoenzyme. The unique phenotypes observed in each these knockout mice, ranging from more subtle (RIIα KO) to embryonic lethal (RIα KO), further reinforced the idea of distinct but overlapping roles for the Type I and Type II isoforms.

The identification and expansion of a family of scaffolding proteins that target PKA to distinct subcellular locations added additional complexity to the cAMP-PKA signaling axis. The PKA RII subunit is tethered to bovine brain microtubules through its interaction with neuronal anchoring proteins, including MAP2[139, 162], thus providing a molecular basis for the separation of RI and RII into cytosolic and particulate fractions, respectively. What followed was the explosion of a new field as more than 50 distinct A-Kinase Anchoring Proteins (AKAPs) have been identified to date. AKAP-mediated targeting of PKA in close proximity to it substrate proteins has been implicated in many diverse physiological

processes[176]. In addition to anchoring PKA, these scaffolding proteins also form multivalent complexes that can shape signaling dynamics for multiple second messengers including cAMP, Ca^{2+}, and phospholipids[91]. The central regulatory role of AKAP complexes has expanded to include components of both second messenger signal initiation and termination through their interaction with GPCRs, adenylyl cyclases, additional kinases, phosphatases, and phosphodiesterases[144].

The RI and RII subunits of PKA are not functionally redundant and genetic disruption of any R subunit (RIβ, RIIβ, RIIα) leads to substantial compensatory increases in the ubiquitously expressed RIα subunit. Using traditional knockout approaches to study PKA function in mice thus inherently alters cellular cAMP signaling dynamics. Furthermore, specific roles for the RII subunits have been well characterized due to their subcellular targeting by AKAPs; the functional role(s) of RIα are decidedly less clear. Using mice that express mutated RIα proteins as described here allows us to disrupt PKA activity while removing the confounding factor of R subunit compensation and may ultimately inform us as to previously undefined roles for RIα. The ubiquitous expression of RIα also allows us to analyze the effects of these mutations in any tissue or cell type, providing us with unique tools to experimentally assess the role of PKA *in vivo*.

1.2 Physiological roles for PKA subunits

The PKA holoenzyme is composed of two catalytic (C) subunits maintained in an inactive conformation by their association with a regulatory (R) subunit dimer. Each R subunit binds two molecules of cAMP causing the PKA holoenzyme to dissociate and release active C subunits that phosphorylate a diverse group of proteins that includes transcription factors, metabolic enzymes, ion channels, receptors, cytoskeletal proteins, cell cycle, and chromosome-associated proteins. The R subunits are acidic, modular proteins that range in size from 380-400 residues and the RI- and RII-containing holoenzymes likely maintain highly diverse functional roles across tissues and among different species. The C subunits are basic, bilobular proteins of approximately 350 residues that phosphorylate serine (S) or threonine (T) residues, usually within the identified consensus sequence, -R-R-X-S/T[88].

The PKA R subunits derive from four separate gene products and display differential

expression levels across tissues. RIα and RIIα are ubiquitously expressed while the RIβ and RIIβ subunits show a more limited expression pattern as both are expressed in brain and RIIβ is highly expressed in adipose tissue. The RI and RII subunits have the same general domain organization, however, while maintaining distinct structural and biochemical differences. RI and RII each have an N-terminal docking/dimerization (D/D) domain that is connected to two highly homologous cAMP binding sites by a flexible linker region. The D/D domains are required for formation of the RI and RII dimers and a hydrophobic surface formed by the dimer interface allows R subunits to bind to the amphipathic helix found in virtually all AKAPs[27]. RII subunits are largely targeted to intracellular organelles and membranes through their interaction with AKAPs. The D/D of RI also contains two cysteine residues that form intermolecular, antiparallel disulfide bonds; these bonds, however, are not required for dimerization[9, 97].

RI and RII isoforms show the highest variability in the flexible linker region, which contains a PKA consensus motif and serves as the docking and inhibitor site for the C subunit. RI contains a pseudosubstrate site (-R-R-G-A-) where the S/T is replaced by a glycine (G) or alanine (A), which requires ATP and magnesium to form a tight complex with the C subunit[65, 182]. RII functions as both an inhibitor and substrate as its consensus sequence is an autophosphorylation site (-R-R-A-S-). Additionally, the two isoforms differ in their sensitivity to cAMP, as the RI holoenzyme requires roughly four-fold less cAMP for half-maximal activation[24].

The distinct functional roles of RI and RII are likely reflected by *in vivo* formation of PKA holoenzyme. Although RI and RII have similar binding affinities for the C subunit *in vitro*[69], the Type II holoenzyme has been shown to form preferentially *in vivo*[124]. In NIH3T3 cells, overexpression of RII results in complete elimination of the RIα subunit; overexpression of Cα or Cβ does not affect RII subunits, but does drives increased expression of RIα[124, 166]. Total RIα mRNA levels do not change under these conditions and the half-life of RIα protein fluctuates based on the relative amount that is bound with C subunit in the holoenzyme. RIα half-life increases 4-5 fold when bound in the holoenzyme; in S49 lymphoma cells that lack C subunit (*kin*-), the RIα half-life is decreased 10-fold[153]. Although the precise mechanism is not clear, preferential formation of RII holoenzyme could

ensure that PKA is targeted to distinct subcellular locations through AKAP binding; RIα then acts to sequester any additional C subunit in order to maintain cAMP-dependent control over kinase activity.

Genetic disruption of PKA R subunits in mice generates distinct phenotypes through altered functionality of the Type I or Type II kinase in specific tissues. The severity of the phenotype in each R subunit knockout mouse directly correlates with both the holoenzyme complement and the ability of additional R subunits to compensate in a particular cell or tissue type. As such, more subtle phenotypes were observed in mice lacking RIβ[17] or RIIα[22] subunits. RIβ is only expressed in brain and knockout mice display defects in hippocampal LTP; RIα levels are increased through post-translational stabilization as discussed previously, however, neither RIα nor RIIβ are able to fully compensate in order to restore this form of synaptic plasticity. Although RIIα is ubiquitously expressed, RIα compensates in peripheral tissues while RIα, RIβ, and RIIβ subunits compensate for any loss of RIIα holoenzyme in the central nervous system. RIIβ knockout mice are lean, hyperactive, and resistant to diet-induced obesity, owing to the predominant role that the RIIβ holoenzyme plays in adipose tissue and the central nervous system[39, 117, 121, 181].

RIα plays an essential role in buffering C subunit activity, supported by its compensatory increase when any other R subunit is disrupted *in vivo*. In contrast, no other R subunit can completely fill the role of RIα as genetic disruption in mice results in early embryonic lethality[4]. Loss of RIα results in increased basal and reduced cAMP-stimulated kinase activity, the former due to the loss of RIα-mediated buffering of C subunit and increased cAMP sensitivity, while the latter results from decreased total C subunit due to increased turnover while no longer protected in the RIα holoenzyme. The unregulated PKA activity in the RIα knockout mouse disrupts development of mesoderm-derived structures including the heart and RIα KO embryos are resorbed by E11.5. Consistent with the role of RIα in buffering PKA activity, embryonic development is continued past E11.5 when RIα KO mice are interbred with mice lacking the Cα catalytic subunits.

1.3 Alterations in RIα expression in human disease

Consistent with mouse genetics studies in our lab and elsewhere, RIα acts primarily as a cytosolic buffer for PKA activity, and pathophysiology correlates with reduced levels of RIα and attenuated kinase activity as observed in systemic lupus erythematosus (SLE), Carney complex, and acrodysostosis. Kammer and colleagues analyzed T lymphocytes from SLE patients and identified deficient cAMP-dependent phosphorylation of membrane substrates[58], and later attributed this to a selective loss of the RIα subunit[84]. Subsequent work, however, also identified decreased RII subunits in conjunction with altered subcellular localization, raising the distinct possibility that a global, rather than RI-specific, disruption in PKA activity may drive altered cAMP signaling in T lymphocytes from SLE patients[112].

Carney complex (CNC) is an autosomal-dominant multiple endocrine neoplasia in which more than 120 mutations have been identified among all coding exons (exons 2-11) in the human gene encoding RIα (PRKAR1A). PRKAR1A mutations are present in up to 70% of CNC patients, and new subpopulations of patients have been identified in which the disease is sporadic, likely due to *de novo* mutations[11]. CNC results in cardiac and other myxomas, spotty skin pigmentation, and endocrine hyperactivity[26]. The adrenal hyperplasia commonly identified in CNC leads to primary pigmented nodular adrenocortical disease (PPNAD) and an ACTH-independent form of Cushing syndrome. CNC patients display PRKAR1A haploinsufficiency and decreased RIα protein; virtually all mutations generate premature stop codons and the resulting truncated mRNAs are degraded through nonsense-mediated decay[90]. This haploinsufficiency gives rise to fertility deficits in male CNC patients as unregulated kinase activity produces abnormal sperm morphology with likely functional deficits[23].

Recently, small cohorts of patients that have been diagnosed with acrodysostosis express mutated RIα proteins that alter PKA signaling dynamics[100, 116]. Acrodysostosis is a rare form of skeletal dysplasia that results in short stature, facial dysmorphisms, severe brachydactyly, and varying levels of mental retardation[53, 174]. Of particular interest with regard to this thesis are the functional deficits observed in these mutant RIα subunits. Each expressed mutation mapped to the cAMP binding sites, and both the A-site (T239A)

and B-site (R368X, truncation) mutants were able to suppress total PKA activity when expressed in cell culture. Neither mutation is as robust as the RIαB mutant described in Chapter 2, but the 10-fold shift in the apparent K_a for cAMP was sufficient to disrupt PKA-dependent CREB phosphorylation (serine 133) as well as CREB-dependent luciferase reporter activity[53, 174].

1.4 Identification of PKA RIα mutants with experimentally useful properties

Traditional mouse genetic knockout approaches have greatly expanded our knowledge of PKA signaling. Early mutational analysis of the RIα subunit has provided us with potential tools to study PKA isoform specific roles and compartmentalization of cAMP signaling. Shortly after the discovery of PKA as an intracellular receptor for cAMP, Tomkins and colleagues screened S49 mouse lymphoma cell populations for clones that were resistant to the cytotoxic effects of dibutyrl-cAMP[40]. S49 lymphoma cells express predominantly, if not exclusively, Type I kinase, and each of the *kin* mutant cell lines identified was deficient in cAMP binding, kinase activity, and growth inhibition when cultured with cAMP[78]. These mutations were subsequently mapped to the cAMP binding sites of RI; the altered subunits acted in a dominant-negative fashion and were able to suppress PKA activity even in the presence of wild type subunits[115, 154].

Our lab cloned two of these dominant-negative mutations and identified single amino acid changes in the N-terminal (A site) and C-terminal (B site) cAMP binding sites[35]. Mutations in site A (G200E) or site B (G324D) result in a dominant negative R-subunit that displays markedly reduced binding affinity for cAMP and remains tightly bound to the C subunit, even in the presence of physiological levels of cAMP. When expressed in Y1 adrenocortical cells, the dominant-negative mutants effectively suppress steroid production[35]. We then generated a mouse line that possessed one wild type allele of *Prkar1a* and one that was altered to incorporate the B site mutation (RIαB). The mutant allele is also expressed in a tissue-specific manner as the full length RIαB protein is only expressed after Cre-mediated recombination. We previously used the RIαB mouse to investigate the role of PKA in intestinal motility and glucose handling *in vivo* by targeting RIαB to the enteric nervous system (*PLP*-Cre, *Hox11L1*-Cre) and liver hepatocytes (*Albumin*-Cre), respectively[72, 173]. As

suitable genetic models are lacking in the study PKA-mediated regulation of AVP signaling, I used the RIαB mutant to investigate the role of PKA in fluid homeostasis (Chapter 2).

In order to assess the role of more discrete PKA signaling events *in vivo*, we generated a mouse with mutations in the D/D domain of RIα that have been demonstrated to disrupt binding to AKAPs *in vitro*. These alanine mutations (V22A, I27A) replace conserved hydrophobic residues found in both mammalian RIα subunits and the regulatory subunit (R_{CE}) of the nematode, *Caenorhabditis elegans*[5, 9]. As discussed in more detail later, there is a growing number of AKAPs that have the ability to anchor RIα at distinct subcellular locations. However, most RI-binding AKAPs are dual-specific and have a much higher affinity for RII versus RI. As such, the precise physiological role(s) that require anchored RIα remains unclear. I used the RIα ALA mouse to investigate physiologically relevant RIα-AKAP interactions *in vivo* (Chapter 3).

1.5 Role of Cyclic AMP-PKA signaling axis in fluid homeostasis

The antidiuretic hormone, arginine vasopressin (AVP), has been well studied, both in terms of its central production in the hypothalamus and its peripheral actions on the kidney in regulating fluid homeostasis[16]. AVP mRNA is produced and translated in discrete nuclei of the hypothalamus, primarily the PVN and SON, where the nonapeptide is then packaged along with a co-translated neurophysin into large, dense core vesicles. The vesicles are trafficked down axonal projections that terminate in the posterior pituitary and stored there until AVP is released into circulation in response to osmotic stimulation[163]. In the kidney, AVP activates renal vasopressin receptors (V2R) to increase water reabsorption in the distal nephron through increased apical localization of the water channel, AQP2. Mutations in the AVP, AVPR2, or AQP2 genes give rise to diabetes insipidus, a condition marked by excessive thirst, an inability to concentrate urine and conserve water, and the potential for stunted development or even mental retardation in children due to repeated bouts of severe dehydration[7].

AVP release into the circulation results from an increased firing rate of neurons within the PVN and SON, and transcriptional regulation of the AVP gene has been extensively linked to the generation of cAMP. An early hypothesis held that magnocellular neurosecretory

cells (MNCs) within the PVN and SON were intrinsically osmosensitive. The PVN/SON are located in the anterior hypothalamus where the blood-brain barrier is less stringent, and could be well placed to quickly respond to changes in serum volume and osmolality through increased neuronal excitability and firing rate. Indeed, studies using isolated hypothalamic slices clearly demonstrated that changes in bath solution osmolality modulated the excitability of MNCs within the PVN/SON[105]. In correlation with the increased activity in MNCs, rats that were subjected to dehydration displayed a marked increase in AVP mRNA levels as assessed by *in situ* hybridization[6, 183]. Furthermore, changes in osmolality also increased cAMP levels in the SON, and to a lesser extent in the PVN, in electrophysiological experiments utilizing cultured brain slices[28].

Using improved molecular genetics approaches, it was further demonstrated that cAMP regulates AVP mRNA levels through increased PKA activity and CREB-dependent transcriptional activation. cAMP response elements (CRE) are present in the $5'$ promoter regions of the *Avp* gene, and pharmacological inhibition of PKA activity or transient expression of a dominant-negative CREB construct (KCREB) attenuated cAMP-dependent promoter activity of the *Avp* gene[49, 79]. Subsequent *in vivo* studies supported a role for PKA- and CREB-dependent transcriptional regulation in AVP-expressing neurons of the hypothalamus. Viral expression of the endogenous PKA inhibitor, PKI, specifically in the PVN virtually abrogates the induction of AVP mRNA when rats are dehydrated for 72 hours [175]. Similarly, Cre-recombinase mediated disruption of CREB1 specifically in the PVN resulted in decreased AVP protein levels[32]. Although these data extend the results of the earlier studies in cell lines, it is important to note that neither viral expression of PKI nor CREB1 deletion were sufficient to induce primary polydipsia or impaired urinary concentration in rats or mice.

Once released into circulation from the posterior pituitary, AVP binds to the renal vasopressin receptor (V2R) and stimulates antidiuresis through regulation of AQP2 trafficking and expression. The V2R is expressed in the basolateral membrane of the renal principal cell while AQP2 localizes to the sub-apical membrane space where the channels reside in intracellular recycling endosomes[119, 122]. V2R activation increases cAMP generation and activates PKA which in turn phosphorylates AQP2 at a critical serine residue (S256) on

its COOH-terminal tail[51]. Apical insertion of AQP2 increases transcellular water flux through the principal cell as water flows along the medullary osmotic gradient and exits through AQP3 and AQP4 channels that are constitutively expressed in the basolateral membrane[46, 160].

AVP regulates AQP2 through short- and long-term process that appear to diverge in their requirement for cAMP production. AVP regulates AQP2 localization through short-term, cAMP- and Ca^{2+}-dependent processes that occur over a period of minutes. The COOH-terminal tail of AQP2 contains four phospho-serine residues (S256, S261, S264, S269) that are differentially regulated by V2R activation[67]. PKA-dependent S256 phosphorylation is required for exocytosis and apical insertion of AQP2 channels; phospho-AQP2 (S269) is only found at the apical membrane and may regulate endocytic retrieval and protein-protein interactions[113]. The phosphorylation state at S256 and S269 thus may regulate the resident time of AQP2 in the apical membrane and thereby altering protein turnover through protection of the channel from proteasome- and/or lysosome-mediated degradation. Accordingly, Fenton and colleagues also demonstrated that alanine mutations mimicking the non-phosphorylated residues reduced half-life while aspartate (phospho-mimetic) mutations at each residue increased half-life. The precise role of phosphorylated S261 and S264 remains unclear although V2R activation increases phosphorylation of S264 and decreases phosphorylation at S261.

In conjunction with increased levels of cAMP, V2R activation also evokes a rise in intracellular Ca^{2+} levels in renal principal cells [152]. The rise in Ca^{2+} appears to be linked to Epac-mediated release from intracellular stores, and Ca^{2+} chelation with BAPTA or inhibition of intracellular release with ryanodine greatly attenuates the action of AVP on collecting duct water permeability[33, 179]. The increased Ca^{2+} further stimulates cAMP production through Ca^{2+}-calmodulin-dependent activation of adenylyl cyclase 3 (AC3)[66]; Ca^{2+}-calmodulin also activates MLCK, which facilitates movement of AQP2-containing endosomes to the apical membrane, by activating non-muscle myosins (II-A and II-B) found in the principal cell [34]. Although Ca^{2+} is required for AQP2 exocytosis and increased collecting duct water permeability, its role in cAMP generation is still somewhat unclear. The vast majority of AVP-stimulated cAMP production appears to occur through AC6,

which operates independent of Ca^{2+}-calmodulin[133].

Long-term regulation of AQP2 takes place over hours to days and occurs through regulation of whole cell abundance of AQP2. Total AQP2 expression levels reflect the balance between gene transcription, mRNA translation, and protein degradation. However, long-term regulation with prolonged dehydration is thought to occur primarily through increased transcription of the *Aqp2* gene[45, 60]. As with *Avp*, there are CREs present in the $5'$ promoter region of the *Aqp2* gene, and transcriptional activity at these sites is regulated in a PKA- and CREB-dependent manner[73, 106, 178]. Although Ca^{2+} is required for short-term regulation and trafficking of AQP2 to the apical plasma membrane, its role in long-term regulation of whole cell AQP2 abundance is less clear. In an AQP2-expressing cell line (mpkCCD cells), calcineurin and NFAT5 (aka TonEBP) were shown to regulate *Aqp2* transcription[59]. Calcineurin is a Ca^{2+}-dependent serine-threonine phosphatase that de-phosphorylates NFAT, driving its translocation to the nucleus. There, NFAT5 binds to an ORE (Osmotic Response Element) that has been identified in the promoter region of the *Aqp2* gene. Calcineurin-NFAT signaling increases transcriptional activity of the *Aqp2* gene in mpkCCD cells, however, the role of osmolality in driving increased AQP2 abundance with dehydration has been called into question. Increased osmolality in the renal interstitium alone does not increase AQP2 abundance[161]; furthermore, water restriction does not drive NFAT5 translocation to the nucleus in cells of the IMCD, although some nuclear localization was observed in cells within the Loop of Henle[29].

1.6 Subcellular targeting of RIα through AKAP interactions

Although most early AKAP studies focused on targeting of the RII holoenzyme to distinct subcellular locations, several key observations can likely be credited in driving the search for AKAPs with the ability to bind RI subunits. First, early studies found that the Type I kinase was tightly associated with the plasma membrane in human erythrocytes[135]. Second, the nematode, *Caenorhabditis elegans*, expresses a single R subunit (R_{CE}) that bears a high degree of similarity to the mammalian RIα subunit. The majority of PKA activity in *C. elegans* segregates with the particulate fraction, suggesting the presence of an AKAP that targets R_{CE} to membrane structures [102]. Indeed, a subsequent study

identified $AKAP_{CE}$, which possesses a putative amphipathic helix and binds R_{CE} with high affinity (K_d=7 nM) in an interaction that could not be competitively inhibited by the introduction of mammalian RII subunits[5]. Lastly, it was found that T cell activation drives RIα from a dispersed localization to highly focalized structures at the capping site containing TCR-CD3 complexes[148]. Based on these findings, Taylor and colleagues conducted a yeast two-hybrid screen using a fusion cDNA (Ret/ptc2) that included the N-terminal D/D of RIα as bait[75]. They identified a novel RIα binding partner and named it D-AKAP1 (AKAP1) based on its ability to bind both RI and RII subunits. Interestingly, this novel RI-binding AKAP was one of several splice variants of the *Akap1* gene that also encodes the sperm-specific AKAP, S-AKAP84, which also targets RII to mitochondria within the flagellum [99]. Although AKAP1 bound RIα with much lower affinity compared to RIIα, this was the first example of a mammalian RI-binding AKAP.

Detailed structural knowledge of the R subunit-AKAP interface has been critical in revealing additional roles for specific targeting of the RI holoenzyme. Similar to RII, the D/D domains of the RIα dimer generate an X-type, antiparallel four-helix bundle that forms a hydrophobic surface capable of interacting with the amphipathic helix found in virtually all AKAPs[10, 118]. Further structural analysis revealed that the RIIα D/D presents a shallow hydrophobic groove for high affinity AKAP interactions[52], while the RIα D/D contains deeper pockets that likely serve to limit AKAP interactions based on the amino acid side chains presented in the helical structure[140]. A number of dual-specific AKAPs were also found to incorporate an additional interaction domain, termed the RI Specifier Region (RISR), that interacts with the RIα D/D and serves to confer binding selectivity to RI over RII subunits[81].

Mass spectrometry and two-hybrid screens have led to the identification of additional RI-binding AKAPs, including the first mammalian RI-selective AKAP, Sphingosine Kinase Interacting Protein (SKIP). Originally identified in a yeast two-hybrid screen, SKIP was deemed an AKAP based on considerable sequence homology with previously identified AKAPs, including AKAP3 and AKAP11[93]. A subsequent mass spectrometry screen of proteins that bound to cAMP resin confirmed SKIP among a number of other AKAPs that are abundantly expressed in mouse ventricular tissue[142]. SKIP is unique among AKAPs

in that is binds RIα selectively, with two distinct PKA binding regions sequestering RIα with higher (K_d=70 nM) and lower affinity (K_d=700 nM)[109]. In heart, SKIP targets the RI holoenzyme to mitochondria where it is anchored in close proximity to an abundant PKA phospho-substrate, the coiled-coil helix protein ChChd3 [109, 41].

Of particular concern with many of the dual-specific AKAPs is the disparity between the binding affinities for RI and RII subunits, as this may call into question the exact physiological role of RIα anchoring. One notable exception occurs in T cells where a dual-specific AKAP, Ezrin, binds to RI (K_d=25 nM) and RII (K_d=1.4 nM) with markedly differing affinities[136]. However, the RI holoenzyme does account for 80% of the total PKA activity present in T cells[147]. PKA RIα is localized to lipid rafts through its interaction with Ezrin where it prevents T cell activation and suppresses IL-2 secretion by phosphorylating Csk at S364[172]. The functional importance of the RIα-Ezrin interaction was further demonstrated through the use of peptides that disrupt PKA-AKAP interactions, including Ht31, the RI-anchoring disruptor (RIAD), as well as a peptide that disrupts interaction at the RISR sequence[81, 25]. Furthermore, mice that express a soluble Ezrin fragment fused to the RIAD sequence display enhanced T cell activation[114].

Dual-specific AKAPs have been shown to bind both RI and RII and localize PKA to distinct subcellular structures including mitochondria and lipid droplets. AKAP1 (also known as S-AKAP84, AKAP121, and AKAP149) anchors PKA, PTPD1, and Src to the outer mitochondrial membrane where this complex serves to regulate oxidative phosphorylation and mitochondrial fission[89, 101]. AKAP10 contains two RGS domains and anchors Rab4 and Rab11 to mitochondria as well. Interestingly, a single-nucleotide polymorphism (SNP) was identified in AKAP10, with the I646V mutation increased binding affinity to RIα by three-fold[85]. More recently, Heck and colleagues identified another dual-specific AKAP with higher affinity for RI than RII. smAKAP (small membrane AKAP) is widely expressed in mouse tissues and appears to be concentrated in filopodia and at cell-cell junctions[21].

Optic atrophy 1 (OPA1) is somewhat unique as an AKAP in that it has roughly equivalent binding affinity for RI (K_d=12.5 nM) and RII (K_d=14.0 nM) subunits. OPA1 is highly expressed in differentiated 3T3-L1 adipocytes where it organizes a complex of PKA and perilipin and regulates lipolysis in the face of β-adrenergic stimulation[125]. However, given

that the vast majority of PKA activity in adipose tissue is associated with RIIβ, it is unclear what role an OPA1-RIα complex plays in regulating lipolysis.

1.7 Significance of this study

The current work utilizes mice with mutations in the PKA RIα subunit to disrupt kinase activity *in vivo*. Chapter 2 focuses on expression of a dominant negative RI subunit, RIαB, specifically in renal principal cells and the role of PKA in AQP2 regulation, both in terms of apical trafficking and whole cell AQP2 abundance. This is the first PKA mutant to display a diabetes insipidus phenotype. Importantly, this mutation has differing effects on AQP2 protein and mRNA abundance when compared to other mouse models of altered cAMP signaling in the renal principal cell. RIαB should prove useful to further study the compartmentalization of cAMP signaling in the principal cell.

In order to study specific roles for anchored RIα, we generated a mouse in which we introduced alanine mutations into the D/D of RIα. While these mutations are highly effective at disrupting RI-AKAP interactions, the results of this study suggest that the D/D also regulates the post-translational stability of RIα. Stabilization of RIα through loss of potential RI-AKAP interactions had profound effects on cAMP-dependent transcriptional regulation. The decreased CREB phosphorylation and lack of c-fos induction observed here support the idea that RIα degradation is necessary to sustain PKA activity in the face of transient cAMP generation in the cell. Further studies will be required to delineate the RIα degradation mechanism(s) that have been disrupted by the RIα ALA mutation.

Chapter 2

EXPRESSION OF A DOMINANT-NEGATIVE PKA MUTATION IN KIDNEY ELICITS A DIABETES INSIPIDUS PHENOTYPE

2.1 Abstract

Protein kinase A (PKA) plays a critical role in water excretion through regulation of both the production and action of the antidiuretic hormone, arginine vasopressin (AVP). The prohormone is first produced in specific regions of the hypothalamus, where its transcription is highly regulated in a cAMP-dependent manner. Once released into circulation from the posterior pituitary, AVP stimulates antidiuresis through its activation of the vasopressin 2 receptor (V2R). In renal collecting duct principal cells, V2R activation increases cAMP and activates PKA, which then phosphorylates a critical serine residue (S256) on aquaporin-2 (AQP2), triggering apical membrane accumulation, increased collecting duct permeability and water reabsorption. Here, we used *Sim1*-Cre-mediated expression of a dominant-negative PKA regulatory subunit (RIαB) to disrupt kinase activity *in vivo* to further investigate the role of PKA in hypothalamic AVP production and its renal action in fluid homeostasis. *Sim1*-Cre directed expression of RIαB to both the hypothalamic AVP-expressing neurons and the renal AQP2-expressing principal cells. Although this RIαB expression in mice gave rise to marked polydipsia and polyuria, neither AVP mRNA expression nor circulating peptide levels were attenuated, indicating a primary physiological effect on the kidney. In water-loaded animals, the acute phosphorylation of AQP2 in response to a V2R agonist is impaired and these mice show a deficit in the ability to concentrate their urine. Dehydration induced *Aqp2* mRNA in the kidney of both control and RIαB-expressing animals, but AQP2 protein levels were reduced in RIαB mutants and the mice are again unable to effectively concentrate their urine and conserve water under either basal or water-restrictive conditions. We conclude that partial PKA inhibition in kidney causes post-translational effects that reduce AQP2 protein levels, impair its phosphorylation, and interfere with apical

membrane localization. These findings demonstrate a distinct physiological role for PKA signaling in both short- and long-term regulation of AQP2 and characterize a novel mouse model of nephrogenic diabetes insipidus.

2.2 Introduction

Impaired fluid homeostasis leads to diabetes insipidus (DI), a condition marked by excessive thirst and polyuria that, if not diagnosed properly, can lead to developmental defects due to repeated bouts of severe dehydration[7]. Central DI results from attenuated production or release of AVP, while nephrogenic DI occurs through impaired activation of renal vasopressin V2 receptor (V2R) or aberrant AQP2 water channel localization in the collecting ducts of the kidney [43, 70, 167]. Central DI is the most common form of the disease and treatment with AVP analogues such as desmopressin is highly effective; however, in nephrogenic DI, the vast majority of patients have inactivating mutations in the gene encoding the V2R and therapeutic options are extremely limited[7, 151]. Both the central production and peripheral action of AVP in regulating fluid homeostasis have been linked to increases in intracellular cAMP levels and activation of Protein Kinase A (PKA). Despite its major role in the expression and action of AVP, suitable genetic models to study the role of PKA in fluid homeostasis are lacking[50].

In response to hypernatremia or hypovolemia, AVP is released into circulation from the posterior pituitary where it acts on the renal V2R to promote water reabsorption in the kidney[16]. The V2R is localized to the basolateral membrane in principal cells of the collecting duct and acts through the stimulatory G-protein (Gsα) to trigger adenylate cyclase (AC) generation of cAMP[122]. Elevation of cAMP in turn activates PKA, which then phosphorylates a critical serine residue (S256) on the carboxy-terminal tail of AQP2 water channels[51], driving channel translocation from intracellular recycling endosomes to the apical plasma membrane[46]. Apical insertion of AQP2 increases permeability of collecting duct epithelium, allowing transcellular water flux along the medullary osmotic gradient, and water then exits through AQP3 and AQP4 water channels that are constitutively present in the basolateral membrane[160].

V2R activation increases collecting duct water permeability through both short- and

long-term regulation of AQP2. The two modes of regulation appear to diverge through the requirement for elevated cAMP levels in the principal cell. Short-term regulation occurs over a period of minutes through AVP-stimulated generation of cAMP and trafficking of AQP2-containing endosomes to the apical plasma membrane[160]. PKA regulates exocytosis of AQP2 through S256 phosphorylation, which is prerequisite for subsequent phosphorylation at S269 and potentiation of AQP2 membrane retention[68]. Long-term regulation, as seen with dehydration, occurs over the course of hours to days through increased whole-cell AQP2 mRNA and protein abundance[45, 60]. *In vivo*, PKA-dependent phosphorylation of CREB increases AQP2 mRNA levels through transcription initiation at cAMP response elements (CRE) that have been identified in the promoter region of the *Aqp2* gene [73, 106, 178]. Water permeability remains high in IMCD tubules isolated from dehydrated rats; however, cAMP levels are extremely low after prolonged dehydration, pointing to PKA-independent regulation of AQP2 levels with long-term V2R activation[95]. More recently, *in vitro* studies using AQP2-expressing mpkCCD cells have implicated changes in osmolality acting through calcineurin and NFAT to increase AQP2 transcription[59, 31] . The *in vivo* effect of increased osmolality on AQP2 expression remains unclear, however, as NFAT5 (TonEBP) does not translocate to the nucleus in the IMCD in dehydrated animals[29].

The PKA holoenzyme is a tetramer composed of a regulatory (R) subunit dimer (RIα, RIβ, RIIα, RIIβ) that sequesters two catalytic (C) subunits (Cα or Cβ) in the absence of cAMP [108]. Upon cAMP binding, the C subunits dissociate from the holoenzyme and phosphorylate many diverse substrates to regulate cellular function. Despite the critical role of PKA in many physiological processes, including fluid homeostasis, *in vivo* studies have been hindered to a certain extent by compensatory changes that occur with genetic disruption of PKA subunits. Knockout mice with targeted disruption of RIβ, RIIα or RIIβ subunits display compensatory increases in the RIα subunit[39, 22], which we have further shown to occur through post-translational stabilization of RIα in the intact holoenzyme[2]. To bypass this compensation, we previously cloned mutant RIα subunits with the ability to suppress C subunit activity, even in the presence of physiological levels of cAMP[35, 155]. The RIαB mutation contains a single amino acid change (G324D) in the carboxy-terminal cAMP-binding site (site B), a mutation that increases the K_a for activation of the RIα

subunit approximately 100-fold. The RIαB mouse harbors a transcriptionally silent mutant allele, and Cre-mediated recombination activates this dominant-negative RIαB allele in a cell type-specific pattern[72, 173].

To further explore the role of PKA activity in fluid homeostasis *in vivo*, we targeted RIαB to the kidney using a BAC transgenic mouse line under the control of *Sim1* regulatory elements[8]. *Sim1*-Cre directs RIαB expression both to the PVN and SON of the hypothalamus and to AQP2-expressing principal cells of the kidney. We found that *Sim1*-directed inhibition of PKA activity leads to severe polydipsia, decreased AQP2 protein levels, and blunted response to short- and long-term V2R activation. These findings are consistent with a greater role for PKA in regulating AQP2 levels during prolonged osmotic stress *in vivo*.

2.3 Experimental Procedures

Experimental Animals. The generation and genotyping of RIαB mice has been described in detail previously[72, 173]. Dr. Brad Lowell (Beth Israel Deaconess Medical Center) kindly provided the *Sim1*-Cre mice[8]. Mice used in this study were housed on a 12-hour light/dark cycle and had free access to standard chow and water, except as noted in the dehydration studies. All experimental studies were conducted using protocols approved by the University of Washington Institutional Animal Care and Use Committee.

Urine collection and osmolality measurements. Urine production and osmolality was determined in mice with free access to water or after 24-hour dehydration. Additionally, mice were subjected to acute water loading (10 mM glucose solution, 3% of body weight) and subsequent intraperitoneal injection of vehicle or the V2R agonist, DDAVP (Ferring Pharmaceuticals). For urine collection, mice were placed in a plastic beaker on a wire platform and spontaneously voided urine was collected over the next 2-4 hours. Urine osmolality was determined using a Vapro 5520 vapor pressure osmometer (Wescor).

Plasma AVP measurements. Mice, with free access to water, were euthanized by intraperitoneal injection of pentobarbital and serum was collected into chilled microcentrifuge tubes containing EGTA. Samples were analyzed for AVP content by ELISA (Cayman Chemical) as per the manufacturers instructions.

X-gal staining and immunohistochemistry. After CO_2 euthanasia, mice were perfused transcardially with PBS followed by PBS-buffered 4% paraformaldehyde solution. Tissues were removed, post-fixed for 2-4 hours in PBS-paraformaldehyde, and then cryopreserved overnight in a 30% sucrose solution (wt/vol). After embedding in Tissue-Tek OCT compound (Sakura Finetek), 20 μm cryosections were taken and processed for X-gal staining or immunohistochemistry. For X-gal staining, sections were washed with PBS and then incubated for 24 hours with X-gal solution (40 mg/ml X-Gal stock solution in DMSO) diluted 1:40 into X-gal buffer (in mM: potassium ferricyanide (crystalline) 5, potassium ferricyanide trihydrate 5, magnesium chloride 2) at 37 °C for 1 hour to overnight in PBS. Sections were washed in PBS, dehydrated in 100% ethanol and counterstained with Eosin Y. Then the sections were mounted with Cytoseal 60 (Thermo Scientific) for imaging by light microscopy on a Nikon Eclipse E600 microscope. For immunohistochemistry, sections were washed in PBS and then incubated in blocking buffer (10% normal goat serum, 0.2% Triton X-100, and 2% BSA in PBS) for 1 hour at room temperature. Primary antibodies (1:250) were diluted into blocking buffer and sections were incubated at 4 °C overnight. After rinsing with PBS-T (PBS with 0.2% Triton X-100), sections were incubated for two hours at room temperature with fluorescence-labeled secondary antibodies (Molecular Probes, 1:500) diluted in blocking buffer. Sections were washed in PBS-T and mounted with Vectashield medium for imaging on a Leica SL confocal microscope. All images were taken at the Keck Microscopy Facility (University of Washington).

Protein sample preparation and western blotting. After CO_2 euthanasia, kidneys were bisected and inner medullae were removed and dounce homogenized in RIPA buffer (5% wt/vol) supplemented with protease and phosphatase inhibitor cocktails (Sigma). Following brief sonication (Branson Sonifier), samples were cleared at 10,000 x g for 10 minutes at $_2$ and supernatant protein concentration was determined by BCA assay (Pierce). Protein samples were diluted in 4X NuPAGE sample buffer (Invitrogen), resolved on 12% polyacrylamide gels, and subsequently transferred to a nitrocellulose membrane (Whatman). Membranes were blocked for 1 hour in 5% BSA or milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T), and incubated overnight in primary antibody. Membranes were then rinsed with TBS-T and incubated with HRP-conjugated secondary antibodies (Jackson ImmunoResearch)

diluted at 1:10,000 in TBS-T with 5% BSA or milk. HRP was detected with Supersignal West Pico chemiluminescent substrate (Pierce).

Kinase activity measurements. PKA activity was calculated through phosphorylation of a PKA substrate peptide (Kemptide, LRRASLG) using ATP-[γ-P32]. Tissue samples were collected and dounce homogenized in kinase activity buffer (in mM: Tris 20, EDTA 0.1, EGTA 0.5, DTT 10, magnesium acetate 5, sucrose 250; pH 7.6 with 1% Triton-X 100), supplemented with 1 μg/ml leupeptin, 3 μg/ml aprotinin, and 5 mM AEBSF. Counts per minute were converted to total enzymatic activity and are expressed as picomoles of phosphate transferred (units-U) per milligram of input protein. Activity measured in the presence of 4 μg/ml Protein Kinase Inhibitor (PKI) was assumed to be non-specific and counts were subtracted from each sample as background.

Immunoprecipitation of RiboTag-labeled polysomes from mouse tissues. Mice were euthanized by CO_2 and hypothalamus and kidneys were collected and immediately processed as described[137, 138], with the exception that the HA antibody (Covance) was incubated with lysate for 4 hr before addition of the protein A/G magnetic beads (Pierce) followed by overnight incubation at 4 °C.

Real-time quantitative RT-PCR. Total RNA was isolated from hypothalamus or kidney using an RNeasy RNA isolation kit (Qiagen). One-step RT-PCR was performed using the Mx3000P QPCR system and Brilliant II QRT-PCR reagent kit (Agilent). Relative amounts of transcripts from each tissue were extrapolated from a four-point standard curve (100, 10, 1, 0.1 ng total RNA) made from a common brain or kidney sample.

Primary Antibodies. All antibodies were used at 1:1000 for western blotting and 1:250-500 for immunohistochemistry. Antibodies purchased were: PKA Cα, RIα subunits (Mouse, BD Biosciences), PKA RIIα (Rabbit, Santa Cruz), α-actin (Mouse, Sigma), AVP (Rabbit, EMD Biosciences), Hemagglutinin (Mouse, Covance), and AQP2 (Rabbit, Novus Biologicals). The phospho-AQP2 (S256) antibody was a kind gift from Dr. Mark Knepper of the National Heart, Lung and Blood Institute.

Data analysis. Data are presented as means ± SEM and statistical significance was determined by a two-tailed Students t test between $Prkar1a^{\mathrm{WT/RI\alpha B}}$ (RIαB-OFF) and *Sim-1*-Cre/$Prkar1a^{\mathrm{WT/RI\alpha B}}$ (RIαB-ON) mice. Analyses were carried out using Prism

4.0c for the Mac OS (Graphpad Software).

2.4 Results

RIαB expression directed by Sim1-Cre causes diabetes insipidus in mice. As shown in Fig. 2.1A, RIαB OFF mice express one wild type *Prkar1a* allele, while a loxP-flanked neomycin cassette placed between exons 10 and 11 keeps the RIαB allele (G324D) transcriptionally silent. *Sim1*-Cre-mediated recombination removes the NEO stop cassette and 5′ loxP site, allowing for the resumption of normal splicing and expression of RIαB transcripts. RIαB-OFF mice ($Prkar1a^{\mathrm{WT/RI\alpha B}}$, Fig. 1B) used in this study are effectively heterozygous for *Prkar1a* and are used as controls for comparison with RIαB-ON mice (*Sim-1*-Cre/$Prkar1a^{\mathrm{WT/RI\alpha B}}$, Fig. 2.1B) with the active mutation. In addition to fluid homeostasis, the PVN is a critical regulatory site for energy homeostasis, receiving input from orexigenic (NPY/AgRP) and anorexigenic (POMC) neurons in the arcuate nucleus (ARC)[38]. POMC neurons are thought to suppress feeding behavior through activation of melanocortin receptors (MC4R) and increased cAMP generation. Gross phenotypic analysis of RIαB-ON mice and littermate controls revealed no significant difference in food intake (Fig. 2.1C) or reproductive fat pad weight (Fig. 2.1D). In contrast with the lack of effect of RIαB activation on these parameters, subtle increases in body weight were observed at eight weeks of age (Fig. 2.1E). Strikingly, we observed a marked increase in water consumption in males and females, a phenotype that was apparent by 6-8 weeks of age (Fig. 2.1F). In conjunction with increased fluid intake, RIαB-ON mice displayed pronounced polyuria when compared to WT and RIαB-OFF littermates (Fig. 2.1G).

Sim1-Cre directs RIαB expression to selective regions of the hypothalamus and kidney. *Sim1*-Cre-dependent reporter activity is known to be concentrated in specific nuclei of the hypothalamus, primarily the PVN and SON[8]. Previously, it was also demonstrated that *Sim1* is expressed in peripheral tissues during early embryonic development, among cell populations that give rise to the pronephros and primitive metanephros[48, 111]. To follow *Sim1*-directed Cre recombinase activity in the hypothalamus and kidney, we crossed *Sim1*-Cre mice to the ROSA26 (R26R) reporter mouse line [150]. Cryosections from brain and kidney were stained with X-Gal to reveal β-galactosidase expression. As expected, *Sim1*-

Cre induced reporter activation in the PVN and SON of the hypothalamus, as shown in Fig. 2.2A (left). Robust β-galactosidase expression was also observed throughout the inner medulla of the kidney (Fig. 2.2A, right).

We next utilized the RiboTag mouse[137, 138] to identify specific cell populations in hypothalamus and kidney that were targeted by *Sim1*-Cre. The RiboTag mouse has a modified exon 4 inserted into the *Rpl22* gene that incorporates three sequential hemagglutinin (HA) epitopes into the RPL22 ribosomal protein after Cre-mediated recombination. Whole tissues were homogenized and ribosomes, with bound mRNAs, were immunoprecipitated with an anti-HA antibody, allowing selective enrichment of transcripts from cell types where Cre recombinase is expressed (Fig. 2.2B). To ensure that both the PVN and SON were included in our analysis, a roughly 3 mm wide coronal section was isolated using a brain matrix (Harvard Apparatus) and the tissue was trimmed dorsal to the hypothalamus and laterally on either side of the amygdala. *Sim*1 was enriched approximately 5-fold in immuno-precipitated mRNA from both hypothalamus and kidney (Figure 2.2C). *Avp* was the most highly enriched hypothalamic transcript when comparing the IP to total input mRNA (Fig. 2.2C) indicating that the *Sim1*-Cre is targeting the neurons known to produce AVP. In the hypothalamus, Cre recombination has occurred primarily in neurons since the oligodendrocyte marker, *Cnp*, was de-enriched when compared to input RNA (Fig. 2.2C). In kidney, *Aqp2* mRNA was enriched approximately 3-fold, while the intercalated cell marker, *Slc26a4*, was found to de-enrich from the immunoprecipitated transcripts (Fig. 2.2D). We conclude that the *Sim1*-Cre is driving expression of RIαB in both the hypothalamic neurons responsible for synthesizing AVP and in the principal cells of the kidney collecting duct where AVP acts to regulate AQP2 expression and function. Since PKA inhibition in either hypothalamic AVP neurons or in kidney AQP2-expressing epithelial cells might be expected to lead to the observed DI phenotypes we examined the relative contribution of neural and kidney expression of RIαB in more detail.

Hypothalamic expression of RIαB does not inhibit AVP production or release. Because *Avp* mRNA was highly enriched in our RiboTag IP, we sought to determine if the DI phenotype in RIαB-ON mice was mediated through attenuated PKA activity in AVP-expressing neurons of the PVN. We used the *Sim1*-Cre mouse to activate RiboTag in hypothalamic

neurons and used antibodies specific for AVP or RPL22-HA. As shown in Fig. 2.3A, AVP and RPL22-HA are co-expressed in a subset of neurons within the PVN. We then isolated 1 mm coronal sections of hypothalamus and took punches of PVN to assess total kinase activity in the lysates. Although RIαB is expressed in the PVN, we were unable to detect any significant decrement in PKA activity in RIαB-ON mice in the absence or presence of cAMP (Fig. 2.3B). We then isolated total mRNA from the hypothalamus and compared *Avp* transcript levels in RIαB-OFF and RIαB-ON mice. *Avp* mRNA was slightly elevated in the RIαB-ON mice (Fig. 2.3C); however, there was no measureable difference in circulating AVP peptide levels after RIαB activation when compared to control groups (Fig. 2.3D). The 1.5 fold increase in *Avp* mRNA suggests a compensatory response to the nephrogenic DI that could be caused by inhibition of PKA in the kidney principal cells.

Inhibition of PKA lowers total AQP2 protein levels in vivo. The lack of RIαB inhibition of *Avp* mRNA or circulating AVP peptide levels suggested that the DI might be primarily due to renal insufficiency in RIαB-ON mice. We crossed *Sim1*-Cre mice to a reporter line that expresses the fluorescent tdTomato protein after Cre-mediated recombination[103]. As shown in Fig. 2.4A, tdTomato localizes to cells of the inner medulla that also express the principal cell-specific water channel, AQP2. Expression of RIαB is sufficient to disrupt the apical membrane localization of AQP2 (Fig. 2.4B), and western blot analysis of inner medulla lysates revealed a dramatic decrease in AQP2 protein (Fig. 2.4C, RIαB OFF=1.02 $\pm$ 0.03 vs. RIαB ON=0.42 $\pm$ 0.01 AU, $P<0.0001$). A small, but not statistically significant, drop in AQP2 mRNA levels (RIαB OFF=0.99 $\pm$ 0.10 vs. RIαB ON=0.82 $\pm$ 0.05 AU, $P>0.05$) was also observed in total RNA isolated from inner medulla (Fig. 2.4D). Kinase activity assays confirmed that inner medulla RIαB expression was sufficient to inhibit PKA activity in both the absence and presence of 5 μM cAMP (Fig. 2.4E). Despite the decrease in kinase activity, there were no detectable changes in the levels of PKA C subunit expression as shown by western blot in Fig. 2.4F.

RIαB expression attenuates the response to V2R activation. To assess the *in vivo* response of the kidney to acute V2R activation, mice expressing the activated RIαB allele (RIαB-ON) were water loaded by oral gavage to suppress circulating AVP levels. Mice were then given an intraperitoneal injection of vehicle or the V2R agonist, DDAVP, (0.1 μg/kg)

and urine was collected for 2 hours. This dose of DDAVP prevents water load-induced diuresis in WT mice[132]. RIαB-ON mice displayed significantly lower urine osmolality with *ad libitum* water consumption; however, acute water loading reduced urine osmolality to a similar level in both RIαB-OFF and RIαB-ON mice (Fig. 2.5A). DDAVP injection of water loaded mice increased urine osmolality in RIαB-OFF mice, while urinary concentrating ability was still significantly impaired in RIαB-ON mice (Fig. 2.5A). However, the localization of AQP2 in RIαB-ON collecting ducts was partially rescued in response to DDAVP (Fig. 2.5B) and this is likely to account for the increased urine osmolality seen in DDAVP treated RIαB-ON mice (Fig. 2.5A). We then examined AQP2 phosphorylation by western blot. Mice were water loaded and, after one hour, received an IP injection of vehicle or DDAVP (0.1 μg/kg). AQP2 phosphorylation was induced after 20 min by DDAVP in the RIαB-OFF controls and in the RIαB-ON mice but the total level of AQP2 in the RIαB-ON mice remained low (Fig. 2.5D).

Prolonged dehydration increases total AQP2 expression in both control and RIαB ON mice. In order to assess the role of PKA in prolonged osmotic stress, we subjected control and RIαB ON mice to 24-hour dehydration and analyzed the localization and expression levels of AQP2. After 24-hour dehydration, AQP2 levels and apical localization were partially restored in RIαB-ON mice (Fig. 2.6A). Consistent with this result, RIαB-ON mice display an improved urinary concentrating ability after 24-hour dehydration (Fig. 2.6B). We isolated total RNA from control and RIαB-ON kidneys and analyzed mRNA content for water channels involved in urinary concentration. Total transcript levels for *Aqp2*, *Aqp3*, *Aqp4*, or *Avpr2* were not significantly different between RIαB OFF and RIαB ON mice with *ad libitum* water consumption and *Aqp2* and *Aqp3* mRNAs were induced 2-fold by dehydration (Fig. 2.6C). Total AQP2 protein levels were still significantly reduced in RIαB ON mice under *ad libitum* and dehydrated conditions (Fig. 2.6D). In concordance with impaired urinary concentrating ability, AQP2 phosphorylation was also reduced under *ad libitum* and dehydration conditions (Fig. 2.6D).

2.5 Discussion

In this study, we demonstrate that *in vivo* suppression of PKA activity through RIαB expression is sufficient to disrupt fluid homeostasis through altered localization and expression of AQP2 protein. Surprisingly, our results suggest a post-translational role for PKA in the maintenance of total AQP2 protein levels with *ad libitum* water consumption and after prolonged dehydration and sustained V2R activation in the renal inner medulla. *Sim1*-Cre-mediated expression of RIαB gives rise to a striking DI phenotype, and more detailed analysis reveals that this pathology is nephrogenic, rather than central, in origin. Although expressed in the PVN and SON, the primary sites of AVP production, RIαB does not appear to induce primary polydipsia through defective osmoregulation of thirst. This is supported by our results indicating decreased urine osmolality in RIαB-ON mice despite a slight elevation in *Avp* mRNA and no change in circulating AVP levels as well as a blunted response to intraperitoneal DDAVP administration.

A potential confounding factor in this study is that *Sim1* is expressed in both the PVN/SON of the hypothalamus and in the developing kidney. Using both *in vivo* and *in vitro* approaches, it was previously demonstrated that elevated serum osmolality or exposure to hyperosmotic solutions resulted in increased cAMP levels in the SON, as well as to a lesser extent in the PVN [28]. cAMP-dependent *Avp* transcriptional regulation likely occurs through PKA-mediated phosphorylation of CREB and its action at putative CREs that have been identified in the *Avp* promoter [79]. Indeed, viral overexpression of the endogenous PKA inhibitor, PKIα, blunted AVP upregulation in the PVN in response to dehydration [175], and *Sim1*-Cre mediated disruption of CREB1 in the PVN reduced basal AVP protein levels [32]. Despite clear *Sim1*-Cre driven reporter activity in the PVN (Figs. 2A and 3A), we were unable to detect any decrement in kinase activity in PVN lysates (Fig. 3B). While the G324D mutation in RIα is remarkably effective at blunting kinase activation, it is expressed as roughly half of the total endogenous RIα expression, and the total complement R-subunit (RIβ, RIIα, RIIβ) expression may mitigate the effects of RIαB in a specific cell types.

AVP-dependent regulation of collecting duct water permeability is regulated through

short- and long-term processes. While PKA has been clearly implicated in short-term regulation through phosphorylation of AQP2 at S256, its role in long-term regulation is not well studied, particularly *in vivo*. PKA has been shown to regulate APQ2 expression through phosphorylation of CREB and increased transcriptional activity at CREs in the *Aqp2* promoter region. As discussed above[32], *Sim1*-Cre mediated disruption of CREB1 results in decreased AVP in neurons of the PVN; however, based on our current work, the authors also likely disrupted CREB1 expression in principal cells of the kidney. As there was no DI phenotype reported, one must assume that diminution of AVP expression combined with loss of CREB1 in collecting duct principal cells is not sufficient to disrupt fluid homeostasis *in vivo*. This view is consistent with our observations that inhibition of PKA activity through RIαB expression did not significantly reduce *Aqp2* mRNA levels in inner medulla (Figure 4F) or whole kidney (Figure 6C).

Surprisingly, the sustained attenuation of PKA activity in RIαB-ON mice was sufficient to reduce total AQP2 protein levels. While not demonstrated directly in this study, a role for PKA in regulating the post-translational stability of AQP2 can be surmised based on phosphorylation events that occur in the carboxy-terminal tail. PKA-mediated phosphorylation of S256 is well accepted as a required event for apical exocytosis of AQP2-containing vesicles. Both *in vitro* and *in vivo*, Knepper and colleagues[68] demonstrated that phospho-S256 is a priming site for subsequent phosphorylation at S269, a residue demonstrated to regulate endocytosis of AQP2 from the apical membrane. Impaired exo- or endocytosis of AQP2 has been shown to alter the stability of the protein in AQP2-expressing cell lines. In Madin Darby Canine Kidney (MDCK) cells, mutational analysis of both serine residues showed that loss of phosphorylation at either site has profound effects on the stability of AQP2 protein as both the S256A and S269A mutants exhibited reduced half-lives [113]. Testing the role of S256 phosphorylation in mediating the stability of AQP2 protein *in vivo* is confounded somewhat by the fact that a mouse model of congenital progressive hydronephrosis (*Cph*), found to have a S256L mutation, also displays marked increases in AQP2 protein in conjunction with higher levels of *Aqp2* mRNA expression.

Phosphorylation of AQP2 also alters its interactions with other proteins including components of the actin cytoskeleton that regulate its trafficking to the apical membrane. AVP

induces depolymerization of F-actin in the subapical cortex of the principal cell, a step proposed to remove the dense actin barrier to the trafficking and apical exocytosis of AQP2 vesicles[146]. PKA-dependent phosphorylation of AQP2 at S256 decreases its interaction with G-actin while producing a high affinity binding site for tropomyosin-5b (TM5b)[120]. Binding to phospho-AQP2 (S256) reduced TM5b interactions with F-actin, thereby enhancing its depolymerization and facilitating movement of AQP2 vesicles to the apical membrane. A direct effect of RIαB expression on actin polymerization also cannot be ruled out as PKA phosphorylates RhoA, causing dissociation, independent of GTP/GDP binding, from membrane-bound proteins including Rho/Rac/Cdc-42-activated kinases[159]. Similarly to the interaction between phospho-AQP2 and TM5b, PKA-mediated inhibition of RhoA would favor depolymerization of F-actin. Indeed, inhibition of RhoA with Clostridium botulinum toxin C3 increased apical accumulation of AQP2 in CD8 collecting duct cells in the absence of V2R activation[158]. The delocalization of AQP2 under *ad libitum* water consumption conditions that we observed in this study may reflect reduced actin depolymerization, through direct or indirect action, and impairment of normal trafficking of AQP2 vesicles.

A clear role for PKA activity in long-term regulation of AQP2 expression has not been demonstrated previously. Our current results suggest a possible role for PKA in regulating whole-cell AQP2 levels in the collecting duct with prolonged dehydration. cAMP generation and activation of PKA are known to be highly compartmentalized, a point illustrated by the fact that both EP4 (53) and calcitonin receptor[15] activation stimulated only modest antidiuresis and increases in AQP2 expression, all despite increasing cAMP to comparable levels as seen with V2R activation. Thus, the RIαB mouse represents a novel model of nephrogenic DI and should extend our knowledge of cAMP signaling in the renal principal cell.

2.6 Acknowledgements

We would like to thank Mark Knepper at the National Heart, Lung, and Blood Institute for helpful discussions regarding phenotypic analysis. This research was supported by National Institutes of Health grant GM032875 (to G.S.M). M.L.G. was supported by the Pharma-

ceutical Sciences Training Grant (5T32GM007750-03) at the University of Washington.

Figure 2.1: Phenotypic analysis after *Sim1*-Cre mediated expression of RIαB. (A) C-terminal exons of *Prkar1a* gene and activation of RIαB mutation (G324D) after Cre-mediated recombination. RIαB OFF mice are heterozygous for *Prkar1a* in all tissues. (B) Breeding strategy and generation of experimental animals. Throughout phenotypic analysis, there was no difference observed between true wild-type (*Prkar1a*$^{\mathrm{WT/WT}}$) and *Sim-1*-Cre/*Prkar1a*$^{\mathrm{WT/WT}}$ animals. Average daily food intake (C), reproductive fat pad weight (D), body weight (E), water intake (F), and urine production (G) in control (WT and RIαB OFF) and RIαB ON mice (N=5-8 for each group). Values were measured in 8 week-old male and female mice and represent mean $\pm$ SEM. *P$<$0.05, **P$<$0.01, ***P$<$0.001 when comparing RIαB OFF to RIαB ON mice by two-tailed Students t test.

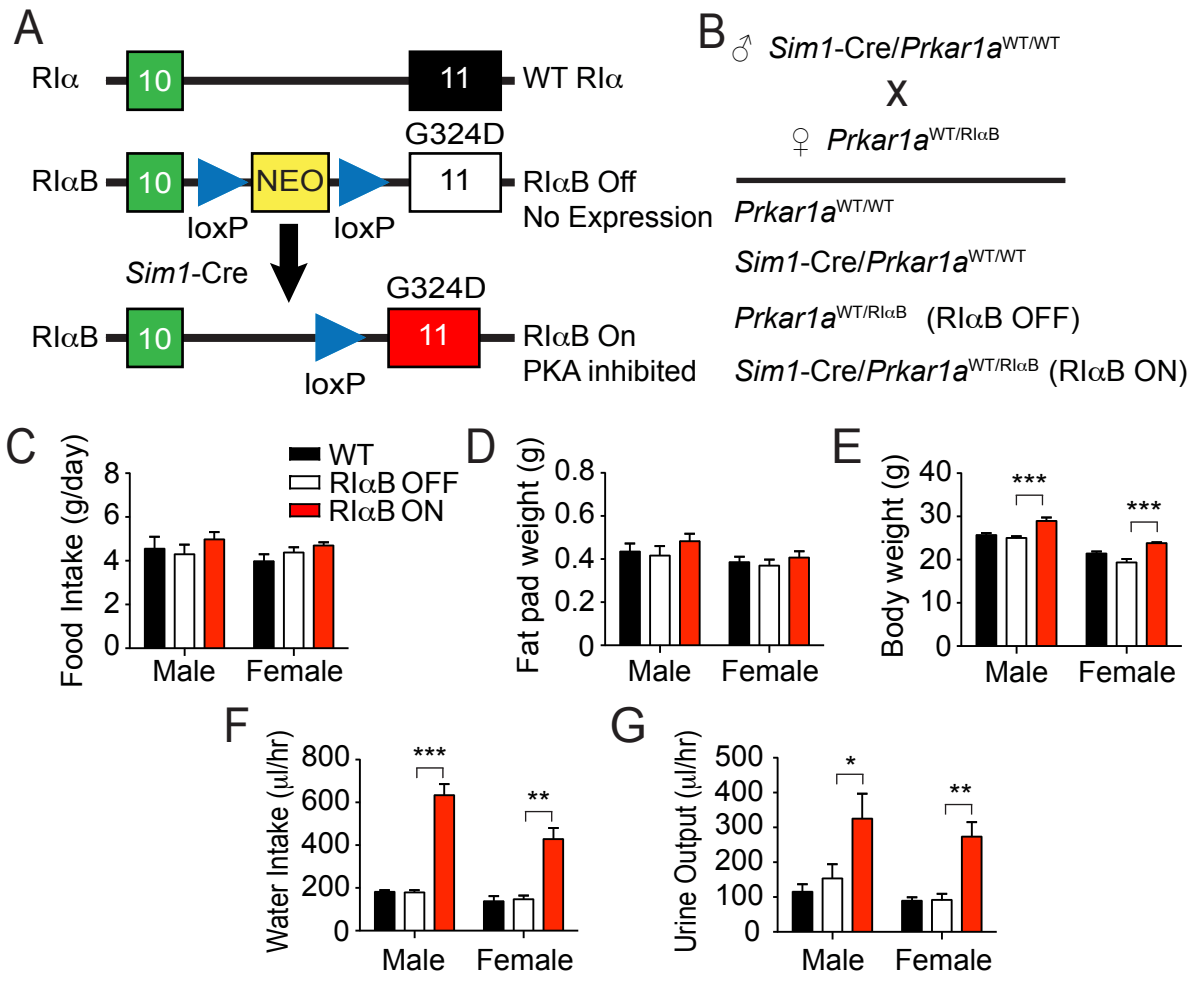

A
RIα
10
11
WT RIα
G324D
RIαB
10
NEO
11
RIαB Off
No Expression
loxP
loxP
Sim1-Cre
G324D
RIαB
10
11
RIαB On
PKA inhibited
loxP
B
♂ Sim1-Cre/Prkar1a WT/WT
X
♀ Prkar1a WT/RIαB
Prkar1a WT/WT
Sim1-Cre/Prkar1a WT/WT
Prkar1a WT/RIαB (RIαB OFF)
Sim1-Cre/Prkar1a WT/RIαB (RIαB ON)
C
Food Intake (g/day)
WT
RIαB OFF
RIαB ON
Male
Female
D
Fat pad weight (g)
Male
Female
E
Body weight (g)
***
***
Male
Female
F
Water Intake (µl/hr)
***
**
Male
Female
G
Urine Output (µl/hr)
*
**
Male
Female

Figure 2.2: Isolation of cell type-specific transcripts from tissues where *Sim1*-Cre is active. (A) *Sim1*-Cre mice were crossed to the ROSA26R reporter mouse line and cryosections from hypothalamus and kidney were stained with X-gal to reveal β-galactosidase expression. (B) Schematic depicting the RiboTag assay. *Sim1*-Cre mice were crossed to the RiboTag mouse and hypothalamus or kidneys were homogenized as described in the methods section. Protein A/G magnetic bead/anti-HA antibody complexes were used to immunoprecipitate polyribosomes and bound mRNAs for gene expression analysis of *Sim1*-positive cells in each tissue. (C) Representative RT-PCR analysis demonstrated enrichment of *Sim1* mRNA in the IP from both hypothalamus and kidney. Furthermore, *Avp* was highly enriched after immunopreciptation from hypothalamus, while the oligodendrocyte-specific transcript, *Cnp*, was de-enriched slightly. Despite widespread reporter expression in the kidney, the principal cell-specific transcript, *Aqp2*, was enriched while *Slc26a4*, found in intercalated cells, was also found to de-enrich. Enrichment shown is the ratio of IP mRNA versus input mRNA from the crude tissue homogenate.

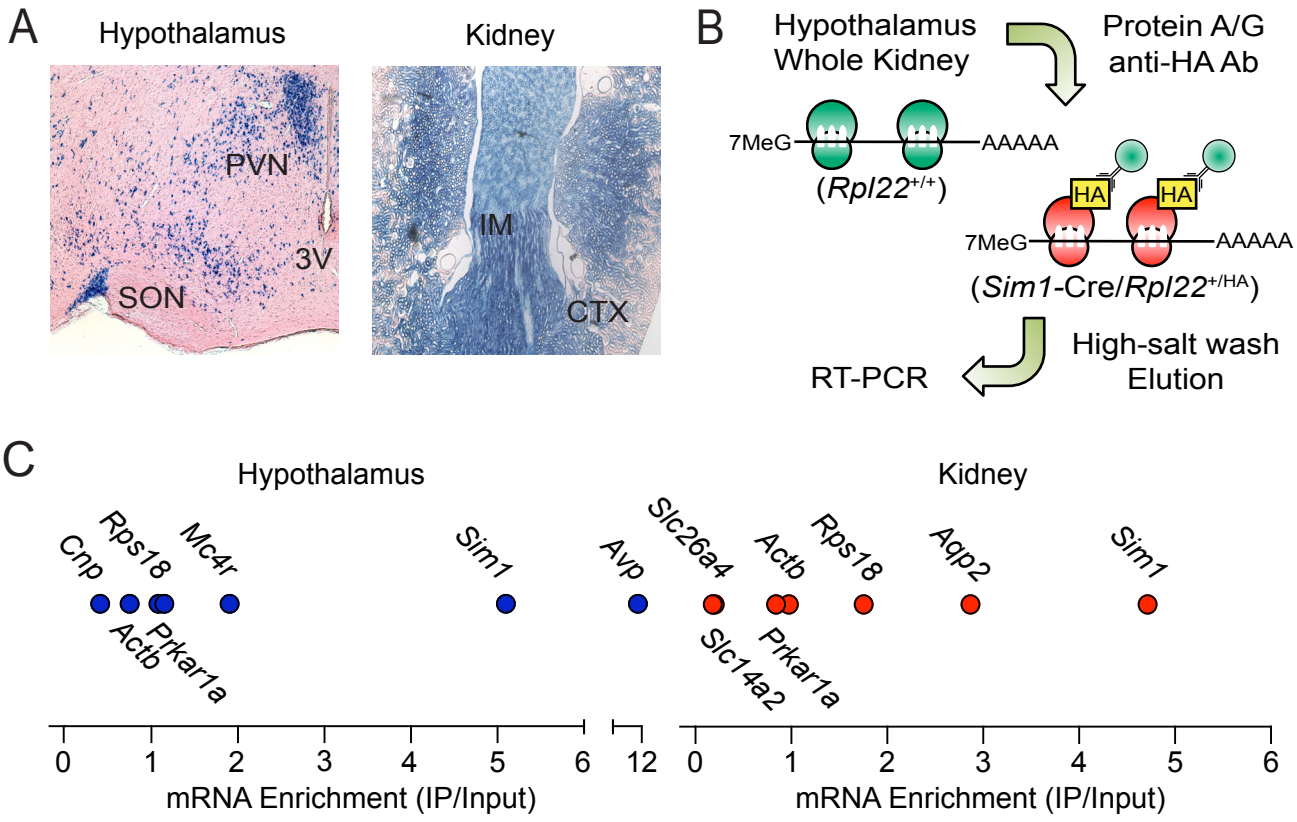

A
Hypothalamus
Kidney
PVN
3V
SON
IM
CTX
B
Hypothalamus
Whole Kidney
Protein A/G
anti-HA Ab
7MeG
AAAAA
(Rpl22+/+)
HA
HA
7MeG
AAAAA
(Sim1-Cre/Rpl22+/HA)
RT-PCR
High-salt wash
Elution
C
Hypothalamus
Kidney
Cnp
Rps18
Mc4r
Actb
Prkar1a
Sim1
Avp
Slc26a4
Actb
Rps18
Aqp2
Sim1
Slc14a2
Prkar1a
0
1
2
3
4
5
6
12
mRNA Enrichment (IP/Input)
0
1
2
3
4
5
6
mRNA Enrichment (IP/Input)

Figure 2.3: AVP mRNA expression and circulating peptide levels. (A) Immunohistochemistry demonstrating co-localization of AVP and HA after *Sim1*-Cre mice were crossed to the RiboTag mouse. (B) Total PKA activity in the presence or absence of 5 μM cAMP (N=3). Homogenates were prepared as described from punches that were taken from coronal sections containing PVN neurons. (C) Total hypothalamic expression of *Avp* mRNA in control and RIαB ON mice (N=4 in each group). (D) Circulating plasma AVP levels as assessed by ELISA (N=5-7 in each group). *P$<$0.05 when comparing RIαB OFF to RIαB ON mice by two-tailed Students t test.

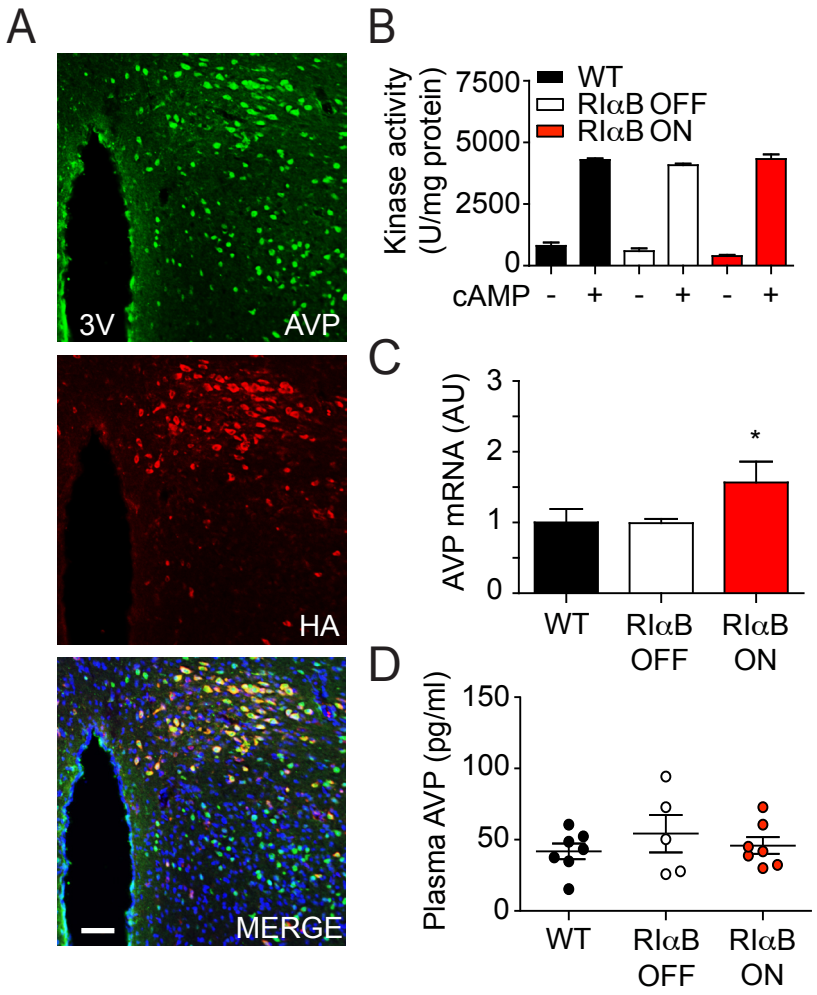

A
3V
AVP
HA
MERGE
B
Kinase activity (U/mg protein)
7500
5000
2500
0
WT
RIαB OFF
RIαB ON
cAMP - + - + - +
C
AVP mRNA (AU)
3
2
1
0
*
WT
RIαB OFF
RIαB ON
D
Plasma AVP (pg/ml)
150
100
50
0
WT
RIαB OFF
RIαB ON

Figure 2.4: Effect of RIαB mutation on AQP2 localization and expression. (A) *Sim1*-Cre driven expression of tdTomato fluorescent protein and co-localization with AQP2 in the inner medulla of the kidney. (B) AQP2 localization in principal cells of RIαB OFF and RIαB ON mice. (C) Representative western blot and quantitation of total AQP2 protein levels in inner medulla. Quantitation includes both glycosylated (Gly-AQP2) and non-glycosylated (NG-AQP2) bands. β-actin was used as a loading control (N=4). (D) Total *Aqp2* mRNA levels in isolated inner medullae from control and RIαB ON mice (N=4). *Actb* transcript levels were used to ensure equivalent loading of RNA. (E) Total inner medulla PKA activity in the presence or absence of 5 μM cAMP (N=4). (F) Representative western blot and quantitation of PKA regulatory and catalytic subunit expression in the inner medulla of control (WT and RIαB OFF) and RIαB ON mice (N=3). **P<0.01, ***P<0.001 when comparing RIαB OFF and RIαB ON mice by two-tailed Students t test.

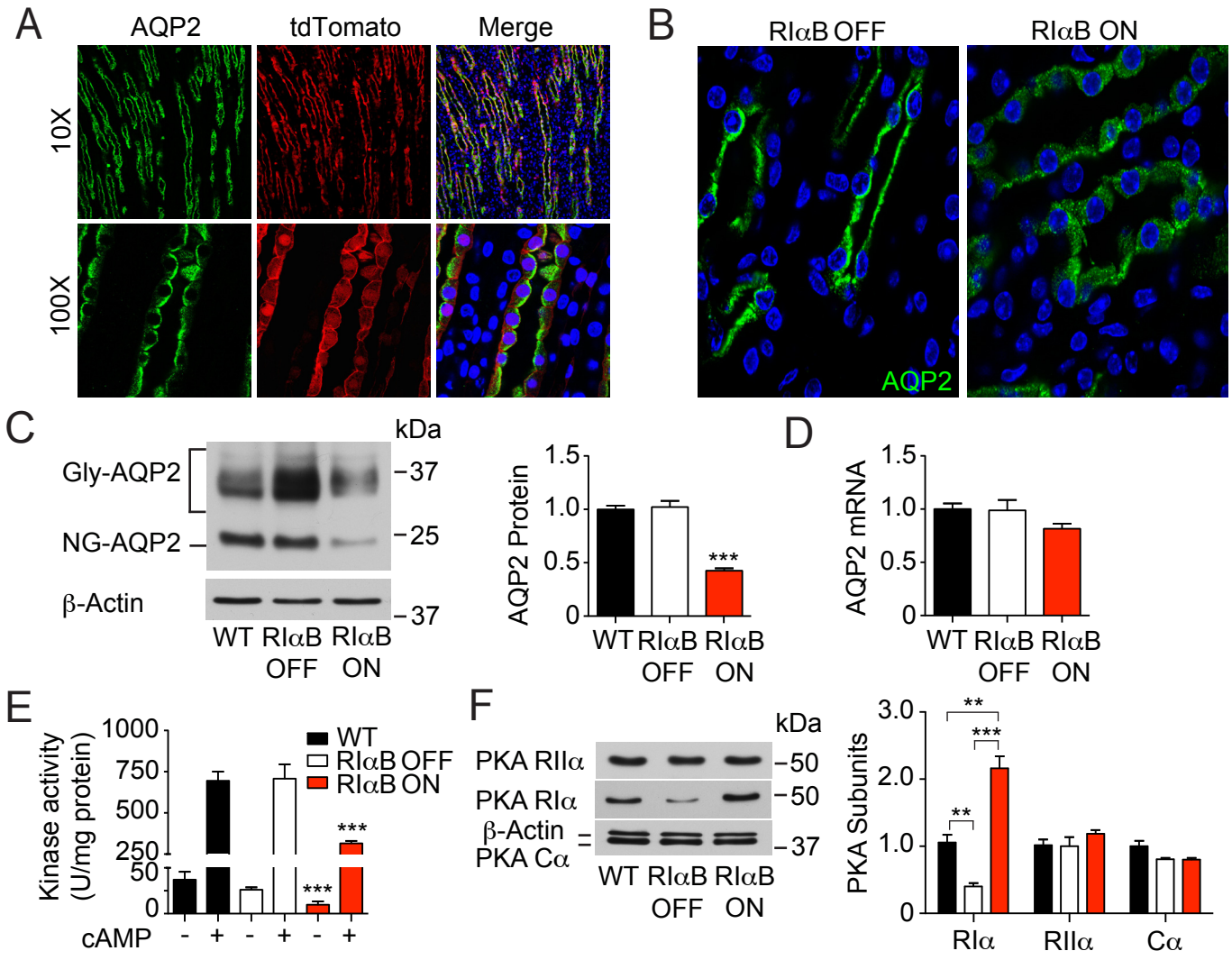

A
AQP2
tdTomato
Merge
10X
100X
B
RIαB OFF
RIαB ON
AQP2
C
kDa
Gly-AQP2
NG-AQP2
β-Actin
37
25
37
WT RIαB OFF RIαB ON
AQP2 Protein
1.5
1.0
0.5
0
***
WT RIαB OFF RIαB ON
D
AQP2 mRNA
1.5
1.0
0.5
0
WT RIαB OFF RIαB ON
E
Kinase activity (U/mg protein)
1000
750
500
250
50
0
WT
RIαB OFF
RIαB ON
***
***
cAMP - + - + - +
F
kDa
PKA RIIα
PKA RIα
β-Actin
PKA Cα
50
50
37
WT RIαB OFF RIαB ON
PKA Subunits
3.0
2.0
1.0
0
**
***
**
RIα
RIIα
Cα

Figure 2.5: Effect of DDAVP administration on urinary concentration and AQP2 phosphorylation and trafficking. (A) Mice were water loaded to suppress endogenous AVP levels and, after 1 hour, injected with vehicle or DDAVP (0.1μg/kg). Urine was collected for 2 hours and osmolality was assessed as described in methods. (B) RIαB OFF and RIαB ON mice were water loaded as in (A), and were euthanized 20 minutes after vehicle or DDAVP injection and AQP2 localization was assessed by immunohistochemistry. (C) Additional groups of mice were treated as in (B), and inner medullae were microdissected and dounce homogenized in RIPA buffer for western blot analysis. A representative western blot and quantitation of total AQP2 and phosphorylated-AQP2 (serine 256) is shown. β-actin was used as a loading control (N=3). (D) Inner medulla lysates were also probed with an antibody to the consensus PKA phosphorylation site (RRX-S/T) in order to assess the general effect of RIαB expression on substrate phosphorylation in the inner medulla. *P<0.05, **P<0.01, ***P<0.001 when comparing RIαB OFF to RIαB ON mice by two-tailed Students t test.

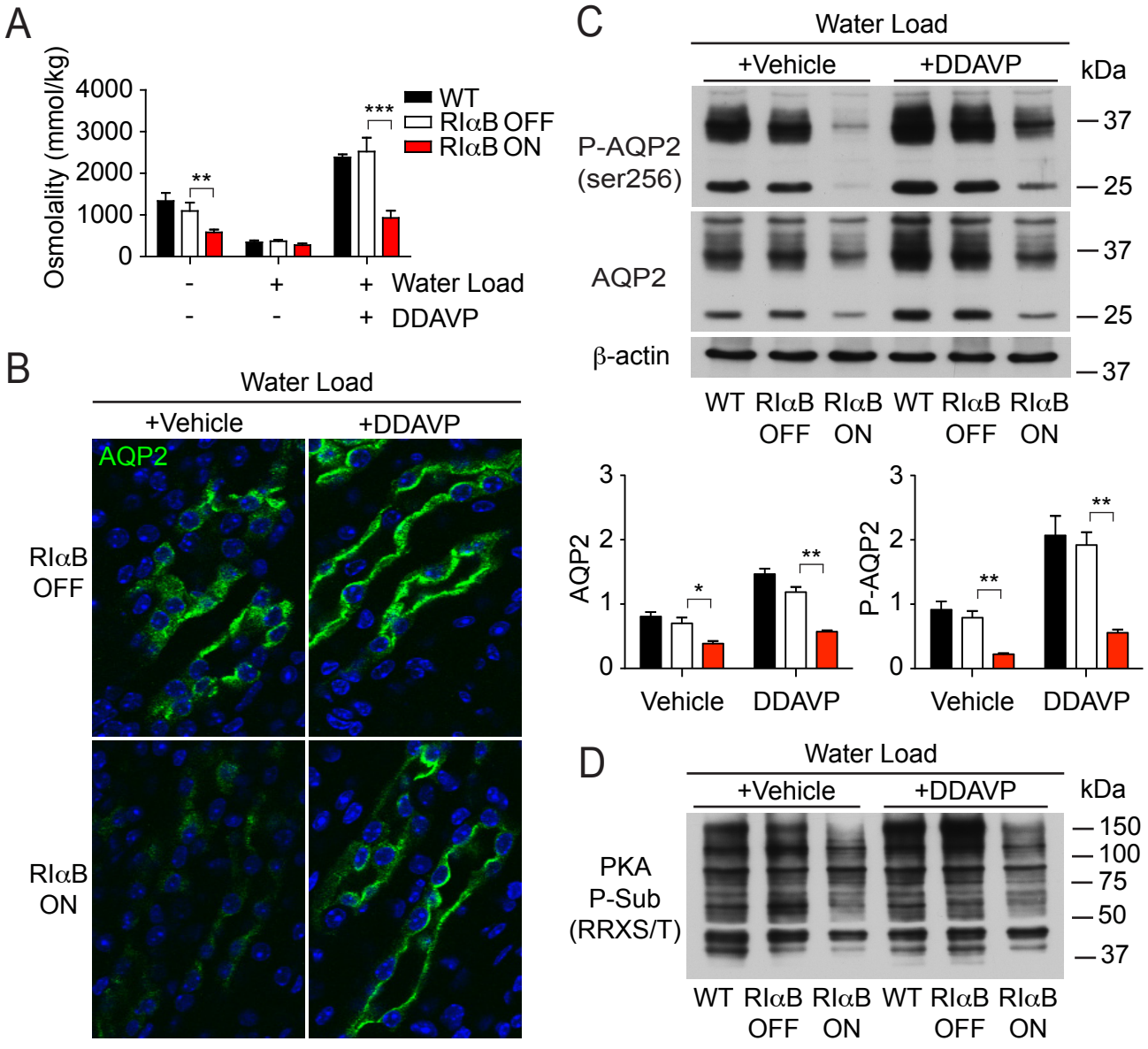
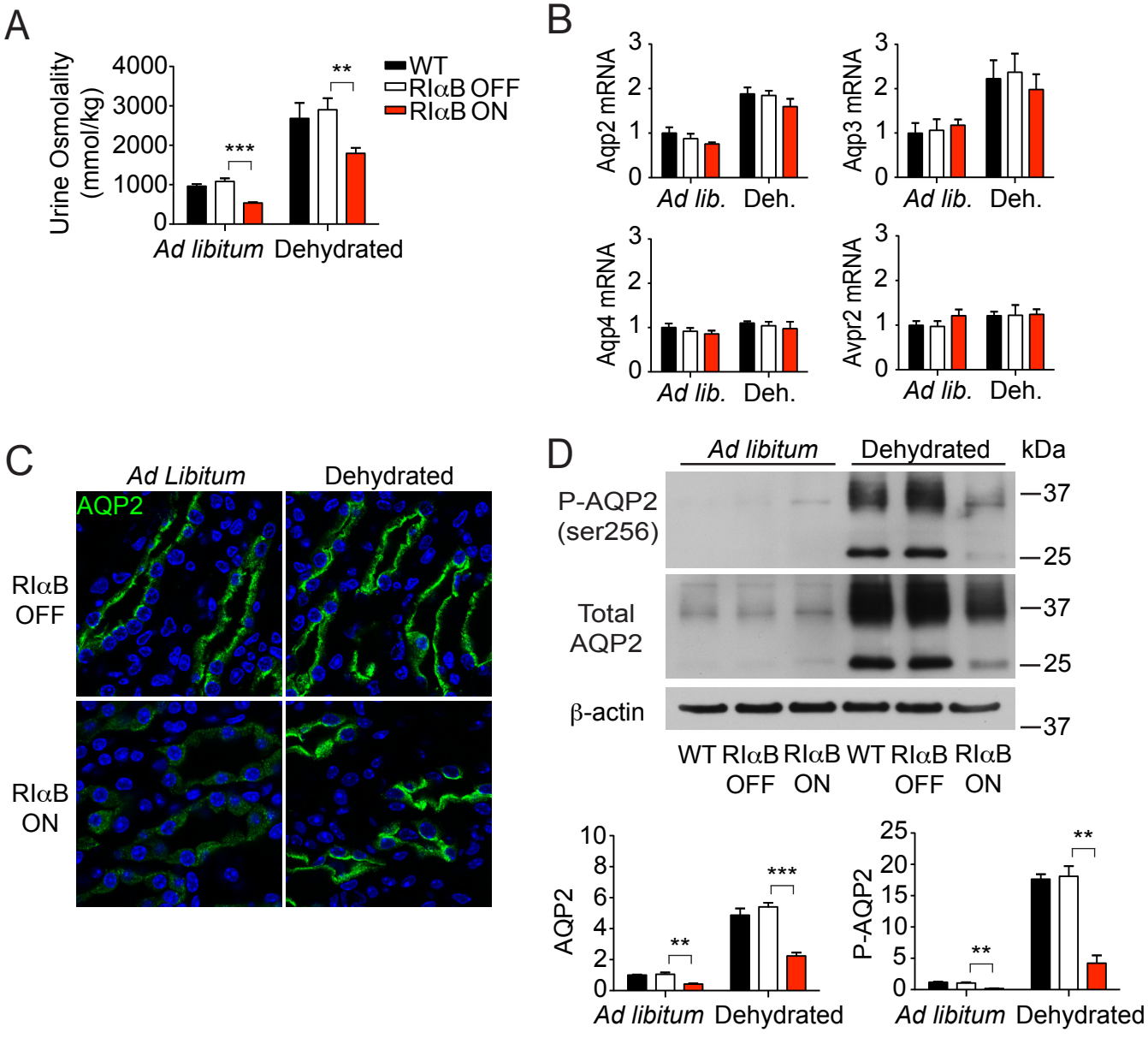

A
Osmolality (mmol/kg)
4000
3000
2000
1000
0
**
***
WT
RIαB OFF
RIαB ON
-
+
+
Water Load
-
-
+
DDAVP
B
Water Load
+Vehicle
+DDAVP
AQP2
RIαB OFF
RIαB ON
C
Water Load
+Vehicle
+DDAVP
kDa
P-AQP2 (ser256)
AQP2
β-actin
37
25
37
25
37
WT
RIαB OFF
RIαB ON
WT
RIαB OFF
RIαB ON
AQP2
P-AQP2
3
2
1
0
*
**
**
**
Vehicle
DDAVP
Vehicle
DDAVP
D
Water Load
+Vehicle
+DDAVP
kDa
PKA P-Sub (RRXS/T)
150
100
75
50
37
WT
RIαB OFF
RIαB ON
WT
RIαB OFF
RIαB ON

Figure 2.6: Effect of 24-hour dehydration on urinary concentration and AQP2 phosphorylation and trafficking. (A) Urine osmolality in control and RIαB ON mice with ad libitum water consumption or after 24-hour dehydration (N=6-8). (B) RT-PCR analysis of water channel (*Aqp2*, *Aqp3*, *Aqp4*) and vasopressin receptor (*Avpr2*) expression in control and RIαB ON mice with or without 24-hour dehydration (N=4 for each group). *Actb* transcript levels were used to ensure equivalent loading of RNA. (C) Total AQP2 expression and localization in RIαB OFF and RIαB ON mice. (D) Representative western blot and quantitation of total AQP2 and phosphorylated AQP2 (serine 256) levels in control and RIαB ON mice with *ad libitum* water consumption or after 24-hour dehydration. β-actin was used as a loading control (N=3). **P<0.01, ***P<0.001 when comparing RIαB OFF to RIαB ON mice by two-tailed Students t test.


A
Urine Osmolality (mmol/kg)
4000
3000
2000
1000
0
***
**
WT
RIαB OFF
RIαB ON
Ad libitum
Dehydrated
B
Aqp2 mRNA
Aqp3 mRNA
Aqp4 mRNA
Avpr2 mRNA
3
2
1
0
Ad lib.
Deh.
C
Ad Libitum
Dehydrated
AQP2
RIαB OFF
RIαB ON
D
Ad libitum
Dehydrated
kDa
P-AQP2 (ser256)
Total AQP2
β-actin
37
25
WT
RIαB OFF
RIαB ON
AQP2
P-AQP2
10
8
6
4
2
0
25
20
15
10
5

Chapter 3

DOCKING/DIMERIZATION DOMAIN MUTATIONS IN RIα DISRUPT PROTEIN TURNOVER AND STABILITY *IN VIVO*

3.1 Abstract

Protein kinase A (PKA) signaling is maintained under strict temporal and spatial control through its interaction with A-Kinase Anchoring Proteins (AKAPs). PKA regulatory subunits form dimers that present a hydrophobic surface that mediates the interaction with the amphipathic helix found in virtually all AKAPs. Although the vast majority of AKAPs identified to date bind RII subunits with low nanomolar affinity, evidence increasingly supports a specific role for targeting of the RIα holoenzyme. In order to assess the role of RIα anchoring *in vivo*, we generated mice with mutations of key hydrophobic residues (V22A, I27A) in the docking/dimerization domain that have previously been shown to disrupt RIα-AKAP interactions *in vitro*. These mutations disrupt RIα binding to the only RI-specific AKAP identified to date, Sphingosine Kinase Interacting Protein (SKIP). Surprisingly, these mutations also result in a marked increase in RIα protein with no corresponding increase in mRNA levels, indicative of altered protein turnover. In MEFs, proteasome inhibition increased wild-type RIα protein levels but had no effect on the mutant subunit while persistent activation of PKA still resulted in degradation of both WT and RIα ALA protein. This stabilization of RIα impaired cAMP-dependent transcriptional responses through decreased CREB phosphorylation, and attenuated induction of the immediate early gene, c-Fos. These results are consistent with a regulated role of RIα degradation in the maintenance of sustained PKA activity, and point to the D/D domain and possibly an AKAP as mediators of RIα turnover *in vivo*.

3.2 Introduction

The PKA holoenzyme is a heterotetramer made up of two regulatory (R) and two catalytic (C) subunits and acts as the principle effector of the intracellular second messenger cAMP. PKA regulates many physiological processes through phosphorylation of a diverse group of proteins that includes receptors, ion channels, metabolic enzymes, cytoskeletal proteins, transcription factors, cell cycle and chromosome-associated proteins. The R subunits (RIα, RIβ, RIIα, RIIβ) are encoded from four separate genes and are not functionally redundant [3]. RII subunits are targeted to membranes through their high affinity interactions with A-kinase Anchoring Proteins (AKAPs); the RI subunits are predominantly cytosolic but can bind AKAPs as well, albeit with greatly reduced affinity when compared to RII subunits [75, 140, 21].

The most clearly defined role for RIα lies in its ability to buffer cytosolic PKA activity. The critical impact of this buffering capacity is apparent as genetic disruption of RIα leads to early embryonic lethality in mice due to unregulated PKA activity and impaired cardiac development[4]. Previously, we demonstrated that RIα protein levels are commensurate with the amount of free C subunit that is available in molar excess of RII subunits, as the Type II holoenzyme forms preferentially *in vivo*. Overexpression of RIIα results in a complete loss of RIα protein, ostensibly due to turnover of the unbound RI subunit, while increased Cα levels lead to a stabilization of RIα in the intact holoenzyme[36, 124]. In tissues where the PKA is mainly comprised of RII holoenzyme, such as in adipose tissue, genetic disruption of RIIβ leads to post-translational stabilization of RIα and a complete isozyme switch[39].

The growing family of AKAPs has expanded to include scaffolding proteins that target the RI holoenzyme to distinct subcellular locations. Dual-specific AKAPs that bind both RI and RII have been found to anchor PKA at mitochondria [76, 170], the cap site of activated T lymphocytes [136], and on lipid droplets in adipocytes[125]. More recently, an RI-specific AKAP, SKIP, was also found to target RIα to mitochondria and this novel AKAP sequesters two molecules of RIα to facilitate phosphorylation of the coiled-coil protein ChChD3[109].

In order to assess the role of RIα anchoring *in vivo*, we generated mice with targeted

alanine substitutions of key hydrophobic residues in the docking and dimerization (D/D) domain. These mutations (I22A, V27A) were previously shown to disrupt the interaction between RIα and two dual-specific AKAPs, AKAP1 and AKAP10 [74, 75]. While highly effective in disrupting RIα-AKAP interactions, these mutations also induced a marked increase in RIα protein that occurred in the absence of changes in mRNA levels, suggesting post-translational effects. Stabilization of the RIα ALA mutant impaired CREB phosphorylation and induction of the IEG c-Fos in both mouse embryo fibroblasts (MEFs) and in the striatum. These findings are consistent with a role for the D/D domain in regulating turnover of the RIα protein and suggest that post-translational regulation of this R subunit may impact cAMP-dependent transcriptional regulation *in vivo*.

3.3 Experimental Procedures

Generation of RIα ALA mice. A 1.8 kb 129svJ mouse RIα genomic fragment containing exon 2 was used to generate the RIα ALA targeting construct. Exon 2 encodes the entire D/D domain and site-directed mutagenesis was performed to introduce two single point mutations. This genomic fragment was then subcloned into a Litmus38 vector and linearized prior to electroporation into ES cells, which were subsequently grown under double selection in G418 and ganciclovir. Resistant clones were screened for homologous recombinants by PCR-based genotyping, and positively targeted clones were expanded, injected into C57BL/6 blastocysts, and subsequently implanted into pseudopregnant females. The resulting male chimeras were mated with C57BL/6 females and offspring were genotyped by PCR analysis (Fig 1B). Heterozygotes were then back-crossed onto C57BL/6 mice for seven generations and then crossed to yield WT and RIα ALA homozygous littermates. All protocols described here were approved by the University of Washington Institutional Animal Care and Use Committee.

Genotyping. Tail DNA was isolated by phenol-chloroform extraction followed by subsequent ethanol precipitation. Samples were analyzed by PCR using DreamTaq polymerase (Fermentas) using the following primers: 5-AGAACCATGGCGTCTGGCAGT-3 (Forward) and 5-CTTCTCCAACCTCTCAAAGTA-3 (Reverse). Primers were designed to amplify a 183 bp fragment of DNA that contained both point mutations. The V22A mutation intro-

duced an Fsp1 restriction site that was used to differentiate between the WT and RIα ALA alleles.

Plasmids. Full length SKIP was cloned into pcDNA3 with an N-terminal FLAG epitope. V5- tagged RIα was cloned into pRetroQ-mCherry (Clontech) and alanine substitutions were introduced by site-directed mutagenesis with QuikChange Lightning Kit (Stratagene).

Cell culture and transfection. HEK293 and MEFs were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin. HEK293 Cells were transiently transfected with plasmids for 48 hours using Transit-LT1 (Mirus).

Immunoprecipitations. Cells were washed once in ice-cold PBS and then scraped into 1 mL phosphate lysis supplemented with protease inhibitors (150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, pH 7.4). Cell lysates were cleared at 16,000 x g for 15 minutes and supernatants protein levels were determined by BCA assay (Pierce). For immunoprecipitation, equal amounts of protein were incubated with V5 antibody and Protein A/G beads (Pierce) overnight at 4 °C. Beads were washed four times with ice-cold lysis buffer, resuspended in NuPAGE sample buffer (Invitrogen), and heated to 95 °C prior to loading on agarose gels.

Generation and culture of primary embryonic fibroblasts. Heterozygous (*Prkar1a* +/ALA) mice were bred to generate wild type and RIα ALA embryos in single litters. Embryos (E8.5) were placed in ice-cold PBS and dissected out from the decidua and Reichardt′s membrane was removed along with the yolk sacs. Prior to dissociation of each embryo, a small tail biopsy was taken for later genotyping. Embryos were placed in the tip of a 1-mL syringe in PBS and then triturated with a 20-gauge needle. Dissociated embryos were placed into individual 10 cm dishes and cultured with 10% FBS/DMEM in a 5% CO2 incubator at 37 °C.

Locomotor activity. Mice, at 8-10 weeks of age, were placed in a plastic activity chamber (47 x 25 x 21 cm) and locomotor activity was assessed using a photobeam apparatus (San Diego Instruments). Ambulations were scored as the interruption of consecutive beams on the apparatus and data from each experimental animal was collected over a 72-hour period.

Immunohistochemistry. After CO_2 euthanasia, mice were perfused transcardially with PBS followed by PBS-buffered 4% paraformaldehyde solution. Brains were removed, post-

fixed for 2-4 hours in PBS-paraformaldehyde, and then cryopreserved overnight in a 30% sucrose solution (wt/vol). After embedding in Tissue-Tek OCT compound (Sakura Finetek), 20 μm cryosections were taken and processed for immunohistochemistry. Sections were washed in PBS, incubated in blocking buffer (10% normal goat serum, 0.2% Triton X-100, and 2% BSA in PBS), and then incubated for 1 hour at room temperature. Primary antibodies (1:250) were diluted into blocking buffer and sections were incubated at 4 °C overnight. After rinsing with PBS-T (PBS with 0.2% Triton X-100), sections were incubated for two hours at room temperature with fluorescence-labeled secondary antibodies (Molecular Probes, 1:500) diluted in blocking buffer. Sections were washed in PBS-T and mounted with ProLong Gold (Invitrogen) medium for imaging on a Zeiss LSM510 microscope. All images were taken at the Keck Microscopy Facility (University of Washington).

Protein sample preparation and western blotting. After CO_2 euthanasia, tissues were removed dounce homogenized in RIPA buffer (5% wt/vol) supplemented with protease and phosphatase inhibitor cocktails (Sigma). Alternatively, MEFs were scraped into RIPA buffer and vortexed followed by brief sonication (Branson Sonifier). Samples were cleared at 10,000 x g for 10 minutes at 4 °C and supernatant protein concentration was determined by BCA assay (Pierce). Protein samples were diluted in 4X NuPAGE sample buffer (Invitrogen), resolved on 12-15% polyacrylamide gels, and transferred to a nitrocellulose membrane (Whatman). Membranes were blocked for 1 hour in 5% BSA or milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T), and incubated overnight in primary antibody. Membranes were then rinsed with TBS-T and incubated with HRP-conjugated secondary antibodies (Jackson ImmunoResearch) diluted at 1:10,000 in TBS-T with 5% BSA or milk. HRP was detected with Supersignal West Pico chemiluminescent substrate (Pierce).

Kinase activity measurements. A PKA activity dose response curve was calculated through phosphorylation of a PKA substrate peptide (Kemptide, LRRASLG) using ATP-[γ-P32]. Brain samples were collected and dounce homogenized in kinase activity buffer (in mM: Tris 20, EDTA 0.1, EGTA 0.5, DTT 10, magnesium acetate 5, sucrose 250; pH 7.6 with 1% Triton-X 100), supplemented with 1 μg/ml leupeptin, 3 μg/ml aprotinin, and 5 mM AEBSF. Counts per minute were converted to total enzymatic activity and are expressed as picomoles of phosphate transferred (units-U) per milligram of input protein. Activity

measured in the presence of 4 μg/ml Protein Kinase Inhibitor (PKI) was assumed to be non-specific and counts were subtracted from each sample as background.

Real-time quantitative RT-PCR. Whole brain or MEF total RNA was extracted using Trizol reagent (Invitrogen) as per the manufacturers protocol. One-step RT-PCR was performed using the Mx3000P QPCR system and Brilliant II QRT-PCR reagent kit (Agilent). Relative amounts of transcripts from cells and tissues were extrapolated from a four-point standard curve (100, 10, 1, 0.1 ng total RNA) made from a common brain or MEF RNA sample.

Primary Antibodies. All antibodies were used at 1:1000 for western blotting and 1:250-500 for immunohistochemistry. Antibodies purchased were: PKA Cα, RIα, and RIIβ subunits (Mouse, BD Biosciences), PKA RIIα (Rabbit, Santa Cruz), β-actin (Mouse, Sigma), FLAG and FLAG agarose (Sigma), and V5 (Invitrogen).

Data analysis. Data are presented as means ± SEM and statistical significance was determined by a two-tailed Students t test between WT (*Prkar1a* +/+) and RIα ALA (*Prkar1a* ALA/ALA) mice or MEFs. Analyses were carried out using Prism 4.0c for the Mac OS (Graphpad Software).

3.4 Results

Generation and genotyping of RIα ALA mice. The primary physiological role of RIα lies in its ability to buffer PKA activity and retain the kinase under cAMP-dependent control. To date, specific functional roles for RIα-AKAP interactions have been assessed through the use of cell permeable peptides that specifically disrupt anchoring of the Type I kinase[25]. In order to assess the role of anchored RIα *in vivo*, we generated mice harboring two alanine substitutions in the D/D domain that were originally shown to abrogate anchoring to dual-specific AKAPs [74, 9]. RIα-anchoring deficient mice were produced as described in the methods section using a targeting vector that replaces two nucleotides within the first coding exon of the *Prkar1a* gene (Fig. 3.1A). WT and homozygous littermates were identified by PCR amplification of a sequence of DNA that spanned both mutations followed by restriction digest analysis of the PCR products (Fig. 3.1B). RIα ALA animals are fertile and healthy and exhibit no obvious developmental defects.

Alanine mutations disrupt RIα interactions with SKIP. Most RI-binding AKAPs are dual-specific and have lower binding affinity for RI compared to RII. To investigate the effects of the dual V22A and I27A mutations, we used an overexpression system to assess the ability of the only RI-selective AKAP, SKIP, to interact with WT and mutant RIα. FLAG epitope-tagged SKIP and either WT or RIα ALA forms of V5 epitope-tagged RIα were co-transfected into HEK293 cells. As shown in Fig. 1C, V5 immune complexes were isolated from cells expressing WT RIα or RIα ALA and copurification of FLAG-SKIP was determined by western blotting. SKIP was readily detected when WT RIα was used coprecipitate the AKAP (Fig. 3.1C, lane 2), but absent when control IgG was used in place of the V5 antibody (Fig. 3.1C, lane 1). RIα ALA displayed virtually no ability to coprecipitate SKIP under the same conditions (Fig. 3.1C, lane 4), indicating the effectiveness of thse alanine substitutions in disrupting RIα-AKAP interactions

RIα ALA displays post-translational stabilization in MEFs. As alanine substitutions in the D/D are effective at abrogating RIα-SKIP interactions, we next decided to assess the *in vivo* role of endogenously expressed RIα ALA. Surprisingly, RIα protein levels were markedly increased in homozygous MEFs, while an intermediate effect was observed in cells that were heterozygous for the RIα ALA mutation (Fig. 3.2A). We previously demonstrated that the RII holoenzyme preferentially forms in cells [124] and the amount of free C subunit remaining determines RIα protein levels through its post-translational stabilization in the intact holoenzyme [2]. RIα also compensates for the loss of other R subunits *in vivo*, and there are no corresponding changes in RIα mRNA. Despite elevated RIα protein levels in the presence of D/D mutations, there was no observable increase in Cα that could drive stabilization of RIα, while RIIα levels were stable across the three genotypes (Fig. 3.2A). PKA subunit mRNA levels were assessed in WT and RIα ALA fibroblasts by quantitative real-time RT-PCR, and the lack of change in any of the subunits confirmed that the elevated RIα was post-translational in nature (Fig. 3.2B). Previous reports by Schwartz and colleagues suggested that post-translational stability of the RIα homolog in *Aplysia californica* was regulated through the ubiquitin-proteasome system[54, 62]. We inhibited the proteasome with cell permeable aldehyde inhibitor, MG132, to assess its impact on RIα protein stability in fibroblasts. As shown in Fig. 2C, WT RIα protein levels gradually increased through-

out the time course of treatment with 10 μM MG132; the known cytotoxic effects of this compound prevented extended observation. In contrast, RIα ALA protein was unaffected by MG132 treatment (Fig. 3.2C) with protein levels remaining constant throughout. These results suggest that the D/D plays a role in the targeting RIα for proteasome-mediated degradation and thus regulating the post-translational stability of RIα protein.

Stabilization of RIα impairs cAMP-dependent transcriptional regulation. PKA-dependent transcriptional regulation is limited by the passive diffusion of the C subunit into the nucleus where it phosphorylates the nuclear transcription factor CREB[57, 171]. Inhibition of PKA or proteasome-mediated degradation of R subunits was previously shown to attenuate CREB-dependent transcriptional activity and the expression of immediate early genes (IEG) in support of LTF[30, 98]. WT and RIα ALA fibroblasts were treated with 1 μM forskolin (FSK) and assessed at varying time points for CREB phosphorylation and induction of the c-Fos, a well-characterized representative of IEGs. As expected, FSK treatment of WT MEFs resulted in increased CREB phosphorylation (ser133) over the first 30 minutes with a gradual decrease observed over the subsequent 90 minutes (Fig. 3.3A), due to enhanced phosphatase activity[56]. The RIα ALA response to FSK treatment followed the same general trend, however peak phosphorylation of CREB was attenuated and returned to basal levels by 60 minutes (Fig. 3A). In parallel experiments, we assessed the induction of c-Fos mRNA in response to FSK treatment by quantitative RT-PCR. As shown in Fig. 3.3B, the total induction of c-Fos mRNA is reduced after 60 minutes in cells expressing RIα ALA protein. These results suggest that stabilization of RIα through D/D mutations negatively impacts PKA- and CREB-dependent transcriptional regulation.

D/D mutations stabilize RIα in brain without corresponding increases in C subunit. Although the alanine mutations stabilize RIα in fibroblasts, we next wanted to assess RIα levels in tissues where there is greater variation in PKA R subunit expression. In brain regions where RIIβ is the major isoform, genetic disruption of this subunit results in defective gene expression and associated behavioral changes that cannot fully be compensated for by RIα[1, 18]. We assessed general expression levels of RIα by immunohistochemistry and PKA subunit levels by western blotting. As shown in Fig. 3.3A, RIα protein levels are increased throughout all brain regions analyzed, with higher expression observed in cell

bodies of the hippocampus and cerebellum. Western blots of whole brain lysates revealed a striking increase in RIα protein and a concomitant minor increase in Cα levels (Fig. 3.3B). Expression levels of the RIβ and RIIβ subunits were unchanged (Fig. 3.3B). RIα mRNA expression levels were reduced to a greater extent in brain when compared to fibroblasts (Fig. 3.3C). Consistent with the increase in Cα protein levels, maximal PKA activity was slightly elevated and there was a slight shift in the activation of PKA in the RIα ALA compared to WT (Fig. 3.3D). Post-translational stabilization of RIα thus has modest effects on C subunit levels in brain, resulting in slight increases in total PKA activity.

RIα ALA mice display impaired locomotor activity. Alterations in PKA activity have been shown to have profound affects on behavioral processes in mice. We previously demonstrated that mice lacking the RIIβ subunit are hyperactive, while they also lack they typical cataleptic response to treatment with the D2 receptor antagonist haloperidol [1, 18]. In order to assess the effects of RIα stabilization on general behaviors in mice, we first measured locomotor activity in WT and RIα ALA littermates. Male and female mice at 8-10 weeks of age were placed in a plastic activity cage and total ambulations were counted by a photobeam apparatus as previously described[181]. Ambulations were tallied over a 72-hour period with light and dark cycles indicated by the white and black boxes, respectively (Fig. 3.5A). Both male and female mice displayed significantly reduced locomotor activity over the 72-hour testing period when total ambulations are summed over that period (Fig. 3.5B). These data are consistent with previous work in our lab demonstrating that altered neuronal PKA activity can have behavioral effects in mice.

Stabilization of RIα attenuates the response to a D2R antagonist. cAMP-dependent transcriptional regulation in the striatum is greatly impacted by the loss of RIIβ, as shown by an attenuated response to D1R activation or D2R inhibition and the induction of *dynorphin*, *neurotensin*, and *c-Fos* transcripts [1, 18]. Inhibition of the G_i-coupled D2R with haloperidol is thought to increase cAMP levels and drive induction of c-Fos and neurotensin in striatum. Based on the effects we observed in MEFs with regard to CREB phosphorylation and c-Fos induction, we next wanted to assess the impact of RIα stabilization on the response to D2R antagonism in WT and RIα ALA mice. Age-matched male mice were given a single injection of vehicle or the D2R antagonist haloperidol (2 mg/kg IP) euthanized after 2 hours, and

then processed for immunohistochemistry. With vehicle injection, few c-Fos positive cells were evident in the striatum of WT and RIα ALA mice. However, 2 hours after haloperidol treatment, numerous c-Fos positive MSNs were observed in WT mice, particularly in the dorsal lateral striatum (Fig. 3.6). In contrast, few MSNs in RIα ALA striatum displayed elevation of c-Fos levels with only a few scattered cells showing c-Fos positive staining.

3.5 Discussion

In this study, we demonstrate that mutations within the D/D domain of RIα originally intended to disrupt AKAP binding also result in marked increases in RIα protein *in vivo*. Furthermore, the enhanced RIα expression occurs in the absence of increased mRNA levels, indicating that post-translational stabilization is occurring as a result of the alanine substitutions. Interestingly, increased RIα levels persist in MEFs and whole brain lysates in the absence of concordant changes in C subunit. We previously demonstrated that RIα protein is more stable when bound to C subunit while the protein half-life fluctuates with the amount free C subunit available for binding [2, 165]. RI and RII have similar binding affinities for C subunit *in vitro*[69], however the Type II holoenzyme preferentially forms *in vivo*[124]. This likely means that the excess RIα that is present in the RIα ALA mutant is not bound to C subunit and yet is still not degraded at a rate comparable to the WT protein.

Although not addressed in this study, AKAP-mediated interactions have been suggested to play a role in the regulated degradation of both RI and RII subunits[98]. There is considerable evidence supporting a role for ubiquitin-mediated degradation of R subunits in *Aplysia*, however the data supporting such a role in mammalian PKA signaling is less complete[62]. This degradation is thought to alleviate inhibition of C subunits, thus prolonging persistent PKA activity during transient increases in cAMP generation. The RIα stabilization that we have observed in this study recapitulates the key findings of Schwartz and colleagues in that sustained RIα levels are sufficient to attenuate CREB phosphorylation as well as subsequent induction of the immediate early gene, c-fos (Fig.3.3A). While we did measure a significant increase in RIα protein in the presence of proteasome inhibition, there was no characteristic laddering on our western blots that would indicate poly-ubiquinated

RIα and subsequent degradation products (Fig. 3.2C).

The striking effects on locomotor activity and c-Fos induction strongly suggest a more detailed behavioral analysis is required for the RIα ALA mice. Genetic disruption of the RIIβ subunit gives rise to behavioral defects and altered gene expression in brain regions such as the striatum. We previously demonstrated that RIα could not fully compensate for the loss of RIIβ as striatum lysates showed a 75% reduction in total kinase activity [18]. The RIIβ knockout mouse is hyperactive and resistant to haloperidol-induced catalepsy while the RIα ALA mice have decreased locomotor activity and also display altered gene induction (Fig. 3.5B and 3.6). Thus the increased RIα we observed in brain (approx. 15-fold) is likely sufficient to disrupt PKA-dependent transcriptional changes that support behavioral modification in the mouse. In the future, it will also be interesting to assess the phosphorylation status of additional PKA substrate proteins. RIα would be required in large molar excess of cAMP to impact global PKA activity in RIα ALA tissues. Clegg and colleagues demonstrated that a 60-fold increase in WT RIα inhibited PKA activity at lower concentrations of cAMP, but had no effect at maximal kinase activation. If subcellular localization is consistent between WT and RIα ALA protein, one might predict that compartmentalized cAMP generation would lead to discrete effects on phosphorylation status among the many PKA substrates. Future studies with these mice should inform us as to RIα-AKAP interaction and post-translational stability of the RIα subunit.

3.6 Acknowledgements

We would like to thank Paul Amieux for helpful discussions of this work and his assistance in generating mouse embryonic fibroblasts. This research was supported by National Institutes of Health grant GM032875 (to G.S.M). M.L.G. was supported by the Pharmaceutical Sciences Training Grant (5T32GM007750-03) at the University of Washington.

Figure 3.1: Generation of RIα ALA mice and disruption of RIα-SKIP interactions. (A) Restriction map of a 1.8 kb fragment of the RIα genomic sequence including exon 2 and the full D/D domain that was subcloned into Litmus38. Mutations used to generate alanine substitutions at Val22 and Ile27 are marked with an asterisk; the Fsp1 restriction site that was gained with mutant RIα is marked with an arrowhead. (B) Genotyping of RIα ALA mice displaying the PCR product before and after Fsp1 treatment. The 183 bp PCR product is cut into 100 bp and 83 bp fragments. (C) HEK293 cells were transfected with vectors encoding FLAG-SKIP and either WT or mutant V5-RIα. V5 immune complexes from HEK293 cells were immunoblotted with antibodies against FLAG (Top) and V5 (Second). Input lysates are shown in the bottom panels.

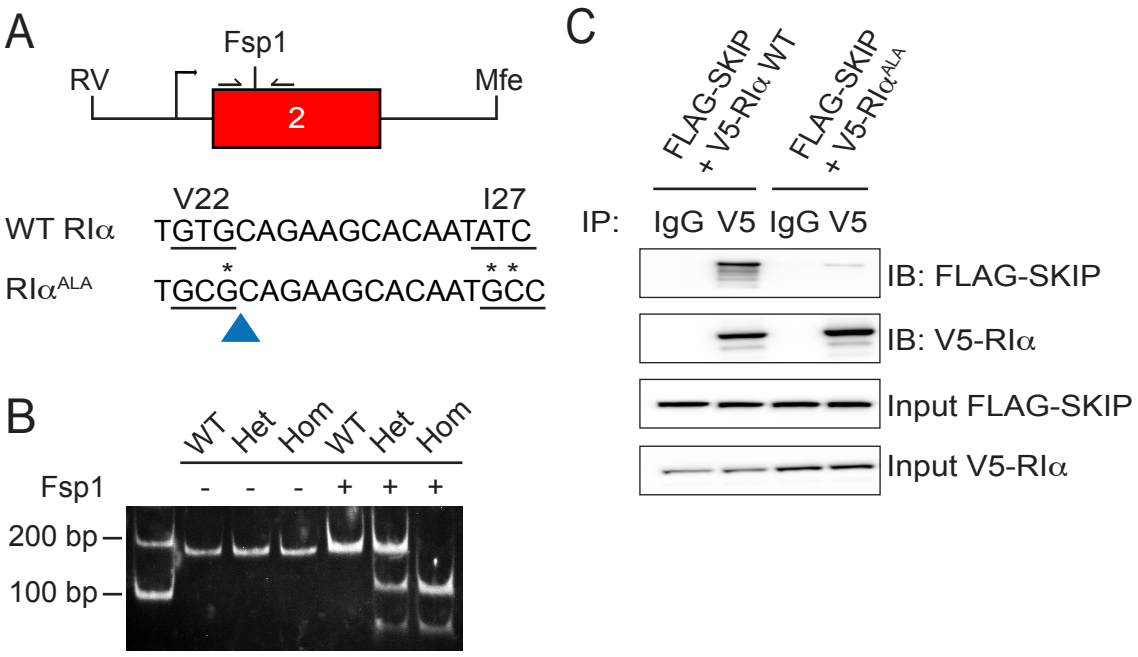

A
Fsp1
RV
Mfe
2
V22
I27
WT RIα
TGTGCAGAAGCACAATATC
RIα^ALA
TGCGCAGAAGCACAATGCC
B
WT
Het
Hom
WT
Het
Hom
Fsp1
- - - + + +
200 bp
100 bp
C
FLAG-SKIP + V5-RIα WT
FLAG-SKIP + V5-RIα^ALA
IP:
IgG V5 IgG V5
IB: FLAG-SKIP
IB: V5-RIα
Input FLAG-SKIP
Input V5-RIα

Figure 3.2: PKA subunit expression and proteasome regulation in MEFs. (A) Representative western blot and quantitation of PKA subunit levels in WT MEFs and MEFs that are heterozygous or homozygous for the RIα ALA mutation. RIIα levels were used to control for equal loading of protein (N=4). (B) Total *Prkar1a* mRNA levels in WT and RIα ALA MEFs (N=4). *Actb* transcript levels were used to ensure equivalent loading of RNA. (C) WT and RIα ALA MEFs were treated with 10 μM MG132 for the times indicated and RIα protein levels were assessed by immunoblotting. RIα quantitation is displayed as an increase over levels measure at T=0. β-actin was used as a loading control (N=3). *P<0.05, **P<0.01, ***P<0.001 when comparing WT and RIα ALA samples by two-tailed Students t test.

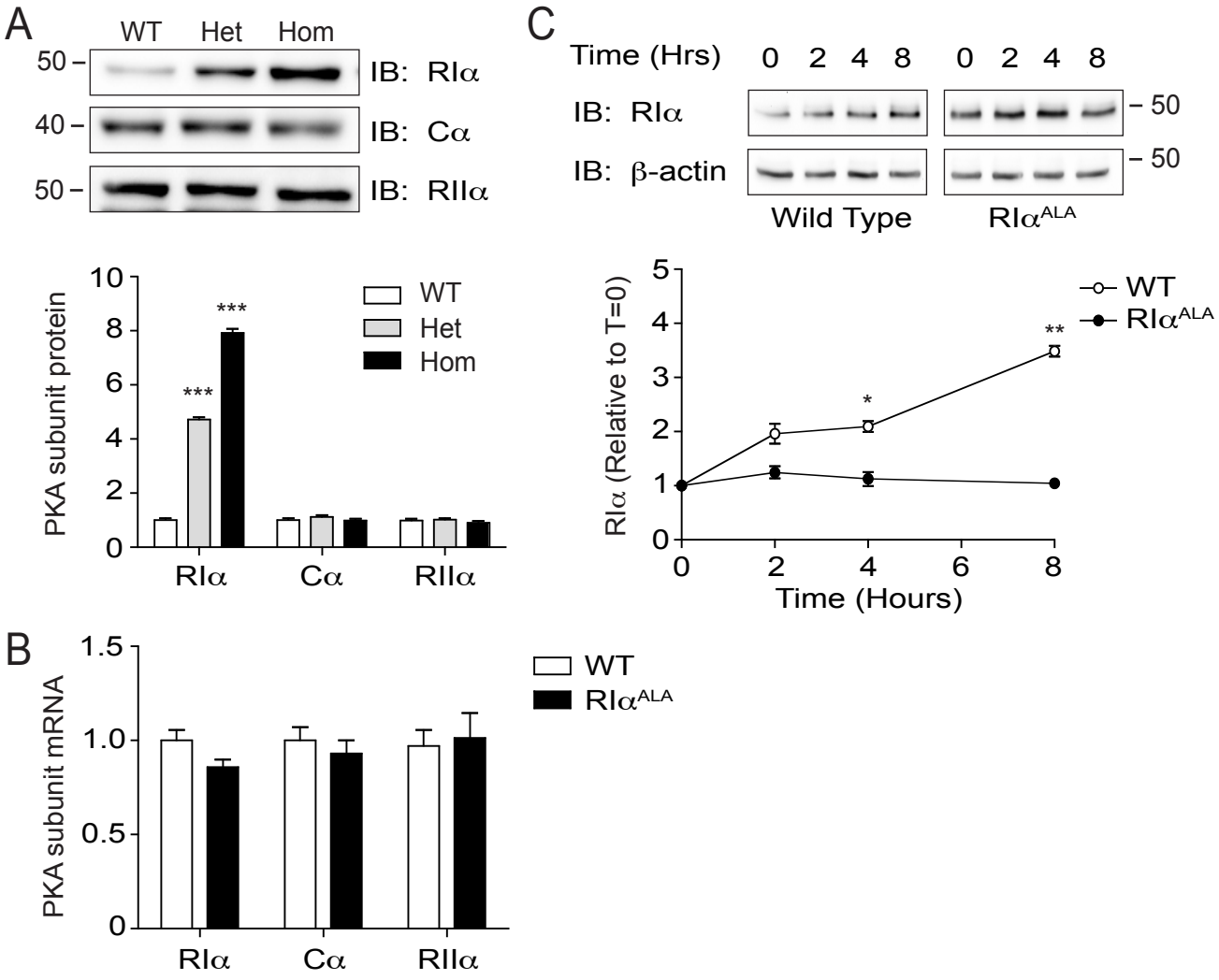

A
WT
Het
Hom
50
IB: RIα
40
IB: Cα
50
IB: RIIα
PKA subunit protein
10
8
6
4
2
0
***
***
WT
Het
Hom
RIα
Cα
RIIα
B
PKA subunit mRNA
1.5
1.0
0.5
0
WT
RIα^ALA
RIα
Cα
RIIα
C
Time (Hrs)
0 2 4 8 0 2 4 8
IB: RIα
IB: β-actin
50
50
Wild Type
RIα^ALA
RIα (Relative to T=0)
5
4
3
2
1
0
*
**
WT
RIα^ALA
0
2
4
6
8
Time (Hours)

Figure 3.3: Effect of FSK on CREB phosphorylation and IEG induction in MEFs. (A) WT and RIα ALA MEFs were treated with 1 μM FSK for the times indicated and phosphorylated CREB (ser133) was assessed using specific antisera. β-tubulin was used as a loading control (N=3). (B) WT and RIα ALA MEFs were treated as in (A) for the times indicated and then total RNA was isolated as described in methods. Induction of c-fos mRNA was assessed by RT-PCR (N=3). *Actb* transcript levels were used to ensure equivalent loading of RNA. *P<0.05, **P<0.01 when comparing WT and RIα ALA samples by two-tailed Students t test.

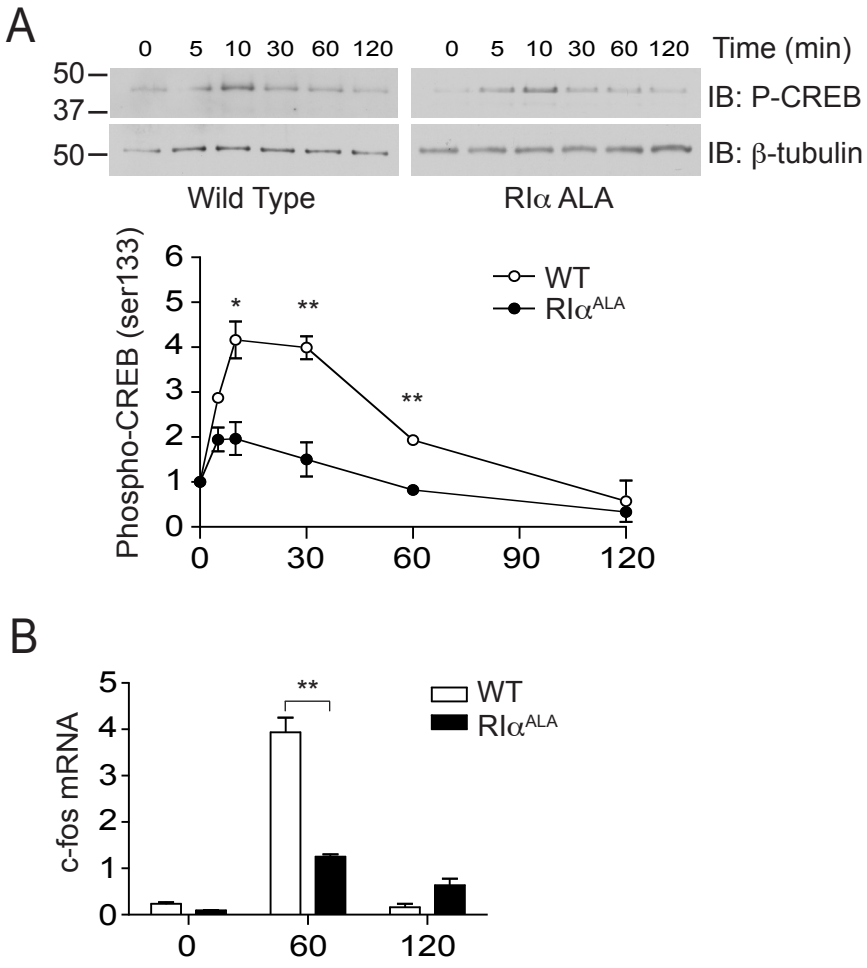

A
0 5 10 30 60 120
0 5 10 30 60 120
Time (min)
50
37
50
IB: P-CREB
IB: β-tubulin
Wild Type
RIα ALA
Phospho-CREB (ser133)
WT
RIα^ALA
*
**
**
0 30 60 90 120
B
c-fos mRNA
**
WT
RIα^ALA
0 60 120

Figure 3.4: PKA subunit expression in WT and RIα ALA brain. (A) Immunohistochemistry for RIα expression in brain regions as indicated. (B) Representative western blot and quantitation of PKA subunit expression in whole brain lysates from WT and RIα ALA mice. β-tubulin was used as a loading control (N=4). (C) Total *Prkar1a* mRNA levels in whole brain from WT and RIα ALA mice (N=4). *Actb* transcript levels were used to ensure equivalent loading of RNA. (D) PKA activation in WT and RIα ALA brain lysates as assessed by cAMP dose response curve and phosphorylation of a PKA substrate peptide (N=3). *P<0.05, **P<0.01 when comparing WT and RIα ALA samples by two-tailed Students t test.

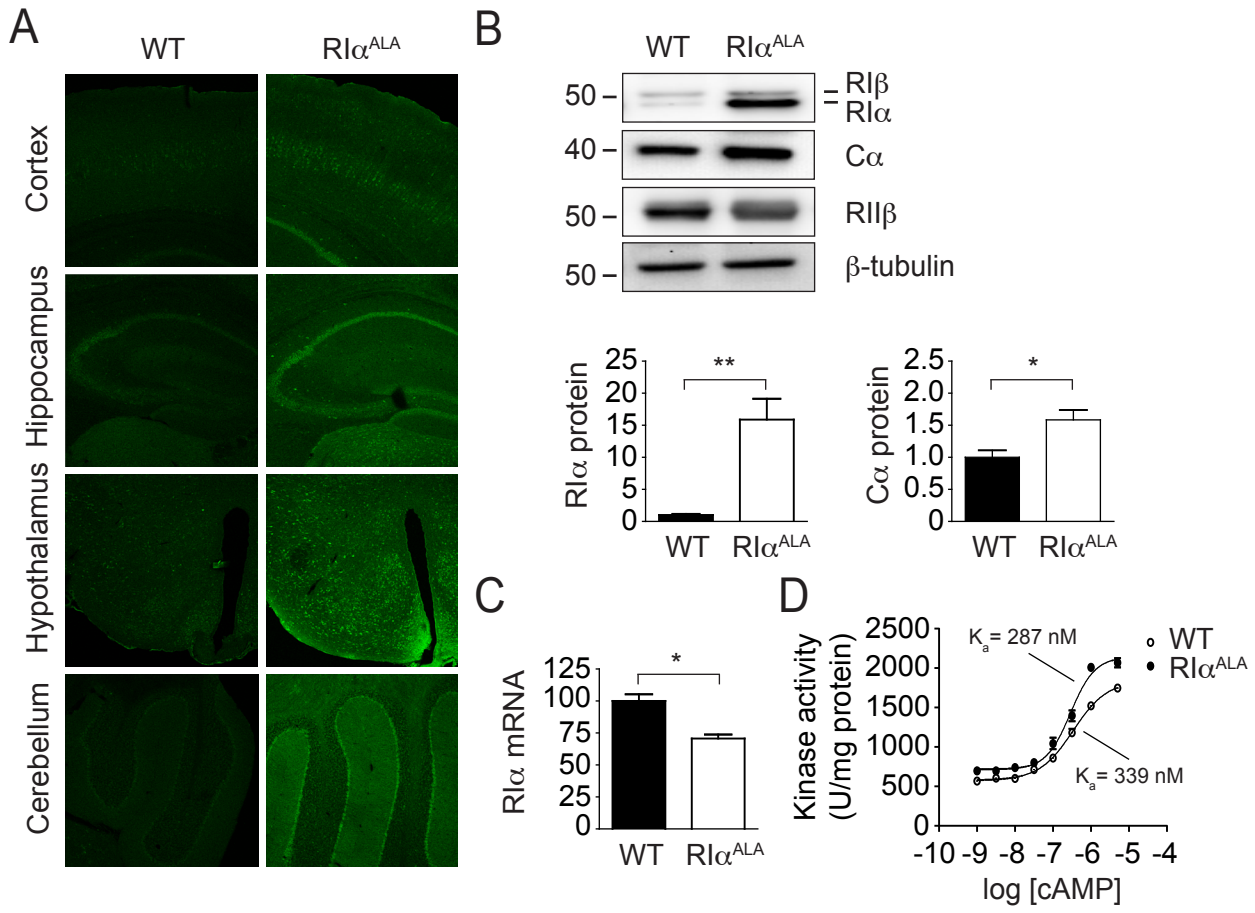

A
WT
RIα^ALA
Cortex
Hippocampus
Hypothalamus
Cerebellum
B
WT
RIα^ALA
50 –
40 –
50 –
50 –
RIβ
RIα
Cα
RIIβ
β-tubulin
RIα protein
25
20
15
10
5
0
**
WT
RIα^ALA
Cα protein
2.5
2.0
1.5
1.0
0.5
0
*
WT
RIα^ALA
C
RIα mRNA
125
100
75
50
25
0
*
WT
RIα^ALA
D
Kinase activity (U/mg protein)
2500
2000
1500
1000
500
0
K_a = 287 nM
K_a = 339 nM
WT
RIα^ALA
-10 -9 -8 -7 -6 -5 -4
log [cAMP]

Figure 3.5: Locomotor activity is impaired in RIα ALA mice. (A) Locomotor activity traces and (B) total ambulations in male and female mice. Black and white boxes above the graphs in (A) indicate dark and light cycles respectively. **P$<$0.01 when comparing WT and RIα ALA mice by two-tailed Students t test.

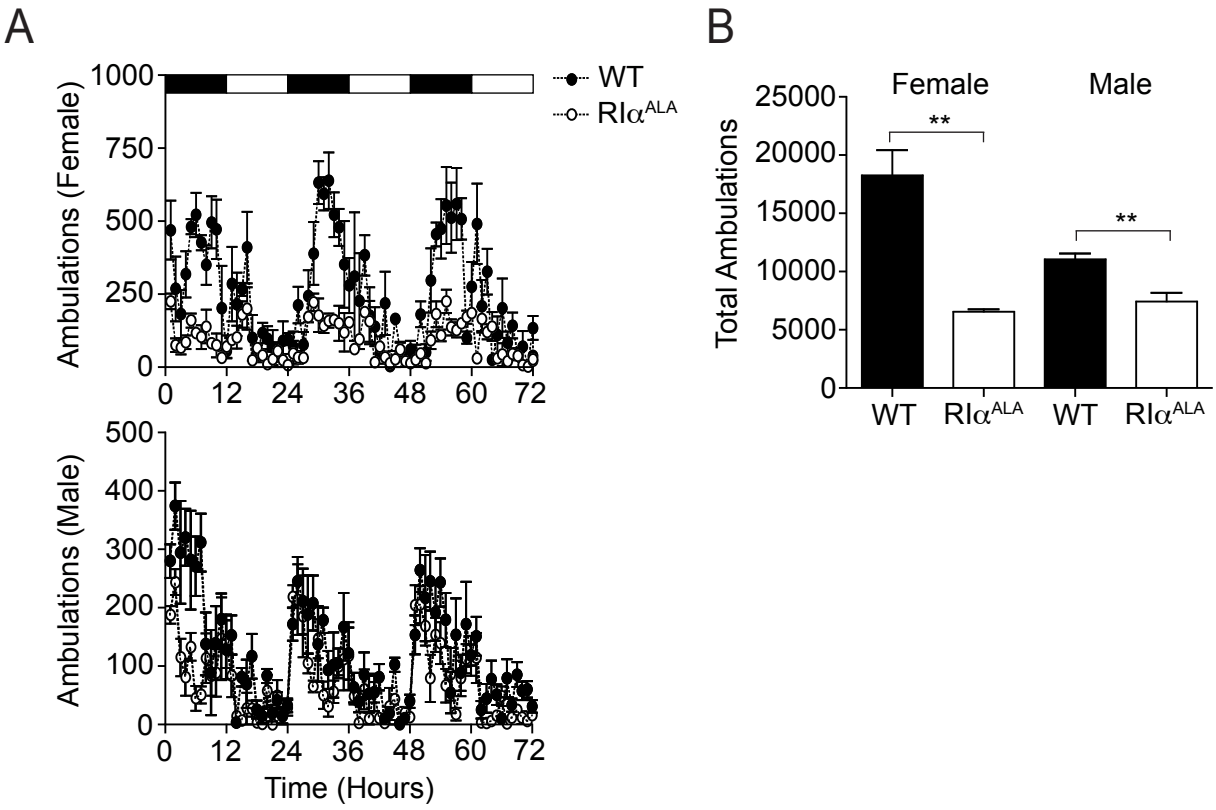
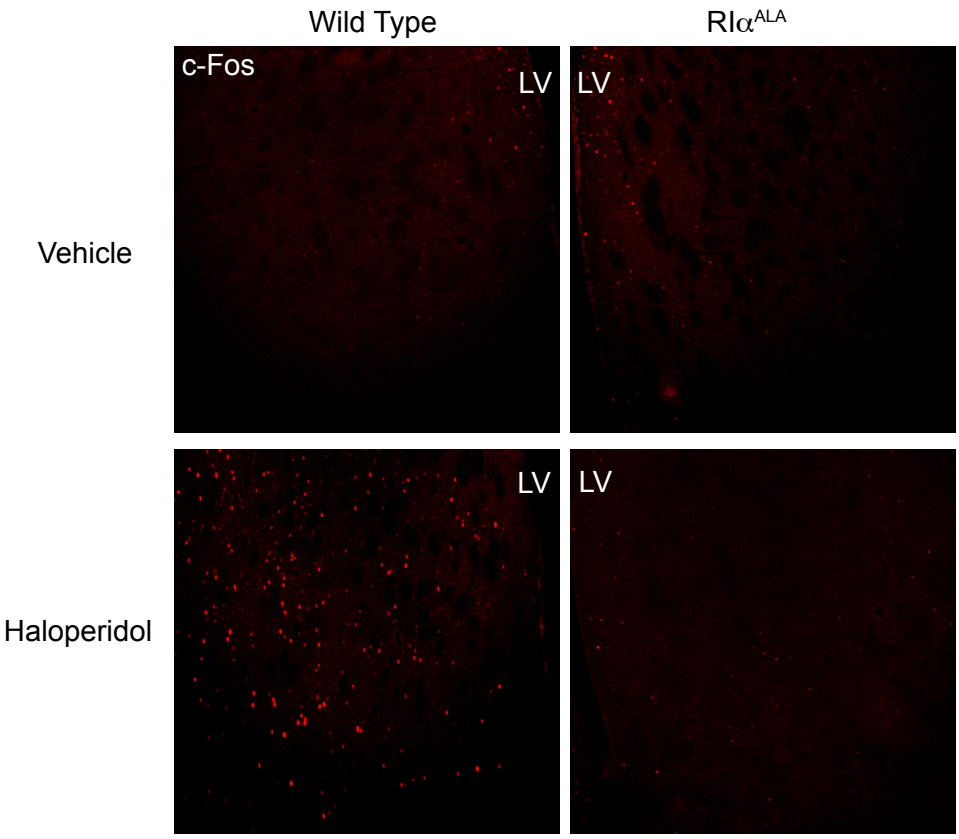

A
B
WT
RIα^ALA
Ambulations (Female)
Ambulations (Male)
Time (Hours)
Female
Male
Total Ambulations
**
**
WT
RIα^ALA
WT
RIα^ALA

Figure 3.6: Immediate early gene induction is attenuated in striatum in response to haloperidol. Immunostaining of c-Fos expression in the striatum of WT and RIα ALA mice. Mice were given a single IP injection of vehicle or the D2R antagonist, haloperidol (2 mg/kg). Mice were euthanized after two hours and processed for immunohistochemistry as described in the methods section.


Wild Type
RIα^ALA
c-Fos
LV
LV
Vehicle
LV
LV
Haloperidol

Chapter 4

CONCLUSIONS

In this study, I have demonstrated disruption of PKA signaling *in vivo* utilizing two different mutations in the RIα regulatory subunit of PKA. The RIαB mutation had little effect on transcriptional regulation but suppressed an acute phosphorylation event through its attenuated cAMP binding. The post-translational stabilization of RIα ALA impaired cAMP-dependent transcriptional regulation and gene induction, likely due to buffering of C subunit diffusion to the nucleus. However, this mutation would be predicted to have little effect on phosphorylation events that required minimal diffusion of C subunit due to its normal cAMP binding affinity.

The RIαB mutation is extremely effective at suppressing kinase activity in cell and tissue types where the mutant subunit has been expressed after Cre-mediated recombination. Half maximal activation of RIαB requires approximately 100-fold more cAMP and it markedly suppresses kinase activity, even in heterogeneous tissue samples where the mutation is not expressed in all cell types. For example, the data in Fig. 2.2C would suggest that RIαB may not be highly expressed in intercalated cells of the inner medulla, evidenced by de-enrichment of the intercalated cell specific marker (*Slc26a4*) in the RiboTag IPs. Inner medulla kinase activity (Fig. 4.3E) was reduced by approximately 60% in inner medulla lysates that included cell types that expressed only WT RIα subunits. This heterogeneous expression effectively dilutes the RIαB subunit present among the total complement of PKA holoenzyme, yet demonstrates the effectiveness of this mutation in suppressing total kinase activity. Renal expression of RIαB also likely affects osmolyte transporters within the inner medullary collecting ducts that are regulated in a cAMP-dependent manner. Among these, PKA regulates urea and sodium transport by modulating the expression and trafficking of both urea transporters (UT-A1) and the epithelial sodium channel (ENaC) in a vasopressin- and cAMP-dependent manner [13, 47, 104, 141]. Further studies will be

required to elucidate the effects of RIαB on each of these critical transport processes and its impact on fluid homeostasis.

The cAMP- and PKA-dependent regulation of gene expression occurs in part through phosphorylation of CREB/ATF transcription factors and their subsequent binding to CREs in the promoter region of target genes. RIαB expression had no observable effects on genes in which CREs are thought to play a major regulatory role, including *Avp* and *Aqp2* (Fig. 2.3C and Fig. 2.4D). This may seem surprising given the effect of RIαB on total kinase activity and the fact that PKA-dependent phosphorylation of CREB (ser133) was previously shown to rely on passive diffusion of the C subunit into the nucleus[57]. However, the effect of RIαB expression on gene induction likely varies across cell types as the mutant subunit has been shown to suppress CRE-dependent transcriptional activity in luciferase reporter assays[110]. Previous work in our lab analyzed fasting-induced transcriptional changes in genes related to gluconeogenesis in liver, many of which are regulated through CREs[173]. When RIαB was expressed in hepatocytes (*Alb*-Cre), 24-hour fasting had no effect on hepatic gene induction of transcripts including PEPCK, glucose-6 phosphatase, and PGC-1α. In both kidney and liver, it is quite possible that there is sufficient PKA activity to maintain transcriptional regulation through elevated cAMP and PKA-dependent phosphorylation of CREB. However, as discussed in the introduction, there also may exist alternative regulatory pathway(s), including calcineurin-NFAT signaling and osmotically sensitive transcription factors, which may play a compensatory role in regulating *Aqp2* transcription. One possible approach to answer this question would be to place control and RIαB ON mice on an RIIα knockout background to further reduce PKA activity. Given our results that suggest that PKA regulates the post-translational stability of AQP2, however, there is a distinct possibility that this reduction of C subunit activity may further reduce AQP2 levels, leading to early postnatal lethality as observed in mice with genetic disruption of AQP2 itself or the vasopressin receptor[134, 180].

If kinase activity is sufficient to maintain principal cell transcriptional regulation of AQP2, then why is RIαB expression so effective in attenuating phosphorylation? PKA activity co-purifies with AQP2-containing endosomes and it has been reported that AKAP-mediated tethering of PKA to these endosomes is required for efficient apical exocytosis and

water reabsorption[64, 94]. However, we subjected RIIα and RIIβ knockout mice to 24-hour dehydration and these mice were able to concentrate their urine to an extent comparable to WT littermates, which suggests that specific anchoring of the Type II kinase is not required for this process. Several AKAPs have been shown to co-localize or co-purify with AQP2[64, 82, 123], and of those, AKAP7γ and AKAP11 have been reported to have some binding affinity for the RI holoenzyme[19, 42]. AKAP-mediated targeting of RIαB in close proximity to AQP2 may enhance its suppressive effects and further impair apical targeting of the water channel. Additional studies are needed to assess the role of AKAP-mediated targeting of PKA *in vivo*, and with particular regard to localization of the Type I kinase.

AQP2 regulation occurs through both short- and long-term mechanisms, and PKA is currently thought to play a predominant role in short-term processes only. Short-term regulation of AQP occurs over the course of minutes and relies on acute phosphorylation events that drive AQP2 translocation to the apical plasma membrane. Long-term regulation relies on changes in whole cell AQP2 abundance, and this is primarily linked to changed in *Aqp2* gene transcription. Long-term regulation appears intact in RIαB ON mice as AQP2 mRNA level are similar to controls after 24-hour dehydration. However, it has been previously demonstrated that cAMP levels are extremely low in collecting tubules isolated from the kidneys of dehydrated rats [45]. If cAMP levels are low in the dehydrated state, but AQP2 mRNA levels and long-term regulation is still intact, why do RIαB ON mice still exhibit greatly reduced AQP2 protein levels and a urinary concentrating defect? Increased intracellular Ca^{2+} is required for AQP2 trafficking and a Ca^{2+}-calmodulin activated adenylyl cyclase (AC3) is one of two predominant AC isoforms found in mouse kidney[126, 133]. Persistent activity by AC3 could sustain cAMP levels so as to maintain PKA-dependent phosphorylation and apical accumulation of AQP2. Under these conditions where cAMP levels decrease with V2R desensitization, it is likely that RIαB would be increasingly effective, and the reduced retention time of AQP2 in the apical membrane would likely reduce its half-life[113]. While AQP2-expressing cell lines have provided valuable information regarding the compartmentalization of cAMP and its role in AQP2 regulation, these processes are still poorly understood *in vivo*.

The fact that RIαB does not disrupt cAMP-dependent transcriptional regulation AQP2

is almost as surprising as the fact that the RIα ALA mutation readily attenuates gene induction. Due to its higher threshold of activation, RIαB likely remains bound to C subunit in the presence of physiological levels of cAMP. This is evident in the stabilization of RIα we observed in the inner medulla of RIαB ON mice (Fig. 2.4F), where there was a two-fold increase in total protein as it is protected from degradation in the holoenzyme state. The RIα ALA mutant has a much lower threshold for activation and, combined with a 15-fold increase in protein in brain, may be more effective at buffering C subunit diffusion to the nucleus and subsequent CREB phosphorylation. This increase in RIα is not able to impair PKA activation in brain lysates (Figure 3.4D), and we have previously demonstrated that a near 60-fold increase would be required to inhibit total PKA activity in whole cell lysates[35]. The ability of RIα expression to suppress cAMP-dependent transcriptional regulation has been clearly demonstrated using over-expression of mutated RIα subunits as well as hybrid hepatoma cell lines that were found to express increased levels of RIα [110, 83, 14]. It still remains to be seen if there are other genes and physiological systems that may be affected by the post-translational stabilization of RIα.

Post-translational regulation of PKA regulatory subunit levels has been proposed as a mechanism for maintaining persistent catalytic subunit activity in the face of transient increases in intracellular cAMP concentration. This escape from second messenger regulation is not unique among kinases, and certainly not among those implicated in memory and learning. Both CaMKII and PKC-ζ possess alternative mechanisms to bypass requirements for Ca^{2+} or calmodulin-dependent activation. CaMKII phosphorylation at threonine 286 prevents interaction between the autoinhibitory and catalytic domains allowing for persistent activation of the kinase in the absence of Ca^{2+}/CaM regulation[177]. PKM-ζ is transcribed from an alternative start site within the PKC-ζ gene (*Prkcz*); this variant encodes only the catalytic domain and lacks the regulatory domain, allowing for persistent kinase activity[168]. Although an analogous system has been proposed for PKA and regulated degradation of the R subunits, evidence has been lacking, especially in mammalian systems. While we have not demonstrated degradation of RIα here, the ability of RIα ALA to exist at high levels without a concomitant increase in C subunit suggests that the normally fragile RIα subunit is protected somewhat by the D/D mutations. This increased

post-translational stabilization of the protein and its effects on c-fos induction in both MEFs and striatum are encouraging for future studies.

Multiple degradation pathways have been proposed in regulating the turnover of PKA subunits, and RIα in particular. Early studies demonstrated that RIα was susceptible to chymotryptic proteolysis in the flexible linker domain between the D/D domain and the cAMP binding domains[127]. More recently, a Ca^{2+}-sensitive mechanism was described in isolated mitochondria in which the protease calpain cleaves PKA subunits that are sequestered on or in mitochondria[145]. However, the best-described degradation pathway for PKA R subunits has been through ubiquitin-mediated degradation. Using neural ganglia isolated from *Aplysia*, Schwartz and colleagues were readily able to demonstrate characteristic laddering of R subunit proteins, indicative of higher molecular weight ubiquitin conjugates as well as lower molecular weight degradation products[62, 63]. These results of these *in vitro* studies have proven difficult to replicate in other genetic models such as mice and likely requires further identification of ubiquitin-proteasome system components that interact directly with PKA subunits *in vivo*.

The role of ubiquitin in the degradation of PKA in mammalian systems has been unclear, until the recent identification of an ubiquitin ligase that also functioned as an AKAP [98]. Praja2 was identified as an ubiquitin ligase and subsequently categorized as a dual-specific AKAP based on its ability to bind both RI and RII subunits. In a potential feed forward mechanism that agreed, in part, with Schwartz model, Praja2 would bind and ubiquinate R subunits signaling their degradation by the proteasome, and the free catalytic subunit would then phosphorylates Praja2 (serine 342, threonine 389) to further increase its E3 ligase activity. As in *Aplysia*, this sustained C subunit activity supported PKA-dependent CREB phosphorylation, induction of immediate early genes, and the induction of LTF. This model diverges from earlier PKA-ubiquitin studies in that both RI and RII subunits are proposed as targets of Praja2, but only RII appears to be ubiquinated in in vitro assays used by Feliciello and colleagues. Based on the ability of the RIα ALA mutation to abrogate binding to SKIP, further studies should focus on protein-protein interactions that are lost with the D/D mutations and potential mediators of RIα degradation *in vivo*.

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